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Engineering of polyhydroxyalkanoate synthase by Ser477X/Gln481X saturation mutagenesis for efficient production of 3-hydroxybutyrate-based copolyesters

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Abstract

Class II polyhydroxyalkanoate synthase from *Pseudomonas sp.* 61-3 (PhaC1_{Ps}) synthesizes 3-hydroxybutyrate (3HB)-based copolyesters, P[3HB-*co*-3-hydroxyalkanoate (3HA)]. Four sites (130, 325, 477 and 481) in PhaC1_{Ps} that affect the cellular content and 3HB fraction of P(3HB-*co*-3HA) produced have been identified. Simple combination of beneficial mutations at the sites successfully increased 3HB fraction in the copolymers (62 mol%). However, polymer content was often largely decreased (0.2 wt%) regardless of an enhancement in 3HB fraction, compared to the wild-type enzyme (14 mol% 3HB and 12 wt%) [Matsumoto et al. (2006) *Biomacromolecules*, 7: 2436-2442]. In the present study, we attempted to explore residues combination at the four sites to overcome the problem. Here, pairwise saturation mutagenesis at the neighboring sites 477 and 481 of PhaC1_{Ps} was performed using single and double mutations at sites 130 and 325 as templates, to increase 3HB fraction in the copolymer without reducing the polymer content in recombinant *Escherichia coli*. These useful PhaC1_{Ps} mutants were screened based on enhanced P(3HB) content, and were subsequently applied to P(3HB-*co*-3HA) production. Among the mutants tested, the Ser325Cys/Ser477Lys/Gln481Leu mutant exhibited increased 3HB fraction in copolymer (63 mol%) and also polymer content (18 wt%), indicating that mutation scrambling was effective for obtaining the desired mutants.

Introduction

Polyhydroxyalkanoates (PHAs) are biodegradable polyesters produced as intracellular carbon storage materials in numerous bacteria (Doi 1990; Rehm 2003). PHAs are of interest because they are thermoplastics that can be processed into a variety of useful plastic materials and because they have the potential to replace petrochemical plastics. To date, approximately 200 PHA-producing bacteria have been isolated (Sudesh et al. 2000).

The most common PHA, the homopolymer of 3-hydroxybutyrate [P(3HB)], is efficiently produced by a variety of wild-type and recombinant bacteria (Madison et al. 1999), but the polymer's brittleness limits its range of applications. However, the potential applications of PHAs could be expanded by introducing medium-chain-length (MCL, C₆-C₁₂) (*R*)-3-hydroxyalkanoate (3HA) units into the polymer chain (Matsusaki et al. 2000). Compared to P(3HB), 3HB-based copolymer including 3HA units, P(3HB-*co*-3HA)s have been shown to be sufficiently flexible and tough for use in a wide range of applications (Noda et al. 2005). Since the properties of P(3HB-*co*-3HA) can be adjusted over a wide range by choosing the ratio of 3HB to 3HA units, regulation of the monomer composition is very important for producing P(3HB-*co*-3HA) with the desired properties.

PHA synthase plays a central role in PHA biosynthesis (Rehm 2003). Substrate specificity of the enzyme is a dominant factor affecting the monomer composition of PHAs. PHA synthases are classified into four groups based on their substrate specificity and subunit structure (Rehm 2003). Class I PHA synthases possess high polymerization activity toward 3HB-CoA. They typically have narrow substrate specificity and synthesize short-chain-length (SCL, C₄ and C₅) PHAs. In contrast, most of the class II PHA synthases exhibit substrate specificity toward MCL monomers and synthesize MCL PHAs. A class II PHA synthase from *Pseudomonas sp.* 61-3 (PhaC1_{Ps}) has

exceptionally broad substrate specificity and synthesizes P(3HB-*co*-3HA) copolymers consisting of monomers 4- to 14-carbons long (Matsusaki et al. 1998). We have investigated 3HB-based P(3HB-*co*-3HA) production using PhaC1_{Ps} (Matsusaki et al. 1998). However, the polymer content and the range of the 3HB fraction [molar ratio of 3HB unit in P(3HB-*co*-3HA)] in the copolymers have been limited. For example, recombinant *E. coli* LS5218 expressing wild-type PhaC1_{Ps} produced 13 wt% P(3HB-*co*-3HA) comprised of only 14 mol% 3HB units when grown on dodecanoate (Takase et al. 2004). The problem is due to the weak activity of PhaC1_{Ps} toward 3HB-CoA (Matsumoto et al. 2002).

