

# Mutations Derived from the Thermophilic Polyhydroxyalkanoate Synthase PhaC Enhance the Thermostability and Activity of PhaC from *Cupriavidus necator* H16

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The thermophile *Cupriavidus* sp. strain 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 polyhydroxyalkanoate (PHA) synthase (PhaC<sub>Csp</sub>) shared 81% identity with mesophilic PhaC of *Cupriavidus necator* H16. The diversity between these two strains was found dominantly on their N and C termini, while the middle regions were highly homologous (92% identity). We constructed four chimeras of mesophilic and thermophilic *phaC* genes to explore the mutations related to its thermostability. Among the chimeras, only PhaC<sub>H16β</sub>, which was PhaC<sub>H16</sub> bearing 30 point mutations derived from the middle region of PhaC<sub>Csp</sub>, accumulated a high content of PHB (65% [dry weight]) at 45°C. The chimera *phaC*<sub>H16β</sub> and two parental PHA synthase genes were over-expressed in *E. coli* BLR(DE3) cells and purified. At 30°C, the specific activity of the chimera PhaC<sub>H16β</sub> (172 ± 17.8 U/mg) was 3.45-fold higher than that of the parental enzyme PhaC<sub>H16</sub> (50 ± 5.2 U/mg). At 45°C, the half-life of the chimera PhaC<sub>H16β</sub> (11.2 h) was 127-fold longer than that of PhaC<sub>H16</sub> (5.3 min). Furthermore, the chimera PhaC<sub>H16β</sub> accumulated 1.55-fold (59% [dry weight]) more PHA content than the parental enzyme PhaC<sub>H16</sub> (38% [dry weight]) at 37°C. This study reveals a limited number of point mutations which enhance not only thermostability but also PhaC<sub>H16</sub> 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.

Polyhydroxyalkanoates (PHAs) are a type of biopolyester. Numerous bacteria accumulate PHAs intracellularly as a 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 method commonly used for the mass production of PHA (49, 50). Numerous bacteria, such as *Cupriavidus necator* H16 (formerly *Ralstonia eutropha* H16) (46), *Alcaligenes latus*, *Methylobacterium organophilum*, and recombinant *Escherichia coli*, have been studied for the production of PHA to a high concentration with a high level of productivity (6). However, the cost of production of PHA with bacterial fermentation is higher than that of 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 use of 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 cost of production of PHA predominantly hampers its applications (6). To resolve this issue, improvements made in the performance of PHA synthase is a promising direction (25). PHA synthase is the key enzyme of PHA biosynthesis (22). Its function correlates with the monomer composition of PHA, the molecular weight of the synthesized polymer, and the PHA contents of bacterial fermentations (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, a rational design method is not applica-

ble for accomplishing this goal. Nonrational design methods, i.e., *in vitro* and *in vivo* evolution approaches, are commonly applied to many PHA synthases, including those of *C. necator* H16 (41), *Aeromonas caviae* (1, 18), *Pseudomonas* sp. strain 61-3 (35, 36), and *Pseudomonas putida* GPo1 (33). The above-mentioned studies succeeded in obtaining point mutations which improve the performance of PHA synthase by changing the substrate specificity or enhancing the enzyme activity.

Once beneficial mutations are found, subsequent saturation mutagenesis of these spots optimizes the beneficial effect (44). For example, in the PHA synthase of *C. necator* H16 (PhaC<sub>H16</sub>), a G4D mutation increases the protein expression level of PHA synthase (26). An F420S mutation enhances the specific activity of PHA synthase (41). A G4D or F420S mutation results in more PHB accumulation in recombinant *E. coli* (26, 41). Another beneficial mutation, A510D(E), leads PhaC<sub>H16</sub> to synthesize a higher-molecular-weight 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 promising applications for *in vitro* PHB polymerization and recombinant *E. coli* fermentation (25). So far, over 59 PHA synthase genes from 45

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TABLE 1 Primers used in this study

| Primer       | Sequence <sup>a</sup>                  |
|--------------|--|
| EcoRI23SDF   | 5'-CGGAATTCGAAGGAGATATACATATG-3'       |
| 23BamHIR     | 5'-AGCGGATCCTGGTGCTCGAGTGC-3'          |
| CspCNdeIF    | 5'-AGAGACACGACCATATGGCGACCCGGC-3'      |
| CspCHindIIIR | 5'-CCGGAAGCTTAAGCCTTGGCCTTGA-3'        |
| CspAB-BamHIF | 5'-CAAGGATCCCAACCGTTACCGAAGGC-3'       |
| CspAB-NotIR  | 5'-ATAGTTTAGCGGCCGCTCGGTCAGCCCATGTG-3' |
| H16AB-BamHIF | 5'-TCCCTCCGGATCCATTGAAAGGACT-3'        |
| H16AB-NotIR  | 5'-TGAACCAGGGCGGCCGCTCAGCCCATAT-3'     |
| CspC505F     | 5'-GATGCGATGTCGCCGGCCAATTCCT-3'        |
| CspC530R     | 5'-AGGAAATTGGCCGGCGACATCGCATC-3'       |
| CspC790F     | 5'-AACAACTACTACATCCTCGACCTGCAG-3'      |
| CspC816R     | 5'-CTGCAGGTCGAGGATGTAGTACTTGT-3'       |
| CspC1253F    | 5'-TGGTCTGGAACACTCGTGGTCGACAACT-3'     |
| CspC1279R    | 5'-AGTTGTGACACAGTAGTTCAGACCA-3'        |
| CspC1582F    | 5'-GTCATCAATCCGCCGGCGAAGAACA-3'        |
| CspC1607R    | 5'-TTGTTCTCGCCGGCGGATTGATGAC-3'        |
| PhaCF1       | 5'-ATCAACAAGTWTACTRCYTSAGCCT-3'        |
| PhaCR2       | 5'-GTSTTCRTSRTSWSCTGGCGCAACCC-3'       |
| G286AF       | 5'-ACGCCAGCATGGCCCGGAGACCTGGGACG-3'    |
| G286AR       | 5'-CGTCCCAGGTGCTCGCGGCCATGCTGGCGT-3'   |
| A341QF       | 5'-GCGAGCACCCGGCCAGAGCGTACGCTGC-3'     |
| A341QR       | 5'-GCAGCGTGACGCTCTGGCCGGTCTCGC-3'      |

<sup>a</sup> The sequence recognized by the restriction enzyme is underlined; the ribosome binding site sequence is double underlined. The sequences of CspC530R, CspC816R, CspC1279R, and CspC1607R are complementary to those of CspC505F, CspC790F, CspC1253F, and CspC1582F, respectively. Gray shading indicates the mutation site.

bacterial strains have been cloned and characterized (29), and most of them are from mesophiles. Few reports have mentioned thermophilic PHA synthases (14). In these limited reports, thermophiles exhibited a more efficient ability for PHA accumulation than mesophiles (16, 17, 31). The results of those studies also support the assumption that thermophilic PHA synthase has a higher level of enzyme activity.

