

# *In Vitro* Evolution of a Polyhydroxybutyrate Synthase by Intragenic Suppression-Type Mutagenesis<sup>1</sup>

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*In vitro* evolution was applied to obtain highly active mutants of *Ralstonia eutropha* polyester synthase (PhbC<sub>Re</sub>), which is a key enzyme catalyzing the formation of polyhydroxybutyrate (PHB) from (*R*)-3-hydroxybutyryl-CoA (3HB-CoA). To search for beneficial mutations for activity improvement of this enzyme, we have conducted multi-step mutations, including activity loss and intragenic suppression-type activity reversion. Among 259 revertants, triple mutant E11S12 was obtained as the most active one *via* PCR-mediated secondary mutagenesis from mutant E11 with a single mutation (Ser to Pro at position 80), which exhibited reduced activity (as low as 27% of the wild-type level) but higher thermostability compared to the wild-type enzyme. Mutant E11S12 exhibited up to 79% of the wild-type enzyme activity. Mutation separation of E11S12 revealed that the replacement of Phe by Ser at position 420 (F420S), located in a highly conserved  $\alpha/\beta$  hydrolase fold region, of the E11S12 mutant contributes to the improvement of the enzyme activity. A purified sample of the genetically engineered mutant, termed E11S12-1, with the F420S mutation alone was found to exhibit a 2.4-fold increase in specific activity toward 3HB-CoA, compared to the wild-type.

**Key words:** activity improvement, error-prone PCR, intragenic suppression-type mutagenesis, *in vitro* evolution, polyhydroxybutyrate synthase.

One of the naturally occurring biopolymers, polyhydroxyalkanoate (PHA), is recognized to be a sink for carbon and reducing equivalents in bacteria producing it. PHAs are bio/environmentally acceptable materials that can be used in place of conventional petrochemical-based plastics (1). During the last 20 years, approximately 150 different PHAs have been isolated from many bacteria, and their biosynthesis genes have been identified and isolated (2). Until now, the improvement of bacterial PHA production has been achieved through fermentation technology of natural PHA-producing bacteria or the gene dosage effect of PHA biosynthesis genes including the PHA synthase (PhaC) one in recombinants (3).

For enhanced production and quality alteration of PHA, we have applied the *in vitro* evolution technique to a key enzyme, PHA synthase, essential for PHA biosynthesis, whose performance is closely related to the properties of the polyesters generated, such as molecular weight, polydispersity, and compositional variation upon copolymerization. *Ralstonia eutropha* synthase (termed PhaC<sub>Re</sub> or PhbC<sub>Re</sub>), which has been the biochemically best-studied

Class I enzyme (4, 5), was chosen as a model target for *in vitro* evolution. Polyhydroxybutyrate (PHB), a typical PHA, can be synthesized and accumulated in *Escherichia coli* through introduction of the *phbC*<sub>Re</sub> gene with monomer supplying enzyme genes, *phbA*<sub>Re</sub> and *phbB*<sub>Re</sub> (3). The latter two genes encode a  $\beta$ -ketothiolase and an acetoacetyl-CoA reductase, respectively. These PHB synthesis genes form an operon in the order of *phbC*<sub>Re</sub>, *phbA*<sub>Re</sub>, and *phbB*<sub>Re</sub>. The difficulty with *in vitro* evolution for PHB synthase lies in the construction of an efficient selection system. Although not positive selection, we have already established a simple *in vivo* assay system for PhbC<sub>Re</sub> based on PCR-mediated random mutagenesis and two analytical procedures for the screening of mutant enzymes (6, 7). This *in vivo* system enables us to readily estimate the synthase activity by monitoring the level of PHB accumulated within the recombinant *E. coli* cells. Until now, however, no mutant enzymes with higher activities could be obtained.

In the present work, intragenic suppression-type mutagenesis was attempted to obtain highly active mutants using an activity lowered but thermostable primary mutant, E11 (6, 7), as a starting template for secondary mutation. Mutant E11 was a good target for investigating the relationship between enzyme activity and thermostability through an evolutionary experiment. One of the activity reverted mutants, E11S12, was found to possess a beneficial mutation (Phe to Ser at position 420, F420S), and exhibited a 2.4-fold activity increase compared to the wild-

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type enzyme. This report presents the first case of the isolation of an evolved PhbC<sub>Re</sub> with higher activity.

