Applied and Environmental Microbiology

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

Kesaven Bhubalan, Jo-Ann Chuah, Fumi Shozui, Christopher J. Brigham, Seiichi Taguchi, Anthony J. Sinskey, ChoKyun Rha and Kumar Sudesh

Appl. Environ. Microbiol. **2011, 77(9):2926. DOI: 10.1128/AEM.01997-10.**
Published Ahead of Print 11 March 2011.

Updated information and services can be found at:
http://aem.asm.org/content/77/9/2926

These include:

REFERENCES

This article cites 43 articles, 7 of which can be accessed free at:
http://aem.asm.org/content/77/9/2926#ref-list-1

CONTENT ALERTS

Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), more»

Information about commercial reprint orders: **http://aem.asm.org/site/misc/reprints.xhtml**
To subscribe to to another ASM Journal go to: **http://journals.asm.org/site/subscriptions/**

Journals.ASM.org

Downloaded from http://aem.asm.org/ on February 3, 2012 by MASS INST OF TECHNOLOGY

Applied and Environmental Microbiology, May 2011, p. 2926–2933 Vol. 77, No. 9
0099-2240/11/$12.00 doi:10.1128/AEM.01997-10
Copyright © 2011, American Society for Microbiology. All Rights Reserved.

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

Kesaven Bhubalan,[1] Jo-Ann Chuah,[1] Fumi Shozui,[2] Christopher J. Brigham,[3] Seiichi Taguchi,[2] Anthony J. Sinskey,[3,4,5] ChoKyun Rha,[6] and Kumar Sudesh[1]*

School of Biological Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia[1]; Division of Biotechnology and Macromolecular Chemistry, Graduate School of Engineering, Hokkaido University, N13-W8, Kita-ku, Sapporo 060-8628, Japan[2]; Department of Biology,[3] Engineering Systems Division,[4] and Division of Health Sciences Technology,[5] Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139; and Biomaterial Science and Engineering Laboratory, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139[6]

Received 24 August 2010/Accepted 21 February 2011

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 ($PhaC_{Cs}$). $PhaC_{Cs}$ 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 $PhaC_{Cs}$ 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 $PhaC_{Cs}$ 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 $PhaC_{Cs}$ 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. $PhaC_{Cs}$ 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 (7). PHA has garnered a great deal of interest for applications in various industries, including medicine, pharmacology, agriculture, packaging, and cosmetics (2, 27, 45). PHA has been produced using wild-type as well as recombinant microorganisms (17–19). The biosynthesis of this bacterial polymer is controlled by both the enzymes that supply usable monomers and PHA synthase (commonly known as PhaC), the key enzyme involved in polymerization (29, 36, 38).

The dominant role played by PhaC in determining polymer composition and properties provided an impetus to extensive investigations of PHA synthases. Four major classes of PHA synthases have been classified with respect to their primary structures, substrate specificities, and subunit composition (28, 29). So far, the PhaC of *Cupriavidus necator* ($PhaC_{Cn}$) (class I) 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 synthase 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 ($phaC_{Cs}$) from an organism termed *Chromobacterium* sp. USM2, isolated from Malaysian sources, and heterologously expressed the synthase gene in a PHA-negative mutant of *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-

* Corresponding author. Mailing address: Ecobiomaterial Research Laboratory, School of Biological Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia. Phone: 60-4-6534367. Fax: 60-4-6565125. E-mail: ksudesh@usm.my.
▽ Published ahead of print on 11 March 2011.

Downloaded from http://aem.asm.org/ on February 3, 2012 by MASS INST OF TECHNOLOGY

TABLE 1. Strains and plasmids used in this study

Bacterial strains and plasmids	Relevant phenotype	Source or reference
Bacterial strains		
Escherichia coli JM109	E14-(*mcrA*) *recA1 gryA96 thi*-1 *hsdR17*(rk^-, mk^+) *supE44 relA1* Δ(*lac-proAB*) [F′ *traD36*, *proAB*, *lacI*^{q}ZΔM15]	Stratagene
BL21(DE3)	F^- *ompT gal dcm lon hsdS*$_B$(r_B^- m_B^-) λ(DE3 [*lacI lacUV5-T7* gene 1 *ind1 sam7 nin5*])	Novagen
Plasmids		
pBBR1MCS-C2	pBBR1MCS-2 derivative harboring an ~2.0-kb fragment of *phaC*$_{Cs}$ from *Chromobacterium* sp. USM2 with putative promoter	5
pGEM′CAB	pGEM-T derivative with *C. necator* promoter and terminator harboring *phaC*$_{Cn}$, *phaA*$_{Cn}$, *phaB*$_{Cn}$	21
pGEM″C1AB	pGEM-T derivative with *C. necator* promoter and terminator harboring *phaC1*$_{Ps}$, *phaA*$_{Cn}$, *phaB*$_{Cn}$	20
pGEM″AB	pGEM-T derivative with *C. necator* promoter and terminator harboring *phaA*$_{Cn}$, *phaB*$_{Cn}$	This study
pGEM″AB(L)	pGEM-T derivative with *C. necator* promoter and terminator harboring *phaA*$_{Cn}$, *phaB*$_{Cn}$, synthetic XbaI-EcoRI-EcoRV-Asp718-HindIII-PstI linker	This study
pGEM″AB(*phaC*$_{Cs}$)	pGEM-T derivative with *C. necator* promoter and terminator harboring *phaC*$_{Cs}$, *phaA*$_{Cn}$, *phaB*$_{Cn}$	This study
pET51b	Protein expression vector for N-terminally Strep2-tagged proteins	Novagen
pET-phaCCs	pET51b derivative with *phaC*$_{Cs}$ open reading frame inserted into BamHI/HindIII restriction site	This study

