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Characterization of the Highly Active Polyhydroxyalkanoate Synthase of *Chromobacterium* sp. Strain USM2

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The synthesis of bacterial polyhydroxyalkanoates (PHA) is very much dependent on the expression and activity of a key enzyme, PHA synthase (PhaC). Many efforts are being pursued to enhance the activity and broaden the substrate specificity of PhaC. Here, we report the identification of a highly active wild-type PhaC belonging to the recently isolated *Chromobacterium* sp. USM2 (PhaC<sub>C</sub>). PhaC<sub>C</sub> showed the ability to utilize 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (3HV), and 3-hydroxyhexanoate (3HHx) monomers in PHA biosynthesis. An in vitro assay of recombinant PhaC<sub>C</sub> expressed in *Escherichia coli* showed that its polymerization of 3-hydroxybutyryl-coenzyme A activity was nearly 8-fold higher (2,462 ± 80 U/g) than that of the synthase from the model strain *C. necator* (307 ± 24 U/g). Specific activity using a Strep2-tagged, purified PhaC<sub>C</sub> was 238 ± 98 U/mg, almost 5-fold higher than findings of previous studies using purified PhaC from *C. necator*. Efficient poly(3-hydroxybutyrate) [P(3HB)] accumulation in *Escherichia coli* expressing PhaC<sub>C</sub> of up to 76 ± 2 weight percent was observed within 24 h of cultivation. To date, this is the highest activity reported for a purified PHA synthase. PhaC<sub>C</sub> is a naturally occurring, highly active PHA synthase with superior polymerizing ability.

Unlike petrochemical polymers, the synthesis of biologically based polymers is very much dependent on the catalytic activities of the various enzymes involved, as well as on the carbon feedstock from which the monomers are produced. Polymerization rates and yields vary based on the biosynthetic pathway of the organism and the available monomer supply. One such biopolymer that has attracted widespread interest is polyhydroxyalkanoate (PHA). Owing to its thermoplastic and biodegradable properties, PHA makes an excellent candidate for the biodegradable replacement of conventional plastics (1). The property of PHA is dependent on its monomeric composition, which is determined in part by PhaC (29, 36). Recently, PhaC has been studied in some mechanistic detail and is the benchmark commonly used to evaluate the performance of other synthases (12, 14, 34). Some studies also have been carried out on the synthesis of *Allochromatium vinosum* (class III) (13, 23). Nevertheless, PhaC is a complex enzyme, and its complete structure and properties are not yet fully understood. It is known that the affinity and polymerization activity toward different hydroxyalkanoate-coenzyme A (CoA) substrates vary based on the different classes of PHA synthases. Efforts have been taken to alter and improve the properties of natural synthase enzymes via enzymatic evolution, with the goal of engineering a more active enzyme with broader substrate specificity (38). Several successful studies have reported the engineering of mutant synthases with up to 4-fold increased activity (1, 26, 42). Nevertheless, the search for a natural synthase with comparable properties still is widespread.

The property of PHA is dependent on its monomeric composition, which is determined in part by PhaC (29, 36). Recently, Bhubalan et al. cloned the PHA synthase gene (*phaC<sub>C</sub>*) from an organism termed *Chromobacterium* sp. USM2, isolated from Malaysian sources, and heterologously expressed the synthase in *C. necator*, PHB-4 (5). In this study, poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] copolymer with the high-3-hydroxyvalerate (3HV) fraction was synthesized from mixtures of fructose and sodium valerate. Furthermore, 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) co-

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polymers are known to possess improved mechanical and thermal properties compared to those of the P(3HB) homopolymer (9, 10). When a combination of sodium valerate, or sodium propionate, with CPKO was fed to the C. necator PHB-4 strain containing heterologously expressed phaC1, high intracellular contents of polymer comprising 3HB, 3HV, and 3HHx monomers were produced (4). This P(3HB-3HV-3HHx) terpolymer produced was found to possess elastomeric properties. However, not many microorganisms express a native PHA synthase with the ability to incorporate both short-chain-length (scl) and medium-chain-length (mcl) monomers. The ability of PhaC1 to produce PHA-containing monomers of mixed chain lengths highlighted the potential of this synthase.