#### MATERIALS AND METHODS

**Bacterial Strains, Plasmids, and Culture Conditions**—*E. coli* strains JM109 (8) and UT5600 (9) were used as hosts for transformation with plasmids and for PHB accumulation. The UT5600 (*ompT*<sup>-</sup>) strain and overexpression plasmid vector for the Phb<sub>Re</sub> gene, pKAS4, were kindly provided by Prof. Sinskey, Massachusetts Institute of Technology. *E. coli* was routinely grown in Luria-Bertani (LB) medium at 37°C. When needed, ampicillin (50 µg/ml) was added to the medium. Growth was monitored by measuring the optical density at 600 nm (*A*<sub>600</sub>). *E. coli* JM109 was used for all standard cloning procedures, and as the host strain for screening mutants of *R. eutropha* H16 PHB synthase (PhbC<sub>Re</sub>) and for PHB accumulation from glucose. Plasmid pGEM'-*phbCAB*<sub>Re</sub> carries PHB polycistronic genes for PhbC<sub>Re</sub> and (*R*)-3-hydroxybutyryl-CoA monomer substrate-supplying enzymes, PhbA<sub>Re</sub> (β-ketothiolase) and PhbB<sub>Re</sub> (NADPH-dependent acetoacetyl-CoA reductase) (10). Single mutant E11S12-1 carrying mutation F420 alone was genetically prepared with mutant E11S12 and the wild-type genes, both of which are placed on pGEM'-*phbCAB*<sub>Re</sub>, by using unique *Bgl*II and *Sse*8387I sites. For overexpression of the genes coding for the wild-type and mutant E11S12-1 synthases, their genes were inserted into the downstream of the IPTG-inducible *trc* promoter of vector pKAS4 (11) using *Not*I and *Sse*8387I sites. For PHB accumulation, recombinant JM109 and UT5600 strains were grown on LB medium containing 2% glucose. All cells were cultivated for 14 h at 37°C. When needed, ampicillin (50 µg/ml) was added to the medium.

**DNA Manipulation and Sequencing**—Standard recombinant DNA manipulations (12) were performed for the isolation of plasmid DNA, restriction digestion, ligation and transformation of *E. coli*. All restriction enzymes and related reagents for DNA manipulation were commercially available and used according to the suppliers' recommendations. All other chemicals were of biochemical analytical grade and were used without further purification. DNA sequencing for analysis of the mutation points was carried out by the dideoxy chain termination method with a Prism 310 DNA sequencer or a Prism 377 DNA sequencer (Applied Biosystems) using a BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems). The nucleotide sequence data and deduced amino acid sequences of PhbC<sub>Re</sub> were analyzed with GENETYX-MAC software (Software Development, Tokyo).

**Random Mutagenesis by Error-Prone PCR**—Random mutations were introduced into the entire region of the *phbC*<sub>Re</sub> gene by error-prone PCR, as described previously (6, 7). pGEM'-*phbCAB*<sub>Re</sub> carrying the single mutation of S80P (clone E11) was used as a template for secondary PCR random mutagenesis with the forward and reverse primers under error-prone conditions with 5 mM MgCl<sub>2</sub> and 10% dimethyl sulfoxide. PCR was carried out using a program of 25 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min with a Gene Amp PCR System 9700 (Perkin-Elmer Applied Biosystems). These conditions should generate an error frequency of approximately one substitution per 1,000 bases, or approximately one or two amino

acid substitutions per *phbC*<sub>Re</sub> gene (approximately 1,770 bases). After amplification, a mixture of the *Csp*451–*Sse*8387I fragments, including the secondarily mutagenized *phbC*<sub>Re</sub> genes, was reintroduced into the same restriction sites of pGEM'-*phbCAB*<sub>Re</sub> carrying the wild-type *phbC*<sub>Re</sub> gene to generate a mutant library. The resultant plasmids were introduced into *E. coli* JM109 cells, and the resulting transformants were spread on LB plates containing 2% glucose, 0.5 µg/ml of Nile red dye, and 50 µg/ml of ampicillin.