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$^-$4 strain containing heterologously expressed *phaC*$_{Cs}$, high intracellular contents of polymer comprising 3HB, 3HV, and 3HHx monomers were produced (4). This P(3HB-*co*-3HV-*co*-3HHx) terpolymer produced was found to possess elastomeric properties. However, not many microorganisms express a native PHA synthase with the ability to incorporate both short-chain-length (scl) and medium-chain-length (mcl) monomers. The ability of PhaC$_{Cs}$ 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, Strep2-tagged version of PhaC$_{Cs}$ to examine the unique abilities of this enzyme. The results obtained in this work showed that PhaC$_{Cs}$ 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 enzymatic 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 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% [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 Strep2-PhaC$_{Cs}$ expression and purification, *E. coli* BL21(DE3) was used as a host strain. The expression of Strep2-PhaC$_{Cs}$ was performed as follows. Cells with Strep2-PhaC$_{Cs}$ expression plasmid were grown in 1 liter of LB broth supplemented with 100 μg/ml ampicillin until an optical density at 600 nm (OD_{600}) 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(*phaC*$_{Cs}$) in Fig. 1 was constructed to determine the expression and activity of PhaC$_{Cs}$ in *E. coli* JM109 via *in vitro* and *in vivo* experiments. First, the plasmid vector pGEM″C1AB 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 (FXbaIPstILK and RXbaIPstIL) (nucleotide sequences are shown in Table 2). The resultant vector was named pGEM″AB(L). The *phaC*$_{Cs}$ gene then 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 gene fragment, flanked with EcoRI and Asp718 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(*phaC*$_{Cs}$). DNA sequencing for the confirmation of new plasmid constructs was carried out by the dideoxy chain termination method with a Prism 310 genetic analyzer DNA sequencer (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-phaC*$_{Cs}$, *phaC*$_{Cs}$ 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*$_{Cs}$ open reading frame and the unique restriction site HindIII 3′ to the *phaC*$_{Cs}$ 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 *phaC*$_{Cs}$ gene was sequenced by MIT Biopolymers Laboratory.

Downloaded from http://aem.asm.org/ on February 3, 2012 by MASS INST OF TECHNOLOGY

