Mutations derived from a thermophilic polyhydroxyalkanoate synthase (PhaC) enhance the thermostability and activity of PhaC from Cupriavidus necator H16

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ABSTRACT

Thermophile Cupriavidus sp. S-6 accumulated polyhydroxybutyrate (PHB) from glucose at 50°C. A 9.0-kbp EcoRI fragment cloned from the genomic DNA of Cupriavidus sp. S-6 enabled Escherichia coli XL1-Blue to synthesize PHB at 45°C. Nucleotide sequence analysis showed a pha locus in the clone. The thermophilic PHA synthase (PhaC_Csp) shared 81% identity with mesophilic PhaC of Cupriavidus necator H16. The diversity between these two strains was dominantly on their N- and C- termini, while the middle regions were highly homologous (92% identity). This study constructed four chimeras of mesophilic and thermophilic phaC genes to explore the mutations related to its thermostability. Among chimeras, only PhaC_H16β, which was PhaC_H16 bearing 30 point mutations derived from the middle region of PhaC_Csp, accumulated high content of PHB (65 wt%) at 45°C. Chimera phaC_H16β and two parental PHA synthase genes were over-expressed in E. coli BLR(DE3) and purified. At 30°C, the specific activity of chimera PhaC_H16β (172 ± 17.8 U/mg) was 3.45-fold higher than parental enzyme PhaC_H16 (50 ± 5.2 U/mg). At 45°C, the half-life of chimera PhaC_H16β (11.2 hour) was 127-fold of PhaC_H16 (5.3 min). Furthermore, chimera PhaC_H16β accumulated 1.55-fold (59 wt%) PHA content of parental enzyme PhaC_H16 (38 wt%) at 37°C. This study reveals a limited number of point mutations, which enhance not only thermostability but also PhaC_H16 activity. The highly thermostable and active PHA synthase
will provide advantages for its promising applications to in vitro PHA synthesis and recombinant E. coli PHA fermentation.

**INTRODUCTION**

Polyhydroxyalkanoates (PHAs) are a type of biopolyester. Numerous bacteria accumulate PHAs intracellularly as carbon source and reducing power sink. PHAs have been intensively studied since Lemoigne discovered poly(β-hydroxybutyrate) (PHB) in the bacterium Bacillus megaterium in 1926 (2, 19, 21, 22). Bacterial fermentation is a commonly used method for PHA mass production (49, 50). Numerous bacteria, such as Cupriavidus necator H16 (formerly known as Ralstonia eutropha H16) (46), Alcaligenes latus, Methylobacterium organophilum, and recombinant E. coli, have been studied for the production of PHA to a high concentration with high productivity (6). However, the production cost of PHA with bacterial fermentation is higher than petroleum-based plastics (6). Transgenic plants may provide a cost-effective solution for PHA production in the future (45), although the feasibility of this approach has been questioned (10). Moreover, in vitro polymerization has been demonstrated for PHA production (11). By using this approach, it is possible to control the properties of PHAs, such as the molecular weight of polymers and the monomer composition of PHAs.
Nowadays, the high production cost of PHA predominantly hampers its applications (6). To resolve this issue, improvement made on the performance of PHA synthase is a promising direction (25). PHA synthase is the key enzyme in PHA biosynthesis (22). Its function correlates with the monomer composition of PHA, the molecular weight of synthesized polymer, and the PHA content in bacteria fermentation (1, 25, 39, 40). There have been many efforts dedicated to improving the enzyme activity of PHA synthase. Because the crystal structure of PHA synthase has not been determined, the rational design method is not applicable to accomplishing this goal. Non-rational designed methods, i.e. the in vitro and in vivo evolution approaches, are commonly applied to many PHA synthases, including that of C. necator H16 (41), Aeromonas caviae (1, 18), Pseudomonas sp. 61-3 (35, 36), and Pseudomonas putida GPo1 (33). Above studies succeed in getting point mutations, which improve the performance of PHA synthase by changing the substrate specificity or enhancing the enzyme activity.

Once beneficial mutations are found, the subsequent saturation-mutagenesis on these spots optimizes the beneficial effect (44). For example, in the PHA synthase of C. necator H16 (PhaC116), mutation G4D increases the protein expression level of PHA synthase (26). Mutation F420S enhances the specific activity of PHA synthase (41). Mutation G4D or F420S results in more PHB accumulation in recombinant E. coli (26, 41). Another beneficial
mutation A510D(E) leads PhaC_{H16} to synthesizing a higher molecular weight of polymer (43).

The thermostability of PHA synthase has never been the aim of protein engineering. A highly thermostable and active PHA synthase will lead to a promising application to in vitro PHB polymerization and recombinant E. coli fermentation (25). So far, over 59 PHA synthase genes from 45 bacteria strains have been cloned and characterized (29), and most of them are from mesophile. Few reports mention the thermophilic PHA synthase (14). In these limited reports, the thermophile exhibit a more efficient ability in PHA accumulation than the mesophile (16, 17, 31). The results of these studies also support the assumption that the thermophilic PHA synthase has higher enzyme activity.

Cupriavidus sp. S-6 is a thermophile isolated from a hot spring in Southern Taiwan. It accumulates PHB from glucose at 50°C. Its 16S rRNA gene is 99% identical to that of mesophile C. necator H16, a well studied PHA synthesis strain formerly known as Ralstonia eutropha H16 (28, 46). A partial phaC gene fragment of Cupriavidus sp. S-6, amplified by colony PCR (34), shows high identity with the phaC gene of mesophile C. necator H16. The PHA synthase of thermophile Cupriavidus sp. S-6 is a proper model to explore the mutations related to the thermostability of PHA synthase.

MATERIALS AND METHODS
Bacterial strains, plasmids, and growth condition. Strains and plasmids used in this study are described below. *Escherichia coli* XL 1-Blue and plasmid pBluescript II KS are used for library construction. Thermophile *Cupriavidus* sp. S-6 and mesophile *C. necator* H16 were grown in Luria-Bertani (LB) medium at 45°C and 30°C, respectively. PCR primers CspCNdeIF and CspChindIIIR (Table 1) amplified the PHA synthase gene of *Cupriavidus* sp. S-6 and cloned to the NdeI and HindIII sites of pET-23a (Novagen). The thermophilic PHA synthase overexpression vector was designated as pECspC. Chimeric PHA synthase gene phaC_{H16}β was amplified with primers EcoRI23SDF and 23BamHIR and was cloned to the EcoRI and BamHI sites of pET-23a. The PhaC_{H16}β overexpression vector was designated as pEH16β.