*Cupriavidus* sp. strain 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 the mesophile *C. necator* H16, a well-studied PHA synthesis strain formerly known as *Ralstonia eutropha* H16 (28, 46). A *phaC* gene fragment of *Cupriavidus* sp. S-6 amplified by colony PCR (34) showed a high level of identity with the *phaC* gene of the mesophile *C. necator* H16. The PHA synthase of the thermophile *Cupriavidus* sp. S-6 is a proper model to explore mutations related to the thermostability of PHA synthase.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The strains and plasmids used in this study are described below. *Escherichia coli* XL1-Blue and plasmid pBluescript II KS were used for library construction. The thermophile *Cupriavidus* sp. S-6 and the 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, which was cloned into the NdeI and HindIII sites of pET-23a (Novagen). The thermophilic PHA synthase overexpression vector was designated pECspC. The chimeric PHA synthase gene *phaC*<sub>H16B</sub> was amplified with primers EcoRI23SDF and 23BamHIR and was cloned into the EcoRI and BamHI sites of pET-23a. The *PhaC*<sub>H16B</sub> overexpression vector was designated pEH16β.

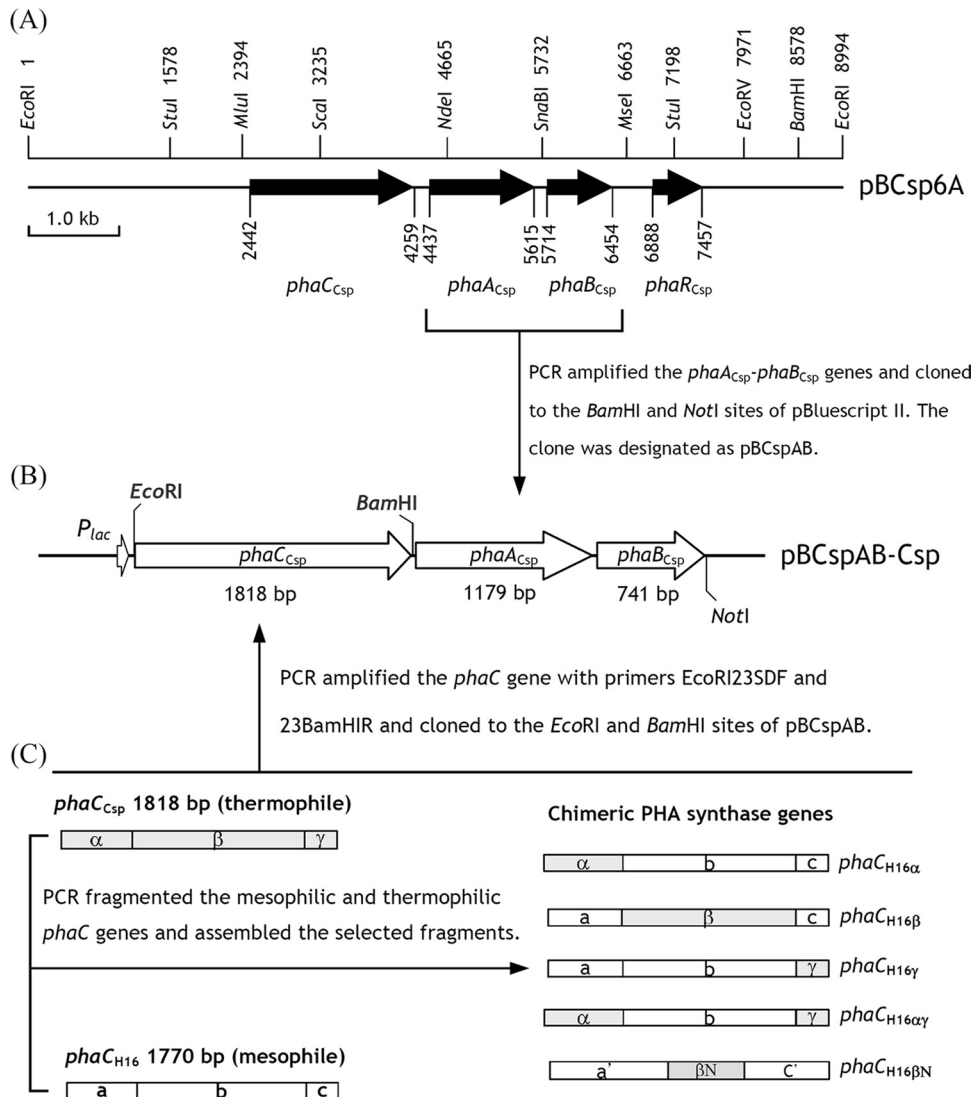
**Construction and screening of a genomic library.** Genomic DNA of *Cupriavidus* sp. S-6 was extracted with an Illustra bacterial genomicPrep Minispin kit (GE Healthcare) and was digested with EcoRI (Fermentas) overnight. One microgram of EcoRI-digested genomic DNA was ligated with 50 ng of EcoRI and alkaline phosphatase (New England BioLabs)-treated pBluescript II KS by T4 DNA ligase (Fermentas) at 8°C. The ligation product was transformed into *E. coli* XL1-Blue cells by electroporation as described previously (33). Transformants were spread onto an LB agar plate containing 1.5% glucose and 100 μg/ml ampicillin and incubated at 37°C for 24 to 48 h. Colonies with PHA accumulation were screened based on an opaque colony appearance.

**Construction of chimeric PHA synthase genes.** The construction of a chimeric *phaC* gene included two parts, the fragmentation of the *phaC* gene and the assembly of the chimeric gene. The PHA synthase genes *phaC*<sub>H16</sub> and *phaC*<sub>Csp</sub> were fragmented into three fragments with corresponding degenerate primer pairs (Table 1 and see Fig. 2). PCR primers EcoRI23SDF and CspC530R amplified the *a* and  $\alpha$  fragments. Primers CspC505F and CspC1607R amplified the *b* and  $\beta$  fragments. Primers CspC1582F and 23BamHIR amplified the *c* and  $\gamma$  fragments. The PCR mixture contained 1× PCR amplification buffer (Finnzymes), 2.2 mM MgCl<sub>2</sub>, 180 μM (each) deoxynucleoside triphosphate (dNTP), 1 μM (each) primers, 2% dimethyl sulfoxide (DMSO) (Sigma), 0.6 U DyNAzyme II DNA polymerase (Finnzymes), 0.5 U *Pfu* DNA polymerase (Fermentas), and plasmid pEReC (pET-23a containing the *phaC*<sub>H16</sub> gene) (32) or pECspC (pET-23a containing the *phaC*<sub>Csp</sub> gene) as the template in a 50-μl reaction mixture volume. The thermal cycle program consisted of 94°C for 5 min, 47°C for 30 s, 72°C for 1 min 40 s, and 35 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 1 min 40 s; the reaction was then followed by incubation at 72°C for 1 min and stopped at 15°C, all attained on a 2720 thermal cycler (Applied Biosystems). The amplified gene fragments were gel purified to remove template DNA and primers (QIAquick gel extraction kit; Qiagen).

