Biotechnology Letters (2005) 27: 705–712
DOI 10.1007/s10529-005-5186-z

Site-directed saturation mutagenesis at residue F420 and recombination with another beneficial mutation of *Ralstonia eutropha* polyhydroxyalkanoate synthase

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Received 19 January 2005; Revisions requested 27 January 2005; Revisions received 24 March 2005; Accepted 25 March 2005

Key words: combined mutations, *in vitro* evolution, PHA synthase, poly(3-hydroxybutyrate), saturation mutagenesis

Abstract

The F420S substitution enhances the specific activity of *Ralstonia eutropha* PHA synthase ($PhaC_{Re}$). We have now carried out site-directed saturation mutagenesis of F420 of $PhaC_{Re}$ and, amongst the F420 mutants, the F420S mutant gave the highest poly(3-hydroxybutyrate) (PHB) content. *In vitro* activity assay showed that the F420S enzyme had a significant decrease in its lag phase compared to that of the wild-type enzyme. Enhancement of PHB accumulation was achieved by combination of the F420S mutation with a G4D mutation, which conferred high PHB content and high *in vivo* concentration of $PhaC_{Re}$ enzyme. The G4D/F420S mutant gave a higher PHB content and *in vivo* concentration of $PhaC_{Re}$ enzyme than the F420S mutant, while the molecular weight of the PHB polymer of the double mutant was similar to that of the F420S mutant.

Introduction

Poly(3-hydroxybutyrate) (PHB), which is a type of polyhydroxyalkanoate (PHA) polymer, is synthesized by various bacteria as carbon and energy storage material under excess carbon and limited nutrient conditions (Madison & Huisman 1999). It has attracted considerable attention due to its similarities of its physical properties, i.e. its tensile strength and stiffness, to that of conventional petrochemical-based plastics (Sudesh *et al.* 2000). Coupled with its biodegradability, these properties make PHB a promising candidate as a replacement for conventional plastics as environmentally favorable plastic in the future.

The key enzyme in PHA biosynthesis is PHA synthase (PhaC). Several studies initiated by our group have established efficient production systems for PHA with desired characteristics through the modifications of PhaC via *in vitro* evolution (Taguchi *et al.* 2001, Taguchi & Doi 2004). For example, the enhancement of PHA accumulation and/or change in PHA composition were successfully achieved by increasing the enzymatic activity and/or altering substrate specificity of PHA synthases (Kichise *et al.* 2002, Takase *et al.* 2003, 2004, Tsuge *et al.* 2004).

Previously, we have obtained an F420S beneficial mutation (in which Phe was replaced with Ser at position 420) of PHA synthase from *Ralstonia eutropha* ($PhaC_{Re}$) via *in vitro* evolution by intragenic suppression mutagenesis (Taguchi *et al.* 2002). The F420S mutation increased the specific activity of $PhaC_{Re}$ compared to that of the

wild-type enzyme (Taguchi *et al*. 2002). Further amino acid substitutions of the F420 residue and their effect on PHB accumulation have not been investigated. In this study, site-directed saturation mutagenesis of F420 has been performed and the effect of the previous F420S substitution and other F420 substitutions on PHB accumulation has been investigated. Next, the $PhaC_{Re}$ enzyme of the F420 mutant, which exhibited the highest PHB content, was purified and *in vitro* activity assay was performed. In addition, the F420 mutation from this mutant was combined with another previously generated beneficial mutation to enhance PHB accumulation further. For this purpose, the G4D mutation (in which Gly was replaced with Asp at position 4 of $PhaC_{Re}$) was chosen. The G4D mutation, also generated via *in vitro* evolution, is one of the most promising mutations among other G4 mutations in conferring high PHB accumulation due to the elevation in the concentration of $PhaC_{Re}$ enzyme *in vivo* as a result of the amino acid substitution (Normi *et al*. 2005). Analysis of the mutational effects of the selected F420 mutation and the constructed G4D/F420S double mutation on PHB content and molecular weight and the concentration of $PhaC_{Re}$ *in vivo* were also investigated.