Construction and screening of genomic library. Genomic DNA of *Cupriavidus* sp. S-6 was extracted with illustra™ bacterial genomicPrep Mini Spin kit (GE Healthcare), and was digested with EcoRI (Fermentas) overnight. One-microgram of EcoRI-digested genomic DNA was ligated with 50 ng of EcoRI and alkaline phosphatase (New England Biolabs) treated pBluescript II KS by T4 DNA ligase (Fermentas) at 8°C. The ligation product was transformed to *E. coli* XL 1-Blue with electroporation as described previously (33). Transformants were spread on the LB agar plate contained 1.5% glucose and 100 μg/ml ampicillin, and incubated at 37°C for 24 to 48 h. PHA-accumulated colony was screened.
Construction of chimeric PHA synthase genes. The construction of chimeric $phaC$ gene included two parts, fragmentation of $phaC$ gene and assembly of chimeric gene. The PHA synthase genes, $phaC_{H16}$ and $phaC_{Csp}$, were fragmented into three fragments with corresponding degenerate primer pairs (Fig. 2 and Table 1). PCR primers EcoRI23SDF and CspC530R amplify the $a$ and $\alpha$ fragments. Primers CspC505F and CspC1607R amplify the $b$ and $\beta$ fragments. Primers CspC1582F and 23BamHIR amplify the $c$ and $\gamma$ fragments. The PCR mixture contained 1x PCR amplification buffer (Finnzymes), 2.2 mM MgCl$_2$, 180 $\mu$M (each) dNTP, 1 $\mu$M (each) primers, 2% DMSO (Sigma), 0.6 U DyNAzyme II DNA polymerase (Finnzymes), 0.5 U Pfu DNA polymerase (Fermentas) and plasmid pEREc (pET-23a vector containing $phaC_{H16}$ gene) (32) or pECspC (pET-23a containing $phaC_{Csp}$ gene) as the template in a 50-$\mu$l reaction volume. The thermal cycle program consisted of 94°C for 5 min, 47°C for 30 sec, 72°C for 1 min 40 sec, and 35 cycles of 94°C for 30 sec, 53°C for 30 sec, and 72°C for 1 min 40 sec; the reaction then followed with incubation at 72°C for 1 min and stopped at 15°C. All attained on a 2720 thermal cycler (Applied Biosystem, USA). The amplified gene fragments were gel-purified to remove template DNA and primers (Qiagen, QIAquick gel extraction kit).

Selected DNA fragments of $phaC_{H16}$ ($a$, $b$, $c$) and $phaC_{Csp}$ ($\alpha$, $\beta$, $\gamma$) were mixed in an equal
molar ratio. Approximately 100 ng of the DNA mixture was added to a 20 μl of PCR mixture containing 1x PCR amplification buffer (Finnzymes), 2.2 mM MgCl₂, 180 μM (each) dNTP, 2% DMSO, 0.3 U of DyNAzyme II DNA polymerase (Finnzymes), and 0.3 U Pfu DNA polymerase (Fermentas) to perform primerless assembly PCR. A PCR program consisted of 94°C for 5 min, then 32 cycles of 94°C for 30 sec, 55°C for 60 sec, 72°C for 40 sec (+ 2 sec/cycle); the reaction then followed with incubation at 72°C for 2 min and stopped at 15°C. Finally, chimeric gene re-amplification: the 50-μl PCR mixture contained 1-μl assembly PCR product, 0.8 μM primers (EcoRI23SDF and 23BamHIR), and 2% DMSO; PCR was performed for 20 cycles (94°C for 30 sec, 52°C for 30 sec, and 72°C for 2 min). The amplified chimeric phaC genes were cloned to pGEM-T easy vector (Promega) for DNA sequence analysis.

**Site-directed mutagenesis.** The site-directed mutagenesis of PHA synthase gene was performed directly on the plasmid pBCspAB-H16 by using QuikChange® Site-Directed Mutagenesis Kit (Stratagene). The OPC purified mutagenic primers and cloning primers were purchased from MB Biotech (Taipei, Taiwan)(Table 1).

**Construction of artificial pha operon.** An artificial pha operon, consisting of phaC-phaA_Csp-phaB_Csp, was constructed downstream from the lac promoter of plasmid pBluescript II (Fig. 1B). Firstly, the phaA_Csp-phaB_Csp gene fragment of Cupriavidus sp. S-6
was amplified with primers CspAB-BamHIF and CspAB-NotIR (Table 1 and Fig. 1B) and was cloned to the BamHI and NotI sites of pBluescript II. The 50-μl PCR mixture contained 1-μl genomic DNA (30 ng), 0.8 μM primers, and 1 M betaine as PCR additive. After heating for 5 min at 94°C, PCR was performed for 2 cycles pre-PCR (94°C for 20 sec, 52°C for 30 sec, and 72°C for 3 min) to be followed by 26 cycles PCR (94°C for 20 sec, 63°C for 30 sec, and 72°C for 3 min). Gel-purified PCR product was cloned to the BamHI and NotI sites of pBluescript II KS. The plasmid contained phaA<sub>Csp</sub>-phaB<sub>Csp</sub> genes was designated as pBCspAB. Plasmid pBH16AB was constructed using the same method, but the phaA<sub>H16</sub>-phaB<sub>H16</sub> gene fragment of <i>C. necator</i> H16 was amplified with primers H16AB-BamHI and H16AB-NotIR (Table 1). Subsequently, PCR amplified the wild type or chimeric phaC genes with primers EcoRI23SDF and 23BamHIR and cloned to the EcoRI and BamHI sites of plasmids pBCspAB or pBH16AB.

**PHA accumulation and analysis.** Recombinant <i>E. coli</i> carrying the artificial pha operon was pre-cultured in the LB medium containing 100 μg/ml ampicillin at 30°C for overnight. The PHA accumulation experiment was carried out by inoculating 2% of overnight culture to a 250 ml Erlenmeyer flask containing 30 ml LB broth, 100 μg/ml ampicillin, 1.5% sodium gluconate and 50 μM IPTG. After 48 h of cultivation at 30°C, 37°C, 42°C, and 45°C respectively in an orbital shaker at 200 rpm, the bacterial cells were harvested, washed twice
with saline, and lyophilized. The lyophilized cells were subjected to methylation. Gas chromatography determined the PHA content based on the previous report (33).

**Overexpression and purification of PHA synthases.** *E. coli* BLR(DE3) (Novagen) harboring overexpression plasmid was pre-cultured overnight in a 2 ml 2x YT medium containing 100 μg/ml ampicillin at 30°C. The overnight culture, 1 ml, was seeded to a 500 ml Erlenmeyer flask containing 100 ml 2x YT medium and 200 μg/ml of ampicillin, and incubated at 30°C. Once the cell density OD$_{600}$ reached approximately 0.6, a final concentration of 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the medium and cultivated at 20°C for an additional 40 h to induce the overexpression of PHA synthase. The crude lysate preparation, protein purification with ammonium sulfate precipitation, and methyl hydrophobic interaction chromatography were performed as described previously (32). After adding the final concentration 20% of glycerol, the purified enzyme was concentrated with Amicon Ultra (30 kDa MWCO) to approximately 1 mg/ml and stored at -70°C. The purified enzymes were separated with SDS-PAGE. Subsequently, Coomassie brilliant blue R-250 staining revealed the electrophoretic homogeneity of purified recombinant PHA synthases (Fig. S1A). Activity staining (Fig. S1B) and Western blotting (Fig. S1C) showed the activity of polymerization and their identity. Anti-PhaC$_{H16}$ antiserum was the first antibody for Western blotting analysis (42). Western blotting and activity staining
were performed as described previously (32, 42).