In this study, the PHA synthase of Chromobacterium sp. USM2 was further characterized by in vitro and in vivo assays using Escherichia coli JM109 to fully understand its PHA-synthesizing ability. We also purified a heterologously expressed, Strept2-tagged version of PhaC1 to examine the unique abilities of this enzyme. The results obtained in this work showed that PhaC1 is a highly active enzyme in its natural form, and it is expressed at high levels in E. coli. The ability to produce high concentrations of active synthase in vivo might facilitate overcoming one of the bottlenecks in the crystallization of the PhaC enzyme, which is producing and isolating an abundant amount of pure protein. Once this is possible, attempts can be made to determine the three-dimensional structure of this complex enzyme that, 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 hydrolysate, 5 g yeast extract, and 10 g NaCl at pH 7.0. To determine the functional expression of the cloned gene in vivo, PHA biosynthesis was carried out by transferring 1.5 ml (3% [vol/vol]) 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% (wt/vol) glucose. The cultures were incubated at 30 and 37°C for 72 h on a reciprocal shaker at 180 rpm. Ampicillin was added at a final concentration of 100 μg/ml to maintain plasmid stability. For maintenance purposes, bacterial cultures from the exponential growth phase were stored at −20°C in 20% (vol/vol) glycerol.

For the expression 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% [vol/vol]) was inoculated to 1.75 ml of fresh LB broth and was incubated at 30°C for 9 h. Ampicillin was added at a final concentration of 100 μg/ml for plasmid maintenance. For Strept2-PhaC1 expression and purification, E. coli BL21(DE3) was used as a host strain. The expression of Strept2-PhaC1 was performed as follows. Cells with Strept2-PhaC1 expression plasmid were grown in 1 liter of LB broth supplemented with 100 μg/ml ampicillin until an optical density at 600 nm (OD600) of 0.6. Enzyme synthesis was induced by the addition of 0.1 mM (final concentration) isopropyl-β-D-thiogalactopyranoside (IPTG) and allowed to incubate for 2 h at 30°C on a reciprocal shaker at 180 rpm. Cells then were pelleted, and protein was purified as described below.

DNA manipulation and plasmid construction. Plasmid isolation and DNA manipulation was carried out according to standard procedures (30). All of the restriction enzymes (TaKaRa, Toyobo, and Roche) were used according to the manufacturers’ protocols. All other chemicals used were of analytical grade. The plasmid pGEM′AB(phaCCs) was digested with XbaI and PstI to remove the Pseudomonas sp. 61-3 phaC1 gene. The vector then was ligated with a synthetic linker, XbaI-EcoRI-EcoRV-Asp718-HindIII-PstI, which was derived by annealing a set of complementary primers (FXbaPstILK and RXbaPstIL) (nucleotide sequences are shown in Table 2). The resultant vector was named pGEM′AB(L). The phaC1 gene then was cloned using the forward primer EcoRIc and the reverse primer Apa718-HindIII-PstI, which was derived by annealing a set of complementary primers (FXbaPstILK and RXbaPstIL) (nucleotide sequences are shown in Table 2). The resultant vector was named pGEM′AB(L). The phaC1 gene then was cloned into the forward primer EcoRIc and the reverse primer Apa718-HindIII-PstI (Table 2) from the plasmid vector pBBR1MCS-C2. The resulting 1.7-kb gene fragment, flanked with EcoRI and Apa718 restriction sites, was purified and then digested with the corresponding enzymes and ligated into the pGEM′AB(L) vector, which was digested with the same enzymes. The resultant vector was named pGEM′AB(phaCCs). DNA sequencing for the confirmation of new plasmid constructs was carried out by the dyeoxy chain termination method with a Prism 310 genetic analyzer DNA sequencing (Applied Biosystems) and the CEQ2000XL DNA analysis system (Beckman Coulter) using a BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems) and Dye terminator cycle sequencing with quick start kit (Beckman Coulter).