Materials and methods

Site-directed saturation mutagenesis of F420 of $PhaC_{Re}$

Site-directed saturation mutagenesis of F420 was performed via PCR using tail-to-tail forward and reverse primers with the pKAS4-SseI plasmid as a template. This plasmid harbors the wild-type PHA synthase gene ($phaC_{Re}$) with an attached linker containing the *Sse*8387I restriction site downstream of the termination codon. The primer pair used was the forward 5pKAS4-F420X primer and the reverse 3pKAS4-sat primer as listed in Table 1. Saturation mutagenesis was carried out based on a previous method (Normi *et al*. 2005), with slight modifications. PCRs involving most of the 5pKAS4-F420X forward primers were carried out using the following three-step cycling program: 94 °C for 2 min, 30 cycles of 94 °C for 15 s, 55 °C for 30 s and 68 °C for 6 min. However, for reactions using forward primers 5pKAS4-F420A/E/M/H, PCR were performed using the following two-step cycling program: 94 °C for 2 min, 30 cycles of 94 °C for 15 s and 68 °C for 6 min. The amplified 6 kbp products were self-ligated and transformed into *Escherichia coli* JM109. The $phaC_{Re}$ gene of the plasmid was sequenced to verify the correctness of the F420X codon change (in which Phe at position 420 of $PhaC_{Re}$ was substituted with other amino acid residues except Phe and Ser, as indicated by an X) and the overall gene sequence.

The plasmids carrying individual F420X mutations were then digested with *Eco*RV and *Sse*8387I restriction enzymes to isolate the respective F420X mutations. The generated 1.5 kbp gene fragments harboring the respective F420X mutations were then ligated with the pGEM′-$phaCAB_{Re}$ expression plasmid, also digested with the same restriction enzymes. These chimeric pGEM′-$phaCAB_{Re}$ plasmids harboring the individual F420X mutations were sequenced and subsequently transformed into *E. coli* JM109.

Construction of the G4D/F420S double mutant

The F420S and G4D mutations were isolated individually by restriction digest from pGEM′-

Table 1. Oligonucleotide primers used in this study.

Primers	Sequence
For saturation mutagenesis at position Phe420	
5pKAS4-F420X[a]	5′-CCGGTGCCG**TTC**GACCTGCTGTTCTGGAACG-3′
3pKAS4-sat	5′-CGTGTTGCCCTTCAGGTAGTTGTCGACTACG-3′

[a]X indicates the 18 amino acid residues other than the original one and Ser. Bold sequences indicate the substitution codons for Phe420 (TTC) in F420X. Codons used to perform site-directed saturation mutagenesis were: Ala (GCG), Cys (TGC), Asp (GAT), Glu (GAA), Gly (GGC), His (CAT), Ile (ATT), Lys (AAA), Leu (CTG), Met (ATG), Asn (AAC), Pro (CCG), Gln (CAG), Arg (CGC), Thr (ACC), Val (GTG), Trp (TGG) and Tyr (TAT).

phaCAB$_{Re}$ plasmids with *Eco*RV and *Sse*8387I restriction enzymes. Both sets of digestion generated two fragments of 1.5 and 6.5 kbp in sizes. The 1.5 kbp fragment carrying the F420S mutation, and the 6.5 kbp fragment carrying the G4D mutation plus the remaining portion of the vector, were gel-purified and ligated. This chimeric pGEM′-*phaCAB*$_{Re}$ plasmid harboring both the G4D and F420S mutations was sequenced and subsequently transformed into *E. coli* JM109 to yield the G4D/F420S double mutant.

PHB content and molecular weight determination

For PHB biosynthesis, recombinant *E. coli* JM109 were either cultivated in 1.75 ml or 100 ml Luria–Bertani (LB) medium containing 2% (w/v) glucose and 100 μg ampicillin ml^{-1} for 14 h at 37 °C. Cells were then harvested by centrifugation, washed with double-distilled water and lyophilized. PHB content was determined by HPLC after converting cellular PHB to crotonic acid by treatment with hot conc. H_2SO_4 (Karr *et al.* 1983). For PHB molecular weight determination, PHB polymer was extracted from dried cells with chloroform and re-precipitated with methanol. The molecular weights of the polymer were determined by multiangle laser light scattering (MALLS) (Wyatt Technology DAWN DSP) and gel permeation chromatography (GPC) (Shimadzu 10 Åsystem), according to the method of Kusaka *et al.* (1998). For GPC, polystyrene standards with low polydispersity were used to construct the standard curve.