**PHA synthase activity assay.** The substrate of PHA synthase, \( \beta \)-hydroxybutyryl-CoA (3-HBCoA), was prepared as reported previously (32, 37). The PHA synthase activity was assayed with a discontinuous method by monitoring the released CoA from the substrate 3-HBCoA (12). A 0.3 ml reaction mixture (100 mM Tricine pH 8.0, 1 mM 3-HBCoA, 0.2% glycerol, and 0.05% Hecameg) in an eppendorf was pre-incubated at 30°C or assigned temperature for 5 min. The addition of 12 to 50 nM PHA synthase to the mixture initiated the reaction. Aliquots (15 \( \mu l \)) were removed at intervals (20 s) and quenched by immediately mixing with 40 \( \mu l \) of 0.5% trichloroacetic acid. The quenched mixture (55 \( \mu l \)) was added to 50 \( \mu l \) of 2 mM DTNB in 150 mM Tris pH 8.0 and incubated at room temperature for 2 min. After adding 300 \( \mu l \) de-ionized water, the optical absorbance of the yellow mixture was measured at 412 nm. The concentration of released CoA was calculated with Beer’s law (\( \varepsilon = 13.6 \text{mM}^{-1}\text{cm}^{-1} \)). Linear range of the increase in absorption OD 412 was taken to calculate the reaction rate. The release of CoA in the blank control, a reaction mixture without PHA synthase, was also monitored to check the stability of 3-HBCoA at temperature 40°C to 60°C.

**Optimal temperature and thermostability measurement.** The optimal temperature for enzyme activity assay was determined using the standard assay at temperature ranging from 15°C to 60°C at 5°C intervals. To determine thermostability, purified PHA synthase (0.14
mg/ml) in 50 mM potassium phosphate buffer pH7.5 containing 20% glycerol (Invitrogen) and 0.005% Hecameg (Calbiochem) was incubated at different temperatures (between 4°C and 60°C) for 30 min. After cooling the samples on ice for 5 min, the residual activity was determined using standard method at their optimal temperature. The pH of the reaction buffer (Tricine, pH8.0) was adjusted at room temperature.

**Half-lives of thermal inactivation.** Purified PHA synthase (0.14 mg/ml) in 50 mM potassium phosphate buffer pH7.5 containing 20% glycerol (Invitrogen) and 0.005% Hecameg was incubated at 45°C. Incubation was carried out in a precisely temperature-controlled water bath (Model-B402H) (Firstek, Taiwan). Aliquots removed at various time intervals were incubated on ice for 5 min prior to enzyme activity measurement. The residual activity was assayed at their optimal temperature (PhaC_H16 at 37°C, PhaC_H16β at 45°C, and PhaC_Csp at 50°C). The heat-inactivation experiment was followed until >80% of the activity was lost. Plot of log (residual activity %) versus time was linear. First order rate constants of thermal inactivation were obtained by linear regression in semi-logarithmic coordinates. Inactivation rate constant (k_inact) was obtained from the slope, and the half-life of a PHA synthase was estimated.

**Nucleotide sequence accession number.** The complete nucleotide sequence of the 8,999-bp *Cupriavidus* sp. S-6 DNA fragment appears in the EMBL, GenBank, and DDBJ
nucleotide sequence database under accession no. HE610111. The accession no. of 16S rRNA gene sequence of strain *Cupriavidus* sp. S-6 in EMBL/GenBank/DBJ database is HE660045.

**RESULTS**

*Cupriavidus* sp. S-6. *Cupriavidus* sp. S-6, a Gram-negative bacterium, was isolated from a hot spring in Southern Taiwan. Its 16S rRNA gene, amplified with primers 27F and 1492R (38), is 99% identical to that of mesophile *Cupriavidus necator* H16. Phylogenetically, *Cupriavidus* sp. S-6 was highly close to the mesophile *C. necator* H16. However, the optimal growth temperature of *Cupriavidus* sp. S-6 was markedly different from that of *C. necator* H16. *Cupriavidus* sp. S-6 was a thermophile. Its optimal growth temperature was around 50°C. *Cupriavidus* sp. S-6 accumulated 49 wt% of PHB from gluconate as carbon source at 50°C. Furthermore, it was also capable of accumulating 18 wt% of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer with 78 mol% 3-hydroxyvalerate monomer from sodium valerate as a sole carbon source at 45°C. The ability of biosynthesizing PHB at 50°C supported that the PHA synthase of *Cupriavidus* sp. S-6 was thermostable. In contrast, *C. necator* H16, a mesophile, was commonly cultivated at 30°C for bacterial growth and PHA accumulation (27, 30). It did not grow over 37°C (data not shown).

Colony-PCR cloned a partial *phaC* gene fragment (806 bp) of *Cupriavidus* sp. S-6 (*phaC*<sub>Csp</sub>) by primers PhaCF1 and PhaCR2 (34). The partial gene of *phaC*<sub>Csp</sub> encoded 268 amino acids,
which exhibited 91% identity and 94% similarity compared with the PHA synthase of mesophile *C. necator* H16. The results of PHA accumulation experiment and PCR cloning supported that the protein sequence of PHA synthase of thermophile *Cupriavidus* sp. S-6 and mesophile *C. necator* H16 was highly similar; however, the thermostability of them was different.

**Cloning the pha operon of *Cupriavidus* sp. S-6.** A genomic DNA library (~10,000 colonies) of *Cupriavidus* sp. S-6 was constructed in *E. coli* XL1-Blue and cultivated on the LB agar plate containing 1.5% glucose and ampicillin. After two days of cultivation at 30°C, an opaque colony was isolated. The positive candidate was further purified by streak culture, and was subjected to PHA accumulation experiment. It accumulated 40 wt% and 12 wt% of PHB content from glucose as a carbon source at 30°C and 45°C, respectively. The plasmid of the candidate clone was designated as pBCsp6A. *Eco*RI digestion and agarose gel electrophoresis analysis showed an approximately 9-kbp of inserted DNA fragment in plasmid pBCsp6A. Colony-PCR detected the existence of PHA synthase gene in pBCsp6A. The DNA sequence was the same as that amplified from the genomic DNA of thermophile *Cupriavidus* sp. S-6 (data not shown). PHA accumulation and PCR results revealed that plasmid pBCsp6A possessed a thermostable PHA synthase gene, which was derived from the thermophile *Cupriavidus* sp. S-6.
Nucleotide sequence of the PHA biosynthesis genes. The nucleotide sequence of pBCsp6A was determined in both strands. Sequence analysis showed a pha operon identified by BLAST (Fig. 1A). The pha locus of Cupriavidus sp. S-6 consisted of a phaCAB operon, in which the organization was the same as that of C. necator H16 (27, 30). PHA synthase (phaC<sub>Csp</sub>), β-ketothiolase (phaA<sub>Csp</sub>), and acetoacetyl-CoA reductase (phaB<sub>Csp</sub>) genes of thermophile Cupriavidus sp. S-6 showed 81%, 92%, and 96% identity, respectively, compared with the correspondents of mesophile C. necator H16 (27, 30). Obviously, β-ketothiolase and acetoacetyl-CoA reductase genes of Cupriavidus sp. S-6 and C. necator H16 were highly homologous; however, PHA synthase genes were not (81% identity). According to the alignment result (Fig. 2), the diversity of the two PHA synthases was dominant on the N-terminal (corresponding to amino acid sequence 1-152 of PhaCH16) and C-terminal (corresponding to amino acid sequence 522-589 of PhaCH16) regions (Fig. 2). The middle regions (corresponding to amino acid sequence 153-521 of PhaCH16) of both were highly homologous (92% identity). The middle region involved the whole α/β hydrolase fold region of PHA synthase analyzed by Pfam (7). In summary, the unique diversity of the thermophilic and mesophilic PHA synthase, which was highly diverse in the N- and C- termini but highly homologous in the middle region, provides an opportunity to explore the correlation between mutations and thermostability.
Establishment of an *in vivo* system for evaluating the thermostability of PHA synthases. In order to evaluate the thermostability of PHA synthases, an artificial *pha* operon, consisting of *phaC-phaA*Csp-*phaB*Csp, was constructed downstream the *lac* promoter of pBluescript II (pBCspAB) (Fig. 1B). The addition of IPTG triggered the expression of the *pha* operon. In the artificial operon, the β-ketothiolase and acetoacetyl-CoA reductase genes of thermophile *Cupriavidus* sp. S-6 were employed. The candidate *phaC* gene was cloned to the *Eco*RI and *Bam*HI sites of the plasmid. The *phaA*H16 and *phaB*H16 genes of mesophile *C. necator* H16 were also evaluated as the component genes of the artificial *pha* operon for PHA accumulation at high temperature. However, mesophile *phaA*H16 and *phaB*H16 genes did not support recombinant *E. coli* accumulating PHB at 45°C even though thermophilic PHA synthase *phaC*Csp was in the artificial *pha* operon (Table 2, pBH16AB-Csp).