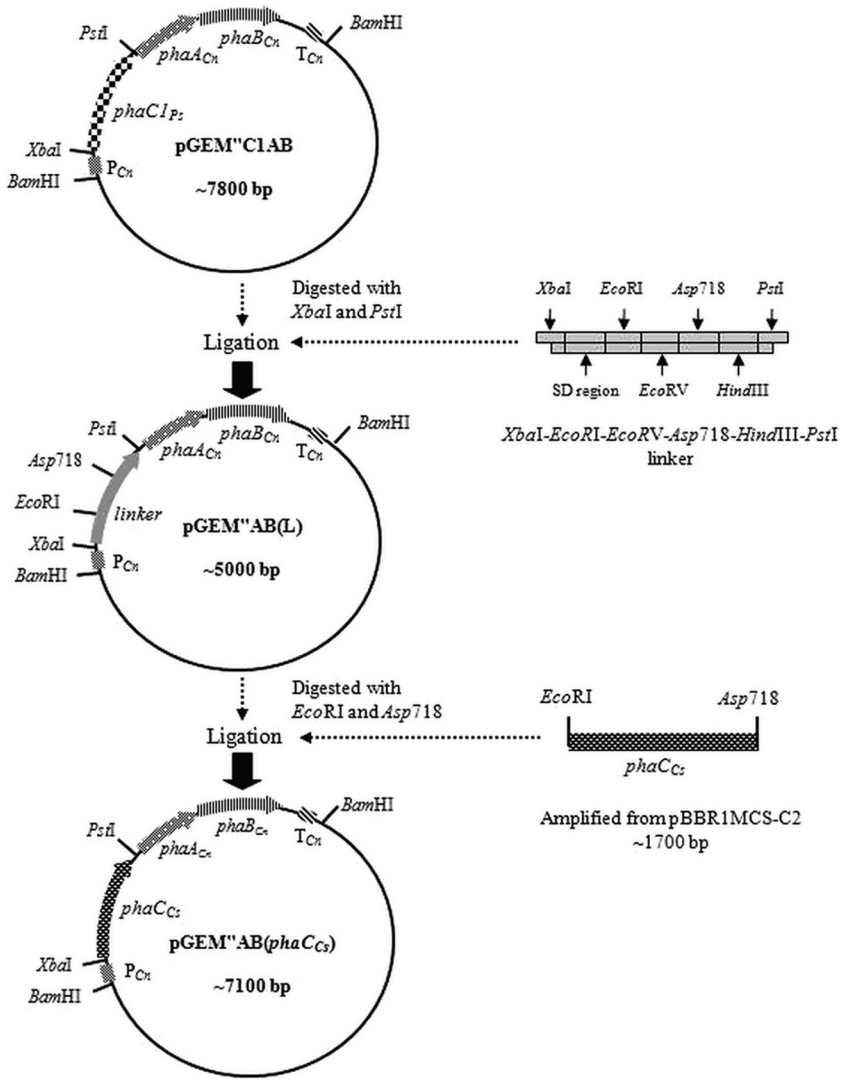

FIG. 1. Construction of the pGEM″AB($phaC_{Cs}$) expression plasmid harboring the PHA synthase gene of *Chromobacterium* sp. USM2 with promoter (P_{Cn})-, terminator (T_{Cn})-, and monomer-supplying genes $phaA_{Cn}$ and $phaB_{Cn}$ of *C. necator*. pGEM″C1AB harbors the PHA synthase gene of *Pseudomonas* 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*.

Preparation of crude protein samples. *E. coli* JM109 harboring either pGEM″AB($phaC_{Cs}$), pGEM′CAB, or pGEM″AB(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).

TABLE 2. Primer sequences used in this study[a]

Primer name	Sequence (5′–3′)
FEcoRICs	GCGGCCAACCAGGAATTCATGC
RAsp718Cs	GGGACGGTACCTTCGGTTCAG
FXbaIPstILK	CTAGATAAGAAGGAGATGAATTCGATATCGGTACCAAGCTTCTGCA
RXbaIPstIL	GAAGCTTGGTACCGATATCGAATTCATCTCCTTCTTAT
Strep2phaCCsFW	CAAGGATCCGATGCAGCAGTTTGTCAATTCCCT
Strep2phaCCsRV	CTTAAGCTTTCAGTTCAAGGCGGCGA

[a] Restriction sites are underlined.

Expression and purification of Strep2-tagged PhaC$_{Cs}$. *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^2). 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 to 20 min. The column was eluted and washed with 5 × 10 ml buffer B. Strep2-PhaC$_{Cs}$ 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

Downloaded from http://aem.asm.org/ on February 3, 2012 by MASS INST OF TECHNOLOGY

using a Vivaspin 15R concentrator (Sartorius AG, Göttingen, 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 spectrometrically at A_{280} using the molar absorption coefficient 110,810 M^{-1} cm^{-1}. Strep2-PhaC$_{Cs}$ purification was performed three separate times.

***In vitro* enzymatic assay of crude PhaC$_{Cs}$.** 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 spectrometrically (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.

***In vitro* enzymatic assay of Strep2-PhaC$_{Cs}$.** Assays were carried out as previously described (43). Final enzyme concentrations of 7.5 to 30 nM Strep2-PhaC$_{Cs}$ and 600 μM 3HB-CoA were used. The concentration of CoA was determined spectrometrically (12) using a molar absorption coefficient of 13,600 M^{-1} cm^{-1} at 412 nm with an Agilent 8453 spectrophotometer. Preparations of Strep2-PhaC$_{Cn}$ were used as a control and purified as described elsewhere (M. Cho, C. Brigham, A. Sinskey, and J. Stubbe, unpublished data). One unit of enzyme activity is defined as described above. 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 then were 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 PhaC$_{Cn}$ as described by Murata et al. (24). PhaC protein was detected using goat anti-rabbit IgG conjugated with alkaline phosphatase as a secondary antibody.

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

GPC. The average molecular weight of P(3HB) produced was estimated using a Shimadzu 10A gel permeation chromatography (GPC) system and a 10A refractive index detector with Shodex K-806 M 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. A sample concentration of 1.0 mg/ml was used. The calibration curve was generated using low-polydispersity polystyrene standards.

TEM. *E. coli* JM109 harboring pGEM″AB(*phaC$_{Cs}$*) was cultured for 24 h in LB supplemented with 2% (wt/vol) glucose as mentioned above. Transmission electron microscopy (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 (22). The cell pellets then were postfixed with 1% osmium tetroxide (OsO_4) 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 to 48 h in Spurr's low-viscosity resin (35). Ultrathin 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$_{Cs}$ in *E. coli*.** The ability of the *C. necator* PHB$^-$4 transformant harboring *phaC$_{Cs}$* (GenBank accession no. HM989943) to utilize CPKO and 3HV precursors for the biosynthesis of PHA polymers containing 3HB, 3HV, and 3HHx monomers (4, 5) served as groundwork to investigate the interesting properties of this synthase further. Hence, in this study, PhaC$_{Cs}$ was characterized using *in vivo* and *in vitro* assays to understand better its PHA-synthesizing ability. The *phaC$_{Cs}$* gene was cloned into plasmid pGEM″AB harboring the monomer-supplying genes of *C. necator* H16 (*phaA$_{Cn}$* and *phaB$_{Cn}$*) and subsequently expressed in *E. coli* JM109. *E. coli* harboring pGEM′CAB plasmid, which contains the PHA biosynthetic genes (*phaCAB*) of *C. necator* H16, was used as the positive control. The construction of pGEM″AB(*phaC$_{Cs}$*) is shown in Fig. 1.

