

# In vitro synthesis of polyhydroxyalkanoate (PHA) incorporating lactate (LA) with a block sequence by using a newly engineered thermostable PHA synthase from *Pseudomonas* sp. SG4502 with acquired LA-polymerizing activity

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**Abstract** Recently, we succeeded in isolating a thermotolerant bacterium, *Pseudomonas* sp. SG4502, which is capable of accumulating polyhydroxyalkanoate (PHA) even at 55 °C, as a source of thermostable enzymes. In this study, we cloned a *pha* locus from the bacterium and identified two genes encoding PHA synthases (PhaC1<sub>SG</sub> and PhaC2<sub>SG</sub>). Two mutations, Ser324Thr and Gln480Lys, corresponding to those of a lactate (LA)-polymerizing enzyme (LPE) from mesophilic *Pseudomonas* sp. 61-3 were introduced into PhaC1<sub>SG</sub> to evaluate the potential of the resulting protein as a “thermostable LPE”. The mutated PhaC1<sub>SG</sub> [PhaC1<sub>SG</sub>(STQK)] showed high thermal stability in synthesizing P(LA-co-3HB) in an in vitro reaction system under a range of high temperatures. Requirement of 3HBCoA as a priming unit for LA polymerization by the LPE has been suggested in both of the in vitro and in vivo experiments. Based on the finding, the PhaC1<sub>SG</sub>(STQK)-mediated synthesis of a LA-based copolymer with a block sequence was achieved in the in vitro system by sequential feeding of the corresponding two substrates. This in vitro reaction system using the thermostable LPE provides us with a versatile way to synthesize the various types of LA-based copolymers with desired sequence patterns,

random or block, depending on the way of supplying hydroxyalkanoates (mixed or sequential feeding).

**Keywords** Polyhydroxyalkanoate (PHA) · Thermal stability · Block copolymer · LA-polymerizing enzyme (LPE) · In vitro polymerization · *Pseudomonas* sp. SG4502

## Introduction

Polyhydroxyalkanoates (PHAs) are a class of aliphatic polyesters that are accumulated as carbon and energy storage materials in numerous bacteria under nutrient-limited conditions (Anderson and Dawes 1990; Lee 1996). The PHAs have attracted much attention because they have properties similar to those of common thermoplastics and can be produced from renewable resources (Jendrossek et al. 1996; Jendrossek and Handrick 2002). Because of their superior properties, PHAs are expected to be applicable to a wide range of agricultural, marine, and medical fields. PHA synthases are the key enzymes for PHA synthesis and produce PHA by polymerizing (*R*)-hydroxyalkanoyl moieties of (*R*)-hydroxyalkanoyl coenzymeAs (HACoAs) with concomitant release of CoA molecules (Rehm 2003; Taguchi and Doi 2004), and the types of repeating units incorporated into PHAs are mainly based on the substrate specificities of the PHA synthases employed. PHA synthases are divided into four classes (classes I to IV) on the basis of their activities toward substrates with different carbon chain lengths and their subunit compositions (Rehm 2003). PHAs are generally produced by in vivo methods

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using bacterial whole-cells, and in vivo methods are suitable for large-scale PHA production from renewable carbon sources. On the other hand, in vitro methods are not suitable for large-scale PHA production, but they have several attractive features, such as incorporation of the unnatural repeating units with controlled fractions and the ability to sensitively monitor this incorporation, which cannot be performed by in vivo methods. In particular, the reactivity and polymerization activity of PHA synthase toward unnatural substrates can be efficiently evaluated by in vitro methods (Han et al. 2011; Taguchi et al. 2008; Tajima et al. 2009) by measuring yield and molecular weight and analyzing the structure of a product.

Like PHA, polylactate (PLA) is also a bio-based polyester produced from biomass. PLA is the most popular biodegradable plastic and has been widely studied all over the world (Carrasco et al. 2010; Fukushima et al. 2009; Inkinen et al. 2011; Nampoothiri et al. 2010; Pang et al. 2010; Saulnier et al. 2004; Tsuji 2005). It is a very attractive material, but its application is limited in part due to its brittle properties, such as poor elongation, slow crystallization rate, and so on. One of the methods to improve the properties of PLA is copolymerization (Abe et al. 1997; Haynes et al. 2007) with another repeating unit. For example, Abe et al. (1997) and Haynes et al. (2007) reported the synthesis of copolymers containing LA and 3HA units by the ring-opening copolymerization of (*S,S*)-lactide with (*R*)- $\beta$ -butyrolactone and by the ring-opening copolymerization of (*S,S*)-lactide using PHA as a macroinitiator. The synthesized copolymers had better properties than those of the PLA homopolymer (Abe et al. 1997; Haynes et al. 2007).

We recently developed a copolymerization method using an LA-polymerizing enzyme (LPE) (Taguchi et al. 2008; Tajima et al. 2009), which was engineered from the PHA synthase from *Pseudomonas* sp. 61-3 by evolutionary engineering (Matsumoto and Taguchi 2010; Taguchi and Doi 2004; Taguchi 2010). In other studies, we attempted to screen the LPE among various natural and mutant PHA synthases belonging to classes I to IV (Rehm 2003) by using a two-phase reaction system (Taguchi et al. 2008; Taguchi 2010; Tajima et al. 2009). As a result of these screenings, a double mutant [PhaC1<sub>ps</sub>(STQK)] of PHA synthase from *Pseudomonas* sp. 61-3, which had two amino acids substitutions, Ser325Thr and Glu481Lys, was found to exhibit LA-polymerizing activity, and *Escherichia coli* carrying an LACoA supply pathway and this engineered enzyme produced LA-based polyesters (Taguchi et al. 2008; Tajima et al. 2009). To date, only class II PHA synthases from *Pseudomonas* species have been reported as LPEs (Matsumoto and Taguchi 2010; Taguchi et al. 2008; Tajima et al. 2009; Yamada et al. 2010; Yang et al. 2011), and active efforts to obtain superior LPEs capable of incorporating LA units with high ratios and of efficiently producing LA-based

copolymers with high yields have been made throughout the world (Yamada et al. 2010; Yang et al. 2011).

Thermostable PHA synthesis enzymes are required in order to develop in vitro systems for sustainable, long-term reactions for producing PHA. However, the isolation of enzymes involved in PHA synthesis has been limited to mesophilic strains thus far. In our efforts to realize this goal, we very recently succeeded in isolating a thermotolerant bacterium, *Pseudomonas* sp. SG4502, which is capable of accumulating PHA even at 55 °C, as a potential source of thermostable enzymes (Satoh et al. 2011). This bacterium was able to grow between 30 °C and 55 °C and to synthesize polymers composed of various repeating units with 4 to 12 carbon atoms, suggesting that it could serve as a thermostable catalyst efficient in incorporating LA unit into the PHA chain. In this study, therefore, we attempted to clone and analyze PHA synthase genes from *Pseudomonas* sp. SG4502 and to evaluate the PHA synthase (PhaC1<sub>SG</sub>) as a thermostable catalyst. Furthermore, we have presented here the first in vitro synthesis of PHA containing LA unit with a block sequence by utilizing the LPE derivative with STQK mutation of the newly isolated PhaC1<sub>SG</sub>. This finding provides us with the attractive potentiality of the in vitro system equipped with thermostable PHA synthase in order to synthesize the various types of any copolymers with desired sequence patterns such as random or block sequence.