Recombinant strain *E. coli* XL 1-Blue/pBCspAB-Csp, harboring the wild type PHA synthase gene of thermophile *Cupriavidus* sp. S-6, accumulated 41 wt% of PHB at 30°C (Table 2). With the elevation of cultivation temperature, the PHB content increased and reached the top at 45°C (75 wt%) (Table 2, pBCspAB-Csp). In contrast, *E. coli* XL 1-Blue/pBCspAB-H16, harboring the PHA synthase of mesophile *C. necator* H16, accumulated the highest content of PHB (49 wt%) at 30°C and then drastically decreased with the increase of cultivation temperature (Table 2, pBCspAB-H16). At 45°C, only 1 wt% PHB
content was detected (Table 2). In this assay system, the thermostability of thermophilic (PhaC<sub>Csp</sub>) and mesophilic (PhaC<sub>H16</sub>) PHA synthase was differentiated distinctly based on the highest PHB content accumulation at different temperatures. The results demonstrated the feasibility of using this system to differentiate the thermostability of PHA synthases.

**The mutations enhanced the thermostability of mesophilic PhaC<sub>H16</sub>.** In this study, α, β, and γ denote the N-terminal (corresponding to 1-152 amino acid of PhaC<sub>H16</sub>), middle (corresponding to 153-521 amino acid sequence of PhaC<sub>H16</sub>), and C-terminal (corresponding to 522-589 amino acid sequence of PhaC<sub>H16</sub>) regions of the thermophilic PHA synthase, PhaC<sub>Csp</sub>, respectively (Fig. 1C). In addition, a, b, and c represent the N-terminal, middle, and C-terminal regions of the mesophilic PHA synthase, PhaC<sub>H16</sub>, respectively (Fig. 1C). In order to explore the mutations of PhaC<sub>Csp</sub> and their relations to thermostability, a region-selected approach constructed four chimeric PHA synthase genes (phaC<sub>H16</sub><sup>α</sup>, phaC<sub>H16</sub><sup>β</sup>, phaC<sub>H16</sub><sup>γ</sup>, and phaC<sub>H16</sub><sup>αγ</sup>) of phaC<sub>H16</sub> and phaC<sub>Csp</sub> (Fig. 1C), in which the N-terminal, middle, and C-terminal regions of the thermophilic PHA synthase gene (phaC<sub>Csp</sub>) replaced the corresponding regions of the mesophilic PHA synthase gene (phaC<sub>H16</sub>). Fig. 1C shows the gene structures of chimeras. The PCR-constructed chimeric gene was cloned to the pha operon position of the artificial pha operon and transformed to *E. coli* XL-1 Blue for PHA accumulation experiment.
The in vivo thermostability of chimeric PHA synthases were evaluated based on the PHB content accumulated at 30°C, 37°C, 42°C, and 45°C. Four chimeras were capable of accumulating PHB at 30°C; the PHB content ranged from 16 to 46 wt% (Table 2). All chimeras were enzymatically active. With the increase of cultivation temperature, chimeras accumulated different amount of PHB in the cell. The temperature under which the cell accumulated the highest content of PHB was the index to differentiate the thermostability of chimeras. In the four chimeras, at 45°C, only PhaC_H16β accumulated a high content of PHB (65 wt%) (Table 2, pBCspAB-H16β); phaC_H16α, phaC_H16γ and phaC_H16αγ accumulated less than 1 wt% of PHB at 45°C, which was similar to the mesophilic parental enzyme PhaC_H16 (Table 2, pBCspAB-H16α, pBCspAB-H16γ, and pBCspAB-H16αγ). The results showed that only chimera PhaC_H16β was thermostable. Moreover, only mutations from the β region of thermophilic PhaC_Csp enhanced the thermostability of the mesophilic PHA synthase, PhaC_H16; mutations from α, γ, or the combined α and γ regions of PhaC_Csp did not improve the thermostability of PhaC_H16. Although the chimera PhaC_H16β accumulated a high content of PHB at 45°C (65 wt%), the PHB content was still lower than PhaC_Csp (75 wt%). Hence, the thermostability of the chimera PhaC_H16β and the parental enzyme PhaC_Csp might be close but not the same. The α, γ, or both regions of PhaC_Csp were still possessing mutations that contribute to the thermostability of PhaC, even though the contribution was minor.
In order to explore the scope of β region that actually contributed to the enhancement of the thermostability of PhaC_H16, another chimeric gene *phaC_H16βN* was constructed. Chimera *phaC_H16βN* gene was the *phaC_H16* gene with a partial β region sequence (βN) of *phaC_Csp*. Fig. 2 shows the βN region in PhaC_Csp (CspC790F to CspC1253F). With the βN region, 17 point mutations surrounding the catalytic site (GXCGG) of PHA synthase were introduced to PhaC_H16 (Fig. 2). Chimera PhaC_H16βN accumulated 41 wt% PHB at 30°C and reached peak at 37°C (47 wt%). Subsequently, the PHB content decreased with the elevation in cultivation temperature (Table 2, pBCspAB-H16βN). Nonetheless, chimera PhaC_H16βN still accumulated 25 wt% of PHB at 45°C (Table 2). Obviously, the point mutations derived from the partial β region (βN) improved the thermostability of PhaC_H16. However, the thermostability of PhaC_H16βN was not equal to that of chimera PhaC_H16. The results suggest that the essential mutations related to the thermostability of PhaC_Csp are mainly scattered on the whole β region rather than on a partial area. Preliminary site-directed mutagenesis experiment showed that the mutant A341Q of PhaC_H16 accumulated 34 wt% PHB at 42°C, and accumulated comparable PHB content with wild type at 30°C and 37°C (Table 2, pBCspAB-H16A341Q). The results suggested that A341Q substitution enhanced the thermostability of PhaC_H16.