Western blot analysis of PhaC$_{Re}$

To 1.75 ml LB medium containing 100 μg ampicillin ml^{-1}, 10 μl of an overnight culture of cells was added, and was grown for a further 9 h at 37 °C. Cells were harvested by centrifugation and disrupted by sonication (TOMY UD-200). The concentration of total cellular proteins of the soluble fraction was determined and 40 μg protein was used for Western blot analysis. Specific rabbit anti-serum raised against the *C*-terminal of PhaC$_{Re}$ was used as the primary antibody. Protein bands were visualized using goat anti-rabbit IgG conjugated to alkaline phosphatase. Band intensities were analyzed on a Macintosh (OS 9.2) computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Thermostability studies of PhaC$_{Re}$

Thermostability studies using soluble fraction from crude cell extracts of recombinant *E. coli* JM109 were performed according to the method of Taguchi *et al.* (2002). Briefly, several aliquots of each crude cell extract were incubated at 50 °C for various time frames and then immediately subjected to PhaC$_{Re}$ activity assay to measure the residual activity of the enzyme. Activity assay was performed by measuring the release of CoA at 412 nm by means of a discontinuous assay using (*R*)-3-hydroxybutyryl-CoA [(*R*)-3HB-CoA] and 5,5′-dithiobis(2-nitrobenzoic acid) (Gerngross *et al.* 1994). The thermostability of the enzyme was defined as the half-life for inactivation of the enzyme at 50 °C.

In vitro *enzymatic assays of purified PhaC*$_{Re}$

Construction and purification of wild-type and mutant (His)$_6$-tagged PhaC$_{Re}$ enzymes were performed as described previously (Normi *et al.* 2005). Enzymatic assays using the purified enzymes were performed using the method of Zhang *et al.* (2000) with slight modifications. Activity assay of PhaC$_{Re}$ was performed at 25 °C by measuring the loss of (*R*)-3HB-CoA at 236 nm. Reaction was carried out in 1 ml total volume of 20 mM potassium phosphate buffer (pH 7) containing 5% (v/v) glycerol, 0.05% (w/v) 6-*O*-(*N*-heptylcarbamoyl)methyl α-D-glucopyranoside (Hecameg), 100 nM purified PhaC$_{Re}$ (M_w = 66 kDa) and 100 μM (*R*)-3HB-CoA. Kinetic parameters were determined based on the velocity at the initial stage of the reaction after the lag phase.

Results and discussion

PHB content of F420 mutants

Previously, the beneficial F420S mutation which enhanced PhaC$_{Re}$ specific activity was acquired via intragenic suppression mutagenesis (Taguchi *et al.* 2002). In this study, the influence of amino