**Screening of Mutants Leading to Reverted PHB Accumulation**—The recombinants harboring *phbC*<sub>Re</sub> secondarily mutagenized genes were grown on LB plates supplemented with 2% glucose, 0.5 µg/ml Nile-red, and 50 µg/ml ampicillin. The change in PHB accumulation resulting from the introduction of secondary mutations into the *phbC*<sub>Re</sub> gene with the mutation of S80P was judged on the basis of the intensity of the pinkish pigmentation of the cells caused by Nile red staining (13) using clone E11 as a reference. For precise quantification of the cellular PHB accumulation, mutants were cultivated in LB medium containing 2% glucose at 37°C for 14 h. The cellular PHB content was determined by analytical high-performance liquid chromatography (HPLC) after the cellular PHB had been converted to crotonic acid by treatment with hot concentrated sulfuric acid (H<sub>2</sub>SO<sub>4</sub>) (14).

**Overproduction and Purification of PhbC<sub>Re</sub>**—For purification of the wild-type and mutant (E11S12-1) synthases, recombinant cells harboring each pKAS4-based expression vector were grown on a 50 ml culture scale at 30°C to an *A*<sub>600</sub> of 0.5 and subsequently induced by the addition of IPTG to a final concentration of 0.4 mM, followed by further 4-h cultivation at 30°C. After centrifugation (5,000 ×g for 10 min), a precipitated sample was suspended in 50 mM phosphate buffer (pH 7.0). Cell disruption was carried out by use of a French press (three times) to yield a crude cell extract. The soluble fraction was obtained by centrifugation (5,000 ×g for 40 min) and then passed through a 0.45 µm filter. Salting out of protein samples was carried out by step-wise addition of two different concentrations, 15% (for removal of the extraneous proteins) and 50% (for concentration of PhbC<sub>Re</sub>), of ammonium sulfate. The precipitate was dissolved in 50 mM phosphate buffer (pH 7.0) containing 1 M ammonium sulfate, 5% glycerol, and 0.05% Hecameg (for enhanced recovery of proteins from the column). Finally, the active fraction was applied directly to a methyl-HIC column (Bio-Rad), followed by elution with a linear gradient of 1 to 0 M ammonium sulfate to yield the purified active fraction of PhbC<sub>Re</sub>.

**Enzyme Assay and Electrophoresis**—Whole cell extracts of recombinant *E. coli* cells were disrupted by sonication (TOMY UD-200). The CoA release activity of PhbC<sub>Re</sub> in the cell extracts or purified protein samples was determined spectrophotometrically by means of a discontinuous assay using chemically synthesized (*R*)-3HB-CoA and 5,5'-dithio-bis(2-nitrobenzoic acid), as described by Gerngross *et al.* (11). One unit of enzyme activity is defined as the amount required to catalyze the transformation of 1 µmol substrate/min·mg of total cellular proteins. The concentration of total cellular proteins was determined by the method of Bradford (15) using a Bio-Rad Protein Assay kit (Hercules, CA) and BSA as the standard.

The amount and purity of PhbC<sub>Re</sub> were analyzed by

sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under the standard conditions (16). Western blot analysis of PhbC<sub>Re</sub> was carried out using the specific anti-serum toward the C-terminal oligopeptide of PhbC<sub>Re</sub> prepared previously (17).

**Thermostability Measurement**—Several aliquots of each crude cell extract were incubated at 50°C for various times and then immediately subjected to the assay system for measuring the residual enzyme activity, as described above. The thermostability of the enzyme was defined as the half-life for inactivation of the enzyme at 50°C.