Selected DNA fragments of *phaC*<sub>H16</sub> (*a*, *b*, and *c*) and *phaC*<sub>Csp</sub> ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) were mixed in an equal molar ratio. Approximately 100 ng of the DNA mixture was added to a 20 μl of PCR mixture containing 1× PCR amplification buffer (Finnzymes), 2.2 mM MgCl<sub>2</sub>, 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. The PCR program consisted of 94°C for 5 min and then 32 cycles of 94°C for 30 s, 55°C for 60 s, and 72°C for 40 s (+ 2 s/cycle); the reaction was then followed by incubation at 72°C for 2 min and was stopped at 15°C. Finally, for chimeric gene reamplification, the 50-μl PCR mixture contained 1 μl of the assembly PCR product, 0.8 μM primers (EcoRI23SDF and 23BamHIR), and 2% DMSO; PCR was performed for 20 cycles (94°C for 30 s, 52°C for 30 s, and 72°C for 2 min). The amplified chimeric *phaC* genes were cloned into the pGEM-T Easy vector (Promega) for DNA sequence analysis.

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

**Construction of an artificial *pha* operon.** An artificial *pha* operon, consisting of *phaC*-*phaA*<sub>Csp</sub>-*phaB*<sub>Csp</sub>, was constructed downstream from the *lac* promoter of the pBluescript II plasmid (Fig. 1B). First, the *phaA*<sub>Csp</sub>-*phaB*<sub>Csp</sub> gene fragment of *Cupriavidus* sp. S-6 was amplified with primers CspAB-BamHIF and CspAB-NotIR (Table 1 and Fig. 1B) and was cloned into 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 a PCR additive. After heating for 5 min at 94°C, PCR was performed for 2 cycles pre-PCR (94°C for 20 s, 52°C for 30 s, and 72°C for 3 min), followed by 26 cycles of PCR (94°C for 20 s, 63°C for 30 s, and 72°C for 3 min). The gel-purified PCR product was cloned to the BamHI and NotI sites of pBluescript II KS. The plasmid carrying the *phaA*<sub>Csp</sub> and *phaB*<sub>Csp</sub> genes was designated pBCspAB. Plasmid pBH16AB was constructed by use of the same method, but the *phaA*<sub>H16</sub>-*phaB*<sub>H16</sub> gene fragment of *C. necator* H16 was amplified with primers H16AB-BamHI and



**FIG 1** Organization of the *pha* locus in *Cupriavidus* sp. S-6 and construction of the artificial *pha* operon and chimeric *phaC* genes. (A) Organization of the *phaC<sub>Csp</sub>*, *phaA<sub>Csp</sub>*, and *phaB<sub>Csp</sub>* genes in the *pha* operon from *Cupriavidus* sp. S-6 and restriction map of the 9-kb *EcoRI*-cloned DNA fragment. (B) Construction of the artificial *pha* operon. (C) Construction of chimeric *phaC* genes. Details of the construction of the artificial *pha* operon and chimeric *phaC* are described in Materials and Methods.

H16AB-NotIR (Table 1). Subsequently, the wild-type or chimeric *phaC* gene was PCR amplified with primers *EcoRI*23SDF and 23BamHIR and cloned into the *EcoRI* and *BamHI* sites of plasmid pBCspAB or pBH16AB.

**PHA accumulation and analysis.** Recombinant *E. coli* cells carrying the artificial *pha* operon was precultured in LB medium containing 100  $\mu$ g/ml ampicillin at 30°C overnight. The PHA accumulation experiment was carried out by inoculating 2% of a culture grown overnight into a 250-ml Erlenmeyer flask containing 30 ml LB broth, 100  $\mu$ g/ml ampicillin, 1.5% sodium gluconate, and 50  $\mu$ M isopropyl- $\beta$ -D-1-thiogalactopyranoside (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. The PHA content was determined by gas chromatography as described in a previous report (33).

**Overexpression and purification of PHA synthases.** *E. coli* BLR(DE3) cells (Novagen) harboring overexpression plasmids were precultured overnight in 2 ml 2 $\times$  YT medium (32) containing 100  $\mu$ g/ml ampicillin at

30°C. One milliliter of the culture grown overnight was seeded into a 500-ml Erlenmeyer flask containing 100 ml 2 $\times$  YT medium and 200  $\mu$ g/ml of ampicillin and incubated at 30°C. Once the optical density at 600 nm ( $OD_{600}$ ) reached approximately 0.6, a final concentration of 0.3 mM IPTG was added to the medium, and the culture was 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 a final concentration of 20% glycerol was added, the purified enzyme was concentrated with an Amicon Ultra instrument (30-kDa-molecular-mass cutoff) to approximately 1 mg/ml and stored at -70°C. The purified enzymes were separated by SDS-PAGE. Subsequently, Coomassie brilliant blue R-250 staining revealed the electrophoretic homogeneity of the purified recombinant PHA synthases (see Fig. S1A in the supplemental material). Activity staining (Fig. S1B) and Western blotting (Fig. S1C) showed the activities of polymerization and their identities. Anti-Pha<sub>C<sub>H16</sub></sub> an-

tiserum was the first antibody used for Western blotting (42). Western blotting and activity staining were performed as described previously (32, 42).

**PHA synthase activity assay.** The substrate of PHA synthase,  $\beta$ -hydroxybutyryl-coenzyme A (CoA) (3-HBCoA), was prepared as reported previously (32, 37). PHA synthase activity was assayed by a discontinuous method by monitoring the CoA released 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 tube was preincubated at 30°C or an 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 immediate 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 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in 150 mM Tris (pH 8.0) and incubated at room temperature for 2 min. After 300  $\mu$ l deionized water was added, the optical absorbance of the yellow mixture was measured at 412 nm. The concentration of CoA released was calculated with Beer's law ( $\epsilon = 13.6 \text{ mM}^{-1} \text{ cm}^{-1}$ ). The linear range of the increase in the OD<sub>412</sub> 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 temperatures of 40°C to 60°C.

**Optimal temperature and thermostability measurements.** The optimal temperature for the enzyme activity assay was determined by using the standard assay at temperatures 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 (pH 7.5) containing 20% glycerol (Invitrogen) and 0.005% Hecameg (Calbiochem) was incubated at different temperatures (between 4°C and 60°C) for 30 min. After the samples were cooled on ice for 5 min, the residual activity was determined by using the standard method at their optimal temperature. The pH of the reaction buffer (Tricine, pH 8.0) was adjusted at room temperature.

**Half-lives of thermal inactivation.** Purified PHA synthase (0.14 mg/ml) in 50 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol (Invitrogen) and 0.005% Hecameg was incubated at 45°C. Incubation was carried out with 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 measurements. The residual activity was assayed at optimal temperatures (PhaC<sub>H16</sub> at 37°C, PhaC<sub>H16 $\beta$</sub>  at 45°C, and PhaC<sub>Csp</sub> at 50°C). The heat inactivation experiment was monitored until >80% of the activity was lost. The plot of the percent log residual activity versus time was linear. First-order rate constants of thermal inactivation were obtained by linear regression with semilogarithmic coordinates. The inactivation rate constant ( $k_{\text{inact}}$ ) was obtained from the slope, and the half-life of PHA synthase was estimated.