Plasmid pET-phaCCs was constructed using pET51b (Novagen) as the parent plasmid. For the construction of strep2-phaCCs, phaC1 on the plasmid pBBR1MCS-C2 was amplified by PCR using the forward primer Strept2phaCCsFW and the reverse primer Strept2phaCCsRV (Table 2) to introduce the unique restriction site BamHI-S to the phaCCs open reading frame and the unique restriction site HindIII-3′ to the phaCCs open reading frame. The amplified gene was digested with BamHI and HindIII, followed by ligation with BamHI- and HindIII-cut pET51b to produce the plasmid pET-phaCCs. The portion of pET-phaCCs containing the tagged phaCCs gene was sequenced by MIT Biopolymers Laboratory.
Preparation of crude protein samples. *E. coli* JM109 harboring either pGEM/H11033AB(phaCCs), pGEM/H11032CAB, or pGEM/H11033AB(L) was cultured as discussed above. Cells were harvested by centrifugation, and whole-cell extracts of each transformant were prepared by resuspending the cells in 2 ml of ice-cold 40 mM potassium phosphate buffer (pH 7.5) and subsequent disruption by sonication (three cycles, 5 s each) on ice using a Tomy UD-200 sonicator. A soluble fraction was obtained from the 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 subsequent precipitate. Protein was measured using the Bradford assay (6).

Expression and purification of Strep2-tagged PhaC<sub>s</sub>. *E. coli* BL21(DE3)/pET-phaCCs was cultured as discussed above. Cells (6.5 to 7.8 g wet weight) were pelleted by centrifugation at 2,988 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 (two passes at 12,000 lb/in<sup>2</sup>). 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, Goettingen, 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 to 20 min. The column was eluted and washed with 5 × 10 ml buffer B. Strep2-PhaC<sub>s</sub> was eluted from the column with six 5-ml fractions of buffer C (buffer B plus 2.5 mM desthiobiotin). The Strep-tactin column was regenerated according to the manufacturer's instructions. The protein concentration of each fraction was determined by Bradford assay. The pooled fractions then were concentrated.

**TABLE 2. Primer sequences used in this study<sup>a</sup>**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FEcoRICs</td>
<td>GCGGCCAACCAACCGAGAAATTCATGC</td>
</tr>
<tr>
<td>RAasp718Cs</td>
<td>GGGACGGTGACCTTCGGTTCAG</td>
</tr>
<tr>
<td>FXbaIPstILK</td>
<td>CTAGATAAGAAGGGAGATGAATTCGAG</td>
</tr>
<tr>
<td>RXbaIPstIL</td>
<td>GAAGCTTGGTACCGATATCGAATTCA</td>
</tr>
<tr>
<td>phCaC&lt;sub&gt;s&lt;/sub&gt;</td>
<td>TATCGGTACCAAGCTTCTGCA</td>
</tr>
<tr>
<td>Strep2phaCCsFW</td>
<td>CAAGGATCCGAGTGCAGCAGCTTTGTCA</td>
</tr>
<tr>
<td>Strep2phaCCsRV</td>
<td>CTTAAGCTTTTAGAGCAGGCGA</td>
</tr>
</tbody>
</table>

<sup>a</sup> Restriction sites are underlined.
using a Vivaspin 15R concentrator (Sartorius AG, Göttin gen, Germany) to 5.5 to 25.5 mg protein/ml and dialyzed twice against 100 mM Tris-HCl (pH 8.0), containing 0.5 mM EDTA and 0.5 mM dithiothreitol, for 12 to 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 spectrophotometrically at A_{280} using the molar absorption coefficient 110,310 M⁻¹ cm⁻¹.

Strep2-PhaC₆₇ purification was performed three separate times.