RESULTS

**Properties of Mutant E11 as a Template for Secondary Mutagenesis**—In our previous study, PhbC<sub>Re</sub> activity was correlated with the level of PHB accumulation (6, 7). For the *E. coli* host dependency of the PHB accumulation, similar relative PHB accumulation values for the wild-type and seven mutants (B1, P5, E11, 1-11, M22, 2-12, and 1-14) characterized were obtained for two *E. coli* hosts, the JM109 and UT5600 strains. However, between the two strains, only single mutant E11 (S80P) with reduced activity exhibited remarkable deviation from the good correlation of PHB accumulation with synthase activity, as shown in Table I. Namely, the relative PHB content of mutant E11 as to those of the others in JM109 was much higher than that in UT5600. This deviation was considered to be attributable to the difference in the physiological environments for the gene products in the two strains.

Then, the amount of the mutant E11 protein yielded from the recombinant cells was compared between the two recombinants by Western blot analysis using the wild-type as a reference. As shown in Fig. 1, a higher amount of mutant E11 compared to the wild-type was observed in JM109, while no difference in the amounts of the two enzymes was observed in UT5600. Proteolytic degradation of recombinant PhbC<sub>Re</sub> by OmpT protease was reported to occur upon cell lysis of *E. coli* JM105 (11). This fact suggests that with the S80P mutation, mutant E11 would acquire higher stability of the protein structure associated with protease resistancy during the cell lysis of *E. coli ompT*<sup>+</sup> strain, JM109.

Thus, we examined the thermostability of mutant E11 along with that of the wild-type enzyme, produced by the *ompT*<sup>-</sup> UT5600 strain. The half-life for enzyme inactivation at 50°C was 2.3 min for mutant E11 and 1.7 min for the wild-type. This higher heat resistancy of mutant E11 could

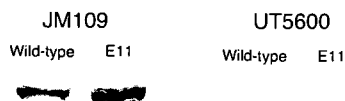


Fig. 1. Western blot analysis for quantification of *Ralstonia eutropha* PHB synthases extracted from recombinant cells of *Escherichia coli* strains JM109 and UT5600. An aliquot of each crude protein sample normalized as to cell density was evenly applied to an SDS-PAGE gel. Western blot analysis was performed using PhbC<sub>Re</sub> C-terminal specific antibodies raised in a rabbit. Indication of each sample is given the corresponding lane. The difference in the intensities of protein bands between the two strains simply depends on the immunological staining conditions employed for each host strain.

be achieved only by single amino acid replacement of S80P. It was of interest to investigate the changes in enzyme activity and also this thermostable character of a template mutant E11 caused by the next round of mutagenesis.

**Secondary Mutagenesis of Mutant E11**—Secondary random mutagenesis was applied to the entire region of the PhbC<sub>Re</sub> gene coding for the template mutant E11. The pGEM<sup>+</sup>-*phbCAB*<sub>Re</sub>-based expression plasmids including heterogeneous mutant enzyme genes were introduced into JM109 host cells, and ampicillin-resistant transformant colonies formed at 37°C on assay plates supplemented with 2% glucose and 0.5 μg/ml of Nile red. As a result, 259 transformant colonies, which exhibited equal or greater pinkish color intensity than that of mutant E11, were screened and picked up from a total of 3,707 colonies. The secondary mutant, E11S12, with the most increased PHB content was investigated in detail to analyze the effect of secondary mutation(s) on the enzyme activity and thermostability of the primary mutant E11.

Figure 2 shows the two-dimensional profile of enzyme activity and thermostability (expressed as the inactivation half-life at 50°C) for the wild-type and its offspring mutant

TABLE I. Accumulation of PHB in *Escherichia coli* recombinants<sup>a</sup> of the JM109 and UT5600 strains.

Mutant (amino acid replacement)	PHB content (wt%) in JM109 <sup>b</sup> (relative to wild-type)	PHB content (wt%) in UT5600 <sup>c</sup> (relative to wild-type)
Wild-type	69.0 (100%)	37.9 (100%)
B1 (N519S)	49.0 (71%)	26.4 (70%)
P5 (A391T, T393A)	31.5 (46%)	20.0 (53%)
E11 (S80P)	13.7 (23%)	2.5 (6.7%)
1-11 (I313T, N426S)	5.6 (20%)	12.1 (32%)
M22 (V470M)	2.6 (8%)	3.1 (8%)
2-12 (L358P)	0.9 (4%)	0.21 (0.5%)
1-14 (I148V, S174P)	0.3 (1%)	0.14 (0.4%)

<sup>a</sup>Cells were cultivated in 1.7 ml of LB medium containing 2% glucose for 14 h at 37°C. <sup>b,c</sup>The data shown are the means for three or four independent experiments.