## Materials and methods

### Materials

Chloroform, methanol, adenosine triphosphate (ATP), and coenzymeA (CoA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). (*R*)-LA lithium salt and (*R*)-3HB sodium salt were products of Sigma-Aldrich Japan (Tokyo, Japan) and MP Bio Japan K.K. (Tokyo, Japan), respectively. All other chemicals were of reagent grade or better.

### Strains, plasmids, primers, and growth conditions

The strains, plasmids, and primers used in this study are shown in Table 1. *E. coli* was grown at 37 °C in Luria–Bertani (LB) medium. When needed, ampicillin (Ap, 100  $\mu\text{g mL}^{-1}$ ) or kanamycin (Km, 25  $\mu\text{g mL}^{-1}$ ) was added to medium. *Pseudomonas* SG4502 (NITE P-578) was grown at 45 °C in LB medium (Satoh et al. 2011).

### Cloning of *pha* locus from *Pseudomonas* sp. SG4502

Two primers, ORF1\_FW and phaD\_RV (Table 1) (Zhang et al. 2001), were used to obtain a PHA operon from *Pseudomonas* sp. SG4502. DNA was amplified by two-step touchdown

**Table 1** Bacterial strains, plasmids, and primers used in this study

Name	Relevant characteristics or genotype	Source or reference
<b>Strains</b>		
<i>Pseudomonas</i> sp. SG4502	Wild type; PHA+	NITE P-578
<i>E. coli</i> JM109	<i>recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, Δ (lac-proAB)/F[traD36, proAB+, lacIq, lacZ</i>	Toyobo
<i>E. coli</i> BL21	F <sup>-</sup> <i>ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup>m<sub>B</sub><sup>-</sup>)gal dcm</i>	Invitrogen
<i>E. coli</i> BL21(DE3)	F <sup>-</sup> <i>ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup>m<sub>B</sub><sup>-</sup>)gal dcm</i> (DE3)	Invitrogen
<b>Plasmids</b>		
pCR2.1-TOPO	TA cloning vector, Ap <sup>r</sup> , Km <sup>r</sup> ; α-lacZ/MCS; P <sub>lac</sub> , 3.9 kb	Invitrogen
pREP4	LaqI <sup>q</sup> expression plasmid, Km <sup>r</sup>	Qiagen
pQE80	N-terminus His-tagged fusion protein expression vector, Ap <sup>r</sup> ; MCS; P <sub>T5</sub> ; <i>lacI<sup>q</sup></i>	Qiagen
pQE30	N-terminus His-tagged fusion protein expression vector, Ap <sup>r</sup> ; MCS; P <sub>T5</sub>	Qiagen
pQEacoE	pQE30 derivative; acetylCoA synthetase gene ( <i>acoE</i> ) from <i>Cupriavidus necator</i> ATCC17699	This study
pQECPP	pQE30 derivative; propionate CoA transferase gene ( <i>pct</i> ) from <i>Clostridium propionicum</i> JCM1430	Han et al. (2009)
pCR-phaSG	pCR2.1-TOPO derivative; <i>pha</i> locus from <i>Pseudomonas</i> sp. SG4502	This study
pQC1SG(WT)	pQE80 derivative; <i>phaC1<sub>SG</sub>(WT)</i> from <i>Pseudomonas</i> sp. SG4502	This study
pQC1SG(STQK)	pQE80 derivative; <i>phaC1<sub>SG</sub>(STQK)</i> from <i>Pseudomonas</i> sp. SG4502	This study
<b>Primers</b>		
ORF1_FW	5'-CCA(C/T)GACAGCGGCTGTTCACCTG-3'	Zhang et al. (2001)
phaD_RV	5'-TCGACGATCAGGTGCAGGAACAGCC-3'	Zhang et al. (2001)
SP( <i>acoE</i> -Bam)	5'-CGGGATCC ATGTCCGCCATCGAATCGGTGA-3'	This study
AP( <i>acoE</i> -Hind)	5'-CCCAAGCTT TCACTGCGCTGCTGAGC-3'	This study
SP( <i>phaC1<sub>SG</sub></i> -Bam)	5'-CGGGATCC AACAAGATCGCCGAAGACCTACAG-3'	This study
AP( <i>phaC1<sub>SG</sub></i> -Kpn)	5'-GGGGTACC TCATCGTTGGTGCACGTAGGTTCC-3'	This study

polymerase chain reaction (PCR) using genomic DNA from *Pseudomonas* sp. SG4502 as a template. Takara LA Taq polymerase (Takara Shuzo, Kyoto, Japan) was used as a DNA polymerase. The temperature protocol consisted of an initial 1-min denaturation at 94 °C, followed by 10 cycles of 94 °C for 30 s and 70 °C for 5 min, followed by 20 cycles of 94 °C for 30 s and 68 °C for 5 min, with a final elongation step of 68 °C for 10 min. The PCR product was ligated with a vector, pCR2.1-TOPO. The ligation mixture was used for the transformation of *E. coli* JM109 and then was plated onto LB agar plates with ampicillin, isopropylthiogalactoside, and 5-bromo-4-chloro-3-indolyl-β-D-galactoside. Approximately 2,000 white colonies with ampicillin resistance were obtained, and candidates for plasmid preparation were selected by confirming DNA insertion into a vector with colony-direct PCR using universal primers. A plasmid containing the PCR product was designated as pCR-phaSG.

#### Sequencing of a DNA fragment from *Pseudomonas* sp. SG4502

The plasmid, pCR-phaSG, was isolated from *E. coli* with a Quantum Prep Plasmid Midiprep Kit (Bio-Rad Laboratories, Hercules, CA, USA). The nucleotide sequence was determined by the dye-terminator method using an ABI PRISM Big-Dye

Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Wellesley, MA, USA) and an ABI PRISM 310 Genetic Analyzer (Perkin Elmer). Primer walking method was used for sequencing the DNA insert and specific primers were designed based on the results of sequencing.

#### Preparation and assay of AcoE, PCT, and PHA synthases

AcoE from *Ralstonia eutropha* (currently *Cupriavidus necator*) ATCC17699 (H16 strain) was used as acetyl-CoA synthetase (ACS) to form acetyl-CoA (AcCoA) (Priefert and Steinbüchel 1992). A gene encoding AcoE was amplified by PCR using a set of specific primers (Table 1), and then the amplified DNA fragment was introduced into pQE80 (Qiagen Japan K.K. Tokyo, Japan). Propionate CoA transferase (PCT) from *Clostridium propionicum* was employed for supplying LACoA and 3HBCoA to a PHA synthase. ACS and PCT were prepared according to the methods previously described (Han et al. 2009). *Pseudomonas* sp. 61-3 and SG4502 were used as sources of PHA synthases. The PHA synthases (PhaC1s) from *Pseudomonas* sp. 61-3 and *Pseudomonas* sp. SG4502 were described as PhaC1<sub>ps</sub> and PhaC1<sub>SG</sub>, respectively. The reactivities of PHA synthase from *Pseudomonas* sp. 61-3 towards LACoA and 3HBCoA were acquired and increased, respectively, by the

introduction of double mutations (Taguchi and Doi 2004; Taguchi et al. 2008; Tajima et al. 2009). A gene encoding PHA synthase with two mutations [PhaC1<sub>SG</sub>(STQK)], Ser324Thr and Gln480Lys, was chemically synthesized by GenScript USA Inc. (NJ, USA). The engineered PHA synthases, PhaC1<sub>PS</sub>(STQK) from *Pseudomonas* sp. 61-3 and PhaC1<sub>SG</sub>(STQK) from *Pseudomonas* sp. SG4502, were prepared by using a His-tag system according to the method described in a previous paper (Satoh et al. 2003; Tajima et al. 2009). The activities towards (*R*, *S*)-3HBCoA of the purified enzymes were measured according to the methods described in previous papers (Han et al. 2009; Satoh et al. 2003; Tajima et al. 2004).