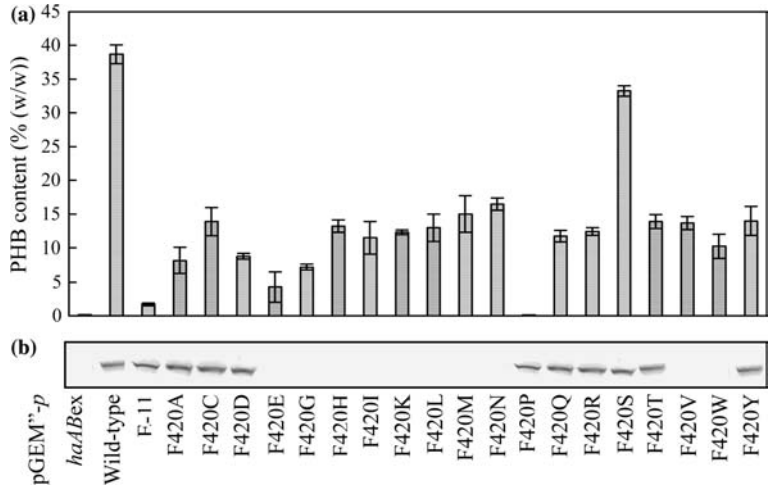

Fig. 1. (a) PHB accumulated by recombinant *E. coli* JM109 harboring wild-type and various F420 mutant $PhaC_{Re}$ enzymes. Recombinant cells were cultivated in 1.75 ml LB medium containing 2% (w/v) glucose and 100 μg ampicillin ml^{-1} for 14 h at 37 °C. All values are averages of three or four independent experiments. (b) Western blot analysis of wild-type and F420 mutant $PhaC_{Re}$ produced in *E. coli* JM109. Total protein of 40 μg was used for the analysis. Representative result of three independent experiments is shown.

acid substitution at position 420 on PHB accumulation was investigated. For this purpose, site-directed saturation mutagenesis at this position was carried out. HPLC results in Figure 1a revealed that all the F420 mutants exhibited lower PHB content than that of the wild-type (39% w/w), with most averaging at 13% (w/w). However, among the F420 mutants, the F420S mutant exhibited the highest PHB content (35% w/w).

Western blot analysis of $PhaC_{Re}$ in Figure 1b showed that the $PhaC_{Re}$ enzyme was produced in all the F420 mutants and the *in vivo* concentrations of the enzyme were similar to that of the wild-type. Since the *in vivo* concentration of the F420S enzyme was similar with those of other F420 mutants, this suggests that the high PHB content caused by the F420S mutation was due to the intrinsic properties of the $PhaC_{Re}$ enzyme.

Enzymatic reaction of F420S mutant enzyme

To evaluate the mutational effect of the F420S mutation on the enzymatic reaction of $PhaC_{Re}$, the wild-type and F420S mutant $PhaC_{Re}$ were purified to homogeneity using a one-step purification with a Ni–NTA column chromatography (Figure 2a).

Based on the reaction profile exhibited by both of the enzymes, the velocity at the initial stage of the polymerization reaction of the F420S mutant enzyme was significantly faster with very minimal lag phase, compared to that of the wild-type enzyme (Figure 2b). However, kinetic studies revealed that the efficiency (k_{cat}/K_m value) of the F420S mutant enzyme was similar to that of the wild-type enzyme (1.9×10^5 and 2.3×10^5 M^{-1} s^{-1}, respectively). Previously, Junker *et al.* (2000) proposed that the conserved W425 residue plays a role in the dimerization of $PhaC_{Re}$ enzyme. Due to the adjacent location of the F420 residue to that of the W425 residue and the increase in the specific activity of the F420S mutant enzyme, it was previously suggested that the F420S mutation might have a positive influence on the dimerization of $PhaC_{Re}$ indispensable for full synthase activity (Taguchi *et al.* 2002). In addition, Zhang *et al.* (2000) reported that the occurrence of the lag phase is due to $PhaC_{Re}$ dimer formation. In our present study, the significantly reduced lag phase exhibited by the F420S mutant enzyme in (*R*)-3HB-CoA polymerization (Figure 2b) further strengthens the suggestion of Taguchi *et al.* (2002) of the positive influence of the single F420S substitution on $PhaC_{Re}$ dimer formation.

PHB content and in vivo *$PhaC_{Re}$ concentration of the G4D/F420S mutant*

Due to the positive effects of the F420S mutation on *in vitro* $PhaC_{Re}$ activity and possibly on the

