In Vivo and in Vitro Characterization of Ser477X Mutations in Polyhydroxyalkanoate (PHA) Synthase 1 from *Pseudomonas* sp. 61−3: Effects of Beneficial Mutations on Enzymatic Activity, Substrate Specificity, and Molecular Weight of PHA

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Evolutionary engineered polyhydroxyalkanoate (PHA) synthases from *Pseudomonas* sp. 61−3 enhance PHA accumulation and enable the monomer composition of PHAs to be regulated. We characterized a newly screened Ser477Arg (S477R) mutant of PHA synthase by in vivo analyses of P(3-hydroxybutyrate) [P(3HB)] homopolymer and P(3HB-*co*-3-hydroxyalkanoate) [P(3HB-*co*-3HA)] copolymer productions in the recombinants of *Escherichia coli*. The results indicated that the S477R mutation contributed to a shift in substrate specificity to smaller monomers containing a 3HB unit rather than to an enhancement in catalytic activity. Multiple mutations of S477R with other beneficial mutations, for example, Ser325Cys, exhibited synergistic effects on both an increase in PHA production (from 9 wt % to 21 wt %) and an alteration of substrate specificity. Furthermore, the effects of complete amino acid substitutions at position 477 were characterized in terms of in vivo PHA production and in vitro enzymatic activity. The five mutations, S477Ala(A)/Phe(F)/His(H)/Arg(R)/Tyr(Y), resulted in a shift in substrate specificity to smaller monomer units. The S477Gly(G) mutant greatly enhanced activity toward all different sizes of substrates with carbon numbers ranging from 4 to 12. These results indicated that the residue 477 contributes to both the catalytic activity and substrate specificity of PHA synthase. In recombinant *E. coli*, the S477A/F/G/H/R/Y mutations consistently led to increases (up to 6 times that of wild-type enzyme) in weight average molecular weights of P(3HB) homopolymers. On the basis of our studies, we created a structural feasibility accounting for the mutational effects on enzymatic activity and substrate specificity of PHA synthase.

Introduction

Polyhydroxyalkanoates (PHAs) are bacterial polyesters that are produced as an intracellular carbon and energy storage material.[1,2] PHAs have attracted research interest because they are biobased and biodegradable thermoplastics that have the potential to replace petrochemical plastics. The development of sustainable materials is an important step toward reducing the consumption of oil. Currently, reduction in the cost of production and regulation of the properties of PHAs are major challenges for commercial production of these polyesters.

PHAs have various physical and thermal properties depending on their monomer composition. PHAs consisting of short-chain-length (SCL, C_4 and C_5) monomers are a highly crystalline and brittle material.[1] However, the development of drawing techniques has enabled processing of P(3-hydroxybutyrate) [P(3HB)] to very strong fibers or films.[3,4] In contrast, PHAs consisting of medium-chain-length monomers (MCL, C_6−C_{14}) are elastomeric in nature. PHA copolymers consisting of SCL and MCL monomer units [P(3HB-*co*-3HA)] have intermediate properties between SCL and MCL PHAs. P(3HB-*co*-3HA) copolymers are flexible and elastic, and their physical and thermal properties vary depending on the molar ratio of SCL versus MCL units. In particular, a P(3HB-*co*-3HA) copolymer consisting of a 90−95 mol % 3HB unit has physical properties that are close to those of polypropylene. In addition, the melting point of the copolymers is lower (140−170 °C) than that of P(3HB) homopolymer (180 °C), which reduces the amount of thermal degradation during the melting process.[5,6] Therefore, regulating the monomer composition is very important to produce SCL/MCL PHAs efficiently and with the desired properties.

PHA synthase (PhaC) is the key enzyme of the PHA biosynthetic pathway. PHA synthase typically polymerizes different (*R*)-3-hydroxyacyl-CoAs to yield various PHAs.[7] The catalytic activity and substrate specificity of PHA synthase greatly affect productivity and its monomer composition. Most PHA synthases belonging to class I have substrate specificity toward SCL substrates, whereas class II PHA synthases have substrate specificity toward MCL substrates.[8] PHA synthase 1 isolated from *Pseudomonas* sp. 61−3 ($PhaC1_{Ps}$) has exceptionally broad substrate specificity and produces P(3HB-*co*-3HA) copolymer consisting of monomers 4−14 carbons long.[9,10] The copolymers produced by $PhaC1_{Ps}$ have the desired properties, such as high flexibility and low melting temperature. However, productivity of the PHAs has been limited because $PhaC1_{Ps}$ has little activity toward 3HB-CoA.[11,12]

Our group addressed this problem by developing the in vitro evolutionary approach[13,14] to generate highly active mutants of PHA synthase.[15−18] Beneficial mutants of PHA synthases from

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Aeromonas caviae,[19] *Pseudomonas* sp. 61−3,[12,20,21] and *Ralstonia eutropha*[15,22,23] were reported. The expression of the mutated PHA synthase genes in recombinant *Escherichia coli* has led to an increase in PHA content and an alteration in the monomer composition of PHAs. Thus, the evolutionary engineering approach is a powerful tool to improve the productivity of PHAs and to regulate the monomer composition of PHAs.