#### Thermal stability test

PHA synthase dissolved in 100 mM sodium phosphate buffer (pH 7.5) was maintained at 30 °C, 37 °C, 45 °C, and 55 °C for 10 min. Then other enzymes and reagents required for enzymatic synthesis of PHA (please refer next section) were added to a reaction mixture for PHA production. After incubation at 30 °C for 72 h, the formed polymer was purified and weighed.

The protein particle size was observed to evaluate thermal stability of PHA synthase (Golub et al. 2007). Four hundred microliters of PHA synthase (0.5 mg mL<sup>-1</sup>) dissolved in 100 mM sodium phosphate buffer (pH 7.5) was put into a sample cell kept at 37 °C. The time course change in a protein particle size at 37 °C was measured by the dynamic light scattering method using a particle size analyzer UPA-UT151 (Nikkiso Co., Ltd., Tokyo, Japan). The same experiment was conducted twice to confirm the reproducibility of the experiments.

#### Enzymatic synthesis of P(LA-*co*-3HB) by an in vitro reaction system using an LPE

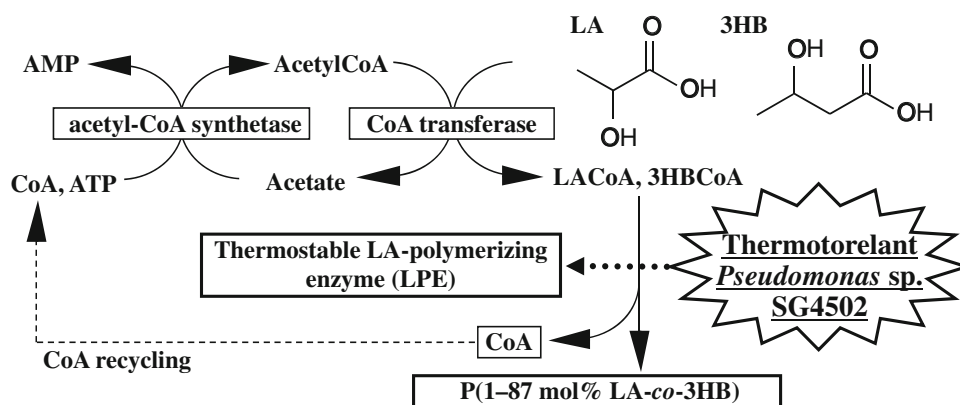
To evaluate PHA synthase as a thermostable LPE under conditions close to those of an in vivo production system (Matsumoto and Taguchi 2010; Taguchi et al. 2008; Taguchi

2010), we attempted to synthesize the P(LA-*co*-3HB) by employing an in vitro PHA synthesis system developed by R. Jossek and A. Steinbüchel (Fig. 1) (Jossek and Steinbüchel 1998). PHA synthesis using the in vitro reaction system was initiated by the addition of the purified PHA synthase [PhaC1<sub>PS</sub>(STQK) or PhaC1<sub>SG</sub>(STQK): 0.5 mg mL<sup>-1</sup>] into a reaction mixture containing 100 mM sodium phosphate buffer (pH 7.5), total 200 mM substrates [(*R*)-LA and (*R*)-3HB], 10 mM acetate, 30 mM ATP, 2 mM CoA, 10 mM MgCl<sub>2</sub>, BSA (0.2 mg mL<sup>-1</sup>), pyrophosphatase (0.4 U), AcoE (0.2 mg mL<sup>-1</sup>), and PCT (0.2 mg mL<sup>-1</sup>). Reactions were performed in 5 mL of total volume at 30 °C, 37 °C, and 45 °C. After incubation for 72 h, 5 mL of chloroform was poured into the reaction tube. The chloroform and aqueous phases were mixed by a vortex mixer, and then the tube was incubated for 3 h at 70 °C to completely dissolve the product into the chloroform. After cooling the solution to room temperature, the chloroform (lower) phase was filtered through a 0.2- $\mu$ m poly(tetrafluoroethylene) (PTFE) membrane to remove insoluble materials such as denatured enzymes, and then the filtrate was concentrated to approximately 1 mL using an evaporator. Ten milliliters of methanol was added, and then the solution was stored at 4 °C for 16 h to precipitate the polymer. The obtained precipitate was collected using a 0.2- $\mu$ m PTFE membrane, and the dried powder was used for the analysis.

#### Measurements

The <sup>1</sup>H NMR spectra of the polymers were obtained using a Bruker MSL400 spectrometer (400 MHz) at a 90° pulse with a 4-ms, 3,000-Hz spectral width and a 4-s repetition rate. The COSY spectrum was recorded in deuterated chloroform (CDCl<sub>3</sub>) at 25 °C using a Bruker MSL400 spectrometer (400 MHz) and the chemical shifts were reported in parts per million using tetramethylsilane (TMS) as an internal reference. The molar ratios of 3HB and LA units in copolymers were calculated from the ratios of the area of methine protons in 3HB units to that in LA units.

**Fig. 1** Enzymatic synthesis of P(LA-*co*-3HB) by an in vitro reaction system using an LPE



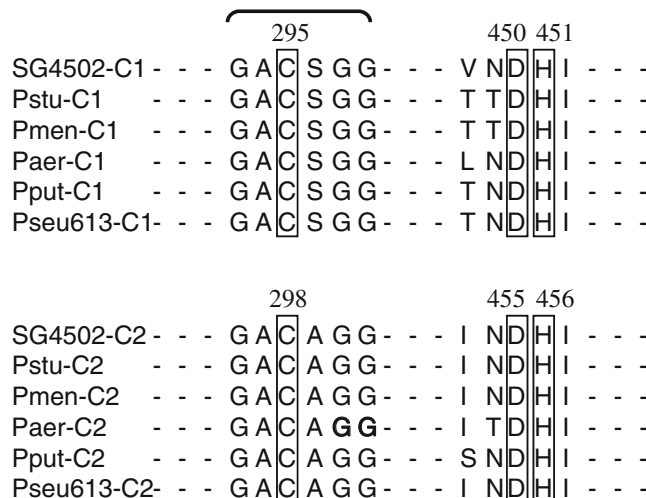
The molecular weights of the obtained polymers were determined by gel-permeation chromatography (GPC) using tandem TSKgel Super HZM-H columns (6.0 mm I.D. × 150 mm; TOSOH, Tokyo) using chloroform as an eluate, and the calibration was performed using polystyrene samples as standards. The formation of CoA derivatives was confirmed HPLC analysis as previously described (Han et al. 2011; Satoh et al. 2003; Tajima et al. 2004).