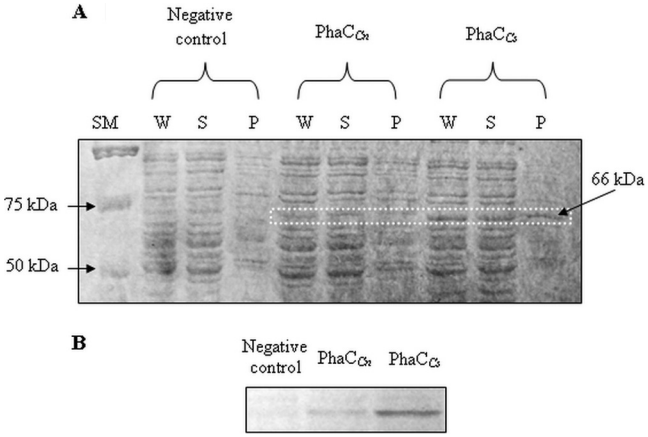

FIG. 2. (A) SDS-PAGE analysis of crude extracts of PhaC$_{Cn}$ and PhaC$_{Cs}$. *E. coli* cells harboring plasmid pGEM″AB(L) were used as a negative control. For each sample, a total of 10 μg of protein was loaded into each well. SM, size marker; W, whole-cell extract; S, supernatant (soluble fraction); 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) were used as a negative control. A total of 10 μg of protein from the supernatant was used for the analysis.

The *in vivo* level of PhaC$_{Cs}$ was evaluated through a series of *in vitro* assays. From the SDS-PAGE analysis of the crude cell lysates (Fig. 2A), it can be seen that the *in vivo* level of PhaC$_{Cs}$ protein appeared to be higher than that of PhaC$_{Cn}$ using the same background strain. In Fig. 2A, distinct bands of approximately 66 kDa in size corresponded to the sizes of the synthases. The detection of similar-size protein bands in the precipitate (cell pellets) confirmed the presence of an insoluble population of synthase protein. A more distinctly observed elevation in the concentration of PhaC$_{Cs}$ was seen through Western blot analysis. As shown in Fig. 2B, the intensity of the PhaC$_{Cs}$ band was much greater than that of PhaC$_{Cn}$. This suggested the presence of a higher concentration of PhaC$_{Cs}$ in the bacterial cells. As expected, no protein band was detected in the negative-control sample, whereby plasmid pGEM″AB(L) harboring only monomer-supplying genes without the presence of PHA synthase gene was used.

To investigate the activity of PhaC$_{Cs}$, 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$_{Cs}$ was measured using the soluble fraction of the crude extract. PhaC$_{Cs}$ demonstrated a superior ability in polymerizing 3HB-CoA compared to that of PhaC$_{Cn}$. The total synthase activity of cell extracts containing PhaC$_{Cs}$ (2,462 ± 80 U/g) was nearly 8-fold higher than that of cells expressing

Downloaded from http://aem.asm.org/ on February 3, 2012 by MASS INST OF TECHNOLOGY

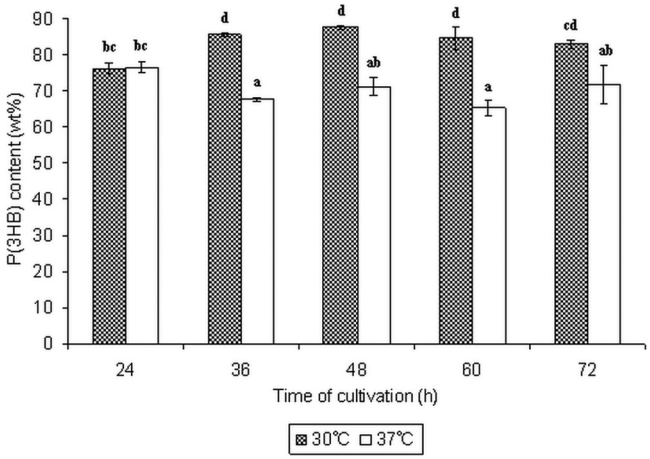

FIG. 3. Comparison of P(3HB) production at 30 and 37°C by an *E. coli* JM109 transformant harboring $phaC_{Cs}$. Cells were incubated for 72 h in LB medium supplemented with 2% (wt/vol) glucose and 100 μg/ml of ampicillin. Data shown are means from triplicates. Means with different letters are significantly different (Tukey's honestly significant difference test; $P < 0.05$).