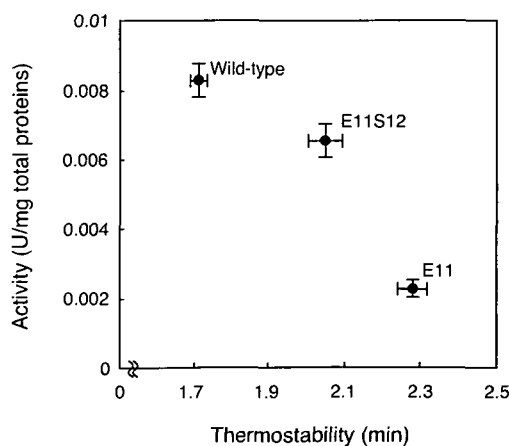
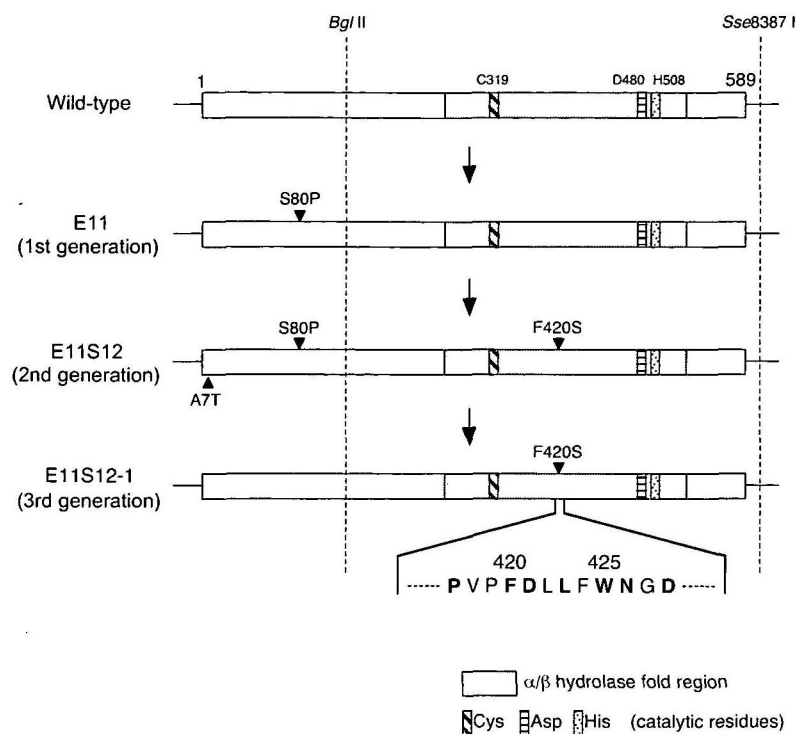
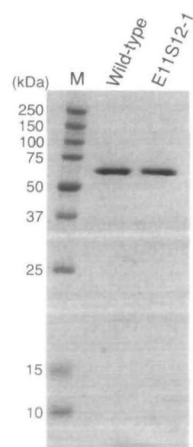


Fig. 2. Two-dimensional presentation of the enzyme activities and thermostabilities of the parent wild-type and its offspring mutant enzymes. Enzyme activity was assayed as CoA-release ability with (*R*)-3HB-CoA as the substrate. The residual enzyme activity after exposure to 50°C for various times was assayed at 25°C by adding protein samples to the reaction system. The details of these measurements are given under “MATERIALS AND METHODS.”