We isolated beneficial mutants of PHA synthases from *Pseudomonas* sp. 61−3 that enhance P(3HB) accumulation. Because the mutants were screened on the basis of the ability to produce P(3HB), we obtained mutated enzymes that have higher activity toward 3HB-CoA. In our previous reports, we detailed how we isolated and characterized six beneficial mutants, Glu130Asp (E130D), Ser325Cys and Ser325Thr (S325C/T), and Gln481Lys, Gln481Met, and Gln481Arg (Q481K/M/R). The E130D and S325C/T mutants led to increases in both P(3HB) content (1.0−3.8 wt %) and P(3HB-*co*-3HA) content (15−22 wt %) in recombinant *E. coli* compared to those of a wild-type enzyme [0.1 wt % P(3HB) and 13 wt % P(3HB-*co*-3HA)]. The monomer compositions of P(3HB-*co*-3HA) produced by these mutants were close to those produced by the wild-type enzyme. The Q481K/M/R mutants also led to increases in P(3HB) content (1.2−2.3 wt %). The difference was that the Q481K/M/R mutants produced P(3HB-*co*-3HA) copolymers with higher 3HB content (32−40 mol %) than the wild-type (14 mol %). These results indicated that the E130D and S325C/T mutations contributed to an increase in the enzymatic activity and that the Q481K/M/R mutations contributed to an alteration in substrate specificity.[12,20,21]

The interesting and industrially important feature of the beneficial mutations is a synergistic effect of multiple mutations. For instance, the double mutant of S325T and Q481K mutations accumulated substantial P(3HB) (40 wt %) and P(3HB-*co*-3HA) (28 wt %) and altered the monomer composition of P(3HB-*co*-3HA) copolymer (31 mol % 3HB). The mechanism of the synergistic effect of multiple beneficial mutations has not been determined precisely. However, the great enhancement in PHA production and alteration in substrate specificity should contribute to reducing the cost and regulating the monomer composition in the industrial system of PHA production. Moreover, the isolation and combination of new beneficial mutations may further improve the activity and substrate specificity of the enzyme. In this study, we isolated new beneficial mutations S477X (saturated mutation at Ser477) and demonstrated that the mutants also exhibited synergistic effects and therefore can serve as a powerful biocatalyst for efficiently producing PHAs with the desired properties. In addition, we created a working model for the synergistic multiple mutational effects on enzymatic activity and substrate specificity of PHA synthase.

Materials and Methods

Construction of the S477X Saturation Mutagenesis. The S477X mutated PHA synthase genes from *Pseudomonas* sp. 61−3 were constructed by PCR using the following primers: 5'-GTGCTGTCCAGCAGTGGGCATATCCAGAGC-3' and 5'-GAATTCGACCTTGCCACCGAACAGTTGCGC-3'. Underline indicates the substitution codons for Ser477(AGT) in S477X. Codons to perform the site-specific saturation mutagenesis were Ala(GTT), Cys(TGC), Asp(GAT), Glu(GAA), Phe(TTC), Gly(GGC), His(CAC), Ile(ATC), Lys(AAG), Leu(CTG), Met(ATG), Asn(AAC), Pro(CCG), Gln(CAG), Arg(CGC), Thr(ACC), Val(GTC), Trp(TGG), and Tyr(TAC), respectively. The expression vectors pGEM″C1AB and pGEM″C1ABJ4 were constructed as described previously.[12] The pGEM″C1AB harbors PHA synthase gene (*phaC1*$_{Ps}$) from *Pseudomonas* sp. 61−3, β-ketothiolase (*phaA*$_{Re}$), and NADPH-dependent acetoacetyl-CoA reductase (*phaB*$_{Re}$) genes from *R. eutropha*. The pGEM″C1ABJ4 harbors enoyl-CoA hydratase 4 gene (*phaJ4*$_{Pa}$) from *P. aeruginosa* in addition to the *phaC1AB* genes.

Analysis of the Content and Monomer Composition of P(3HB) and P(3HB-*co*-3HA) Produced by Recombinant *E. coli* Strains. *E. coli* JM109[24] was used as a host strain for P(3HB) production. Recombinant JM109 cells harboring pGEM″C1AB were grown on 1.5 mL Luria-Bertani (LB) medium containing glucose (2 wt %/v) and ampicillin (100 μg/mL) in a test tube for 96 h at 30 °C. For the data in Table 1, test tubes were shaken at a tilt angle of 60°, whereas we changed the angle to 45° for the data in Figure 1. The change improved aeration of culture and led to an increase in PHA content. The cellular P(3HB) content was determined by HPLC.[12] For accumulation of P(3HB-*co*-3HA) copolymers, recombinant *E. coli* LS5218 [*fadR601*, *atoC*(Con)][25] strains harboring pGEM″C1ABJ4 were cultivated on 100 mL M9 medium[26] containing sodium dodecanoate (0.3 wt %/v) and ampicillin (100 μg/mL) with 0.4% (v/v) Brij-35 at 37 °C for 72 h. Brij-35 is a good detergent, which allows dodecanoate to be solubilized well and recombinant bacteria to utilize dodecanoate. The concentration of Brij-35 was routinely used and suitable for the purpose described above. The cellular P(3HB-*co*-3HA) content and monomer composition were determined by gas chromatography (GC).[27]