**In vitro enzymatic assay of crude PhaC₆₇.** The activity of PHA synthase from crude extract was determined by measuring the amount of CoA released from 3HB-CoA during polymerization (41). The assay mixture contained 2 mM 3HB-CoA, 40 mM potassium phosphate buffer (pH 7.5), 10 mM 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), and 1 mg/ml bovine serum albumin (BSA). The reaction was initiated by adding 35 to 40 μg of protein obtained from the soluble fraction of disrupted cells into the reaction mixture described above, and the absorbance at 412 nm was measured at 30°C. The concentration of CoA was determined spectrophotometrically (12) using a molar absorption coefficient of 13,600 M⁻¹ cm⁻¹ at 412 nm with a Hitachi U-3900H spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme that catalyzed the release of 1.0 μmol CoA/min. Enzyme 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% SDS-PAGE. Separated proteins from the soluble fraction were then transferred onto a polyvinylidene fluoride (PVDF) membrane (Immun-blot PVDF membrane [Bio-Rad]) using a Criterion blotter (Bio-Rad). An immunoblot analysis of PHA synthase was carried out using specific rabbit antiserum raised against the C-terminal region of PhaC₆₇ as described above (M. Cho, C. Brigham, A. Sinskey, and J. Stubble, unpublished data). One unit of enzyme activity is defined as described above. Enzyme assays were performed in triplicate.

**RESULTS**

**In vitro assay of crude PhaC₆₇ in E. coli.** The ability of the C. necator PHB⁺ transformant harboring phaC₆₇ (GenBank accession no. HM989943) to utilize CPKO and 3HV precursors for the biosynthesis of PHA polymers containing 3HB, 3HV, and 3HHX monomers (4, 5) served as groundwork to investigate the interesting properties of this synthase further. Hence, in this study, PhaC₆₇ was characterized using in vivo and in vitro assays to understand better its PHA-synthesizing ability. The phaC₆₇ gene was cloned into plasmid pGEM'AB(L) harboring the monomer-supplying genes of C. necator H16 (phaAC₆₇, and phaB₆₇) and subsequently expressed in E. coli transformants using Western blot analysis. E. coli cells harboring plasmid pGEM'AB(L) were used as a negative control. A total of 10 μg of protein from the supernatant was used for the analysis.

![FIG. 2.](http://aem.asm.org/Downloaded from)
Cs concentrations of PhaC

Given these results, it was assumed that the availability of higher chromo-
temperature for growth and PHA accumulation by E. coli bacterium than at 37°C (Fig. 3). P(3HB) content of up to 88 ± 1 wt% (48 h) was accumulated by this transformant at 30°C, whereas there was a maximum of 76 ± 2 wt% (24 h) at 37°C. The polymerization of P(3HB) by PhaC<sub>Cn</sub> expressed in E. coli appeared to be better at 30°C. As shown in Fig. 4A, the E. coli transformant was packed with granules of various sizes. Some cells contained mainly smaller granules, as shown in Fig. 4B. The molecular mass of P(3HB) produced averaged 5 × 10<sup>5</sup> Da, with a high polydispersity of 6.0.

Enzymatic activity of purified Strep2-PhaC<sub>Cn</sub>. To further investigate the polymerization ability of PhaC<sub>Cn</sub>, we constructed a Strep2-tagged PhaC<sub>Cn</sub> for expression in and purification from E. coli. Strep2-PhaC<sub>Cn</sub> was cloned into E. coli BL21(DE3) and purified as described in Materials and Methods. The specific activity of this highly purified Strep2-PhaC<sub>Cn</sub> was 238 ± 98 U/mg, which is significantly greater than that of purified synthase from C. necator from previously published results (specific activity, 40 U/mg) (43). It could be argued that the difference in N-terminal epitope tags (Strep2 tag for the enzyme purified in this work and an oligonucleotide-HIS tag in reference 43) could result in differences of activity. A Strep2-PhaC<sub>Cn</sub> enzyme was expressed previously and purified from E. coli BL21(DE3), and the activity was determined to be 38.5 ± 8.1 U/mg (M. Cho et al., unpublished), which is similar to that of the previously published His6-PhaC<sub>Cn</sub> activity (43). The purified Strep2-PhaC<sub>Cn</sub> enzyme also exhibited a lag phase in activity (Fig. 5), which is consistent with previous results using purified class I PhaC proteins (such as PhaC from C. necator) isolated from E. coli (12, 43, 44). While the lag phase of Strep2-PhaC<sub>Cn</sub> is more prevalent in Fig. 5, the lag phase in activity of Strep2-PhaC<sub>Cn</sub> is more prevalent when lower concentrations of enzyme are used (data not shown).