Mutant G286A of PhaC_H16 accumulated higher content of PHB (45 wt%) than the wild type (38 wt%) at 37°C, and accumulated 17 wt% PHB at 42°C (Table 2, pBCspAB-H16G286A).
The mutation G286A enhanced the enzyme activity, and slightly the thermostability.

The effects of temperature on the activity of PHA synthases. Parental and chimeric PHA synthase genes \textit{phaC}_{H16}, \textit{phaC}_{Csp}, and \textit{phaC}_{H16β} were over-expressed in \textit{E. coli} BLR(DE3) and purified. Fig. 3A shows the activity-temperature profiles of chimeric and parental PHA synthases. Parental enzymes \textit{PhaC}_{H16} and \textit{PhaC}_{Csp} had temperature optima at 37°C and 50°C, respectively; chimera \textit{PhaC}_{H16β} was at 45°C. At their optimal temperature, \textit{PhaC}_{H16}, \textit{PhaC}_{Csp}, and chimera \textit{PhaC}_{H16β} had specific activity of 70 ± 8.8 U/mg, 236 ± 19.4 U/mg, and 237 ± 11.8 U/mg, respectively. Although the optimal temperature of chimera \textit{PhaC}_{H16β} was 5°C lower than its parental enzyme \textit{PhaC}_{Csp}, its specific activity was the same as \textit{PhaC}_{Csp} at their optimal temperatures.

Obviously, \textit{PhaC}_{H16β} inherited the high enzyme activity from its parental enzyme \textit{PhaC}_{Csp}, despite having a lower optimal temperature. At 30°C, the specific activity of \textit{PhaC}_{H16}, \textit{PhaC}_{Csp}, and chimera \textit{PhaC}_{H16β} was 50 ± 5.2, 142 ± 11.7, and 172 ± 17.8 U/mg, respectively. The specific activity of chimera \textit{PhaC}_{H16β} was 1.21 folds of \textit{PhaC}_{Csp} (142 ± 11.7 U/mg) and 3.45 folds of \textit{PhaC}_{H16} (50 ± 5.2 U/mg). At 15°C, \textit{PhaC}_{H16}, \textit{PhaC}_{Csp}, and chimera \textit{PhaC}_{H16β} showed nearly the same specific activity (Fig. 3A). Obviously, chimera \textit{PhaC}_{H16β} showed better activity than its parental enzymes \textit{PhaC}_{H16} and \textit{PhaC}_{Csp}, measuring at 30°C (Fig. 3A).

At temperature 30°C, high cell density fermentation for PHB mass-production is reported (49,
In this temperature, chimera PhaC_{H16β} expresses higher specific activity than its parental enzymes, PhaC_{Csp} and PhaC_{H16}. This suggests that chimera PhaC_{H16β} will accumulate higher content of PHB than PhaC_{Csp} and PhaC_{H16} in recombinant *E. coli* fermentation.

At 37°C, PhaC_{H16β} accumulated 1.55-fold PHB content compared with PhaC_{H16} (Table 2). This is in good agreement with the correlation between specific activity of PHA synthase and PHB content (40). However, at 30°C, PhaC_{H16} accumulated higher content of PHB than the higher activity enzymes PhaC_{H16β} and PhaC_{Csp} (Table 2). This conflicts with the above results.

We postulate that the conflict may be due to the use of thermophilic genes *phaA*_{Csp} and *phaB*_{Csp} in the artificial operon. Some reports suggest that a thermophilic enzyme shows a more rigid structure but less flexibility than a mesophilic enzyme (47, 48). This means that thermophilic enzymes will present lower activity at lower temperatures than mesophilic enzymes. If the inference was correlative with our case, then the thermophilic PhaA_{Csp} and PhaB_{Csp} provided lower concentration of PHA synthase substrate than the mesophilic PhaA_{H16} and PhaB_{H16}.

To further investigate this observation, another artificial *pha* operon was constructed, in which the β-ketothiolase and acetoacetyl-CoA reductase genes were derived from the mesophile *C. necator* H16. In the pBH16AB-X based plasmids (X = H16, Csp, or H16β), thermostable PHA synthases PhaC_{Csp} and chimera PhaC_{H16β} accumulated higher content of
PHB than PhaC H16 at 30°C and 37°C (Table 2). In the pBH16AB serial plasmids, the PHB content and specific activity of PHA synthase revealed a positive correlation at 30°C and 37°C. Furthermore, the PHB content accumulated by *E. coli* /pBH16AB-X serial strains was higher than *E. coli* /pBCspAB-X serial strains (Table 2). The results suggest that not only PHA synthase but also β-ketothiolase and acetoacetyl-CoA reductase are also critical for efficient PHA accumulation.

**Thermostability of PHA synthases.** With regard to heat stability, mesophilic parental enzyme PhaC H16 was stable at 30°C for 30 min (Fig. 3B). It retained about 70% of its maximum activity at 42°C and became inactive at 45°C. Thermophilic parental enzyme PhaCCsp and chimera PhaC H16β were stable up to 45°C and retained about 70% of their maximum activity at 47°C and 52°C, respectively; both became inactive at 55°C (Fig. 3B).

The kinetics of heat inactivation, reported as the rate of thermal inactivation (half-life of heat inactivation, *t*₁/₂), was also measured at 45°C. As shown in Fig. 3C, parental enzymes PhaC H16 and PhaCCsp and chimera PhaC H16β exhibited first-order inactivation kinetics with a *t*₁/₂ of 5.3 min, 15.06 h, and 11.20 h, respectively. Substantially, the half-life of PhaC H16 (5.3 min) was enhanced 127 folds (11.20 h, PhaC H16β) when the β region of PhaCCsp was introduced. Nonetheless, the half-life of chimera PhaC H16β (11.20 h) was a little shorter than thermophilic parental enzyme PhaCCsp (15.06 h). This suggests that the amino acid residues contributing to
the thermostability of PhaCsp were not all in the β region; some were located in the α or γ region. This result is in good agreement with the results of PHA accumulation experiment (Table 2, pBCspAB-Csp and pBCspAB-H16β).

DISCUSSION

This study presents a thermostable PHA synthase (PhaCsp) from the thermophile Cupriavidus sp. S-6, which is of high activity and high thermostability. The most important characteristic of PhaCsp is its primary structure, which is highly similar to the PHA synthase of mesophile C. necator H16. Using the region-selected method, 30 point mutations derived from the thermophilic PHA synthase are verified having association with the enhancement of thermostability and the activity of mesophilic PHA synthase PhaC_{H16}. This study establishes an in vivo system in E. coli, which is applicable to differentiating the thermostability of PHA synthases. The system differentiated the thermostability of PHA synthases based on the PHA content accumulation at various temperatures. It did not require tedious works on protein purification and biochemical analysis. Thus, it was suitable for the rapid screening of thermostable PhaC candidates.

This study employed E. coli XL 1-Blue, a commonly used strain for PHA fermentation (4, 49, 50), as the host cell for the PHA accumulation experiment. Fotadar et al. report that E. coli
DH5α is capable of growth up to 49°C, although the growth is prohibitive beyond 40°C (8, 15). The effect of temperature on the growth of E. coli XL 1-Blue was similar; however, E. coli XL 1-Blue had no growth when the cultivation temperature went over 45°C (data not shown). At 42°C and 45°C, the growth and PHA accumulation of recombinant E. coli XL 1-Blue were monitored for four days. The turbidity and PHA content of the recombinant E. coli reached the plateau on the second day, and slightly decreased with the time at 42°C and 45°C (data not shown). In this study, recombinant E. coli XL 1-Blue strains accumulated PHA at the temperature 30°C to 45°C. Theoretically, a more thermostable PHA synthase should peak the PHB content at higher temperatures. Accordingly, the thermostable PHA synthase is discriminated.