**Nucleotide sequence accession numbers.** 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 the 16S rRNA gene sequence of *Cupriavidus* sp. strain S-6 in the 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 the mesophile *Cupriavidus necator* H16. Phylogenetically, *Cupriavidus* sp. S-6 was very closely related 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% (dry weight) of PHB from gluconate as the carbon source at 50°C. Furthermore, it was also capable of accumulating

18% (dry weight) 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 for the biosynthesis of 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 at temperatures over 37°C (data not shown). Colony PCR was used to clone a *phaC* gene fragment (806 bp) of *Cupriavidus* sp. S-6 (*phaC*<sub>Csp</sub>) by use of primers PhaCF1 and PhaCR2 (34). The partial gene of *phaC*<sub>Csp</sub> encoded 268 amino acids, which exhibited 91% identity and 94% similarity to the PHA synthase of the mesophile *C. necator* H16. The results of PHA accumulation experiments and PCR cloning supported that the protein sequences of the PHA synthases of the thermophile *Cupriavidus* sp. S-6 and the mesophile *C. necator* H16 were highly similar; however, their thermostabilities were different.

**Cloning of the *pha* operon of *Cupriavidus* sp. S-6.** A genomic DNA library (~10,000 colonies) of *Cupriavidus* sp. S-6 was constructed with *E. coli* XL1-Blue and cultivated on an LB agar plate containing 1.5% glucose and ampicillin. After 2 days of cultivation at 30°C, an opaque colony was isolated. The positive candidate was further purified by streak culturing and was subjected to PHA accumulation experiments. It accumulated 40% (dry weight) and 12% (dry weight) PHB contents from glucose as a carbon source at 30°C and 45°C, respectively. The plasmid of the candidate clone was designated pBCsp6A. EcoRI digestion and agarose gel electrophoresis analysis showed an approximately 9-kbp inserted DNA fragment in plasmid pBCsp6A. Colony PCR detected the existence of the PHA synthase gene in pBCsp6A. The DNA sequence was the same as that amplified from the genomic DNA of the 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 sequences of the PHA biosynthesis genes.** The nucleotide sequence of pBCsp6A in both strands was determined. 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). The PHA synthase (*phaC*<sub>Csp</sub>),  $\beta$ -ketothiolase (*phaA*<sub>Csp</sub>), and acetoacetyl-CoA reductase (*phaB*<sub>Csp</sub>) genes of the thermophile *Cupriavidus* sp. S-6 showed 81%, 92%, and 96% identity, respectively, to the corresponding genes of the mesophile *C. necator* H16 (27, 30). Obviously, the  $\beta$ -ketothiolase and acetoacetyl-CoA reductase genes of *Cupriavidus* sp. S-6 and *C. necator* H16 were highly homologous; however, the PHA synthase genes were not (81% identity). According to the alignment results (Fig. 2), the diversity of the two PHA synthases was dominant on the N-terminal (corresponding to amino acids 1 to 152 of PhaC<sub>H16</sub>) and C-terminal (corresponding to amino acids 522 to 589 of PhaC<sub>H16</sub>) regions (Fig. 2). The middle regions (corresponding to amino acids 153 to 521 of PhaC<sub>H16</sub>) of both PHA synthases were highly homologous (92% identity). The middle region involved the whole  $\alpha/\beta$ -hydrolase fold region of PHA synthase analyzed by Pfam (7). In summary, the unique diversity of the thermophilic and mesophilic PHA synthases, which were 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.

|             |     |  |     |
|-------------|-----|--|-----|
| Thermophile | 1   | MATGKGAASGQEEKTTPFSSTPGPFDPATWLEWSRQAQA---NGRAAG-GMPGADAFAA    | 56  |
|             |     | MATGKGAAS QE K+ PF TPGPFDPATWLEWSRQ Q NG AA G+PG DA A          |     |
| Mesophile   | 1   | MATGKGAASSTQEGKSQPFKVTGPFDPATWLEWSRQWQGTGNGHAAASGIPGLDALA-     | 59  |
| Thermophile | 57  | LGAFPGGAFPGAGFPGTAFPGIKIAPAQLAEIQQRFMQGFTDLWRAMAAGDQQV-QLTD    | 115 |
|             |     | G+KIAPAQL +IQQR+M+ F+ LW+AMA G + L D                           |     |
| Mesophile   | 60  | -----GVKIAPAQLGDIQQRYMKDFSALWQAMAEGKAEATGPLHD                  | 99  |
|             |     | CspC505F   |     |
| Thermophile | 116 | RRFAGDAWRSNAPYRYAAAFYLLTARAMSEMADAVEADAKTRQIRIRFAVTOQVVDAMSPAN | 175 |
|             |     | RRFAGDAWR+N PYR+AAAFYLL ARA++E+ADAVEADAKTRQIRIRFA++QVVDAMSPAN  |     |
| Mesophile   | 100 | RRFAGDAWRNLPYRFAAAFYLLNARALTELADAVEADAKTRQIRIRFAISQVVDAMSPAN   | 159 |
| Thermophile | 176 | FLATNPEAQRRLIESNGESLRAGLRNMLEDLTRGKISQTDSESAFEVGRNAVTEGAVVYE   | 235 |
|             |     | FLATNPEAQR LIES GESLRAG+RNM+EDLTRGKISQTDSESAFEVGRNAVTEGAVV+E   |     |
| Mesophile   | 160 | FLATNPEAQRLLIESGGESLRAGVRNMMEDLTRGKISQTDSESAFEVGRNAVTEGAVVFE   | 219 |
|             |     | CspC790F   |     |
| Thermophile | 236 | NEYFQLLOQKPLTAKVHARPLLMVPPCINKYYILDLQPESSLVRHIVEQGHTVFLVSWRN   | 295 |
|             |     | NEYFQLLOQKPLT KVHARPLLMVPPCINKYYILDLQPESSLVRH+VEQGHTVFLVSWRN   |     |
| Mesophile   | 220 | NEYFQLLOQKPLTDKVHARPLLMVPPCINKYYILDLQPESSLVRHIVEQGHTVFLVSWRN   | 279 |
|             |     | *  |     |
| Thermophile | 296 | PDASMAARTWDDYIEHGAIARAIEVARAISGQPRINVLGFCVGGTIVSTALAVMAGRGERP  | 355 |
|             |     | PDASMA TWDDYIEH AIRATEVAR ISGQ +INVLGFCVGGTIVSTALAV+A RGE P    |     |
| Mesophile   | 280 | PDASMA GSTWDDYIEHAARAIEVARDISGQDKINVLGFCVGGTIVSTALAVLAARGEHP   | 339 |
| Thermophile | 356 | AQSLTLLTLLDFSDTGLDVVFDEAHVQLREATLGGAGAPCALLRGIELANTFSFLRP      | 415 |
|             |     | A S+TLLTLLDF+DTG+LDVVFDE HVQLREATLGG AGAPCALLRG+ELANTFSFLRP    |     |
| Mesophile   | 340 | AA SVTLLTLLDFADTGILDVVFDEAHVQLREATLGGAGAPCALLRGIELANTFSFLRP    | 399 |
|             |     | CspC1253F  |     |
| Thermophile | 416 | NDLVWNYVVDNYLKGNTVPVFDLLFWNGDATNLPGPWYCWYLRHTYLODELKVPKLTVC    | 475 |
|             |     | NDLVWNYVVDNYLKGNTVPVFDLLFWNGDATNLPGPWYCWYLRHTYLO+ELKVPKLTVC    |     |
| Mesophile   | 400 | NDLVWNYVVDNYLKGNTVPVFDLLFWNGDATNLPGPWYCWYLRHTYLODELKVPKLTVC    | 459 |
|             |     | CspC1582F  |     |
| Thermophile | 476 | GVPVDLLEKIDVPTYLYGSREDHIVPWTAAAYASTRLSNDLRFVLGASGHIAGVINPPAKN  | 535 |
|             |     | GVPVDL IDVPTY+YGSREDHIVPWTAAAYAST LL+N LRFVLGASGHIAGVINPPAKN   |     |
| Mesophile   | 460 | GVPVDLASIDVPTYLYGSREDHIVPWTAAAYASTALLANKLRFVLGASGHIAGVINPPAKN  | 519 |
| Thermophile | 536 | KRSHWLNEDLPDNDWLAGATEAPGSWWPDWFAWLKGHAGAKKAAPTQYGSRDYPAIEP     | 595 |
|             |     | KRSHW N+ LP+SP WLAGA E GSWWPDW AWL AGAK+AAP YG+ Y AIEP         |     |
| Mesophile   | 520 | KRSHWNTDALPESPQQWLAGAIEHHSWWPDWTAWLAGQAGAKRAAPANYGNARYRAIEP    | 579 |
| Thermophile | 596 | APGRYVKAKA 605   |     |
|             |     | APGRYVKAKA   |     |
| Mesophile   | 580 | APGRYVKAKA 589   |     |