We therefore attempted to create a novel PHA synthase capable of producing P(3HB-*co*-3HA) with a larger 3HB fraction through evolutionary engineering of PhaC1_{Ps} (Takase et al. 2003; Taguchi et al. 2004; Nomura et al. 2007; Taguchi et al. 2008). Random mutagenesis of PhaC1_{Ps} previously addressed the four useful sites, Glu130(E130), Ser325(S325), Ser477(S477), and Gln481(Q481), which contribute to enhanced activity and alteration of substrate specificity of the enzyme (Takase et al. 2004; Matsumoto et al. 2005). *In vivo* and *in vitro* analyses of the beneficial mutants revealed that single and double mutations at sites 130 and 325 (E130D, S325C, S325T, E130D/S325C and E130D/S325T) enhanced enzymatic activity toward all monomer substrates tested (C₄ to C₁₀). In contrast, mutations at sites 477 and 481 shifted substrate specificities to smaller monomers, mainly 3HB-CoA (Matsumoto et al. 2006; Matsumoto et al. 2009).

We previously created 54 engineered enzymes by simply combining beneficial mutations at the four useful sites to increase 3HB fraction in P(3HB-*co*-3HA). In fact, these engineered enzymes increased 3HB fraction from 14 to 62 mol%. However, polymer content was significantly decreased compared with wild-type PhaC1_{Ps} with elevation of 3HB fraction in the copolymer. As a typical example, Ser477Arg/Gln481Arg (S477R/Q481R) double mutants synthesized P(3HB-*co*-3HA) with 62 mol% 3HB, but polymer content was only 0.2 wt% (Matsumoto et al.

2006). These amino acid substitutions at individual sites were effective for enhanced 3HB fraction, but polymer content was reduced upon their combination, suggesting complexity of mutational combination between the two neighboring sites 477 and 481. Indeed, the three-dimensional structure prediction of PhaC1_{Ps} suggested that residues 477 and 481 could be adjoining in the tertiary structure (Fig. 1). Therefore, in this study, we attempted to explore mutational combinations that achieve an enhancement in 3HB fraction without reducing polymer content, through saturation mutagenesis at sites 477 and 481 using beneficial mutations at the other two sites (130 and 325) as templates.

Materials and Methods

S477X/Q481X saturation mutagenesis

The S477X/Q481X mutated PHA synthase genes from *Pseudomonas* sp. 61-3 were constructed by oligonucleotide-directed mutagenesis using the following primers: 5'-AAGGTCGAATTCGTGCTGTCCAGCNNNGGGCATATCNNNAGC-3' and 5'-CCAGTTCGGTGCCGGGATCTTGGCCAGCG-3'. Underlined sequences, which encode codons at sites 477 and 481, were randomized for saturation mutagenesis. A 0.3 kb DNA fragment including restriction enzyme sites of *Eco*RI and *Pst*I was ligated with pGEM⁺C1AB (Takase et al. 2003) vectors harboring the wild-type, E130D, S325C, S325T, E130D/S325C, and E130D/S325T mutated PHA synthase genes (Fig. 2). Sequencing of the amplified region was performed for selected pGEM⁺C1AB vectors. pGEM⁺C1ABJ4 vectors were constructed from selected pGEM⁺C1AB vectors, as described previously (Takase et al. 2004). pGEM⁺C1AB vectors harbor the PHA synthase gene (*phaC*_{Ps}), β -ketothiolase gene (*phaA*_{Re}) and NADPH-dependent acetoacetyl-CoA reductase gene (*phaB*_{Re}) isolated from *Ralstonia eutropha* (*Cupriavidus necator*). pGEM⁺C1ABJ4 vectors harbor the enoyl-CoA hydratase 4 gene (*phaJ*_{Pa}) isolated from *Pseudomonas aeruginosa* (Tsuge et al. 2003) in addition to the *phaC*_{1AB} genes.