In Vitro Enzyme Assay. The wild-type and recombinant strains of *E. coli* JM109 harboring pGEM″C1ABs were grown on 100 mL LB medium containing ampicillin (50 μg/mL) for 12 h at 30 °C. The harvested cells were disrupted by sonication, and the supernatants were used to assay enzymatic activity.[12] PHA synthase activity was determined by a modified spectroscopic assay based on the method described previously.[12,28] (*R*,*S*)-3-Hydroxyacyl-CoAs were prepared as described previously.[20] One unit of enzymatic activity is defined as the amount required to catalyze the transformation of 1 μmol substrate in 1 min.

Molecular Weight Analysis of P(3HB). For P(3HB) production, cells were grown in 100 mL LB medium containing glucose (2 wt %/v) and ampicillin (100 μg/mL) at 30 °C for 96 h. The harvested cells were lyophilized, and PHA was extracted with chloroform for 72 h at room temperature and purified by reprecipitation with hexane. Molecular masses of the polymers were determined by gel permeation chromatography (GPC) calibrated using polystyrene standards.[29]

Results

Effect of the S477R Mutation in PHA Synthase on in Vivo PHA Production. We isolated several positive mutants containing the S477R substitution from the random mutant library of the PHA synthase gene of *Pseudomonas* sp. 61−3.[21] Different codons encoding Arg (AGA and AGG) were found at the 477 position using positive screening, suggesting that the S477R amino acid substitution affected the function of PHA synthase. Thus, we characterized the activity and substrate specificity of the S477R mutant using the in vivo and in vitro assay system. First, we constructed the S477R single substitution and found that the S477R mutation contributed to the enhancement in P(3HB) accumulation (Table 1). Next, the in vivo substrate specificity of PHA synthase was investigated by examining unit composition in the generated copolymer. The monomer composition of the P(3HB-*co*-3HA) copolymer produced by the S477R mutant shifted toward smaller monomers compared to that of the wild-type enzyme (Table 1). However, the content of the PHA copolymer produced by the S477R mutant (9 wt %) was lower than that of the wild-type enzyme (11 wt %). This result combined with the enhanced P(3HB) accumulation observed when the cells were grown with glucose suggested that the S477R mutation contributed to a change in substrate specificity toward smaller substrates rather than to an increase in the total enzymatic activity.

Table 1. PHA Contents and Monomer Compositions of P(3HB-*co*-3HA) in the Recombinant *E. coli* Harboring the Wild-Type and Mutated PHA Synthase Genes

PHA synthase	P(3HB) content[a] (wt %)	P(3HB-*co*-3HA) content[b] (wt %)	monomer composition[b] (mol %) 3HB (C_4)	3HHx (C_6)	3HO (C_8)	3HD (C_{10})	3HDD (C_{12})
wild-type	0.1	11	13	18	39	19	11
Mutants							
S477R	0.5	9	30	35	24	8	3
E130D/S477R	6	27	29	31	24	11	5
S325C/S477R	37	21	35	31	21	9	4
S325T/S477R	36	17	30	30	23	11	6
S477R/Q481K	1	1	**56**	25	11	5	3
S477R/Q481M	5	6	**55**	27	12	4	2
S477R/Q481R	0.8	0.2	**62**	24	14	-	-
E130D/S325C/S477R	42	26	31	32	22	10	5
E130D/S325T/S477R	43	17	22	31	25	14	8
E130D/S477R/Q481K	3	10	47	27	15	7	4
E130D/S477R/Q481M	**25**	15	48	29	14	6	3
E130D/S477R/Q481R	3	4	53	26	13	5	3
S325C/S477R/Q481K	8	17	50	27	14	6	3
S325C/S477R/Q481M	**39**	10	53	27	13	5	2
S325C/S477R/Q481R	6	8	55	26	12	5	2
S325T/S477R/Q481K	39	23	41	28	18	9	4
S325T/S477R/Q481M	41	13	47	29	15	6	3
S325T/S477R/Q481R	39	23	45	28	16	7	4
E130D/S325C/S477R/Q481K	40	11	51	26	14	6	3
E130D/S325C/S477R/Q481M	42	7	54	27	12	5	2
E130D/S325C/S477R/Q481R	40	12	52	26	13	6	3
E130D/S325T/S477R/Q481K	46	13	39	30	18	9	4
E130D/S325T/S477R/Q481M	42	3	44	34	15	5	2
E130D/S325T/S477R/Q481R	42	5	47	28	15	7	3

[a] Cells harboring pGEM″C1AB were grown on glucose. P(3HB) content in dry cells is presented as an average of three independent experiments. [b] Cells harboring pGEM″C1ABJ4 were grown on dodecanoate. P(3HB-*co*-3HA) content and monomer compositions in dry cells are presented as an average of three independent experiments.