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 the *a* fragment is from the 1st amino acid to the 152nd amino acid of the mesophile Pha<sub>C<sub>H16</sub></sub>. The *b* fragment is from the 153rd to the 510th amino acid of Pha<sub>C<sub>H16</sub></sub>. The *c* fragment is from the 511th to the 589th amino acid of Pha<sub>C<sub>H16</sub></sub>. The βN fragment is from the 248th to the 410th amino acid of Pha<sub>C<sub>H16</sub></sub>. Cysteine (C), marked by an 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.

**Establishment of an *in vivo* system for evaluation of the thermostability of PHA synthases.** In order to evaluate the thermostability of PHA synthases, an artificial *pha* operon, *phaC<sub>Csp</sub>-phaA<sub>Csp</sub>-phaB<sub>Csp</sub>*, was constructed downstream of 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 the thermophile *Cupriavidus* sp. S-6 were employed. The candidate *phaC* gene was cloned into the EcoRI and BamHI sites of the plasmid. The *phaA<sub>H16</sub>* and *phaB<sub>H16</sub>* genes of the mesophile *C. necator* H16 were also evaluated as the component genes of the artificial *pha* operon for PHA accumulation at high temperatures. However, the mesophilic *phaA<sub>H16</sub>* and *phaB<sub>H16</sub>* genes did not support the accumulation of PHB in recombinant *E. coli* cells at 45°C, even though the thermophilic PHA synthase gene *phaC<sub>Csp</sub>* was in the artificial *pha* operon (pBH16AB-Csp) (Table 2).

Recombinant *E. coli* strain XL1-Blue/pBCspAB-Csp, harboring the wild-type PHA synthase gene of the thermophile *Cupria-*

*vidus* sp. S-6, accumulated 41% (dry weight) PHB at 30°C (Table 2). With the elevation of the cultivation temperature, the PHB content increased and reached its highest level at 45°C (75% [dry weight]) (pBCspAB-Csp) (Table 2). In contrast, *E. coli* XL1-Blue/pBCspAB-H16, harboring the PHA synthase of the mesophile *C. necator* H16, accumulated the highest content of PHB (49% [dry weight]) at 30°C, and the content then drastically decreased with the increase of the cultivation temperature (pBCspAB-H16) (Table 2). At 45°C, only 1% (dry weight) PHB content was detected (Table 2). In this assay system, the thermostabilities of thermophilic (Pha<sub>C<sub>Csp</sub></sub>) and mesophilic (Pha<sub>C<sub>H16</sub></sub>) PHA synthases were differentiated distinctly based on the highest level of PHB accumulation at different temperatures. The results demonstrated the feasibility of using this system to differentiate the thermostabilities of PHA synthases.

**Mutations enhance the thermostability of mesophilic Pha<sub>C<sub>H16</sub></sub>.** In this study, α, β, and γ denote the N-terminal (corresponding to amino acids 1 to 152 of Pha<sub>C<sub>H16</sub></sub>), middle (corre-

TABLE 2 PHA accumulation in recombinant *E. coli* at different temperatures<sup>a</sup>

| Structure of <i>phaC</i> gene | Plasmid          | Mean PHB content<br>(% [dry wt]) ± SD <sup>a</sup> |          |                    |          |
|-------------------------------|------------------|--|----------|--------------------|----------|
|                               |                  | 30°C   | 37°C     | 42°C               | 45°C     |
|                               | pBCspAB-H16      | 49 ± 3.0   | 38 ± 1.2 | 10 ± 0.7           | 1 ± 0.0  |
|                               | pBCspAB-Csp      | 41 ± 1.3   | 42 ± 4.0 | 56 ± 4.2           | 75 ± 3.8 |
|                               | pBCspAB-H16α     | 16 ± 1.2   | 34 ± 1.7 | 4 ± 0.0            | 1 ± 0.0  |
|                               | pBCspAB-H16β     | 46 ± 2.3   | 59 ± 3.5 | 52 ± 1.2           | 65 ± 2.8 |
|                               | pBCspAB-H16βN    | 41 ± 0.7   | 47 ± 2.2 | 37 ± 1.3           | 25 ± 3.1 |
|                               | pBCspAB-H16γ     | 30 ± 1.2   | 1 ± 0.0  | Trace <sup>b</sup> | Trace    |
|                               | pBCspAB-H16αγ    | 33 ± 2.6   | 48 ± 3.2 | 1 ± 0.2            | Trace    |
|                               | pBCspAB-H16G286A | 49 ± 1.5   | 45 ± 1.5 | 17 ± 1.7           | 4 ± 2.5  |
|                               | pBCspAB-H16A341Q | 49 ± 2.5   | 41 ± 1.6 | 34 ± 3.7           | 4 ± 0.4  |
|                               | pBH16AB-H16      | 50 ± 2.7   | 49 ± 3.4 | 3 ± 0.8            | Trace    |
|                               | pBH16AB-H16β     | 51 ± 2.3   | 57 ± 2.5 | 3 ± 0.2            | Trace    |
|                               | pBH16AB-Csp      | 57 ± 2.7   | 54 ± 2.6 | 11 ± 1.2           | Trace    |

<sup>a</sup> The data shown are the means of data from three individual experiments. Plasmid pBCspAB-H16 carried the mesophilic *phaC*<sub>H16</sub> gene from *C. nector* H16; pBCspAB-Csp carried the thermophilic *phaC*<sub>Csp</sub> gene from *Cupriavidus* sp. S-6. Others carried the chimera of the *phaC*<sub>H16</sub> and *phaC*<sub>H16</sub> genes or the mutants. The pBCspAB serial plasmids carried the thermophilic *phaA* and *phaB* genes of the 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. nector* H16.