DISCUSSION

In vitro and in vivo characterization of PhaC<sub>Cn</sub> in E. coli was carried out by the heterologous expression of phaC<sub>Cn</sub> along with phaAC<sub>Cn</sub> and phaBC<sub>Cn</sub> under the control of the C. necator promoter in a pGEM<sup>®</sup>-AB(phaC<sub>Cn</sub>) expression plasmid. The heterologously expressed synthase showed an increased level of expression and enzyme activity. As seen in Fig. 2A in the
crude cell lysates, a distinct band of approximately 66 kDa in size was observed that corresponded to the class I PHA synthases (28, 29). In SDS-PAGE gel analysis, a more distinct band exhibited by PhaC<sub>Cn</sub> compared to that of PhaC<sub>Ac</sub> suggests that PhaC<sub>Cn</sub> was expressed at a higher level in this system. This finding was further confirmed by Western blot analysis (Fig. 2B). The total activity of cell extracts containing PhaC<sub>Cn</sub> toward the polymerization of 3HB-CoA was nearly 8-fold higher than those containing PhaC<sub>Ac</sub>. This suggested that the total enzymatic activity of PhaC<sub>Cn</sub> can be partially correlated with its elevated level of expression in vivo.

The ability to polymerize 3HB-CoA varies among the different classes of PHA synthases, with class I, III, and IV synthases showing higher preference toward the polymerization of 3HB-CoA (36, 38). 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> from cell extracts of E. coli was comparable to that observed in extracts of wild-type C. necator, whereby its activity is known to range from 180 to 330 U/g during PHA accumulation stages (11, 15, 31).

The activities of several PHA synthases recombinantly expressed in E. coli have been documented previously. Alterations in the expression level and specific activity of some of these enzymes were achieved through enzyme evolution studies. The PhaC<sub>Cn</sub> enzyme harboring an F420S mutation has a 2.4-fold higher specific activity for the polymerization of 3HB-CoA than wild-type PhaC<sub>Cn</sub> (39). Meanwhile, PhaC<sub>Cn</sub> harboring a double mutation (G4D and F420S) exhibited an increased synthase concentration in vivo and enhanced polymer accumulation (26). In a similar study, the synthase activities in cell extracts of Aeromonas punctata wild-type and mutant strains were found to be in the range of 118 to 768 U/g (1). The mutant synthases exhibited up to 5-fold-increased activity compared to that of the wild-type synthase. On the other hand, wild-type and mutant synthases of Pseudomonas sp. 61-3, which belongs to the class II PHA synthase, exhibited activity of less than 50 U/g toward the polymerization of 3HB-CoA (42).

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<sub>4</sub> grown on fructose, were reported to be in the range of 18 to 249 U/g (41). Compared with the activity levels of these wild-type and mutant PHA synthases, PhaC<sub>Cn</sub> clearly exhibited a much higher 3HB-CoA polymerizing activity (2,462 ± 80 U/g).

It is interesting that PhaC<sub>Cn</sub> revealed a homology of 46% with PhaC<sub>Ac</sub> (GenBank accession no. P23608) but only 34% with PhaC<sub>Cs</sub> (GenBank accession no. BAA21815), even though PhaC<sub>Cn</sub> is also known to incorporate the 3HHx monomer into PHA (4, 5). As mentioned earlier, engineered PhaC<sub>Cn</sub> synthases are known to exhibit improved levels of synthase activity and polymer accumulation. The mutant synthases that harbor mutations at F420S, G4D, or G4D/F420S showed improved activities and higher in vivo concentrations of enzyme (25, 26, 39). It was found that the amino acid sequences at positions 4 and 420 in PhaC<sub>Cn</sub> did not contain the altered residues mentioned above. The amino acids at these positions both were identified as phenylalanine. This indicated that the PHA synthase of Chromobacterium sp. USM2 was highly active in its natural form. The characterization of PhaC<sub>Cn</sub> in vitro showed that this enzyme was produced at high concentrations in E. coli cells. Such a high level of expression exhibited by PhaC<sub>Cn</sub> might be correlated with the efficient translation capability of E. coli due to optimal codon usage (33).