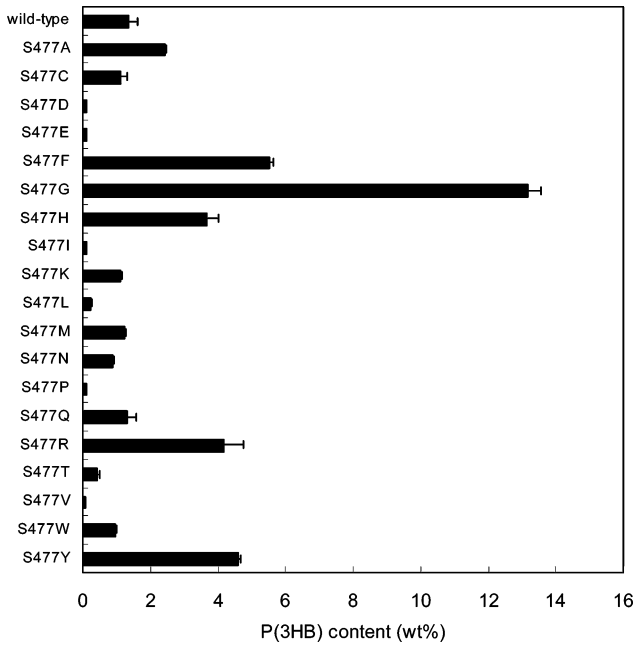

Figure 1. Effect of saturation mutagenesis of the amino acid residue 477 on P(3HB) accumulation in the recombinant *E. coli* JM109.

Our previous studies demonstrated that the combination of beneficial mutations of PHA synthase leads to a synergistic increase in the enzymatic activity and/or change in substrate specificity. Therefore, a recombination of these beneficial mutations with the S477R mutation was expected to exhibit stronger activity. In this study, we characterized the recombinants of S477R with E130D, S325C/T, Q481K/M/R, and multiple combinations of all the mutations. Table 1 summarizes in vivo P(3HB) and P(3HB-*co*-3HA) productions in the recombinant *E. coli* harboring the recombinant mutant genes of PHA synthase. The (S325C/T)/S477R (S325C/S477R and S325T/S477R) double mutations strongly enhanced P(3HB) accumulation and shifted monomer composition toward C_4 and C_6 units. However, the combination of S477R and E130D had little effect on P(3HB) production, suggesting that this mutation causes an unfavorable interaction at both sites for enzymatic activity. The double mutations of S477R and Q481K/M/R also did not enhance the PHA production. However, they synergistically changed the monomer composition of PHAs. The mutations at the positions of 477 and 481 seemed to cooperate to hinder the incorporation of the MCL substrate into a polymer chain. For the triple mutants, the addition of the S477R substitution to the mutants containing the E130D substitution had a negative effect on P(3HB) accumulation and little effect on monomer composition. This result also suggested the improper orientation of Asp130 and Arg477 residues. The E130D/S477R/Q481M mutant produced an exceptionally high 25 wt % of P(3HB). The Q481M mutation may rescue the unfavorable interaction caused by the E130D/S477R combination. All of the triple recombinants of (S325C/T)/S477R/(Q481K/M/R) showed a large shift in monomer composition toward incorporating smaller monomers, although they also simultaneously decreased the P(3HB-*co*-3HA) content compared to the (S325C/T)/(Q481K/M/R) double recombinants. The quadruple recombinants [E130D/(S325C/T)/S477R/(Q481K/M/R)] had similar properties for the triple mutants; that is, they

Table 2. PHA Contents and Monomer Compositions of P(3HB-*co*-3HA) Copolymers Produced by the Recombinant *E. coli* LS5218 Expressing the S477X Mutant Genes

		monomer composition (mol %)				
PHA synthase	PHA content[a] (wt %)	3HB (C_4)	3HHx (C_6)	3HO (C_8)	3HD (C_{10})	3HDD (C_{12})
wild-type	11	13	18	39	19	11
S477A mutant	11	16	23	35	17	9
S477C mutant	1	15	23	39	16	7
S477F mutant	19	27	30	26	12	5
S477G mutant	15	18	32	29	15	6
S477H mutant	11	23	24	32	14	7
S477K mutant	1	36	33	19	8	4
S477M mutant	1	15	27	36	15	7
S477N mutant	6	13	20	40	18	9
S477Q mutant	1	17	30	34	14	5
S477R mutant	9	30	35	24	8	3
S477Y mutant	6	28	29	26	12	5