**Results**

**Cloning of the *pha* locus from *Pseudomonas* sp. SG4502**

To date, class II *pha* gene loci have been cloned from several *Pseudomonas* strains, including *P. stutzeri* (Chen et al. 2004), *P. mendocina* (Hein et al. 2002), *P. aeruginosa* (Timm and Steinbüchel 1992), *P. putida* (Kim et al. 2003), and *Pseudomonas* sp. 61-3 (Matsusaki et al. 1998). In the *pha* loci, there are two PHA synthase genes (*phaC1* and *phaC2*) and one PHA depolymerase gene (*phaZ*), which is located between *phaC1* and *phaC2*. An expected gene fragment of about 5.2 kbp was obtained by PCR using genomic DNA of *Pseudomonas* sp. SG4502 as a template. A plasmid containing the 5.2 kbp DNA fragment was designated as pCR-*pha*SG. The nucleotide sequencing analysis of the inserted gene fragment revealed three ORFs of 1680 bp, 906 bp, and 1692 bp in length (accession no. AB448740). The polypeptides designated as PhaC1<sub>SG</sub>, PhaZ<sub>SG</sub>, and PhaC2<sub>SG</sub> were composed of 559, 301, and 563 amino acid residues with molecular masses of 62.6 kDa, 33 kDa, and 63.0 kDa, respectively. A database search with the Protein BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) for the ORFs showed that their amino acid sequences had high homology with those of PhaC1, PhaZ, and PhaC2 from *Pseudomonas* species (Chen et al. 2004; Hein et al. 2002; Kim et al. 2003; Matsusaki et al. 1998; Timm and Steinbüchel 1992) (Table 2). Figure 2 shows the partial sequence alignments of PhaC1s and PhaC2s from *Pseudomonas* strains. It has been reported that all PHA synthases contain a putative lipase box, [Gly–Xaa–(Ser/Cys)–Xaa–Gly–Gly], in which the essential active site serine is replaced with a cysteine in the PHA synthase (Rehm et al.

**Putative lipase box**



**Fig. 2** Partial alignments of the deduced amino acid sequence of the PHA synthases from *Pseudomonas* sp. SG4502 (SG4502-C1, C2) with those from *P. stutzeri* (Pstu-C1, C2), *P. mendocina* (Pmen-C1, C2), *P. aeruginosa* PAO1 (Paer-C1, C2), *P. putida* (Pput-C1, C2), and *Pseudomonas* sp. 61-3 (Pseu613-C1, C2). PhaC1s and PhaC2s refer to PhaC1<sub>SG</sub> and PhaC2<sub>SG</sub>, respectively

2002; Steinbüchel et al. 1992). In both PhaC1<sub>SG</sub> and PhaC2<sub>SG</sub>, the putative lipase boxes including a cysteine residue were also conserved (Fig. 2). Furthermore, Amara et al. have reported that the highly conserved Cys-296, Asp-452, and His-453 in PhaC1 from *P. aeruginosa* ATCC 15692 were considered to constitute the catalytic triad (Amara and Rehm 2003). Since these amino acids (Fig. 2: Cys-295, Asp-450, and His-451 for PhaC1<sub>SG</sub>; Cys-298, Asp-455, and His-456 for PhaC2<sub>SG</sub>) were conserved in the sequences deduced from *phaC1<sub>SG</sub>* and *phaC2<sub>SG</sub>*, they may constitute a catalytic triad in each PHA synthase.

**Assay of PhaC1<sub>SG</sub>(STQK)**

It has been reported that the reactivities of PHA synthase from *Pseudomonas* sp. 61-3 towards LACoA and 3HBCoA were acquired and increased, respectively, by the introduction of the double mutations (Taguchi and Doi 2004; Taguchi et al. 2008; Tajima et al. 2009). Therefore, the corresponding mutations, Ser324Thr and Gln480Lys, were

**Table 2** Homologies of amino acid sequences deduced from the ORF1, 2, and 3 to those of PHA synthases and depolymerases from other *Pseudomonas* species

Bacterial species	ORF1 PhaC1 (%)	ORF2 PhaZ (%)	ORF3 PhaC2 (%)	Reference
<i>Pseudomonas stutzeri</i>	89	95	89	Chen et al. (2004)
<i>Pseudomonas mendocina</i>	89	94	89	Hein et al. (2002)
<i>Pseudomonas aeruginosa</i> PAO1	89	93	88	Timm and Steinbüchel (1992)
<i>Pseudomonas putida</i>	87	92	84	Kim et al. (2003)
<i>Pseudomonas</i> sp. 61-3	86	92	86	Matsusaki et al. (1998)

introduced into PhaC<sub>1SG</sub> from *Pseudomonas* sp. SG4502 to make PhaC<sub>1SG</sub>(STQK). As expected, the reactivity of PhaC<sub>1SG</sub>(STQK) towards 3HBCoA was increased in the same manner as that of PhaC<sub>1Ps</sub>(STQK) (Table 3, Run 2) (Matsumoto et al. 2009; Taguchi and Doi 2004; Taguchi 2010). The specific activities of PhaC<sub>1SG</sub>(STQK) and PhaC<sub>1Ps</sub>(STQK) were examined at 30 °C, 37 °C, and 45 °C. The specific activity of PhaC<sub>1SG</sub>(STQK) at 45 °C reached almost four times that at 30 °C and was temperature dependent (Table 3, Runs 2–4). We also tried to measure the specific activity at 55 °C, but the substrate, 3HBCoA, decomposed at this high temperature. On the other hand, the specific activity of PhaC<sub>1Ps</sub>(STQK) decreased at 37 °C and was completely diminished at 45 °C (Table 3, Runs 5–7), probably because of the low thermal stability of PhaC<sub>1Ps</sub>(STQK).

#### Thermal stability of PhaC<sub>1SG</sub>(STQK)

To compare the thermal stabilities of PhaC<sub>1SG</sub>(STQK) and PhaC<sub>1Ps</sub>(STQK), polymers were synthesized by the in vitro reaction system (Fig. 1) after heat treatment at various temperatures for 10 min. The yields of polymers are shown in Fig. 3 as relative yields compared to those produced by PHA synthases treated at 30 °C. While the yields by PhaC<sub>1Ps</sub>(STQK) drastically decreased above 37 °C, the yields by PhaC<sub>1SG</sub>(STQK) were constant up to 45 °C, suggesting PhaC<sub>1SG</sub>(STQK) had a higher thermal stability than PhaC<sub>1Ps</sub>(STQK).

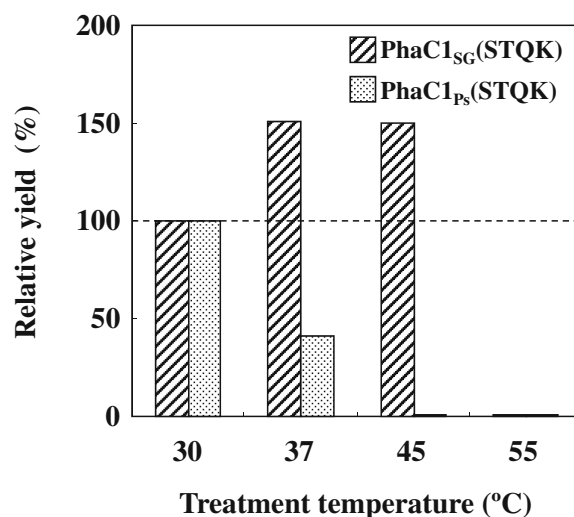
In addition, the time course changes in protein particle sizes of PhaC<sub>1SG</sub>(STQK) and PhaC<sub>1Ps</sub>(STQK) at 37 °C were also measured by the dynamic light scattering method as an index of the thermal stabilities of PHA synthases. The protein particle size of PhaC<sub>1Ps</sub>(STQK) rapidly increased and then decreased (Fig. 4). This drastic change in the

**Table 3** Specific activities of PhaC<sub>1SG</sub>(STQK) and PhaC<sub>1Ps</sub>(STQK) at various temperatures

Run	PHA synthase	Temperature (°C)	Specific activity <sup>a</sup> (U g protein <sup>-1</sup> )
1	PhaC <sub>1SG</sub> (WT)	30	1.0
2	PhaC <sub>1SG</sub> (STQK)	30	2.5
3		37	7.2
4		45	9.5
5	PhaC <sub>1Ps</sub> (STQK)	30	6.7
6		37	3.8
7		45	n.d.