Besides the high level of expression, PhaC<sub>Cn</sub> also exhibited a very high level of activity, approximately 8-fold higher than that of PhaC<sub>Cn</sub>. Furthermore, the activity of purified Strep2-PhaC<sub>Cn</sub> was shown to be at least three to five times greater than the activity of pure Strep2-tagged PhaC<sub>Cn</sub>. Preliminary enzymatic assay experiments using 3HV-CoA also suggested that the specific activity of Strep2-PhaC<sub>Cn</sub> is roughly twice as great as that of Strep2-PhaC<sub>Cn</sub> using this substrate (data not shown).

It was reported previously that a mutant synthase of A. caviae had an increased specific activity toward 3HB-CoA of approximately 1.6-fold compared to that of the wild-type (0.016 U/mg) (16). PhaC<sub>Cs</sub> exhibits a much higher preference toward 3HB-CoA than to other class I PHA synthases, such as PhaC<sub>Cn</sub> and PhaC<sub>Ac</sub>. The characteristics of PhaC<sub>Cn</sub> in its native strain, Chromobacterium sp. USM2, or in the C. necator PHB<sub>4</sub> transformant have yet to be investigated to determine if these elevated levels of gene 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>Cn</sub> correlated with results obtained from the in vitro experiments. The synthase efficiently polymerized P(3HB) when glucose was the carbon source. Cells were able to rapidly accumulate P(3HB) to 76 ± 2 wt% within 24 h of cultivation. The highest P(3HB) content of 88 ± 1 wt% was accumulated at 48 h. This resulted in a P(3HB) concentration of 7.2 ± 0.2 g/liter. Previously, E. coli transformants harboring phaC<sub>Cn</sub> were shown to accumulate P(3HB) in the range of 60 to 70 wt% (37). The residual cell biomass was in the range of 1 ± 0.1 to 1.4 ± 0.1 g/liter throughout 72 h of cultivation. An increase in total cell biomass of up to 8.2 ± 0.2 g/liter at 60 h was caused by increasing amounts of intracellular P(3HB) accumulation. The average molecular mass of P(3HB) synthesized by PhaC<sub>Cn</sub> (5 × 10<sup>5</sup> Da) was found to be lower than that produced by some E. coli transformants harboring different PHA synthases, such as PhaC<sub>Ac</sub> (9.7 × 10<sup>5</sup> Da) and mutant PHA synthase of Pseudomonas sp. 61-3 (7.2 × 10<sup>5</sup> Da) (3, 40). The smaller molecular size of the resulting polymer could be attributed to the high in vivo concentration of the synthase (32). As observed in Fig. 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 shorter P(3HB) chains, thus increasing the polydispersity.

The Chromobacterium sp. USM2 synthase produced larger amounts of P(3HB) at 30°C than at 37°C. A significant difference was noticed in the polymer accumulation compared to that at 30°C, whereby a reduction in P(3HB) content of approximately 18% was noticed at the end of cultivation at 37°C (Fig. 3). Nevertheless, residual biomass values (data not shown) indicated that cell growth was not affected by the different cultivation temperatures. The higher accumulation of P(3HB) at a lower temperature could be correlated with the
temperature optimum of PhaC<sub>C<sub>sp</sub></sub>. Since wild-type Chromobacterium sp. USM2 is known to grow and accumulate PHA at an optimum temperature of 30°C, the synthase potentially is more active at this temperature. The performance of PHA synthases is known to be affected by various temperatures (26).

The PHA synthase of Chromobacterium sp. USM2 has been successfully characterized via in vitro and in vivo assays in E. coli. The synthase exhibited high levels of expression and specific activity toward the polymerization of 3HB-CoA compared to that of the PHA synthase of model strain C. necator. The activity of this natural synthase was found to be higher than that of some of the engineered mutant synthases. This finding raises the possibility that organisms with higher than that of some of the engineered mutant synthases.

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