[a] Cells were grown on M9 medium containing 0.3% sodium dodecanoate at 37 °C for 72 h.

enhanced P(3HB) production, caused a decrease in P(3HB-*co*-3HA) production, and changed the monomer composition. In summary, the S477R mutation synergistically enhanced the PHA production by recombining with S325C/T mutations but not with the E130D mutation. Although the recombinants with multiple mutations containing the S477R mutation produced less P(3HB-*co*-3HA), the polymer had a higher 3HB and 3HHx molar ratio when compared to other copolymer samples (Table 1). The S477R and Q481K/M/R mutations had a similar effect, and the combination of these mutations further changed the monomer composition. However, the combinatorial effect of S477R and Q481K/M/R exhibited diversified patterns when the PHA copolymer was synthesized. The Arg477 and Met481 seemed to be a synergistic combination in terms of effective in vivo PHA production, whereas the combination of S477R with E130D, Q481K, and Q481R mutations exhibited a negative effect on PHA production. However, the addition of the other mutations reverted the phenotype exhibited by the S477R/E130D and S477R/Q481K recombinants, strongly suggesting that the four sites, 130, 325, 477, and 481, are located close together in the 3-D structure of PHA synthase.

Saturation Mutagenesis at the Position of 477. The effect of amino acid substitution at position 477 was further investigated by saturation mutagenesis (S477X). The levels of produced protein of the wild-type and mutant PHA synthases were nearly identical based on an immuno-blot analysis (data not shown). Figure 1 shows the P(3HB) content in the recombinant *E. coli* expressing the S477X mutants. Six mutants exhibited higher PHA content than the wild-type synthase, and the other thirteen mutants retained the ability to accumulate PHAs. The substitution of S477 with aromatic residues (Phe and Tyr) and basic residues (His and Arg) resulted in increased P(3HB) production. Interestingly, the S477Gly(G) mutant exhibited an especially high P(3HB) content (13 wt %). However, the substitution with Ile, Leu, Pro, or Val residues, which have aliphatic side chains, led to a loss of the function of the PHA synthase. The acidic amino acid residues (Asp and Glu) also decreased the function of the enzyme.

Substrate specificities of the eleven mutants, which were chosen on the basis of the ability to accumulate P(3HB), were investigated by in vivo copolymer production. Table 2 shows the content and monomer composition of P(3HB-*co*-3HA) copolymers accumulated in the recombinant *E. coli* grown on dodecanoate. P(3HB-*co*-3HA) contents of *E. coli* harboring the

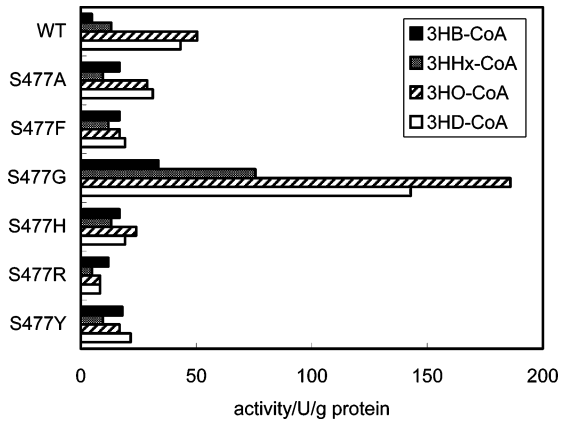

Figure 2. In vitro activity of the wild-type and S477X mutated PHA synthases produced by the recombinant *E. coli*. 3HB-CoA, 3-hydroxybutyryl-CoA; 3HHx-CoA, 3-hydroxyhexanoyl-CoA; 3HO-CoA, 3-hydroxyoctanoyl-CoA; and 3HD-CoA, 3-hydroxydocanoyl-CoA.

S477Asp(D)/Glu(E)/Ile(I)/Leu(L)/Pro(P)/Thr(T)/Val(V)/Trp(W) mutants were not detectable. The monomer composition of P(3HB-*co*-3HA) produced by the S477Ala(A)/His(H)/Arg(R)/Try(Y) mutants was shifted toward the smaller monomer units (3HB and 3HHx), but these mutants accumulated approximately the same or lower amounts of PHAs compared to the wild-type enzyme, indicating that the S477A/H/R/Y mutations did not increase the total activity but rather altered the substrate specificity of the enzyme. The S477Cys(C)/Lys(K)/Met(M)/Gln(Q) mutants produced only a trace amount of P(3HB-*co*-3HA) (1 wt %). However, these same substitutions did not affect P(3HB) production, suggesting that the S477C/K/M/Q mutations reduced the affinity toward MCL substrates. In contrast, the S477F/G mutations led to an increase in P(3HB-*co*-3HA) production (19 and 15 wt %, respectively) compared to the wild-type enzyme. In addition, the S477F/G mutants synthesized P(3HB-*co*-3HA) with higher molar ratios of C_4 and C_6, indicating that the S477F/G mutations enhanced the enzymatic activity and also slightly altered substrate specificity.