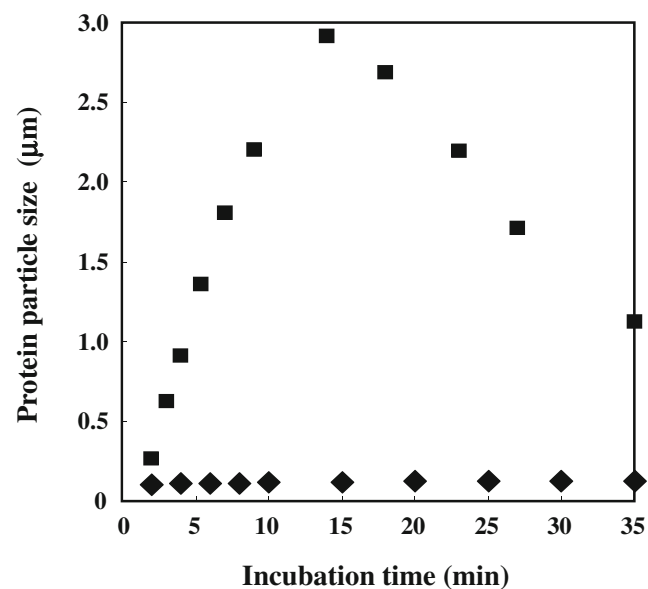
n.d. not determined

<sup>a</sup> Determined by measuring concentration of CoA released during the polymerization reaction of 3HBCoA



**Fig. 3** Comparison of thermal stabilities between PhaC<sub>1SG</sub>(STQK) and PhaC<sub>1Ps</sub>(STQK)

particle size of PhaC<sub>1Ps</sub>(STQK) could be explained by the denaturation and aggregation of the protein molecules. In contrast, the particle size of PhaC<sub>1SG</sub>(STQK) remained virtually constant, suggesting that PhaC<sub>1SG</sub>(STQK) was more stable than PhaC<sub>1Ps</sub>(STQK). From these results, it was suggested that PhaC<sub>1SG</sub>(STQK) had higher thermal stability than that of PhaC<sub>1Ps</sub>(STQK) from a mesophilic *Pseudomonas* and could be used as a thermostable PHA synthase.



**Fig. 4** Time course changes in protein particle sizes of PhaC<sub>1SG</sub>(STQK) (solid rhombus) and PhaC<sub>1Ps</sub>(STQK) (solid square) at 37 °C. The protein particle sizes were determined by dynamic light scattering method

Enzymatic synthesis of P(LA-*co*-3HB) by an in vitro reaction system using PhaC1<sub>SG</sub>(STQK) or PhaC1<sub>PS</sub>(STQK) as a catalyst

To analyze the potential of PhaC1<sub>SG</sub>(STQK) as a thermostable LPE, in vitro synthesis of P(LA-*co*-3HB) was performed at various temperatures. Before the in vitro synthesis, a reaction mixture without PHA synthase was prepared and then incubated at 30 °C to confirm the formation of (*R*)-LACoA and (*R*)-3HBCoA in the in vitro reaction system. HPLC analyses clearly showed the formations of (*R*)-3HBCoA and (*R*)-LACoA (data not shown). Then, PhaC1<sub>SG</sub>(STQK) or PhaC1<sub>PS</sub>(STQK) was added to a reaction mixture containing 100 mM each (*R*)-LA and (*R*)-3HB as substrate precursors. The reaction mixtures became turbid after several hours, and precipitates formed in all the reaction mixtures. <sup>1</sup>H NMR spectra and GPC analyses of the products confirmed the production of P(LA-*co*-3HB) by both PhaC1<sub>SG</sub>(STQK) and PhaC1<sub>PS</sub>(STQK) (Table 4). The molar ratios of LA units in the polymers were around 65 mol% (Table 4, Runs 2, 3, 5, and 6), and the temperature-dependent LA incorporation ratios were not observed for either PhaC1<sub>SG</sub>(STQK) or PhaC1<sub>PS</sub>(STQK). The yields of P(LA-*co*-3HB)s by PhaC1<sub>SG</sub>(STQK) were higher than those by PhaC1<sub>PS</sub>(STQK) (Table 4, Runs 2, 3, 5, and 6). Whereas PhaC1<sub>SG</sub>(STQK) had the polymerizing activity at 45 °C (Table 3, Run 4), polymer was not obtained at 45 °C (Table 4, Run 4). Since ACS and PCT originated from mesophilic bacteria, therefore, this could be due to the denaturation of ACS and/or PCT during a reaction. From the results of the characterization and in vitro synthesis, PhaC1<sub>SG</sub>(STQK) and PhaC1<sub>PS</sub>(STQK) would have similar reactivities towards LACoA and 3HBCoA.

#### Control of molar ratios of LA and 3HB units in polymers

In general, the physical properties of PHA vary with the repeating unit compositions in the polymers. It is easy to control the repeating unit composition in PHA in vitro, that is one advantage of in vitro PHA synthesis. To confirm the

possibility of control in the repeating unit composition, (*R*)-LA and (*R*)-3HB were added to the reaction mixture at various molar ratios (LA/3HB—0:100, 50:50, 75:25, 90:10, and 100:0). The proton NMR spectra of the copolymers obtained from the reaction mixtures with PhaC1<sub>SG</sub>(STQK) are shown in Fig. 5. The intensities of methine (5.16 ppm) and methyl (1.59 ppm) proton peaks in LA-LA\*-LA increased with the increase of LA fraction in the copolymers. The methine and methylene proton peaks (LA-3HB\*-LA) were shifted to the low magnetic field side by the direct linkages with LA units. These data provide direct evidence for the increased concentration of LA repeating unit in the copolymers. The 3HB unit molar ratios in copolymers were calculated from the ratios of the area of methine protons in 3HB units to that in LA units. The repeating unit compositions, yields, and molecular weights of products synthesized by PhaC1<sub>SG</sub>(STQK) or PhaC1<sub>PS</sub>(STQK) at various LA/3HB ratios are summarized in Table 5. The molar ratios of LA in the polymer increased with the increase of the (*R*)-LA concentration in the reaction mixture, suggesting that it is possible to control the repeating unit ratios in the polymers. The maximal molar ratios of LA in the polymers synthesized by the PhaC1<sub>PS</sub>(STQK) and PhaC1<sub>SG</sub>(STQK) were 60 mol% (Table 5, Run 7) and 87 mol% (Table 5, Run 4), respectively. The yields of the polymers decreased with the increase in the ratio of (*R*)-LA (Table 5, Runs 1–4). In the experiment using only (*R*)-LA, the PLA homopolymer could not be synthesized (Table 5, Runs 5 and 10).