<sup>b</sup> Trace indicates a PHB content of <0.2%.

sponding to amino acids 153 to 521 of PhaC<sub>H16</sub>), and C-terminal (corresponding to amino acids 522 to 589 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 mutations of PhaC<sub>Csp</sub> and their relationships to thermostability, a region-selected approach was used to construct four chimeric PHA synthase genes (*phaC*<sub>H16α</sub>, *phaC*<sub>H16β</sub>, *phaC*<sub>H16γ</sub>, and *phaC*<sub>H16αγ</sub>) 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>). Figure 1C shows the gene structures of the chimeras. The PCR-constructed chimeric gene was cloned in to the *phaC* gene position of the artificial *pha* operon and transformed into *E. coli* XL-1 Blue for PHA accumulation experiments.

The *in vivo* thermostabilities 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% (dry weight) (Table 2). All chimeras were enzymatically active. With the increase of the cultivation temperature, chimeras accumulated different amounts of PHB in the cell. The temperature at which the cells accumulated the highest contents of PHB was the index to differentiate the thermostabilities of chimeras. In the four chimeras, at 45°C, only PhaC<sub>H16β</sub> accumulated a high content of PHB (65% [dry weight]) (pBCspAB-H16β) (Table 2); *phaC*<sub>H16α</sub>, *phaC*<sub>H16γ</sub>, and *phaC*<sub>H16αγ</sub> accumulated less than 1% (dry weight) PHB at 45°C, which was similar to the amount accumulated with the mesophilic parental enzyme PhaC<sub>H16</sub> (pBCspAB-H16α, pBCspAB-H16γ, and pBCspAB-H16αγ) (Table 2). The results showed that only chimeric PhaC<sub>H16β</sub> was thermostable. Moreover, only mutations from the β region of thermophilic PhaC<sub>Csp</sub> enhanced the thermostability of the mesophilic PHA synthase PhaC<sub>H16</sub>; mutations from α, γ, or the combined α

and  $\gamma$  regions of PhaC<sub>Csp</sub> did not improve the thermostability of PhaC<sub>H16</sub>. Although the chimera PhaC<sub>H16 $\beta$</sub>  accumulated a high content of PHB at 45°C (65% [dry weight]), the PHB content was still lower than that with PhaC<sub>Csp</sub> (75% [dry weight]). Hence, the thermostabilities of the chimera PhaC<sub>H16 $\beta$</sub>  and the parental enzyme PhaC<sub>Csp</sub> might be close but not the same. The  $\alpha$  region,  $\gamma$  region, or both regions of PhaC<sub>Csp</sub> were still possessing mutations that contributed to the thermostability of PhaC, even though the contribution was minor.

In order to explore the scope of the  $\beta$  region that actually contributed to the enhancement of the thermostability of PhaC<sub>H16</sub>, another chimeric gene, phaC<sub>H16 $\beta$ N</sub>, was constructed. The chimeric phaC<sub>H16 $\beta$ N</sub> gene was the phaC<sub>H16</sub> gene with a partial  $\beta$  region sequence ( $\beta$ N) of phaC<sub>Csp</sub>. Figure 2 shows the  $\beta$ N region of PhaC<sub>Csp</sub> (CspC790F to CspC1253F). With the  $\beta$ N region, 17 point mutations surrounding the catalytic site (GXCXGG) of PHA synthase were introduced into PhaC<sub>H16</sub> (Fig. 2). The chimera PhaC<sub>H16 $\beta$ N</sub> accumulated 41% (dry weight) PHB at 30°C and reached a peak at 37°C (47% [dry weight]). Subsequently, the PHB content decreased with the elevation of the cultivation temperature (pBCspAB-H16 $\beta$ N) (Table 2). Nonetheless, chimeric PhaC<sub>H16 $\beta$ N</sub> still accumulated 25% (dry weight) PHB at 45°C (Table 2). Obviously, the point mutations derived from the partial  $\beta$  region ( $\beta$ N) improved the thermostability of PhaC<sub>H16</sub>. However, the thermostability of PhaC<sub>H16 $\beta$ N</sub> was not equal to that of chimeric PhaC<sub>H16 $\beta$</sub> . The results suggest that the essential mutations related to the thermostability of PhaC<sub>Csp</sub> are mainly scattered on the whole  $\beta$  region rather than on a partial area. Preliminary site-directed mutagenesis experiments showed that the A341Q mutant of PhaC<sub>H16</sub> accumulated 34% (dry weight) PHB at 42°C and accumulated a PHB content comparable to that of the wild type at 30°C and 37°C (pBCspAB-H16A341Q) (Table 2). The results suggested that the A341Q substitution enhanced the thermostability of PhaC<sub>H16</sub>. The G286A mutant of PhaC<sub>H16</sub> accumulated a higher content of PHB (45% [dry weight]) than the wild type (38% [dry weight]) at 37°C and accumulated 17% (dry weight) PHB at 42°C (pBCspAB-H16G286A) (Table 2). The G286A mutation enhanced the enzyme activity and slightly enhanced the thermostability.

**Effects of temperature on the activity of PHA synthases.** The parental and chimeric PHA synthase genes phaC<sub>H16</sub>, phaC<sub>Csp</sub>, and phaC<sub>H16 $\beta$</sub>  were overexpressed in *E. coli* BLR(DE3) cells and purified. Figure 3A shows the activity-temperature profiles of chimeric and parental PHA synthases. The parental enzymes PhaC<sub>H16</sub> and PhaC<sub>Csp</sub> had temperature optima of 37°C and 50°C, respectively; the optimal temperature of the chimera PhaC<sub>H16 $\beta$</sub>  was 45°C. At their optimal temperatures, PhaC<sub>H16</sub>, PhaC<sub>Csp</sub>, and the chimera PhaC<sub>H16 $\beta$</sub>  had specific activities of  $70 \pm 8.8$  U/mg,  $236 \pm 19.4$  U/mg, and  $237 \pm 11.8$  U/mg, respectively. Although the optimal temperature of chimeric PhaC<sub>H16 $\beta$</sub>  was 5°C lower than that of its parental enzyme PhaC<sub>Csp</sub>, its specific activity was the same as that of PhaC<sub>Csp</sub> at their optimal temperatures.