$PhaC_{Cn}$ (307 ± 24 U/g). The high activity of $PhaC_{Cs}$ could be associated with its elevated level of expression in *E. coli*. Given these results, it was assumed that the availability of higher concentrations of $PhaC_{Cs}$ in the cells would ensure more efficient and faster accumulation of polymer upon the addition of a carbon substrate. Results obtained from the *in vivo* evaluation of $PhaC_{Cs}$ confirmed this hypothesis, as shown below.

***In vivo* evaluation of $PhaC_{Cs}$.** The *E. coli* transformant harboring $phaC_{Cs}$ was found to accumulate large amounts of intracellular P(3HB) (76 ± 2 weight percent [wt%]) within 24 h of cultivation using glucose as the carbon source. The total P(3HB) concentration reached a maximum value of 7.2 ± 0.2 g/liter after 48 h of cultivation. It is well known that the optimal growth temperature for *E. coli* is 37°C. However, the optimal temperature for growth and PHA accumulation by *Chromobacterium* sp. USM2 previously had been identified as 30°C (5). Therefore, an *E. coli* transformant harboring $phaC_{Cs}$ was cultivated at 30°C to determine the effect of lowered temperature on the overall growth and productivity. A significant difference in the P(3HB) accumulation was noticed at 30°C compared to that 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 $PhaC_{Cs}$ 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 5×10^5 Da, with a high polydispersity of 6.0.

Enzymatic activity of purified Strep2-$PhaC_{Cs}$. To further investigate the polymerization ability of $PhaC_{Cs}$, we constructed a Strep2-tagged $PhaC_{Cs}$ for expression in and purification from *E. coli*. Strep2-$PhaC_{Cs}$ was cloned into *E. coli* BL21(DE3) and purified as described in Materials and Methods. The specific activity of this highly purified Strep2-$phaC_{Cs}$ was 238 ± 98 U/mg, which is significantly greater than that of purified synthase from *C. necator* from previously published

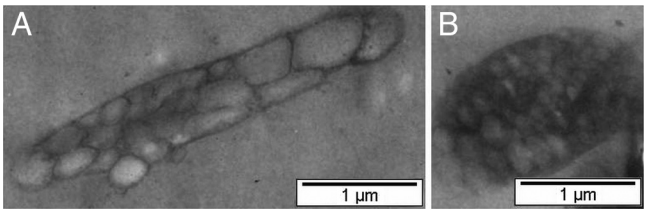

FIG. 4. TEM showing P(3HB) granules in an *E. coli* JM109 transformant harboring pGEM″AB($phaC_{Cs}$). Cells were cultured for 24 h at 30°C in LB supplemented with 2% (wt/vol) glucose and 100 μg/ml ampicillin. It could be observed that some cells contained granules of various sizes (A), while others contained a large number of smaller granules (B).

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-$PhaC_{Cn}$ 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-$PhaC_{Cn}$ activity (43). The purified Strep2-$PhaC_{Cs}$ 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-$PhaC_{Cn}$ is more prevalent in Fig. 5, the lag phase in activity of Strep2-$PhaC_{Cs}$ is more prevalent when lower concentrations of enzyme are used (data not shown).

DISCUSSION

In vitro and *in vivo* characterization of $PhaC_{Cs}$ in *E. coli* was carried out by the heterologous expression of $phaC_{Cs}$ along with $phaA_{Cn}$ and $phaB_{Cn}$ under the control of the *C. necator* promoter in a pGEM″AB($phaC_{Cs}$) expression plasmid. The heterologously expressed synthase showed an increased level of expression and enzyme activity. As seen in Fig. 2A in the

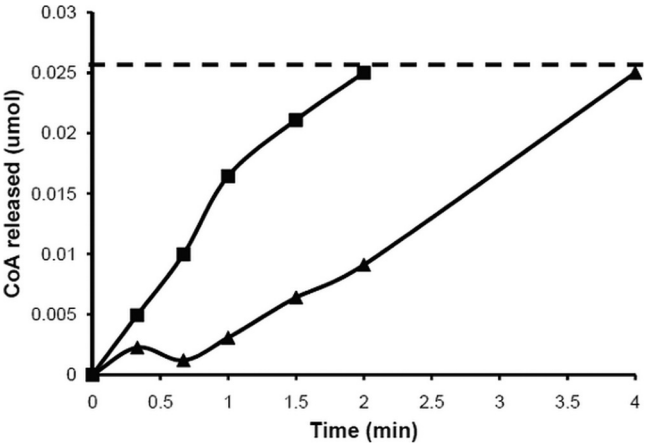

FIG. 5. Time course of CoA release from 3HB-CoA catalyzed by purified Strep2-$PhaC_{Cs}$ (black boxes) and purified Strep2-$PhaC_{Cn}$ (black triangles). In the experiment represented by this figure, 30 nM Strep2-$PhaC_{Cs}$ and 30 nM Strep2-$PhaC_{Cn}$ were used. The dashed line indicates when the 3HB-CoA substrate was completely used up.