#### Synthesis of a copolymer with a block sequence by PhaC1<sub>SG</sub>(STQK) treated with 3HBCoA

Currently, the active polyester synthase has been suggested to form a homodimer considering class I and II polyester synthases (Rehm 2007), and dimerization of PHA synthase is significantly induced in the presence of substrate (Rehm et al. 2001) or trimeric CoA analogs (3-hydroxybutyryl)<sub>3</sub>-CoA (Wodzinska et al. 1996), respectively. To date, complete LA-homopolymer has not been synthesized in

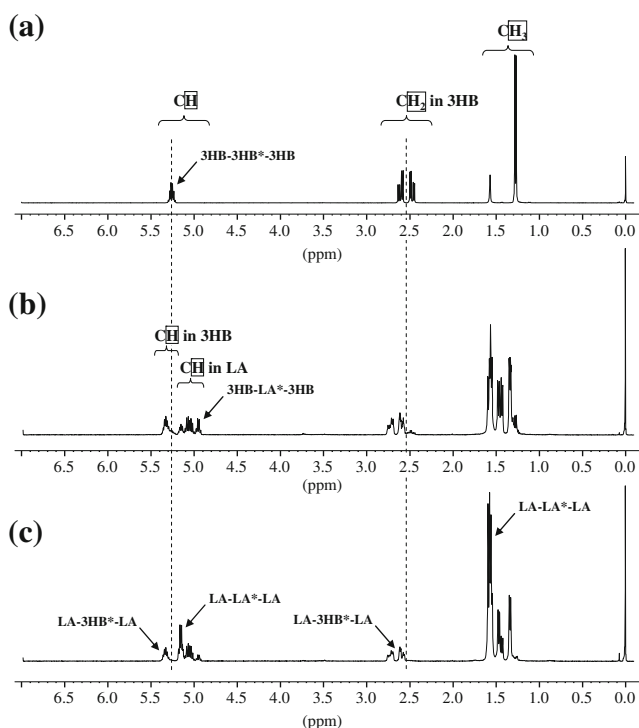
**Table 4** Yields of P(LA-*co*-3HB)s produced by PhaC1<sub>SG</sub>(STQK) and PhaC1<sub>PS</sub>(STQK) at various temperatures

Run	PHA synthase	Temperature (°C)	Molar ratio in a reaction mixture ( <i>R</i> )-LA/( <i>R</i> )-3HB	Molar ratio in a polymer <sup>a</sup> ( <i>R</i> )-LA/( <i>R</i> )-3HB	Yield (g L <sup>-1</sup> )	$M_w \times 10^{-5b}$	$M_w/M_n$
1	PhaC1 <sub>SG</sub> (WT)	30	50:50	n.d.	n.d.	n.d.	n.d.
2	PhaC1 <sub>SG</sub> (STQK)	30	50:50	60:40	0.51	1.5	2.1
3		37	50:50	63:37	0.77	1.5	2.0
4		45	50:50	n.d.	n.d.	n.d.	n.d.
5	PhaC1 <sub>PS</sub> (STQK)	30	50:50	64:36	0.32	1.7	1.9
6		37	50:50	67:33	0.13	n.d.	n.d.
7		45	50:50	n.d.	n.d.	n.d.	n.d.

n.d. not determined

<sup>a</sup>Determined by NMR

<sup>b</sup>Determined by GPC using polystyrene samples as standards in chloroform



**Fig. 5**  $^1\text{H}$  NMR spectra of **a** P(3HB), **b** P(62 mol% LA-co-3HB) (Table 5, Run 2), and **c** P(80 mol% LA-co-3HB) (Table 5, Run 3) produced by PhaC1<sub>SG</sub>(STQK).  $\text{CDCl}_3$  and TMS were used as a solvent and an internal standard

our in vitro experiments, in which only LACoA was supplied to a LPE (Taguchi et al. 2008; Tajima et al. 2009). Therefore, synthesis of a polymer by PhaC1<sub>SG</sub>(STQK) treated with 3HBCoA was performed to confirm that a priming step by a favorable substrate such as 3HBCoA was necessary for LA polymerization by a LPE. PhaC1<sub>SG</sub>(STQK) was incubated with 1 mM (*R*)-3HBCoA for 9 h in 100 mM sodium phosphate buffer (pH 7.5). After the confirmation of a disappearance of (*R*)-3HBCoA by HPLC analysis, LA and other components (ATP and enzymes) were added into

the reaction mixture. After additional incubation (total 72 h), a product was purified by the same method described in the “Materials and methods” section.

The proton NMR spectra of P(3HB), a chemically synthesized block copolymer P(3HB-*b*-LA), and a product obtained from a reaction mixture including PhaC1<sub>SG</sub>(STQK) pretreated with 3HBCoA are shown in Fig. 6. The proton NMR spectrum of the product obviously differs from that of P(LA-co-3HB) with a random sequence (Fig. 5b and c) and is the same with that of the chemically synthesized block copolymer (Fig. 6b). A unimodal peak was observed in the GPC chart of the product. In addition, no polymer was synthesized by PhaC1<sub>SG</sub>(STQK) untreated with 3HBCoA. The repeating unit composition and weight average molecular weight of the product were 3HB/LA=30/70 and  $2.7 \cdot 10^5$ , respectively. From these results, it was suggested that the product synthesized by PhaC1<sub>SG</sub>(STQK) treated by 3HBCoA was a copolymer with a block sequence.

## Discussion

For efficient in vitro enzymatic production of PHA, the stability of an enzyme is essential because denaturation of the enzyme occurs during the course of the reaction. The thermal stability of the enzyme is a key factor. In general, the reaction rate increases depending on the reaction temperature. It is therefore significantly important to obtain thermostable enzyme for efficient enzymatic production with high yields. In this study, we have succeeded in cloning the genes encoding PHA synthases from the previously isolated thermotolerant bacterium *Pseudomonas* sp. SG4502, which was capable of producing PHA even at 55 °C (Satoh et al. 2011). Moreover, the thermal stability test of the recombinant enzyme clearly showed the high thermal stability. To date, various PHA synthases have been obtained from *Pseudomonas* species, but there have been no