Obviously, PhaC<sub>H16 $\beta$</sub>  inherited high enzyme activity from its parental enzyme PhaC<sub>Csp</sub> despite having a lower optimal temperature. At 30°C, the specific activities of PhaC<sub>H16</sub>, PhaC<sub>Csp</sub>, and chimeric PhaC<sub>H16 $\beta$</sub>  were  $50 \pm 5.2$ ,  $142 \pm 11.7$ , and  $172 \pm 17.8$  U/mg, respectively. The specific activity of chimeric PhaC<sub>H16 $\beta$</sub>  was 1.21-fold higher than that of PhaC<sub>Csp</sub> ( $142 \pm 11.7$  U/mg) and 3.45-fold higher than that of PhaC<sub>H16</sub> ( $50 \pm 5.2$  U/mg). At 15°C, PhaC<sub>H16</sub>, PhaC<sub>Csp</sub>, and chimeric PhaC<sub>H16 $\beta$</sub>  showed nearly the

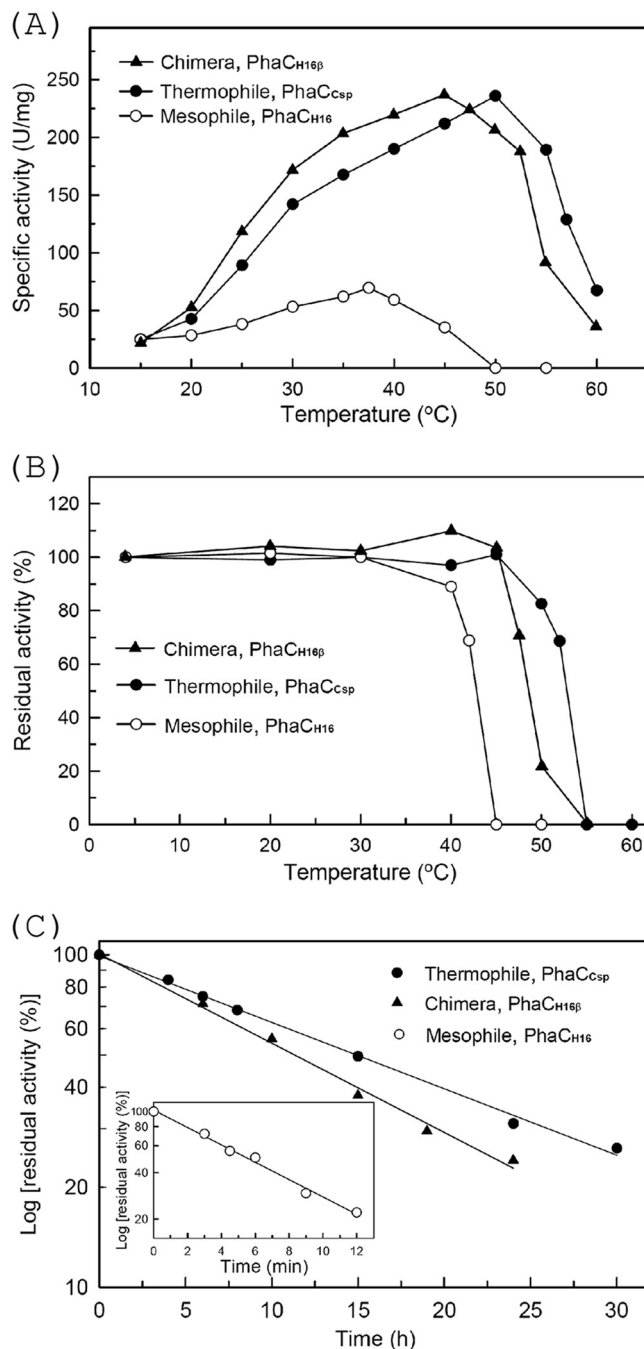


FIG 3 Optimal temperature, thermal stability, and half-lives of thermal inactivation of PHA synthases. (A) Activity-temperature profiles of wild-type and chimeric PHA synthases. (B) Thermal stability of PHA synthases. (C) Kinetics of thermal inactivation of PHA synthases at 45°C. To estimate the thermal stabilities of PHA synthases, the enzyme was preincubated at the indicated temperatures for 30 min and then incubated on ice for 5 min. The residual activities were then determined by using a discontinuous method at 37°C (mesophile, PhaC<sub>H16</sub>), 45°C (chimera, PhaC<sub>H16 $\beta$</sub> ), or 50°C (thermophile, PhaC<sub>Csp</sub>). PhaC<sub>Csp</sub> and PhaC<sub>H16</sub> are PHA synthases from the thermophile *Cupriavidus* sp. S-6 and the mesophile *C. necator* H16, respectively. PhaC<sub>H16 $\beta$</sub>  is a chimera of PhaC<sub>H16</sub> and PhaC<sub>Csp</sub>. The data are the means of data from three individual experiments.

same specific activities (Fig. 3A). Obviously, chimeric PhaC<sub>H16β</sub> showed better activity than those of its parental enzymes PhaC<sub>H16</sub> and PhaC<sub>Csp</sub> at 30°C (Fig. 3A). At a temperature of 30°C, high-cell-density fermentation for the mass production of PHB was reported (49, 50). At this temperature, chimeric PhaC<sub>H16β</sub> has a higher specific activity than those of its parental enzymes PhaC<sub>Csp</sub> and PhaC<sub>H16</sub>. This finding suggests that chimeric PhaC<sub>H16β</sub> will accumulate higher levels of PHB than PhaC<sub>Csp</sub> and PhaC<sub>H16</sub> in recombinant *E. coli* fermentations.

At 37°C, PhaC<sub>H16β</sub> accumulated 1.55-fold more PHB than did PhaC<sub>H16</sub> (Table 2). This is in good agreement with the correlation between the specific activity of PHA synthase and the PHB content (40). However, at 30°C, PhaC<sub>H16</sub> accumulated a higher level of PHB than those of the higher-activity enzymes PhaC<sub>H16β</sub> and PhaC<sub>Csp</sub> (Table 2). This finding conflicts with the above-described results. We postulate that the conflict may be due to the use of the thermophilic genes *phaA*<sub>Csp</sub> and *phaB*<sub>Csp</sub> in the artificial operon. Previous reports suggested 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 levels of activity at lower temperatures than mesophilic enzymes. If the inference was correlative in our case, then thermophilic PhaA<sub>Csp</sub> and PhaB<sub>Csp</sub> provided lower concentrations of PHA synthase substrate than did mesophilic PhaA<sub>H16</sub> and PhaB<sub>H16</sub>.

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 pBH16AB-X-based plasmids (where X is H16, Csp, or H16β), the thermostable PHA synthases PhaC<sub>Csp</sub> and chimeric PhaC<sub>H16β</sub> accumulated higher levels of PHB than did PhaC<sub>H16</sub> 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 that accumulated by *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 critical for efficient PHA accumulation.