In Vitro Assay of the Enzymatic Activity of the S477X Mutations. The effects of the S477X mutations were further characterized by the in vitro assay of enzymatic activity. The S477A/F/G/H/R/Y mutants, which accumulated P(3HB-*co*-3HA), were chosen for the in vitro analysis (Figure 2). The S477A/F/H/R/Y mutants exhibited enhanced activity toward the C_4 substrate (12.0−18.0 U/g) and decreased activity toward C_8 (8.4−28.8 U/g) and C_{10} (8.4−31.2 U/g) substrates compared to the wild-type enzyme. A similar phenomenon was observed with the Q481K mutant.[12] Overall, the in vitro substrate specificity of PHA synthase was consistent with the in vivo monomer composition of PHAs. However, an exception to this trend was that the activity of the S477F mutant toward MCL substrates was lower than the activity of the wild-type enzyme in vitro, despite the fact that this mutant exhibited an overall increase in P(3HB-*co*-3HA) accumulation in vivo (19 wt %). The S477G mutant had enhanced activity toward all substrates tested, and the relative activities toward C_4 and C_6 substrates versus C_8 and C_{10} substrates were also increased. The in vitro activities of the S477G mutants were similar to those of the E130D mutant.[20] This result is consistent with the increase in P(3HB) and P(3HB-*co*-3HA) content and the alteration in the monomer composition in vivo by the S477G mutant. On the basis of these in vivo and in vitro results, we concluded that the substitutions at the 477 position have two distinct effects. One is an alteration in substrate specificity to C_4 and C_6 substrates that is probably due to a hindrance of the incorpora-

Table 3. Molecular Weights of P(3HB) Homopolymer Produced by the Recombinant *E. coli* Harboring the Wild-Type and Mutated PHA Synthase Genes

	molecular weight[a]		
PHA synthase	M_n (10^4)	M_w (10^4)	M_w/M_n
wild-type	19	43	2.2
S477A mutant	28	95	3.4
S477F mutant	48	187	3.9
S477G mutant	31	92	3.0
S477H mutant	61	264	4.3
S477R mutant	60	279	4.7
S477Y mutant	40	237	6.0

[a] M_n, number-average molecular weight; M_w, weight-average molecular weight; M_n/M_w, polydispersity.

tion of MCL substrates. Another is an increase in the total enzymatic activity by the S477G mutation.

Molecular Weights of PHA Synthesized by the S477X Mutants. Several mutated PHA synthases were reported to have synthesized PHAs with higher molecular weights.[20] This result prompted us to investigate the effect of the S477X mutations on the molecular weight of PHAs. Table 3 shows the molecular weights and polydispersities of P(3HB) synthesized by the wild-type and S477X mutated PHA synthases. The molecular weights of P(3HB) synthesized by the S477/A/F/G/H/R/Y mutants (M_n = 280 000−610 000 and M_w = 920 000−2 790 000) were higher than those synthesized by the wild-type PHA synthase (M_n = 190 000 and M_w = 430 000). The PHAs produced by the S477X mutants also had very high polydispersities (3.0−6.0), compared to the wild-type and E130D, S325T, and Q481K mutated PHA synthases (2.0−3.0).[20]

Discussion

In vitro evolved PHA synthases enable us to control the activity and substrate specificity of PHA synthase: critical factors for producing PHAs with desirable properties.[5] In this study, we characterized new beneficial S477X mutants of PHA synthase and revealed several interesting features of the mutants. Previously, the beneficial mutations in PHA synthase were classified into two types: E130D and S325C/T mutations, which increase the enzymatic activity toward all substrates, and Q481K/M/R mutations, which alter the substrate specificity of the enzyme.[12,20] The in vivo and in vitro analyses of the S477X mutations revealed that S477A/F/H/R/Y mutations altered substrate specificity as well as the Q481K/M/R mutations. However, the S477G mutations enhanced total enzymatic activity that was similar to the E130D mutants, indicating that two distinct effects occurred because of the S477X mutations. These new beneficial mutations at position 477 are very useful, because the activity of PHA synthase can be synergistically enhanced by combining it with other beneficial mutations; for example, the S325T/S477R double mutant produced more PHAs than each single mutant did. The S477R mutations, on the other hand, exhibited a negative combinatorial effect with the Q481K/R mutations. However, they had better synergy when a Met residue was located at the 481 position (Table 1). This result suggested that the combinatorial effect of residue 477 and 481 is very delicate. Therefore, we then investigated the shuffling of S477X/Q481X mutations to examine all possible multiple mutations and found several activity-enhanced mutants that will be reported in the future.