**Table 5** Control of molar ratios of LA and 3HB in polymers

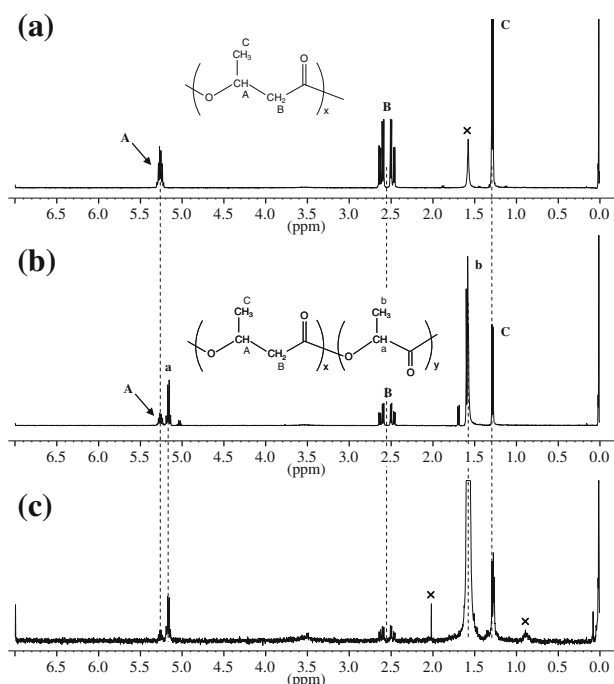
Run	PHA synthase	Temperature (°C)	Molar ratio in a reaction mixture (R)-LA/(R)-3HB	Molar ratio in a polymer <sup>a</sup> (R)-LA/(R)-3HB	Yield (g L <sup>-1</sup> )	$M_w \times 10^{-5b}$	$M_w/M_n$
1	PhaC1SG(STQK)	37	0:100	0:100	1.10	5.3	2.3
2		37	50:50	62:38	0.68	1.5	2.0
3		37	75:25	80:20	0.70	1.4	1.7
4		37	90:10	87:13	0.30	n.d.	n.d.
5		37	100:0	n.d.	n.d.	n.d.	n.d.
6	PhaC1Ps(STQK)	30	0:100	0:100	1.74	5.1	3.8
7		30	50:50	60:40	0.30	1.8	2.0
8		30	75:25	n.d.	n.d.	n.d.	n.d.
9		30	90:10	n.d.	n.d.	n.d.	n.d.
10		30	100:0	n.d.	n.d.	n.d.	n.d.

n.d. not determined

<sup>a</sup>Determined by NMR

<sup>b</sup>Determined by GPC using polystyrene samples as standards in chloroform





**Fig. 6** <sup>1</sup>H NMR spectra of **a** P(3HB), **b** chemically synthesized block copolymer, P(3HB-*b*-59 mol% LA), and **c** a product synthesized by PhaC1<sub>SG</sub>(STQK) treated with 3HBCoA

reports on a PHA synthase with high thermal stability. Therefore, this is the first report of a thermostable PHA synthase from *Pseudomonas* species.

At this stage, we have not addressed the exact reason why the PHA synthase of *Pseudomonas* sp. SG4502 is thermostable. As mentioned above, the result of the homology search using the BLAST program showed that PhaC1<sub>SG</sub>

had high homology to PhaC1<sub>PS</sub> (86% amino acid identity; Table 2). Therefore, we next compared the amino acid compositions of PhaC1<sub>SG</sub> and PhaC1<sub>PS</sub> to reveal the reasons for the differences in their thermal stabilities. PhaC1<sub>SG</sub> has more hydrophobic residues, especially proline and leucine (Fig. 7). A plausible hydrophobic interaction formed by these amino acids could be effective to gain resistance against high temperature-induced protein denaturation. In addition, proline has also been reported to play crucial roles in the thermal stability because of its rigid structure (Watanabe et al. 1997). On the other hand, the numbers of cationic and anionic amino acids and cysteine residues were the same in both PHA synthases (PhaC1<sub>SG</sub> and PhaC1<sub>PS</sub>), suggesting that electrostatic interactions and disulfide linkages are not related to the differences in thermal stability between PhaC1<sub>SG</sub> and PhaC1<sub>PS</sub>.

Since the two amino acid substitutions, Ser325Thr and Gln481Lys, in a PHA synthase from *Pseudomonas* sp. 61-3 were previously demonstrated to gain reactivity towards LACoA and enhance reactivity towards 3HBCoA, the corresponding mutations were introduced into PhaC1<sub>SG</sub> to evaluate the potential of the resulting protein as a thermostable LPE. As expected, the reactivities of the thus constructed PhaC1<sub>SG</sub>(STQK) towards 3HBCoA and LACoA were increased and acquired, respectively, in the same manner as those of the PHA synthase from *Pseudomonas* sp. 61-3 (Tables 3 and 4). Since the repeating unit compositions in the polymers produced by PhaC1<sub>SG</sub>(STQK) and PhaC1<sub>PS</sub>(STQK) were almost the same (Table 4), PhaC1<sub>SG</sub>(STQK) and PhaC1<sub>PS</sub>(STQK) would have similar substrate specificities towards 3HBCoA and LACoA. Considering the high homologies between PhaC1<sub>SG</sub> and PhaC1<sub>PS</sub>

**Fig. 7** Alignments of the amino acid sequence of the engineered PHA synthases from *Pseudomonas* sp. SG4502 [PhaC1<sub>SG</sub>(STQK)] and *Pseudomonas* sp. 61-3 [PhaC1<sub>PS</sub>(STQK)]. The asterisks mean the mutation points

PhaC1 <sub>SG</sub> (STQK)	1: MNKIAE-DLQ	ROQASEHTL	SLNPNV	GLRGKDLLS	TRCQV	LVQALR	QPLHSTR	VHAFG	VQ	59
PhaC1 <sub>PS</sub> (STQK)	1: MSKNSSD	DLNRQASEHTL	GLNPNV	GLRGKDLLS	SARMV	LQAI	KQPLHSTR	VHAFG	IEL	60
PhaC1 <sub>SG</sub> (STQK)	60: KNVLL	QADL	KPE	DGRRFP	ADPAW	SHNPLY	RRYMOLY	LAWR	QELHD	WI
PhaC1 <sub>PS</sub> (STQK)	61: KNVMP	KSKLQ	PE	SDRRFP	ADPAW	SHNPLY	RRYMOLY	LAWR	QELHD	WI
PhaC1 <sub>SG</sub> (STQK)	120: GHFV	INLL	TEAL	APS	NSLAN	PAAL	KRFP	FETGGK	SLLD	GL
PhaC1 <sub>PS</sub> (STQK)	121: AHFV	ITL	TEAL	MAP	NSLAN	PAAV	KRFP	FETGGK	SLLD	GL
PhaC1 <sub>SG</sub> (STQK)	180: FEVGN	LAL	TEGAV	VFRND	LLE	L	IQYR	PT	TEQV	HAR
PhaC1 <sub>PS</sub> (STQK)	181: FEVGK	SL	GT	TEGAV	VFRND	VLE	L	IQYR	PT	TEQV
PhaC1 <sub>SG</sub> (STQK)	240: RFAL	RSGL	QTF	IL	SWRN	PTR	Q	REWGL	STYI	EAL
PhaC1 <sub>PS</sub> (STQK)	241: RFCT	SNNQ	QTF	IV	SWRN	PTR	Q	REWGL	STYI	DAL
PhaC1 <sub>SG</sub> (STQK)	300: ITA	ALLGH	YAA	RGE	PI	HAL	TLL	VT	VLD	TE
PhaC1 <sub>PS</sub> (STQK)	301: ICT	ALLGH	YAA	RG	E	CKV	NAL	TLL	VT	VLD
PhaC1 <sub>SG</sub> (STQK)	360: NLAK	LFAW	MRP	NDL	IWN	VWV	NNYLL	GR	PP	FD
PhaC1 <sub>PS</sub> (STQK)	361: DMAK	VFAW	MRP	NDL	IWN	VWV	NNYLL	GR	NE	PP
PhaC1 <sub>SG</sub> (STQK)	420: LFP	PGALE	VCG	T	PID	LKQ	V	KSD	LY	CV
PhaC1 <sub>PS</sub> (STQK)	421: LVR	ANALE	VSG	T	PID	LKQ	V	TAD	LY	SL
PhaC1 <sub>SG</sub> (STQK)	480: KAL	LNPP	GNPK	AR	F	MT	T	NGEL	P	TE
PhaC1 <sub>PS</sub> (STQK)	481: KSI	LNPP	GNPK	SR	F	MT	T	STD	M	P
PhaC1 <sub>SG</sub> (STQK)	540: LGNK	QYPS	GEA	AP	GT	YV	V	H		
PhaC1 <sub>PS</sub> (STQK)	540: LGNK	AYPS	GEA	AP	GT	YV	V	H		

(Table 2), the alteration of the substrate specificity in PhaC<sub>1SG</sub>(STQK) could be due to conformational changes identical to those occurring in PhaC<sub>1PS</sub>(STQK).