Screening of highly active mutants

Screening of highly active mutants of PHA synthase was carried out based on previously established *in vivo* screening system (Spiekermann et al. 1999; Taguchi et al. 2001). The recombinant *E. coli* JM109 (Yanisch-Perron et al. 1985) harboring pGEM⁺C1AB encoding

pairwise saturation mutants were grown on LB plates supplemented with glucose [2% (w/v)], Nile red (0.5 µg/ml), and ampicillin (100 µg/ml). The colonies producing large amount of P(3HB) were selected based on intensity of the pinkish pigmentation of the cells.

Analyses of the content and monomer composition of P(3HB) and P(3HB-*co*-3HA)

E. coli JM109 was used as a host cell for producing P(3HB). Recombinant JM109 strains harboring pGEM[™]C1ABs were cultured in 1.75 ml Luria-Bertani (LB) medium in a glass test tube containing glucose [2% (w/v)] and ampicillin (100 µg/ml) for 48 h at 30°C. The cellular P(3HB) content was determined using HPLC as described previously (Karr et al. 1983). For accumulating P(3HB-*co*-3HA), *E. coli* LS5218 [*fadR601*, *atoC*(Con)] was used (Spratt et al. 1981). Recombinant LS5218 strains harboring pGEM[™]C1ABJ4s were cultured in M9 medium (Sambrook et al. 1989) containing sodium dodecanoate [0.3% (w/v)] and ampicillin (100 µg/ml) with brij-35 [0.4% (w/v)] (Kishida Chemical Co., LTD, Japan) for 72 h at 37°C. Brij-35 is a good detergent, which aids solubilization of dodecanoate and recombinant bacteria to utilize dodecanoate. Content and monomer composition of P(3HB-*co*-3HA) were analyzed using gas chromatography (GC) as described previously (Kato et al. 1996). HPLC analysis is a simple and quick method to quantify P(3HB) whereas GC is suitable for copolymer analysis.

Molecular weight Analysis of the P(3HB-*co*-3HA)

P(3HB-*co*-3HA)s accumulated in the cells were extracted with chloroform and precipitated by adding 10-fold volume of methanol to purify the polymer. Molecular weight of P(3HB-*co*-3HA)s was determined using gel permeation chromatography (GPC) (Jasco, Japan) equipped with

KF-804L columns (Shodex, Japan). The molecular weight and polydispersity of the polymers were calculated on the basis of a polystyrene calibration.

Immunoblot Analysis

Expression levels of PhaA_{Re}, PhaB_{Re}, and PhaC1_{Ps} in recombinant *E. coli* JM109 were confirmed by immunoblot analysis with rabbit antisera (anti-PhaA_{Re}, anti-PhaB_{Re}, and anti-PhaC1_{Ps}) as described previously (Takase et al. 2004; Jo et al. 2007). Recombinant *E. coli* JM109 strains harboring pGEM⁺C1ABs were grown on LB medium containing ampicillin (100 µg/ml), in which no PHA is accumulated. The crude extracts of harvested strains were prepared by sonication as described previously (Takase et al. 2004).

Results

Pairwise saturation mutagenesis of PHA synthase at sites 477 and 481

Initially, tertiary structure prediction was performed by homology modeling through the PHYRE server (<http://www.sbg.bio.ic.ac.uk/~phyre/>) as described previously (Arias et al. 2008). The structural prediction made feasible the systematic functional mapping that allowed exploration of the useful sites. PhaC1_{Ps} has no strong similarity to any proteins of known structure. However, remote homologues with similar global folding were found by PHYRE. The partial sequence of PhaC1_{Ps} corresponding to the putative α/β hydrolase fold region, which includes sites 325, 477, and 481, was threaded onto an epoxide hydrolase from human (SCOP encodes d1zd3a2) (Fig. 1). In the structure predicted, the S325, S477, and Q481 residues are closely located to Cys296, which is inferred to be a catalytic center site of the enzyme. In addition, the sites S477 and Q481 are located proximally to another plausible catalytic dyad residue, H479 (Amara et al. 2003). These findings suggest that residues at sites 477 and 481 have to be finely adjusted rather than simple combinations of beneficial single mutations at each site to alter the properties of the enzyme. Therefore, we applied pairwise saturation mutagenesis at these sites to explore the desired mutations increasing 3HB fraction while retaining polymer content.