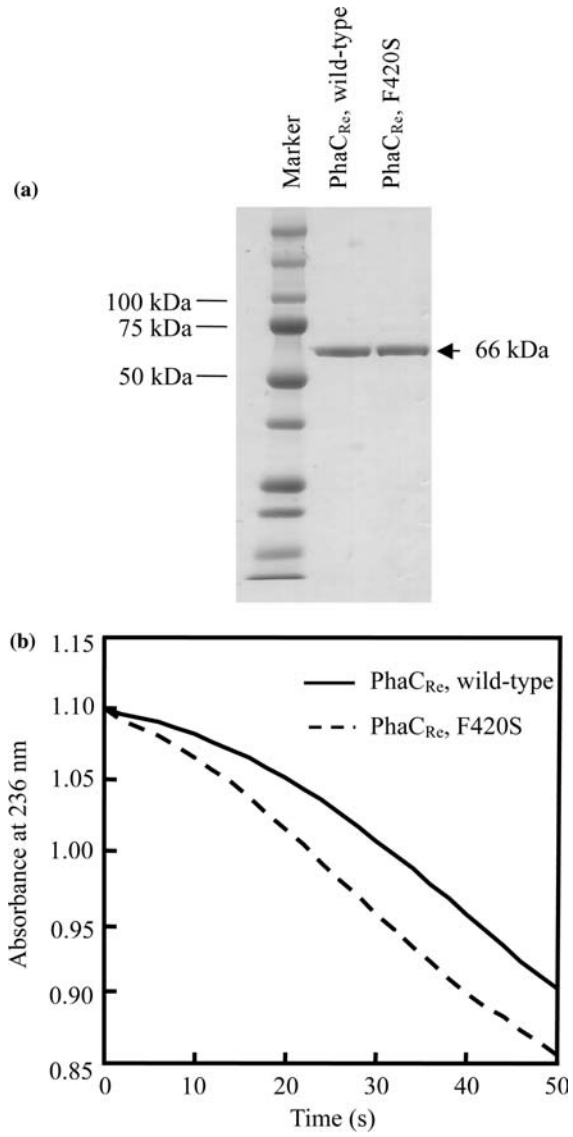

Fig. 2. (a) SDS-PAGE analysis of purified $(His)_6$-tagged wild-type and mutant $PhaC_{Re}$. (b) Profile of *in vitro* enzymatic reaction catalyzed by purified wild-type and mutant $PhaC_{Re}$ at 25 °C. The reaction mixture contained 100 μM (*R*)-3HB-CoA and 100 nM $PhaC_{Re}$ in 20 mM potassium phosphate buffer (pH 7) containing 5% (v/v) glycerol and 0.05% (w/v) Hecameg.

dimerization of the enzyme, this mutation was selected to combine with another beneficial mutation to enhance PHB accumulation further. The G4D mutation, which was obtained previously by *in vitro* evolution, is one of the most promising mutations in enhancing PHB accumulation due to its positive influence on elevating $PhaC_{Re}$ enzyme concentration *in vivo* (Normi *et al.* 2005). Therefore, the F420S mutation was combined with the G4D mutation and the effect of the G4D/F420S double mutation on PHB accumulation was investigated.

The G4D/F420S mutant accumulated 56% (w/w) PHB, which was 1.4-fold higher than that of the wild-type and F420S mutant (Figure 3b). Interestingly, the PHB content of the G4D/F420S mutant was similar to that of the G4D mutant (58% w/w), while the F420S mutant had a similar PHB content to that of the wild-type (39% w/w and 35% w/w, respectively). Similar tendencies were also observed in the PHB yields, cell dry weights and the residual biomasses of the G4D/F420S and G4D mutants, and of the F420S mutant and the wild-type (Figure 3).

Western blot analysis of $PhaC_{Re}$ enzyme in Figure 3e revealed that the *in vivo* concentration of $PhaC_{Re}$ of the F420S mutant was indistinguishable from that of the wild-type, similar to the result obtained in Figure 1b. However, an elevation in the concentration of $PhaC_{Re}$ of the G4D/F420S mutant was observed, similar to that of the G4D mutant. Analysis on the band intensities using the NIH Image program revealed that the concentrations of $PhaC_{Re}$ of both the G4D and G4D/F420S mutants were 3-fold higher compared to that of the wild-type. Therefore, it is clear that the G4D mutation had elevated the concentration of $PhaC_{Re}$ of the G4D/F420S mutant, suggesting that the G4D mutation is dominant over the F420S mutation in determining the *in vivo* concentration of $PhaC_{Re}$ in the double mutant. As a result of the elevation in the concentration of the $PhaC_{Re}$ enzyme, the G4D/F420S mutant was able to accumulate higher PHB content and hence, gave higher PHB yield than the F420S mutant and wild-type. Nevertheless, what constituted the increase in the dry cell weights and residual biomasses of the G4D and G4D/F420S mutants is still unknown. Further biochemical studies into this matter will have to be done.