Downloaded from http://aem.asm.org/ on February 3, 2012 by MASS INST OF TECHNOLOGY

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 $PhaC_{Cs}$ compared to that of $PhaC_{Cn}$ suggests that $PhaC_{Cs}$ 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 $PhaC_{Cs}$ toward the polymerization of 3HB-CoA was nearly 8-fold higher than those containing $PhaC_{Cn}$. This suggested that the total enzymatic activity of $PhaC_{Cs}$ 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 $PhaC_{Cn}$ 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 $PhaC_{Cn}$ enzyme harboring an F420S mutation has a 2.4-fold higher specific activity for the polymerization of 3HB-CoA than wild-type $PhaC_{Cn}$ (39). Meanwhile, $PhaC_{Cn}$ 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* ($PhaC_{Ac}$) and some of its mutants, when expressed in *C. necator* $PHB^{-}4$ 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, $PhaC_{Cs}$ clearly exhibited a much higher 3HB-CoA polymerizing activity (2,462 ± 80 U/g).

It is interesting that $PhaC_{Cs}$ revealed a homology of 46% with $PhaC_{Cn}$ (GenBank accession no. P23608) but only 34% with $PhaC_{Ac}$ (GenBank accession no. BAA21815), even though $PhaC_{Cs}$ also is known to incorporate the 3HHx monomer into PHA (4, 5). As mentioned earlier, engineered $PhaC_{Cn}$ 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 $PhaC_{Cs}$ 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 $PhaC_{Cs}$ *in vitro* showed that this enzyme was produced at high concentrations in *E. coli* cells. Such a high level of expression exhibited by $PhaC_{Cs}$ might be correlated with the efficient translation capability of *E. coli* due to optimal codon usage (33).

Besides the high level of expression, $PhaC_{Cs}$ also exhibited a very high level of activity, approximately 8-fold higher than that of $PhaC_{Cn}$. Furthermore, the activity of purified Strep2-$PhaC_{Cs}$ was shown to be at least three to five times greater than the activity of pure Strep2-tagged $PhaC_{Cn}$. Preliminary enzymatic assay experiments using 3HV-CoA also suggested that the specific activity of Strep2-$PhaC_{Cs}$ is roughly twice as great as that of Strep2-$PhaC_{Cn}$ 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). $PhaC_{Cs}$ exhibits a much higher preference toward 3HB-CoA than to other class I PHA synthases, such as $PhaC_{Cn}$ and $PhaC_{Ac}$. The characteristics of $PhaC_{Cs}$ in its native strain, *Chromobacterium* sp. USM2, or in the *C. necator* $PHB^{-}4$ 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 $PhaC_{Cs}$ 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 $phaC_{Cn}$ 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 $PhaC_{Cs}$ (5×10^5 Da) was found to be lower than that produced by some *E. coli* transformants harboring different PHA synthases, such as $PhaC_{Cn}$ (9.7×10^5 Da) and mutant PHA synthase of *Pseudomonas* sp. 61-3 (7.2×10^5 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

Downloaded from http://aem.asm.org/ on February 3, 2012 by MASS INST OF TECHNOLOGY

temperature optimum of $PhaC_{Cs}$. 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 other such PHA synthases are present in nature and have yet to be discovered. The naturally active $PhaC_{Cs}$ can be developed as a model synthase to compare the activity of other synthases.

ACKNOWLEDGMENTS

This study was supported by the Techno Fund, provided by The Ministry of Science, Technology and Innovation, Malaysia (MOSTI). K.B. and J.C. acknowledge a 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 Ken'ichiro Matsumoto and Miwa Yamada. We also thank JoAnne Stubbe and Ping Li for the generous gift of some of the 3HB-CoA used in this study and Jingnan Lu for helpful assistance with protein purification.