Very recently, Shozui et al. reported that a copolymer containing 96 mol% LA was produced in recombinant *E. coli* expressing PhaC<sub>1PS</sub>(STQK) (Shozui et al. 2010a, b). However, in our in vitro study, the maximal molar ratios of LA in the polymers synthesized by the PhaC<sub>1PS</sub>(STQK) and PhaC<sub>1SG</sub>(STQK) were up to 60 mol% (Table 5, Run 7) and 87 mol% (Table 5, Run 4), respectively. Since PhaC<sub>1SG</sub>(STQK) and PhaC<sub>1PS</sub>(STQK) had similar substrate specificities towards 3HBCoA and LACoA, the higher production of P(87 mol% LA-co-3HB) by PhaC<sub>1SG</sub>(STQK) in the in vitro reaction system may have been due to the increase in the reaction rate with the reaction temperature elevation. Therefore, the increase in the reaction rate is essential for the high yield production of LA-enriched polymers, and the high thermal stability of PhaC<sub>1SG</sub>(STQK) could be a significantly useful feature as an LPE. Furthermore, LPEs are derived from class II PHA synthases that can polymerize various kinds of 3HAs. In fact, the in vivo synthesis of P[LA-co-3HB-co-3-hydroxyvalerate], P[LA-co-3HB-co-3-hydroxyhexanoate (3HHx)], and P[LA-co-3-hydroxyalkanoates (3HAs)] has been achieved by PhaC<sub>1PS</sub>(STQK) (Matsumoto et al. 2011; Shozui et al. 2010a, b, 2011). For PhaC<sub>1SG</sub>, it has been confirmed that *P. putida* Gpp104 expressing PhaC<sub>1SG</sub> was able to produce various PHAs, including 3HB, 3HHx, 3-hydroxyoctanoate, 3-hydroxydecanoate, and 3-hydroxydodecanoate (unpublished data), and it would be possible to synthesize various polyesters including LA by using PhaC<sub>1SG</sub>(STQK) as an LPE. Thus, the synthesis of various kinds of polyesters including LA is one of the advantages of the enzymatic synthesis using an LPE (Taguchi 2010), and more efficient enzymatic production will be achieved by using the thermostable LPE from *Pseudomonas* sp. SG4502.

However, to date, a polyester consisting of the complete 100 mol% LA (PLA) has not been produced by PhaC<sub>1SG</sub>(STQK) and PhaC<sub>1PS</sub>(STQK) either in vitro or in vivo (Matsumoto and Taguchi 2010; Shozui et al. 2010a, b, 2011; Song et al. 2011; Taguchi et al. 2008; Tajima et al. 2009). Notably, the fact that a polymer was not produced in an in vitro system that contained only LACoA without other HACoAs strongly suggests the necessity of coexistence of preferable substrates such as 3HBCoA in LA-based polyester production by LPEs. So far, copolymers containing 96 mol% LA and 99.3 mol% LA were produced in recombinant *E. coli* (Shozui et al. 2010a, b) and *Corynebacterium glutamicum* (Song et al. 2011) by using PhaC<sub>1PS</sub>(STQK), respectively. As demonstrated in these bacterial systems, a small amount of an intrinsic HACoA exists within a cell; therefore, it may act as a priming molecule for the reaction of LPE in vivo also.

Currently, the trial synthesis of homopolymers and block copolymers has been attempted by using natural or artificially mutated bacterial strains (Hu et al. 2011; Penderson et al. 2006). In microbial polymer production system, however, finely regulated synthesis of the repeating units might be difficult because of a significant effect of metabolic background of the microbial cells. On the other hand, the in vitro polymerization system has a potential for generation of various copolymers finely incorporating unnatural repeating units with a block sequence. Considering the necessity of 3HBCoA for LA incorporation, we attempted to make LA-based copolymer with PhaC<sub>1SG</sub>(STQK) by supplying 3HBCoA as a primary substrate and followed by LACoA as a secondary substrate into the reaction system. Based on the identical NMR profiling with chemically synthesized P(3HB-*b*-LA), we have finally demonstrated the first synthesis of the block-type polymer containing LA (Fig. 6). For this achievement, requirement of 3HBCoA as a priming unit for LA incorporation into the polymer chain is very advantageous to prepare structurally finely regulated P(3HB-*b*-LA), while P(LA-*b*-3HB) generation seems to be not easy. Most recently, the in vitro system provided us with the first finding that 2-hydroxybutyrate can be incorporated into the polymer by class I PHA synthase from *Ralstonia eutropha* (Han et al. 2011) as well as LPE [PhaC<sub>1PS</sub>(STQK)] (will be soon published elsewhere). Moreover, another unnatural 2-hydroxyalkanoate, glycolate, was found to be a repeating unit incorporated into the polymer chain using the same LPE (Matsumoto et al. 2011). To our knowledge, this is the first report to synthesize a copolymer with block sequence by an in vitro reaction system, and it will be possible to produce novel biopolyesters incorporating natural and unnatural repeating units with random or block sequences, depending on the way of supplying substrates.

In conclusion, we cloned a *pha* locus from thermotolerant *Pseudomonas* sp. SG4502 and revealed that the locus contained two genes encoding PHA synthases (PhaC<sub>1SG</sub> and PhaC<sub>2SG</sub>) to establish a PHA production system with long-term stability and high yield. The two amino acid substitutions, Ser324Thr and Gln480Lys, which were previously demonstrated to confer the reactivity towards LACoA with enhanced 3HBCoA reactivity in a PHA synthase from *Pseudomonas* sp. 61-3, were introduced to construct a mutated PHA synthase [PhaC<sub>1SG</sub>(STQK)] (Ser324Thr/Gln480Lys). The enzyme also showed high activity even at 45 °C and was able to synthesize P(LA-co-3HB)s with high yields. The molar ratios of LA in the copolymers were controllable in the range of 0 to 87 mol%, and the yield and weight average molecular weight of P(80 mol% LA-co-3HB) were 0.70 gL<sup>-1</sup> and 1.4 · 10<sup>5</sup>, respectively. To date, although several mutated PHA synthases from mesophilic *Pseudomonas* species have been reported as LPEs (Matsumoto and Taguchi 2010; Taguchi

et al. 2008; Taguchi 2010; Tajima et al. 2009; Yamada et al. 2010; Yang et al. 2011), there have been no reports of LPEs with high thermal stabilities. Therefore, PhaC1<sub>SG</sub>(STQK) is the first thermostable LPE. The P(LA-co-3HB) production at higher temperature (45 °C) could not be performed, probably because of the low thermal stabilities of the enzymes, ACS and PCT, employed for HACoAs supply; however, such production will be possible with thermostable ACSs and PCTs. Such studies of this line are now in progress and will be reported in the near future.

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