The saturation mutagenesis experiment of residue 477 demonstrated that substitutions occurring in fifteen mutants were enzymatically active (Figure 1). This result presents a sharp

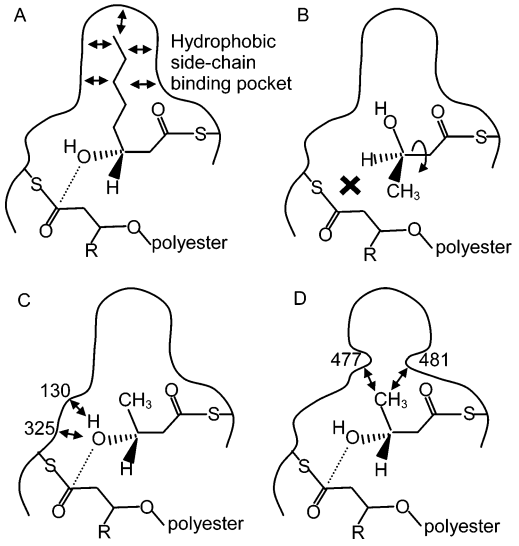

Figure 3. Proposed mechanism accounting for the effect of amino acid substitutions in the PHA synthase on activity and substrate specificity. (A) Binding of MCL side chain may hold the 3-hydroxyl group of the incoming substrate in the proper orientation. (B) Class II PHA synthase has little activity toward SCL substrates, since mobility of the enzyme-bound substrate causes a low rate of elongation. (C) Interaction with the 3-hydroxyl group, which holds the 3-hydroxyl group to the proper orientation, leads to an enhancement in total enzymatic activity. (D) Blockage of the MCL substrate and/or stabilization of SCL side chain leads to a change in substrate specificity toward smaller substrates.

contrast to the observed phenotypes of other beneficial mutations in which only a few amino acid substitutions retained activity. For example, only Glu and Asp residues were active at the 130 position,[20] indicating that a carboxyl group is essential for activity of the enzyme, whereas polar hydrogen was required at the 325 position.[12] The tolerance of the activity toward the number of substitutions at residue 477 suggested that the residue does not directly contribute to the formation of an ester bond, but rather, this position contributes to the structure of the enzyme. This idea is consistent with the fact that most of the S477X mutations altered the substrate specificity of the enzyme but not the activity.

From the in vivo and in vitro analyses of the mutated PHA synthase, we created a possible mechanism for the synergy of the beneficial mutations (Figure 3). It is odd that most class II PHA synthases have little or no activity toward SCL substrates even though these substrates are smaller than MCL 3HA-CoAs. A possible interpretation is that the binding of the side chain of the incoming monomer substrate into the hydrophobic pocket holds the 3-hydroxyl group in a particular position to drive the polymerization of MCL 3HA-CoAs (Figure 3A). If the 3-hydroxyl group of the SCL substrate is highly mobile, the rate of ester formation might decrease (Figure 3B). The orientation of the 3-hydroxyl group must be greatly restricted because PHA synthase does not polymerize any S-form substrates. Therefore, a possible mechanism for enhancing enzymatic activity toward SCL substrates is an increase in the ability to properly position and hold the 3-hydroxyl group in place for catalysis (Figure 3C). On the basis of this model, the enhancement in the enzymatic activity should be independent of the side chain length of the substrates. The E130D and S325C/T mutations may work by this manner because these mutants increased the total enzymatic activity.

However, the substrate specificity of the enzyme can be changed by reducing the access of the incoming substrate to

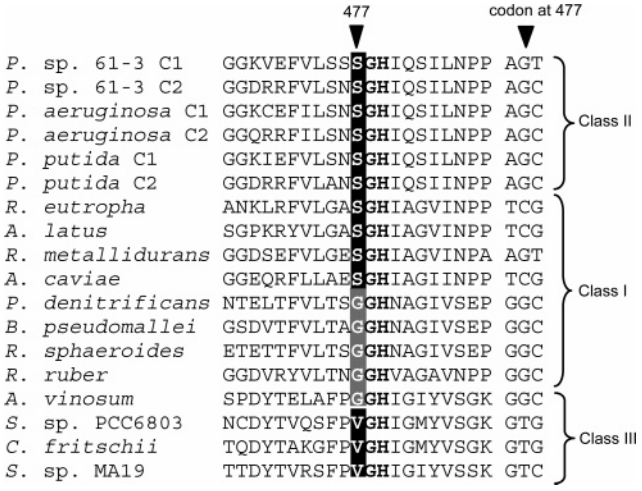

Figure 4. Partial alignment of the amino acid sequences of PHA synthase. The black box indicates the Ser477 from *Pseudomonas* sp. 61−3 PhaC1 and corresponding amino acids from other PHA synthases. The bold letters indicate conserved GH dyad. *P.* sp. 61−3, *Pseudomonas* sp. 61−3; *A. latus*, *Alcaligenes latus*; *R. metallidurans*, *Ralstonia metallidurans*; *P. denitrificans*, *Paracoccus denitrificans*; *B. pseudomallei*, *Burkholderia pseudomallei*; *R. sphaeroides*, *Rhodobacter sphaeroides*; *R. ruber*, *Rhodococcus ruber*; *A. vinosum*, *Allochromatium vinosum*; *S.* sp. PCC6803, *Synechocystis* sp. PCC6803; *S.* sp. MA19, *Synechococcus* sp. MA19.