The gene structure of chimera phaC_{H16β} is the phaC_{H16} gene possessing the β region sequence of phaC_{Csp}. In the β region, 30 point mutations are introduced to mesophilic PhaC_{H16}. The 30 point mutations derived from PhaC_{Csp} were naturally evolved, not artificially generated. Fig. 2 exhibits the point mutations in PhaC_{H16β}. Taguchi et al. report a point mutation S80P of PhaC_{H16} that enhances the thermostability of PhaC_{H16} but results in 27% enzyme activity (41). In this study, mutation S80P is not included in PhaC_{H16β}; position S80 is not in the middle range of PhaC_{H16}. Hence, the effect of S80P on the thermostability of PhaC_{H16β} is ruled out. Kumar et al. indicate that the appearance of arginine (Arg) and tyrosine
(Tyr) are significantly more frequent in thermophilic proteins based on the comparison of sequences of thermophile-mesophile homologous protein pairs (20). In the original sequence of 30 point mutations (PhaC₁₁₆), there are no Arg and Tyr residues. However, five Arg (L170R, S287R, K312R, H338R, and A493R) and one Tyr (F218Y) mutations are observed in PhaC₁₁₆β (Fig. 2). Accordingly, the Arg and Tyr mutations should be highly promising related to the thermostability of PhaC₁₁₆β. Watanabe and Suzuki propose the proline rule for protein thermostabilization (51). The authors suggest that the replacements with proline residues reduce the conformational freedom in the polypeptide chain and thus increase the protein thermostabilization (51). In PhaC₁₁₆β, one amino acid substitution D311P is generated. Based on the proline rule, mutation D311P might relate to the thermostability of PhaC₁₁₆β.

Margarit et al. find the mutants G147A and G189A of a neutral protease from Bacillus subtilis to be more stable towards irreversible thermal inactivation (23). Ganter and Plückthun show one Gly to Ala substitution (G316A) in a glyceraldehyde-3-phosphate dehydrogenase strongly stabilizing mutant in irreversible heat denaturation (9). Menéndez-Arias and Argos compare the amino acid sequences of thermophilic and mesophilic molecules from six different protein families. They show that Gly→Ala is the top residue substitution for helices and strands from mesophile to thermophile (24). These references strongly suggest that position-specific Gly→Ala substitution in an enzyme is highly related to thermostability.
enhancement. In this study, three Gly→Ala substitutions, G286A, G365A, and G377A, were found in PhaC_{H16β} (Fig. 2). The Gly→Ala substitutions in PhaC_{H16β} should be correlative to its thermostability. In contrast, three Ala→Gly substitutions, A296G, A334G, and A466G, were also observed in PhaC_{H16β} (Fig. 2). The Ala→Gly substitution in the helix is helix-destabilizing (5). This means that Ala→Gly substitution is promising to decrease the thermostability of protein. Thus, the role of Ala→Gly substitutions, A296G, A334G, and A466G, in PhaC_{H16β} needs to be further investigated.

Previous researches show a mutation F420S on PhaC_{H16}, which enhances 2.4-fold specific activity. However, the thermostability of mutant F420S is lower than the wild type (41). In the sequence of PhaC_{H16β}, F420 is conservative. The high activity of PhaC_{H16β} does not relate to mutation F420S. PhaC_{H16β} should bear beneficial mutations related to activity enhancement that are not yet reported. Recently, Bhubalan, et al. report a highly active PHA synthase from Chromobacterium sp. USM2, PhaC_{Cs}, which reveals a homology of 46% with PhaC_{H16β} and exhibits 238 ± 98 U/mg of specific activity at 30°C (3). That is the highest activity reported for a purified PHA synthase (3). In this study, chimera PhaC_{H16β} has a specific activity of 172 ± 17.8 U/mg at 30°C. Despite lower activity than Chromobacterium sp. USM2 at 30°C, chimera PhaC_{H16β} exhibits a comparable activity at its optimal temperature of 45°C (237 ± 11.8 U/mg). Furthermore, chimera PhaC_{H16β} is thermostable and only 30 amino acids different
from PhaC_{H16}. Thus, chimera PhaC_{H16β} is a proper model to explore the activity- and thermostability- enhancing substitutions in the PHA synthase.

The \textit{in vitro} synthesis approach is an ideal method to produce a tailor-made PHA polymer; however, the availability of a stable and catalysis-efficient PHA synthase and substrate 3-hydroxyacyl-CoA limits its application (11). The thermostable and highly active PHA synthase will give a partial answer to \textit{in vitro} PHA synthesis (13). Chimera PhaC_{H16β} shows a long half-life at 45°C (11.2 h) and possesses high activity at 30°C and 45°C. Thus, it is a good candidate for \textit{in vitro} PHA polymerization. In addition, PhaC_{H16β} possesses higher activity than the parental enzymes mesophilic PhaC_{H16} and thermophilic PhaC_{Csp} from 20°C to 40°C (Fig. 3A). Chimeric phaC_{H16β} gene is more appropriate for \textit{E. coli} PHA fermentation than mesophilic phaC_{H16} gene.

Regarding the optimal temperature, enzyme activity, thermostability, or kinetics of heat inactivation, chimera PhaC_{H16β} shows better characteristics than the parental enzyme PhaC_{H16}. The high thermostability and enzyme activity of PhaC_{H16β} are the effect of introducing 30 point mutations derived from the thermophilic enzyme PhaC_{Csp}. The introduced point mutations should include the activity-improving and the thermostability-enhancing residues. Due to the limited numbers of mutations generated in PhaC_{H16β}, it is possible to explore the role of each mutation by site-directed mutagenesis in future studies.
ACKNOWLEDGEMENTS

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REFERENCES

   *Aeromonas punctata* polyhydroxyalkanoate (PHA) synthase: isolation and 


   Rha, and K. Sudesh. 2011. Characterization of the highly active polyhydroxyalkanoate 

   high-efficiency plasmid transforming *recA* *Escherichia coli* strain with beta-galactosidase 


polyhydroxyalkanoate-accumulated bacteria isolated from the environment by colony PCR. Microbiology-SGM 146:2019–2025.


system by “enzyme evolution”: successful case studies of directed evolution. Macromol. Biosci. 4:146–156.


FIG. 1. Organization of pha locus in Cupriavidus sp. S-6 and the construction of artificial pha operon and chimeric phaC genes.

(A) The organization of phaCsp, phaAasp, and phaBsp genes in the pha operon from Cupriavidus sp. S-6 and the restriction map of the 9-kb EcoRI cloned DNA fragment. (B) The construction of artificial pha operon. (C) The construction of chimeric phaC genes. The details of artificial pha operon and chimeric phaC construction were shown in materials and methods section.

FIG. 2. Alignment of PHA synthases from C. necator H16 and Cupriavidus sp. S-6.