**Fig. 3. Evolutionary lineage of the PHB synthase through *in vitro* evolution.** A region consisting of approximately 250 amino acids carrying an “ $\alpha/\beta$  hydrolase fold” is commonly shared by all synthases belonging to classes I to III, and is assigned to positions 270 to 526 of PhbC<sub>Re</sub>. The corresponding regions are shaded. The active site residue, cysteine-319, is located within a lipase box-like sequence (G-F-C319-V-G-G) in the  $\alpha/\beta$  hydrolase fold region (5). This residue forms a catalytic diad with histidine-508. Aspartic acid-480 is proposed to be a general basic catalyst for the activation of 3HB-CoA. The N-terminal non-conserved region (positions 1 to 98) is dotted. Amino acid replacements are indicated by closed triangles. For mutant E11S12-1, the surrounding amino acid sequence of F420 is given for discussion related to the structure-function relationship. Bold-faced letters represent highly conserved amino acids among the PHA synthases isolated so far (2). Two unique sites, *Bgl*II and *Sse*8387I, are available for the generation of single mutant E11S12-1 with the wild-type and mutant E11S12 genes.



**Fig. 4. Electrophoretic analysis of the purified PHB synthases.** Five microgram aliquots of the purified enzyme proteins were subjected to SDS-PAGE.

enzymes, E11 and E11S12. Reverted mutant E11S12 exhibited activity improvement of up to 2.9-fold compared to mutant E11, although it was accompanied by a slight reduction in thermostability. Thus, thermostabilizing mutations tended to deactivate, while activating mutations tended to destabilize.

DNA sequencing revealed that double mutations (A7T and F420S) were introduced to generate second-site revertant E11S12, as shown in Fig. 3. The amino acid replaced positions, A7T and F420S, correspond to the non-conserved region and the  $\alpha/\beta$  hydrolase fold region, respectively.

**Generation and Characterization of Mutant E11S12-1**—To begin with, we decided to construct a new mutant derivative (termed E11S12-1) possessing only an F420S replace-

ment using DNA fragments coding for the wild-type and mutant E11S12 by the use of intervening restriction sites, *Bgl*II and *Sse*8387I. After overexpression of the mutant E11S12-1 gene using an *E. coli* host-vector system (*ompT*<sup>-</sup>UT5600-pKAS4), synthase activity toward 3HB-CoA was measured with a cell-free crude extract as the enzyme source as well as the wild-type. When normalized as to the concentration of extracted proteins, mutant E11S12-1 was found to be approximately 2-fold more active than the wild-type enzyme.

Finally, both enzymes were purified to electrophoretic homogeneity (see Fig. 4) from 50 ml of culture medium according to the purification procedure described under “MATERIALS AND METHODS.” It was demonstrated that mutant E11S12-1 exhibits 2.4-fold specific activity of the wild-type. As for thermostability, this mutant was slightly lower than the wild-type.

## DISCUSSION

In this study, for the first time, we obtained an evolved mutant (E11S12-1) with increased activity of *R. eutropha* PHB synthase (PhbC<sub>Re</sub>). Since it is not easy to distinguish subtle differences in activity among many activity-positive mutants and the wild-type under the screening conditions employed here, a more reliable evolutionary program was required to explore the beneficial mutations for activity improvement. The intragenic suppression-type mutagenesis (18–22) attempted here was very useful for this requirement. This approach is technically advantageous in that it allows us to obtain, in a positive selection manner, improved mutant PHB synthases from the primary mutant enzyme with reduced activity, compared to the screening from the library including the wild-type molecules and molecules with apparently increased activity. In short, on rare

occasions, revertants can arise through mutation at a second site with retention of the original mutation. Hence, it should be noted that the primary mutation seems crucial in the sense that each mutational event determines both the possibility and character of the secondary mutation. Indeed, it was observed for another enzyme, protease subtilisin BPN', that some primary mutants gave secondary mutants with reverted activity and others not (18–21). In this sense, single mutant E11 (S80P) was an adequate template for generating the next mutation to seek beneficial mutations such as F420S obtained here.

As for intragenic suppression-type mutagenesis, second-site reversion is dependent on or independent of primary mutation in the activity. Secondary mutation of F420S was the latter case, being independent of the primary mutation of S80P. A similar case was demonstrated for the thermostabilization of yeast iso-1-cytochrome *c* (22). On the other hand, one of the present authors previously experienced the former case when working on the cold-adaptation of an industrial protease, subtilisin BPN' (18–21). It is of interest to determine which is the case for another secondary mutation (A7T) of E11S12. Possibly, this mutation might not have a remarkable influence on the enzyme activity, judging from the evidence that the highly variable N-terminus is not essential for the synthase activity (23). Thus, it can be imagined that there may be diverse pathways for enzyme improvement, which prompts us to use a lot of primary mutants of PhbC<sub>Re</sub> as a set for secondary mutagenesis to efficiently increase the possibility for exploring beneficial mutations.