At present, it is not known how the *in vivo* concentration of $PhaC_{Re}$ was elevated in the G4D/F420S mutant. As reported previously, the GAC codon coding for Asp of the G4D mutation has a rare codon usage in *E. coli* (Normi *et al.* 2005). Hence, the possibility that the G4D mutation enhanced $phaC_{Re}$ mRNA translation in the double mutant was ruled out. However, it can be suggested that the G4D mutation might have a

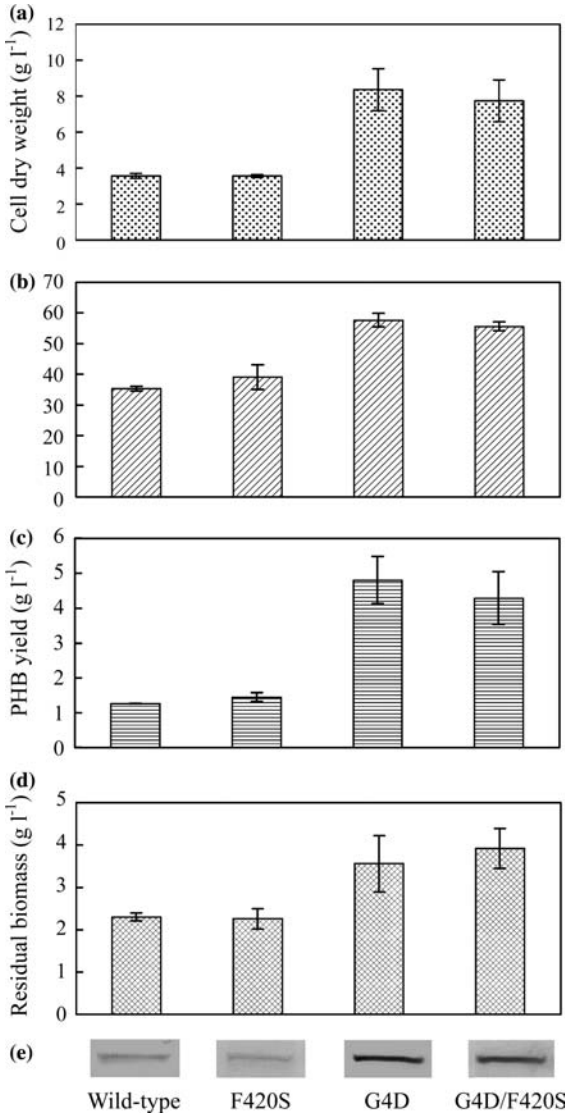

Fig. 3. (a) Dry cell weight, (b) PHB content, (c) PHB yield and (d) residual biomass of recombinant *E. coli* JM109 harboring wild-type and various mutants $PhaC_{Re}$. Recombinant cells were cultivated in 100 ml LB medium containing 2% (w/v) glucose and 100 μg ampicilin ml^{-1} for 14 h at 37 °C. All values are averages of three independent experiments. (e) Western blot analysis of wild-type and mutants $PhaC_{Re}$ produced in recombinant *E. coli* JM109. Total protein of 40 μg was used for the analysis. Representative result of three independent experiments is shown.

positive influence at the transcription or protein level (Normi *et al.* 2005). In this study, thermostability studies revealed that the G4D/F420S mutant and wild-type enzyme had identical half-lives of 1.2 min (data not shown), ruling out the possibility that the G4D/F420S enzyme was heat-stable. However, the possibility that the G4D substitution enhanced the stability of the G4D/F420S $PhaC_{Re}$ against cellular proteases in *E. coli* could not be dismissed. Hence, there are two possibilities how the G4D substitution has resulted in the elevation in the *in vivo* concentration of $PhaC_{Re}$: the G4D substitution might have enhanced (1) the expression and/or the stability of mRNA transcripts or (2) the stability of $PhaC_{Re}$ against proteases in *E. coli* (Normi *et al.* 2005).