Thermophile, PHA synthase of Cupriavidus sp. S-6; Mesophile, PHA synthase of C. necator H16. The range of a fragment is from the first amino acid to the 152nd amino acid of mesophile PhaC_{H16}. The b fragment is from the 153rd to the 510th amino acid of PhaC_{H16}. The c fragment is from the 511th to 589th amino acid of PhaC_{H16}. The βN fragment is from the 248th to the 410th amino acid of PhaC_{H16}. Cysteine (C) marked by asterisk is the catalytic site of PHA synthase. The gray box indicates the difference between the middle regions of mesophile and thermophile PHA synthases. The underlined sequences are the primer recognition sites for chimera construction.
FIG. 3. The optimal temperature, thermal stability, and the half-lives of thermal inactivation of PHA synthases.

(A) Activity-temperature profiles of wild type and chimeric PHA synthases. (B) Thermal stability of PHA synthases. (C) Kinetics of thermal inactivation of PHA synthases at 45°C. To estimate the thermal stabilities of PHA synthases, the enzyme was preincubated at designated temperatures for 30 min and then incubated on ice for 5 min. The residual activities were then determined using the discontinuous method at 37°C (mesophile, PhaC_H16), 45°C (chimera, PhaC_H16\β), or 50°C (thermophile, PhaC_Csp). PhaC_Csp and PhaC_H16 are PHA synthases from thermophile Cupriavidus sp. S-6 and mesophile C. necator H16, respectively. PhaC_H16\β is a chimera of PhaC_H16 and PhaC_Csp. The data are the means of three individual experiments.
Table 1. Primers used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoRI23SDF</td>
<td>5’– CGGAATTCGAAGGAGATATACATATG–3’</td>
</tr>
<tr>
<td>23BamHIR</td>
<td>5’– AGCGGATCTCTGGTGCTGAGTG–3’</td>
</tr>
<tr>
<td>CspCNdeIF</td>
<td>5’– CCGGAAGCTTAAGCCTTGCGCTTT–3’</td>
</tr>
<tr>
<td>CspCHindIIIR</td>
<td>5’– CCGGAAGCTTAAGCCTTGCGCTTT–3’</td>
</tr>
<tr>
<td>CspAB-BamHIF</td>
<td>5’– CAAAGATCCGACGTTACCGAAGGC–3’</td>
</tr>
<tr>
<td>CspAB-NotlR</td>
<td>5’– ATAGGTTTAGCGGCGCTCGGTCAAGGCCCAT–3’</td>
</tr>
<tr>
<td>H16AB-BamHIF</td>
<td>5’– TCCCTCCGGATCCCATTGAAAGGACT–3’</td>
</tr>
<tr>
<td>H16AB-NotlR</td>
<td>5’– TGAACCACTCCGCGGCAATCTCGACATGC–3’</td>
</tr>
<tr>
<td>CspC505F</td>
<td>5’– GATGCGATGTCGCGCCGCAATTCT–3’</td>
</tr>
<tr>
<td>CspC530R</td>
<td>5’– AGAAATTCGGCGCGACATGC–3’</td>
</tr>
<tr>
<td>CspC790F</td>
<td>5’– AACAAGTAACACTACATCCTCGACCTGC–3’</td>
</tr>
<tr>
<td>CspC816R</td>
<td>5’– TGTCAGGTGGGATGTAGTACCTG–3’</td>
</tr>
<tr>
<td>CspC1253F</td>
<td>5’– TGTTCTGGGACTACATGCTGAC–3’</td>
</tr>
<tr>
<td>CspC1279R</td>
<td>5’– AGTTGTGCGACGAGTATGTTCCAGACCA–3’</td>
</tr>
<tr>
<td>CspC1582F</td>
<td>5’– GCGAGCACGCCGGCGACGGAGAGA–3’</td>
</tr>
<tr>
<td>CspC1607R</td>
<td>5’– TTGTTCTTCTCGCGCGGATGGAC–3’</td>
</tr>
<tr>
<td>PhaCF1</td>
<td>5’– ATCAACAGTWCTACRTGCYSGACCT–3’</td>
</tr>
<tr>
<td>PhaCR2</td>
<td>5’– GTSTTCRSTSRTSWGCTGCGGACCA–3’</td>
</tr>
<tr>
<td>G286AF</td>
<td>5’– ACGCCAGCTGTCGCGACGAGCTGCGG–3’</td>
</tr>
<tr>
<td>G286AR</td>
<td>5’– CTGCCCAGGTGCTGCGGCGACGAGCTGCG–3’</td>
</tr>
<tr>
<td>A341QF</td>
<td>5’– GCAGCCTGGGACGCTGCGGCGACGAGCTG–3’</td>
</tr>
<tr>
<td>A341QR</td>
<td>5’– GCAGCCTGGGACGCTGCGGCGACGAGCTG–3’</td>
</tr>
</tbody>
</table>

*Underlined is the sequence recognized by restriction enzyme; double underlined is the ribosome binding site sequence. The sequences of CspC505R, CspC816R, CspC1279R, and Csp1607R are complimentary to those of CspC505F, CspC790F, CspC1253F, and CspC1582F, respectively. The gray box is the mutation site.*
Table 2. The PHA accumulation of recombinant *E. coli* at different temperatures\(^{a}\).

<table>
<thead>
<tr>
<th>The structure of phaC genes</th>
<th>Plasmids</th>
<th>PHB content (%, wt/dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>30°C</td>
</tr>
<tr>
<td>a a a</td>
<td>pBCspAB-H16</td>
<td>49 ± 3.0</td>
</tr>
<tr>
<td>b b b</td>
<td>pBCspAB-Csp</td>
<td>41 ± 1.3</td>
</tr>
<tr>
<td>a a a</td>
<td>pBCspAB-H16(\alpha)</td>
<td>16 ± 1.2</td>
</tr>
<tr>
<td>b b b</td>
<td>pBCspAB-H16(\beta)</td>
<td>46 ± 2.3</td>
</tr>
<tr>
<td>a a a</td>
<td>pBCspAB-H16(\gamma)</td>
<td>41 ± 0.7</td>
</tr>
<tr>
<td>b b b</td>
<td>pBCspAB-H16(\alpha)</td>
<td>30 ± 1.2</td>
</tr>
<tr>
<td>a a a</td>
<td>pBCspAB-H16G286A</td>
<td>49 ± 1.5</td>
</tr>
<tr>
<td>b b b</td>
<td>pBCspAB-H16A341Q</td>
<td>49 ± 2.5</td>
</tr>
<tr>
<td>a a a</td>
<td>pBH16AB-H16</td>
<td>50 ± 2.7</td>
</tr>
<tr>
<td>b b b</td>
<td>pBH16AB-H16(\beta)</td>
<td>51 ± 2.3</td>
</tr>
<tr>
<td>a a a</td>
<td>pBH16AB-Csp</td>
<td>57 ± 2.7</td>
</tr>
</tbody>
</table>

\(^{a}\)The data shown are the means of three individual experiments. Plasmid pBCspAB-H16 carried the mesophilic *phaC*\(_{H16}\) gene from *C. nector* H16; pBCspAB-Csp carried the thermophilic *phaC*\(_{Csp}\) gene from *Cupriavidus* sp. S-6. Others carried the chimera of *phaC*\(_{H16}\) and *phaC*\(_{Csp}\) genes or the mutants. The pBCspAB serial plasmids carried the thermophilic *phaA* and *phaB* genes of thermophile *Cupriavidus* sp. S-6 to build the metabolic pathway for the provision of *R*-3-hydroxybutyryl-CoA; the pBH16AB serial plasmids carried the mesophilic *phaA* and *phaB* genes of *C. necator* H16.