REFERENCES

1. **Amara, A. A., A. Steinbüchel, and B. H. A. Rehm.** 2002. *In vivo* evolution of the *Aeromonas punctata* polyhydroxyalkanoate (PHA) synthase: isolation and characterization of modified PHA synthases with enhanced activity. Appl. Microbiol. Biotechnol. **59:**477–482.
2. **Anderson, A. J., G. W. Haywood, and E. A. Dawes.** 1990. Biosynthesis and composition of bacterial poly(hydroxyalkanoates). Int. J. Biol. Macromol. **12:**102–105.
3. **Antonio, R. V., A. Steinbüchel, and B. H. A. Rehm.** 2000. Analysis of *in vivo* substrate specificity of the PHA synthase from *Ralstonia eutropha*: formation of novel copolyesters in recombinant *Escherichia coli*. FEMS Microbiol. Lett. **182:**111–117.
4. **Bhubalan, K., D. N. Rathi, H. Abe, T. Iwata, and K. Sudesh.** 2010. Improved synthesis of P(3HB-*co*-3HV-*co*-3HHx) terpolymers by mutant *Cupriavidus necator* using the PHA synthase gene of *Chromobacterium* sp. USM2 with high affinity towards 3HV. Polym. Degrad. Stab. **95:**1436–1442.
5. **Bhubalan, K., K. H. Yong, Y. C. Kam, and K. Sudesh.** 2010. Cloning and expression of the PHA synthase gene from a locally isolated *Chromobacterium* sp. USM2. Malays. J. Microbiol. **6:**81–90.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
7. **Braunegg, G., G. Lefebvre, and K. F. Genser.** 1998. Polyhydroxyalkanoates, biopolyesters from renewable resources: physiological and engineering aspects. J. Biotechnol. **65:**127–161.
8. **Braunegg, G., B. Sonnleitner, and R. M. Lafferty.** 1978. A rapid gas chromatographic method for the determination of poly-β-hydroxybutyric acid in microbial biomass. Eur. J. Appl. Microbiol. **6:**29–37.
9. **Doi, Y., S. Kitamura, and H. Abe.** 1995. Microbial synthesis and characterization of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate). Macromolecules **28:**4822–4828.
10. **Du, G. C., J. Chen, J. Yu, and S. Lun.** 2001. Feeding strategy of propionic acid for production of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) with *Ralstonia eutropha*. Biochem. Eng. J. **8:**103–110.
11. **Fukui, T., and Y. Doi.** 1997. Cloning and analysis of the poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) biosynthesis genes of *Aeromonas caviae*. J. Bacteriol. **179:**4821–4830.
12. **Gerngross, T. U., et al.** 1994. Overexpression and purification of the soluble polyhydroxyalkanoate synthase from *Alcaligenes eutrophus*: evidence for a required posttranslational modification for catalytic activity. Biochemistry **33:**9311–9320.
13. **Jia, Y., T. J. Kappock, T. Frick, A. J. Sinskey, and J. Stubbe.** 2000. Lipases provide a new mechanistic model for polyhydroxybutyrate (PHB) synthases: characterization of the functional residues in *Chromatium vinosum* PHB synthase. Biochemistry **39:**3927–3936.
14. **Jia, Y., et al.** 2001. Mechanistic studies on class I polyhydroxybutyrate (PHB) synthase from *Ralstonia eutropha*: class I and III synthases share a similar catalytic mechanism. Biochemistry **40:**1011–1019.
15. **Kichise, T., T. Fukui, Y. Yoshida, and Y. Doi.** 1999. Biosynthesis of polyhydroxyalkanoates (PHA) by recombinant *Ralstonia eutropha* and effects of PHA synthase activity on *in vivo* PHA biosynthesis. Int. J. Biol. Macromol. **25:**69–77.
16. **Kichise, T., S. Taguchi, and Y. Doi.** 2002. Enhanced accumulation and changed monomer composition in polyhydroxyalkanoate (PHA) copolyester by in vitro evolution of *Aeromonas caviae* PHA synthase. Appl. Environ. Microbiol. **68:**2411–2419.
17. **Lee, S. Y.** 1996. Bacterial polyhydroxyalkanoates. Biotechnol. Bioeng. **49:**1–14.
18. **Lee, S. Y.** 1996. Plastic bacteria? Progress and prospects for polyhydroxyalkanoate production in bacteria. Trends Biotechnol. **14:**431–438.
19. **Li, R., H. Zhang, and Q. Qi.** 2007. The production of polyhydroxyalkanoates in recombinant *Escherichia coli*. Bioresour. Technol. **98:**2313–2320.
20. **Matsumoto, K., K. Takase, E. Aoki, Y. Doi, and S. Taguchi.** 2005. Synergistic effects of Glu130Asp substitution in the type II polyhydroxyalkanoate (PHA) synthase: enhancement of PHA production and alteration of polymer molecular weight. Biomacromolecules **6:**99–104.
21. **Matsusaki, H., H. Abe, K. Taguchi, T. Fukui, and Y. Doi.** 2000. Biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyalkanoates) by recombinant bacteria expressing the PHA synthase gene phaC1 from Pseudomonas sp. 61-3. Appl. Microbiol. Biotechnol. **53:**401–409.
22. **McDowell, E. M., and B. F. Trump.** 1976. Histologic fixatives suitable for diagnostic light and electron-microscopy. Arch. Pathol. Lab. Med. **100:** 405–414.
23. **Müh, U., A. J. Sinskey, D. P. Kirby, W. S. Lane, and J. Stubbe.** 1999. PHA synthase from *Chromatium vinosum*: cysteine 149 is involved in covalent catalysis. Biochemistry **38:**826–837.
24. **Murata, T., K. Takase, I. Yamato, K. Igarashi, and Y. Kakinuma.** 1997. Purification and reconstitution of Na^+-translocating vacuolar ATPase from *Enterococcus hirae*. J. Biol. Chem. **272:**24885–24890.
25. **Normi, Y. M., et al.** 2005. Characterization and properties of G4X mutants of *Ralstonia eutropha* PHA synthase for poly(3-hydroxybutyrate) biosynthesis in *Escherichia coli*. Macromol. Biosci. **5:**197–206.
26. **Normi, Y. M., et al.** 2005. Site-directed saturation mutagenesis at residue F420 and recombination with another beneficial mutation of *Ralstonia eutropha* polyhydroxyalkanoate synthase. Biotechnol. Lett. **27:**705–712.
27. **Park, S. J., J. I. Choi, and S. P. Lee.** 2005. Short-chain-length polyhydroxyalkanoates: synthesis in metabolically engineered *Escherichia coli* and medical applications. J. Microbiol. Biotechnol. **15:**206–215.
28. **Pötter, M., and A. Steinbüchel.** 2005. Poly(3-hydroxybutyrate) granule-associated proteins: impacts on poly(3-hydroxybutyrate) synthesis and degradation. Biomacromolecules **6:**552–560.
29. **Rehm, B. H. A.** 2003. Polyester synthases: natural catalysts for plastics. Biochem. J. **376:**15–33.
30. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
31. **Schubert, P., A. Steinbüchel, and H. G. Schlegel.** 1988. Cloning of the *Alcaligenes eutrophus* genes for synthesis of poly-β-hydroxybutyric acid (PHB) and synthesis of PHB in *Escherichia coli*. J. Bacteriol. **170:**5837–5847.
32. **Sim, S. J., et al.** 1997. PHA synthase activity controls the molecular weight and polydispersity of polyhydroxybutyrate *in vivo*. Nat. Biotechnol. **15:**63–67.
33. **Slater, S. C., W. H. Voige, and D. E. Dennis.** 1988. Cloning and expression in *Escherichia coli* of the *Alcaligenes eutrophus* H16 poly-β-hydroxybutyrate biosynthetic pathway. J. Bacteriol. **170:**4431–4436.
34. **Song, J. J., S. Zhang, R. W. Lenz, and S. Goodwin.** 2000. *In vitro* polymerization and copolymerization of 3-hydroxypropionyl-CoA with the PHB synthase from *Ralstonia eutropha*. Biomacromolecules **1:**433–439.
35. **Spurr, A. R.** 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26:**31–43.
36. **Steinbüchel, A., and T. Lütke-Eversloh.** 2003. Metabolic engineering and pathway construction for biotechnological production of relevant polyhydroxyalkanoates in microorganisms. Biochem. Eng. J. **16:**81–96.
37. **Taguchi, K., et al.** 2002. Metabolic pathways and engineering of PHA biosynthesis, p. 217–247. *In* Y. Doi and A. Steinbüchel (ed.), Biopolymer handbook. Wiley-VCH, Weinheim, Germany.
38. **Taguchi, S., and Y. Doi.** 2004. Evolution of polyhydroxyalkanoate (PHA) production system by "enzyme evolution": successful case studies of directed evolution. Macromol. Biosci. **4:**145–156.
39. **Taguchi, S., H. Nakamura, T. Hiraishi, I. Yamato, and Y. Doi.** 2002. *In vitro* evolution of a polyhydroxybutyrate synthase by intragenic suppression-type mutagenesis. J. Biochem. **131:**801–806.
40. **Taguchi, S., et al.** 2008. A microbial factory for lactate-based polyesters using a lactate-polymerizing enzyme. Proc. Natl. Acad. Sci. U. S. A. **105:**17323–17327.