PHB molecular weights analysis

Next, we investigated the effect of the mutations on PHB molecular weight. MALLS and GPC results in Table 2 show that the molecular weight of the PHB polymer synthesized by the G4D mutant was the highest among all the samples. On the other hand, the PHB polymer synthesized by the G4D/F420S mutant had a lower molecular weight than that of the G4D mutant, but similar to that of the F420S mutant (Table 2). It was previously suggested that the G4D mutation might have stabilized and/or enhanced polymer chain elongation, thus, allowing higher molecular weight polymer to be synthesized (Normi *et al.* 2005). However, this effect was not observed in combination with the F420S mutation. Hence, this suggests that the F420S mutation is dominant over the G4D mutation in determining the molecular weight of the polymer synthesized by the G4D/F420S mutant. In fact, the dominance of the F420S mutation was further reflected by the identical polydispersity values of the polymers isolated from the F420S and G4D/F420S mutants compared to that of the G4D mutant (Table 2).

It was previously suggested that chain transfer events result in the synthesis of lower molecular weight polymer with broader polydispersity (Kawaguchi & Doi 1992). Judging from the low molecular weights and broad polydispersity values of the PHB polymers of both the F420S and G4D/F420S mutants to that of the G4D mutant, there is a possibility that the F420S mutation has an effect on chain transfer events.

In addition, it is worthy to note that the molecular weights of the PHB polymers (Table 2) are independent of the *in vivo* concentrations of $PhaC_{Re}$ (Figure 3e). Results showing that: (1)

Table 2. Molecular weights of PHB homopolymers produced by recombinant *E. coli* JM109 harboring wild-type and mutants $PhaC_{Re}$.

Sample[a]	$M_{w\ (MALLS)}$[b] ($\times 10^6$)	$M_{n\ (GPC)}$[c] ($\times 10^6$)	$M_w/M_{n\ (GPC)}$[d]
Wild-type	2.9	3.4	2.2
F420S	2.7	2.8	2.6
G4D	4.1	5.1	1.5
G4D/F420S	2.8	2.4	2.6

[a]Cells were cultivated in 100 ml LB medium (pH 7.0) containing 2% (w/v) glucose and 100 μg ampicillin ml^{-1} as the final concentration for 14 h at 37 °C.
[b]Absolute weight-average molecular weight determined by multiangle laser light scattering (MALLS) in 2,2,2-trifluoroethanol.
[c,d]Number average (M_n) and weight-average molecular weight (M_w) determined by gel permeation chromatography relative to polystyrene standard in chloroform.

both the F420S and G4D/F420S mutants have different *in vivo* concentrations of $PhaC_{Re}$ but synthesized PHB polymers with similar molecular weights, (2) both G4D and G4D/F420S mutants have similar *in vivo* concentrations of $PhaC_{Re}$ but synthesized PHB polymers with different molecular weights clearly indicate that the molecular weights of the polymers were determined by the effect of the mutational point(s) involved, not the *in vivo* concentrations of $PhaC_{Re}$.

In conclusion, the G4D mutation is beneficial for the enhancement of both the *in vivo* concentration of $PhaC_{Re}$ enzyme and PHB content in the G4D/F420S double mutant. On the other hand, the F420S mutation is beneficial for lag phase reduction of $PhaC_{Re}$, by possibly encouraging the dimerization of the enzyme. Obtaining such a mutation conferring lag phase reduction is indeed desirable for various *in vitro* biochemical studies in the future as the presence of lag phase in the reaction hinders accurate kinetic characterization of the enzyme. In addition, the molecular weights of the PHB polymers synthesized by the G4D, F420S and G4D/F420S mutants were determined by the mutational effect of the mutation(s) involved, not by the *in vivo* concentrations of $PhaC_{Re}$ enzyme. The F420S mutation is postulated to play a role in chain transfer events, based on the lower molecular weight and broader polydispersity values of the PHB polymers synthesized by both the F420S and G4D/F420S mutants.