\(^{b}\)Trace (< 0.2%)
Figure 1 (Sheu)

(A) EcoRI 1 StuI 1578 HhaI 2394 SstI 3225 NdeI 4665 NsiI 5712 SpeI 6663 SphI 7198 BamHI 8578 EcoII 8954

-1.0 kb 4297 2442 567 940 6484 6888 7467

pBCsp6A

phaC<sub>Cup</sub> phaA<sub>Cup</sub> phaB<sub>Cup</sub> phaR<sub>Cup</sub>

PCR amplified the pha<sub>Cup</sub>-pha<sub>B<sub>Cup</sub></sub> genes and cloned to the BamHI and NsiI sites of pBluescript II. The clone was designated as pBCspAB.

(B) ProE<sub>ら</sub> pB<sub>Cup</sub> phaC<sub>Cup</sub> phaA<sub>Cup</sub> phaB<sub>Cup</sub> phaR<sub>Cup</sub> pBCspAB-Csp

EcoRI BamHI

1818 bp 1179 bp 741 bp

PCR amplified the phaC gene with primers EcoRl235DF and 238BamHR and cloned to the EcoRI and BamHI sites of pBCspAB.

(C) phaC<sub>Cup</sub> 1818 bp (thermophile) Chimeric PHA synthase genes

\[ \begin{array}{ccc}
\alpha & \beta & \gamma \\
\alpha & \beta & \gamma \\
\end{array} \]

phaC<sub>Hsl</sub> 1770 bp (mesophile)

\[ \begin{array}{ccc}
a & b & c \\
\alpha & \beta & \gamma \\
\end{array} \]

PCR fragmented the mesophile and thermophile phaC genes and assembled the selected fragments.

phaC<sub>Hsl</sub> 1770 bp (mesophile)

\[ \begin{array}{ccc}
a & b & c \\
\alpha & \beta & \gamma \\
\end{array} \]

phaC<sub>Hsl</sub> 1770 bp (mesophile)

\[ \begin{array}{ccc}
a & b & c \\
\alpha & \beta & \gamma \\
\end{array} \]
Figure-2 (Sheu)

Thermophile 1
MATGKGAASQGKQEETTSFGSPFGDPAHMLENR[QQA]---NGRAAG-GMPGADAFAA
MATGKGAAS QR K+ PF TP[GGPDPAHMLENRQGA] G+GA GA+PA DA

Mesophile 1
MATGKGAASQGKQEETTSFGSPFGDPAHMLENR[QQA]---NGRAAG-GMPGADAFAA
MATGKGAAS QR K+ PF TP[GGPDPAHMLENRQGA] G+GA GA+PA DA

Thermophile 57
LGAFPGGAPFGGAPFGPFGAPFGAPFGAPFGAPFGAPFGAPFGAPFGAPFGAPFGAPFGAPFGAPFGAPFGAPFGAPFGAPFGA

Mesophile 60
--------------------------GVIKAFAQSLDGIQYKMDPSALQAMSEKPARSETPLHD

Thermophile 116
RRFADGWHSNAPTAYAFAYAATFLLTARLWEMDAADEADAKTRQIRFATVNGVTMDSAPAH
RRFADGWHSNAPTAYAFAYAATFLLTARLWEMDAADEADAKTRQIRFATVNGVTMDSAPAH

Mesophile 100
RRFADGWHSNAPTAYAFAYAATFLLTARLWEMDAADEADAKTRQIRFATVNGVTMDSAPAH
RRFADGWHSNAPTAYAFAYAATFLLTARLWEMDAADEADAKTRQIRFATVNGVTMDSAPAH

Thermophile 176
FLATNFAYQRILIESGSLRAG+RNR+EDLTRS[QG]QDSEA[EV]SVEVSTGAV+V
FLATNFAYQRILIESGSLRAG+RNR+EDLTRS[QG]QDSEA[EV]SVEVSTGAV+V

Mesophile 160
FLATNFAYQRILIESGSLRAG+RNR+EDLTRS[QG]QDSEA[EV]SVEVSTGAV+V
FLATNFAYQRILIESGSLRAG+RNR+EDLTRS[QG]QDSEA[EV]SVEVSTGAV+V

CspC505F

Thermophile 236
NEYFQYQLQYKFLTNTVHARPLMVPPCINKY[QG]ILPDQSLVRH+QGQHTVLPSWKN
NEYFQYQLQYKFLTNTVHARPLMVPPCINKY[QG]ILPDQSLVRH+QGQHTVLPSWKN

Mesophile 220
NEYFQYQLQYKFLTNTVHARPLMVPPCINKY[QG]ILPDQSLVRH+QGQHTVLPSWKN
NEYFQYQLQYKFLTNTVHARPLMVPPCINKY[QG]ILPDQSLVRH+QGQHTVLPSWKN

* CspC790F

Thermophile 296

Mesophile 280

CspC1253F

Thermophile 356
A[QG]LITTTLTLDLPDTGLDVDDQ+HQLREATLGGAGAPCALL+KIELANTFSFLRP
A[QG]LITTTLTLDLPDTGLDVDDQ+HQLREATLGGAGAPCALL+KIELANTFSFLRP

Mesophile 340
A[QG]LITTTLTLDLPDTGLDVDDQ+HQLREATLGGAGAPCALL+KIELANTFSFLRP
A[QG]LITTTLTLDLPDTGLDVDDQ+HQLREATLGGAGAPCALL+KIELANTFSFLRP

CspC1582F

Thermophile 416

Mesophile 400

CspC492F

Thermophile 476
GVFDLTADVDFT[$QG]SREDHVNT[QG]TAYASTLLLNGLRFLVAGASHGAIJVPNFPAG
GVFDLTADVDFT[$QG]SREDHVNT[QG]TAYASTLLLNGLRFLVAGASHGAIJVPNFPAG

Mesophile 460
GVFDLTADVDFT[$QG]SREDHVNT[QG]TAYASTLLLNGLRFLVAGASHGAIJVPNFPAG
GVFDLTADVDFT[$QG]SREDHVNT[QG]TAYASTLLLNGLRFLVAGASHGAIJVPNFPAG

Thermophile 536
KRSHWRLDPFDFLHAGAY[QG]PSWDF[QG]WFLHAKGAGATFQQYGRVYPAIRP
KRSHWRLDPFDFLHAGAY[QG]PSWDF[QG]WFLHAKGAGATFQQYGRVYPAIRP

Mesophile 520
KRSHWRLDPFDFLHAGAY[QG]PSWDF[QG]WFLHAKGAGATFQQYGRVYPAIRP
KRSHWRLDPFDFLHAGAY[QG]PSWDF[QG]WFLHAKGAGATFQQYGRVYPAIRP

Thermophile 596
APGTYXKARA 605
APGTYXKARA

Mesophile 580
APGTYXKARA 589
APGTYXKARA

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Figure-3 (Sheu)