Downloaded from http://aem.asm.org/ on February 3, 2012 by MASS INST OF TECHNOLOGY

41. **Takase, K., K. Matsumoto, S. Taguchi, and Y. Doi.** 2004. Alteration of substrate chain-length specificity of type II synthase for polyhydroxyalkanoate biosynthesis by in vitro evolution: *in vivo* and *in vitro* enzyme assays. Biomacromolecules **5:**480–485.
42. **Tsuge, T., et al.** 2007. Variation in copolymer composition and molecular weight of polyhydroxyalkanoate generated by saturation mutagenesis of *Aeromonas caviae* PHA synthase. Macromol. Biosci. **7:**846–854.
43. **Yuan, W., et al.** 2001. Class I and III polyhydroxyalkanoate synthases from *Ralstonia eutropha* and *Allochromatium vinosum*: characterization and substrate specificity studies. Arch. Biochem. Biophys. **394:**87–98.
44. **Zhang, S., T. Yasuo, R. W. Lenz, and S. Goodwin.** 2000. Kinetic and mechanistic characterization of the polyhydroxybutyrate synthase from *Ralstonia eutropha*. Biomacromolecules **1:**244–251.
45. **Zinn, M., B. Witholt, and T. Egli.** 2001. Occurrence, synthesis and medical application of bacterial polyhydroxyalkanoate. Adv. Drug Deliv. Rev. **53:**5–21.

Downloaded from http://aem.asm.org/ on February 3, 2012 by MASS INST OF TECHNOLOGY