The second-site, F420, for reversion of the enzyme activity, is located in the relatively hydrophobic region, as shown in Fig. 3. A Phe at this position is almost completely conserved among class I and II synthases isolated so far (2). Activity improvement resulting from the replacement of Phe by Ser can not be readily predicted by a rational protein design, and indeed successful *in vitro* evolution of PhbC<sub>Re</sub> has not been achieved by means of a site-specific mutagenesis approach (24). From the evidence reported by Junker *et al.* (23), mutation of highly conserved W425 resulted in remarkably reduced dimerization indispensable for full synthase activity. In contrast, amino acid replacement at F420 adjacent to W425 might have a positive influence as to dimer formation closely related to the synthase activity.

Recently, through an *in vitro* evolution system, we succeeded in acquiring evolved mutants with higher activities of *Aeromonas caviae* synthase (PhaC<sub>Ac</sub>), which led to enhanced accumulation and changed monomer compositions of PHA copolyesters (25). Unfortunately, at present, a detailed biochemical study can not be performed *in vitro* because of difficulty in the overproduction of the active form of PhaC<sub>Ac</sub>. In contrast, the beneficial mutant, E11S12-1, of PhbC<sub>Re</sub> would be a good target for a kinetic study on the *in vitro* polymerization of monomer substrates using the purified active enzyme. The next project is directed to further accumulation of beneficial mutations, site-specific saturation mutagenesis (26, 27) at hot spots (such as F420) for enzyme improvement, and recombination of sets of improved genes.

## REFERENCES

1. Sudesh, K., Abe, H., and Doi, Y. (2000) Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Prog. Polym. Sci.* **25**, 1503–1555
2. Steinbüchel, A. and Hein, S. (2001) Biochemical and molecular basis of microbial synthesis of polyhydroxyalkanoates in microorganisms. *Adv. Biochem. Eng. Biotechnol.* **71**, 81–123
3. Madison, L.L. and Huisman, G.W. (1999) Metabolic engineering of poly(3-hydroxyalkanoates): From DNA to plastic. *Microbiol. Mol. Biol. Rev.* **63**, 21–53
4. Zhang, S., Takagi, Y., Lenz, R.W., and Goodwin, S. (2000) Kinetic and mechanistic characterization of the polyhydroxybutyrate synthase from *Ralstonia eutropha*. *Biomacromolecules* **1**, 244–251
5. Jia, Y., Yuan, W., Wodzinska, J., Park, C., Sinskey, A.J., and Stubbe, J. (2001) Mechanistic studies on class I polyhydroxybutyrate (PHB) synthase from *Ralstonia eutropha*: class I and III synthases share a similar catalytic mechanism. *Biochemistry* **40**, 1011–1019
6. Taguchi, S., Maehara, A., Takase, K., Nakahara, M., Nakamura, H., and Doi, Y. (2001) Analysis of mutational effects of polyhydroxybutyrate (PHB) polymerase on bacterial PHB accumulation using an *in vivo* assay system. *FEMS Microbiol. Lett.* **198**, 65–71
7. Taguchi, S., Maehara, A., Takase, K., Nakahara, M., Nakamura, H., and Doi, Y. (2001) *In vivo* assay system of the polymerase as a key enzyme for PHA biosynthesis. *RIKEN Rev.* **42**, 3–6
8. Yanisch-Perron, C., Vieira, C., and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119
9. Earhart, C.F., Lundrigan, M., Pickett, C.L., and Pierce, J.R. (1979) *Escherichia coli* K-12 mutants that lack major outer membrane protein a. *FEMS Microbiol. Lett.* **6**, 277–280
10. Matsusaki, H., Abe, H., Taguchi, K., Fukui, T., and Doi, Y. (2000) Biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyalkanoates) by recombinant bacteria expressing the PHA synthase gene *phaC1* from *Pseudomonas* sp. 61-3. *Appl. Microbiol. Biotech.* **53**, 401–409
11. Gerngross, T.U., Snell, K.D., Peoples, O.P., Sinskey, A.J., Csuhi, E., Masamune, S., and Stubbe, J. (1994) Overexpression and purification of the soluble polyhydroxyalkanoate synthase from *Alcaligenes eutrophus*: evidence for a required posttranslational modification for catalytic activity. *Biochemistry* **33**, 9311–9320
12. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed. (Laboratory, C.S.H., ed.) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
13. Spiekermann, P., Rehm, B.H.A., Kalscheuer, R., Baumeister, D., and Steinbüchel, A. (1999) A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. *Arch. Microbiol.* **171**, 73–80
14. Karr, D.B., Waters, J.K., and Emerich, D.W. (1983) Analysis of poly-β-hydroxybutyrate in *Rhizobium japonicum* bacteroids by ion-exclusion high-pressure liquid chromatography and UV detection. *Appl. Environ. Microbiol.* **46**, 1339–1344
15. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254
16. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
17. Taguchi, S., Kumagai, I., Nakayama, J., Suzuki, A., and Miura, K. (1989) Efficient extracellular expression of a foreign protein in *Streptomyces* using secretory protease inhibitor (SSI) gene fusions. *Bio/Technol.* **7**, 1063–1066
18. Tange, T., Taguchi, S., and Momose, H. (1994) Improvement of a useful enzyme (subtilisin BPN') by an experimental evolution system. *Appl. Microbiol. Biotech.* **41**, 239–244