Acknowledgements

We would like to specially thank Dr Ken'ichiro Matsumoto for his kind supervision and technical assistance in (*R*)-3HB-CoA substrate synthesis. This work was supported by grants from RIKEN Asia Program (AP) to YMN, Solution Oriented Research for Science and Technology (SORST) and Ecomolecular Science Research to RIKEN Institute.

References

Gerngross TU, Snell KD, Peoples OP, Sinskey AJ, Csuhai E, Masamune S, 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.

Junker B, York G, Park C, Jia Y, Rha C, Stubbe J, Sinskey AJ (2000) Genetic manipulation of polyhydroxybutyrate synthase activity in *Ralstonia eutropha*. In: *The 8th International Symposium of Biological Polyester*, September 11–15, Cambridge, Massachusettes.

Karr DB, Waters JK, Emerich DW (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.

Kawaguchi Y, Doi Y (1992) Kinetics and mechanism of synthesis and degradation of poly(3-hydroxybutyrate) in *Alcaligenes eutrophus*. *Macromolecules* **25**: 2324–2329.

Kichise T, Taguchi S, 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.

Kusaka S, Iwata T, Doi Y (1998) Microbial synthesis and physical properties of ultra-high-molecular-weight poly[*(R)*-3-hydroxybutyrate]. *J. Macromol. Sci., Pure Appl. Chem.* **A35**: 319–335.

Madison L, Huisman GW (1999) Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic. *Microbiol. Mol. Biol. Rev.* **63**: 21–53.

Normi YM, Hiraishi T, Taguchi S, Abe H, Sudesh K, Najimudin N, Doi Y (2005) Characterization and properties of G4X mutants of *Ralstonia eutropha* PHA synthase for poly(3-hydroxybutyrate) biosynthesis in *Escherichia coli*. *Macromol. Biosci.* **5**: 197–206.

Sudesh K, Abe H, Doi Y (2000) Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Prog. Polym. Sci.* **25**: 1503–1555.

Taguchi S, Doi Y (2004) Evolution of polyhydroxyalkanoate (PHA) production system by 'enzyme evolution': successful case studies of directed evolution. *Macromol. Biosci.* **4**: 145–156.

Taguchi S, Maehara A, Takase K, Nakahara M, Nakamura H, 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.

Taguchi S, Nakamura H, Hiraishi T, Yamato I, Doi Y (2002) *In vitro* evolution of a polyhydroxybutyrate synthase by intragenic suppression-type mutagenesis. *J. Biochem.* **131**: 801–806.

Takase K, Matsumoto K, Taguchi S, Doi Y (2004) Alteration of substrate chain-length specificity of type II synthase for polyhydroxyalkanoate biosynthesis by *in vitro* evolution: *in vivo* and *in vitro* enzyme assays. *Biomacromolecules* **5**: 480–485.

Takase K, Taguchi S, Doi Y (2003) Enhanced synthesis of poly(3-hydroxybutyrate) in recombinant *Escherichia coli* by means of error-prone PCR mutagenesis, saturation mutagenesis, and *in vitro* recombination of the type II polyhydroxyalkanoate synthase gene. *J. Biochem.* **133**: 139–145.

Tsuge T, Saito Y, Kikkawa Y, Hiraishi T, Doi Y (2004) Biosynthesis and compositional regulation of poly[(3-hydroxybutyrate)-*co*-(3-hydroxyhexanoate)] in recombinant *Ralstonia eutropha* expressing mutated polyhydroxyalkanoate synthase genes. *Macromol. Biosci.* **4**: 238–242.

Zhang S, Yasuo T, Lenz RW, Goodwin S (2000) Kinetic and mechanistic characterization of the polyhydroxybutyrate synthase from *Ralstonia eutropha*. *Biomacromolecules* **1**: 244–251.