**Thermostability of PHA synthases.** With regard to heat stability, mesophilic parental enzyme PhaC<sub>H16</sub> 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. The thermophilic parental enzyme PhaC<sub>Csp</sub> and chimera PhaC<sub>H16β</sub> were stable at 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, the parental enzymes PhaC<sub>H16</sub> and PhaC<sub>Csp</sub> and chimera PhaC<sub>H16β</sub> exhibited first-order inactivation kinetics, with  $t_{1/2}$  values of 5.3 min, 15.06 h, and 11.20 h, respectively. Substantially, the half-life of PhaC<sub>H16</sub> (5.3 min) was enhanced 127-fold (11.20 h for PhaC<sub>H16β</sub>) when the β region of PhaC<sub>Csp</sub> was introduced. Nonetheless, the half-life of the chimera PhaC<sub>H16β</sub> (11.20 h) was a little shorter than that of thermophilic parental enzyme PhaC<sub>Csp</sub> (15.06 h). This suggests that the amino acid residues contributing to the thermostability of PhaC<sub>Csp</sub> were not all in the β region and that some were located in the α or γ region. This result is in good agreement with the results of the PHA accumulation experiments (pBCspAB-Csp and pBCspAB-H16β) (Table 2).

## DISCUSSION

This study presents a thermostable PHA synthase (PhaC<sub>Csp</sub>) from the thermophile *Cupriavidus* sp. S-6 that has a high level of activity and a high level of thermostability. The most important characteristic of PhaC<sub>Csp</sub> is its primary structure, which is highly similar to that of the PHA synthase of the mesophile *C. necator* H16. By use of the region-selected method, 30 point mutations derived from the thermophilic PHA synthase were verified to have an association with the enhancement of thermostability and the activity of the mesophilic PHA synthase PhaC<sub>H16</sub>. This study establishes an *in vivo* system in *E. coli* which is applicable for differentiating the thermostability of PHA synthases. The system differentiated the thermostability of PHA synthases based on the accumulation of PHA at various temperatures. It did not require tedious work with protein purification and biochemical analyses. Thus, it was suitable for the rapid screening of thermostable PhaC candidates.

This study employed *E. coli* XL1-Blue, a strain commonly used for PHA fermentation (4, 49, 50), as the host cell line for the PHA accumulation experiment. Fotadar et al. and Hoffman et al. reported previously that *E. coli* DH5α is capable of growth at temperatures of up to 49°C, although growth is prohibitive beyond 40°C (8, 15). The effects of temperature on the growth of *E. coli* XL1-Blue were similar; however, *E. coli* XL1-Blue had no growth when the cultivation temperature went above 45°C (data not shown). At 42°C and 45°C, the growth and PHA accumulation of recombinant *E. coli* XL1-Blue were monitored for 4 days. The turbidity and PHA content of recombinant *E. coli* reached a plateau on the second day and slightly decreased with time at 42°C and 45°C (data not shown). In this study, recombinant *E. coli* XL1-Blue strains accumulated PHA at temperatures of 30°C to 45°C. Theoretically, a more thermostable PHA synthase should cause a peak in the PHB content at higher temperatures. Accordingly, the thermostable PHA synthase can be discriminated.

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



was generated. Based on the proline rule, the D311P mutation might be related to the thermostability of PhaC<sub>H16β</sub>.

Margarit et al. found previously that G147A and G189A mutations of a neutral protease from *Bacillus subtilis* were more stable against irreversible thermal inactivation (23). Ganter and Plückthun showed previously that one Gly-to-Ala substitution (G316A) in glyceraldehyde-3-phosphate dehydrogenase strongly stabilized a mutant under conditions of irreversible heat denaturation (9). Menéndez-Arias and Argos compared the amino acid sequences of thermophilic and mesophilic molecules from six different protein families. They showed that the Gly→Ala substitution is the top residue substitution for helices and strands from mesophiles to thermophiles (24). Those studies strongly suggest that a position-specific Gly→Ala substitution in an enzyme is highly related to the enhancement of thermostability. In this study, three Gly→Ala substitutions, G286A, G365A, and G377A, were found in PhaC<sub>H16β</sub> (Fig. 2). The Gly→Ala substitutions in PhaC<sub>H16β</sub> should be correlative with its thermostability. In contrast, three Ala→Gly substitutions, A296G, A334G, and A466G, were also observed for PhaC<sub>H16β</sub> (Fig. 2). The Ala→Gly substitution in the helix is helix destabilizing (5). This means that the Ala→Gly substitution is promising to decrease the thermostability of proteins. Thus, the role of the Ala→Gly substitutions A296G, A334G, and A466G in PhaC<sub>H16β</sub> needs to be further investigated.

Previous research described an F420S mutation in PhaC<sub>H16</sub> which enhanced specific activity 2.4-fold. However, the thermostability of the F420S mutant is lower than that of the wild type (41). In the sequence of PhaC<sub>H16β</sub>, F420 is conservative. The high level of activity of PhaC<sub>H16β</sub> does not relate to the F420S mutation. PhaC<sub>H16β</sub> should bear beneficial mutations related to activity enhancement that have not yet been reported. Recently, Bhubalan et al. reported a highly active PHA synthase from *Chromobacterium* sp. strain USM2, PhaC<sub>Cs</sub>, which revealed a homology of 46% with PhaC<sub>H16</sub> and exhibited a specific activity of 238 ± 98 U/mg at 30°C (3). This is the highest specific activity reported for a purified PHA synthase (3). In this study, the chimera PhaC<sub>H16β</sub> has a specific activity of 172 ± 17.8 U/mg at 30°C. Despite a lower specific activity than that of *Chromobacterium* sp. USM2 at 30°C, the chimera PhaC<sub>H16β</sub> exhibited comparable activity at its optimal temperature of 45°C (237 ± 11.8 U/mg). Furthermore, the chimera PhaC<sub>H16β</sub> is thermostable and only 30 amino acids different from PhaC<sub>H16</sub>. Thus, the chimera PhaC<sub>H16β</sub> is a proper model to explore the activity- and thermostability-enhancing substitutions in PHA synthase.

The *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 the substrate 3-hydroxyacyl-CoA limits its application (11). The thermostable and highly active PHA synthase will give a partial answer to questions regarding *in vitro* PHA synthesis (13). The chimera PhaC<sub>H16β</sub> showed a long half-life at 45°C (11.2 h) and possessed high specific activity at 30°C and 45°C. Thus, it is a good candidate for *in vitro* PHA polymerization. In addition, PhaC<sub>H16β</sub> possesses a higher level of activity than the mesophilic PhaC<sub>H16</sub> and thermophilic PhaC<sub>Csp</sub> parental enzymes at 20°C to 40°C (Fig. 3A). The chimeric phaC<sub>H16β</sub> gene is more appropriate for *E. coli* PHA fermentation than the mesophilic phaC<sub>H16</sub> gene.

Regarding the optimal temperature, enzyme activity, thermostability, or kinetics of heat inactivation, the chimera PhaC<sub>H16β</sub>

shows better characteristics than the parental enzyme PhaC<sub>H16</sub>. The high levels of thermostability and enzyme activity of PhaC<sub>H16β</sub> are the effect of the introduction of 30 point mutations derived from the thermophilic enzyme PhaC<sub>Csp</sub>. The introduced point mutations should include activity-improving and thermostability-enhancing residues. Due to the limited numbers of mutations generated in PhaC<sub>H16β</sub>, it is possible to explore the role of each mutation by site-directed mutagenesis in future studies.

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