19. Kano, H., Taguchi, S., and Momose, H. (1997) Cold adaptation of a mesophilic serine protease, subtilisin, by in vitro random mutagenesis. *Appl. Microbiol. Biotech.* **47**, 46–51
20. Taguchi, S., Ozaki, A., and Momose, H. (1998) Engineering of a cold-adapted protease by sequential random mutagenesis and a screening system. *Appl. Environ. Microbiol.* **64**, 492–495
21. Taguchi, S., Ozaki, A., Nonaka, T., Mitsui, Y., and Momose, H. (1999) A cold-adapted protease engineered by experimental evolution system. *J. Biochem.* **126**, 689–693
22. Das, G., Hickey, D.R., McLendon, D., McLendon, G., and Sherman, F. (1989) Dramatic thermostabilization of yeast iso-1-cytochrome *c* by an asparagine→isoleucine replacement at position 57. *Proc. Natl. Acad. Sci. USA* **86**, 496–499
23. Junker, B., York, G., Park, C., Jia, Y., Rha, C., Stubbe, J., and Sinskey, A.J. (2000) Genetic manipulation of polyhydroxybutyrate synthase activity in *Ralstonia eutropha* in *The 8th International Symposium of Biological Polyester*, Abstract, Cambridge, MA
24. Rehm, B.H.A., Antonio, R.V., Spiekermann, R., Amara, A.A., and Steinbüchel, A. (2002) Molecular characterization of the poly(3-hydroxybutyrate) (PHB) synthase from *Ralstonia eutropha*: in vitro evolution, site-specific mutagenesis and development of a PHB synthase protein model. *Biochim. Biophys. Acta* **1594**, 178–190
25. Kichise, T., Taguchi, S., and Doi, Y. (2002) Enhanced accumulation and changed monomer composition in polyhydroxyalkanoate (PHA) copolyester by in vitro evolution of *Aeromonas caviae* PHA synthase. *Appl. Environ. Microbiol.* **68**, 2411–2419
26. Taguchi, S., Komada, S., and Momose, H. (2000) The complete amino acid substitutions at position 131 that are positively involved in cold adaptation of subtilisin BPN'. *Appl. Environ. Microbiol.* **66**, 1410–1415
27. Miyazaki, K. and Arnold, F.H. (1999) Exploring nonnatural evolutionary pathways by saturation mutagenesis: rapid improvement of protein function. *J. Mol. Evol.* **49**, 716–720