the side chain binding pocket or by creating an environment to stabilize the short side chains of the monomer substrates (Figure 3D). As the size of the side chain binding pocket decreases, incorporation of MCL substrates would be inhibited. In addition, a small hydrophobic pocket may stabilize the enzyme-bound SCL substrates that may accelerate the polymerization of SCL substrates. The mutations at the residues 477 and 481 may change the substrate specificity of the enzyme by this hypothetical mechanism. Moreover, this model suggests that combining the proper positioning of the 3-hydroxyl group and stabilizing the short side chain can accelerate the polymerization of SCL substrates in tandem. This may be the mechanism for the synergistic enhancement in P(3HB) production due to the combination of beneficial mutations. The model is also consistent with the mutational effect of class I PHA synthase. Tsuge et al. reported that most substitutions at amino acid position 510 of PHA synthase from *R. eutropha* ($PhbC_{Re}$), which corresponds to position 481 of $PhaC1_{Ps}$, had no effect on or negatively affected the P(3HB) production.[30] We presumed that the A510X mutations may not affect the mobility of the enzyme-bound substrate, because the substrate binding site of the Class I $PhbC_{Re}$ is likely small compared to the class II PHA synthases, and thus, the A510X mutations had little effect on P(3HB) production.

The S477G mutation surprisingly had the highest total enzymatic activity among the S477X saturation mutations, and many PHA synthases have a Gly residue at the corresponding position. Figure 4 shows a partial alignment of PHA synthases. The Ser residue at the 477 position is conserved among class II PHA synthases. The PHA synthases from *R. eutropha* and *A. caviae*, which belong to class I, also have a Ser residue at the corresponding position. Because the codon for the Ser residue of class I PHA synthases is different from that of class II enzymes, we presumed that the Ser residue was not directly inherited from the ancestral PHA synthase but was instead likely the result of convergent evolution. However, when this position is aligned between class I and class III PHA synthases, a Gly residue is predominant. At this position, Ser and Gly seem to be most common, although these residues are not essential for the activity of the enzyme, suggesting that these residues could have another advantage aside from enzymatic activity, such as stability of the enzyme or substrate affinity. The exceptions are PHA synthases from cyanobacteria. The PHA synthases from *Synechocystis* sp. PCC6803,[30] *Chlorogloeopsis fritschii* PCC 6912, and *Synechococcus* sp. MA19[31] have a Val residue at their corresponding position. Our results showed that the S477V mutant of $PhaC1_{Ps}$ was inactive, at least toward 3HB-CoA, suggesting that PHA synthases from cyanobacteria have a different structure in this local space contacting the substrate from class II PHA synthases.

The molecular weight of PHAs is a very important factor that affects their physical strength. However, little is known about the mechanism for determining their molecular weight.[32,33] We previously found that the E130D mutation in PHA synthase led to an alteration in the PHA molecular weight.[20] The S477X mutated PHA synthases also produced PHAs with various molecular weights. The S477H/R/Y mutants produced P(3HB)s with very high M_w, whereas the extremely active S477G mutant synthase produced P(3HB) with relatively lower M_w compared to all other mutants tested. The Q481K mutation was also shown to increase the M_w of PHAs, whereas highly active mutants, such as E130D/S325T, produced low M_w PHAs. This phenomenon is counterintuitive, because, if the rates of termination of polymerization and the number of PHA synthases were constant, the molecular weight of polymer would be commensurate with the turnover of the enzyme. Two possible interpretations can explain the phenomenon. One is that the mutations affected the stability of the enzyme and changed the number of PHA synthases in the active form. According to our immuno-blot analysis, the concentrations of the enzymes were nearly identical, but the immuno-blot analysis does not distinguish between the active and inactive forms of PHA synthase enzymes. A second possible explanation is an activation of water by the residues 130 and 325. Stubbe et al. suggested that Asp residue near the active center activated water and caused the PHAs to hydrolyze.[34] If the mutations promoted the activation of water, they would lead to low molecular weight of PHAs due to hydrolysis.

The evolutionary engineered PHA synthases increased the productivity of PHA copolymers and enabled the regulation of the monomer composition. These PHA synthases, which have been selected on the basis of the PHA content in *E. coli*, exhibited enhanced activity also in the recombinant *R. eutropha*[35] and the transgenic *Arabidopsis thaliana*,[36] indicating that the evolutionary engineered PHA synthases are functional under various conditions and have a wide range of potential hosts and applications. The S477X mutants have shown further potential to increase the activity of the PHA synthase using the recombination with the other mutations. The highly active multiple mutant PHA synthases will improve the efficiency of SCL/MCL PHA production in various recombinant organisms.

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