As a mutation-scrambling program, six sets of pairwise saturation mutants at sites 477 and 481, S477X/Q481X, of PHA synthase were constructed using the wild-type, E130D, S325C, S325T, E130D/S325C, and E130D/S325T as templates (Fig. 2). The mutated PHA synthase genes were introduced into *E. coli* along with the *phaAB* genes. Approximately 9200 clones were screened by colony formation on selection plates containing glucose and Nile red. Out of these, 41 clones were selected based on P(3HB) accumulation, because PhaC1_{Ps} activity toward 3HB-CoA should be a dominant factor for productivity of 3HB-based P(3HB-co-3HA) and 3HB

fraction in the copolymers. Figure 3 summarizes P(3HB) content in the recombinant *E. coli* harboring the selected mutants. We did not find any positive colonies - *i.e.*, colonies exhibiting enhanced pigmentation compared with those harboring the template plasmid - in S477X/Q481X mutants (wild-type derivatives), indicating that the wild-type derivatives hardly increased P(3HB) content. Several highly active mutants generated from E130D and S325T were isolated, but most of them were identical to those obtained previously (Matsumoto et al. 2006). However, many new positive mutants were obtained from S325C, E130D/S325C, and E130D/S325T derivatives.

Thirteen triple mutants and three double mutants of S325C derivatives accumulated greater amounts of P(3HB) than the original S325C mutant (2.0 wt%). P(3HB) content varied widely, between 3.9 and 66 wt%. Site 477 was occupied by various amino acids, aliphatic residues (Ala, Gly, and Val), aromatic residues (Phe and Tyr), positively charged residues (Lys and Arg), and Asn, suggesting that particular functional groups are not required at the position as described previously (Matsumoto et al. 2006). Among these, Gly and Arg residues at site 477 led to high P(3HB) accumulation. Site 481 was frequently occupied by a restricted selection of amino acids: positively charged residues (Arg and Lys) and aliphatic residues (Leu, Met, and Val).

In contrast to the S325C-derived mutants, most of the selected E130D/S325T-derived quadruple mutants had Gly at site 477. Trends in the E130D/S325C-derived mutants were similar to those in E130D/S325T-derived mutants in terms of selected amino acid residues. Many highly active mutants were obtained by pairwise saturation mutagenesis at sites 477 and 481. Of these mutants, E130D/S325T/S477G/Q481L exhibited the highest P(3HB) content (68 wt%).

Production of P(3HB-*co*-3HA) using highly active mutants

We investigated P(3HB-*co*-3HA) production using the selected mutants. Figure 4 summarizes P(3HB-*co*-3HA) content and monomer composition of P(3HB-*co*-3HA)s produced in recombinant *E. coli* grown on dodecanoate. These selected mutants exhibited a wide range of

monomer composition (14 to 70 mol%). The S325C-derived mutants incorporated a relatively large amount of 3HB. For example, the S325C/S477K/Q481L mutant produced P(3HB-*co*-3HA) containing 63 mol% 3HB, which was the highest 3HB content (cellular content of 3HB unit in the copolymer) among the mutants obtained in this study. In contrast, most of the E130D/S325T-derived mutants produced small amounts of P(3HB-*co*-3HA) (1 to 4 wt%; compared with 12 wt% from the wild-type enzyme). The E130D/S325C-derived mutants exhibited similar results to the E130D/S325T-derived mutants in terms of P(3HB-*co*-3HA) content.

We chose three focusing mutants for molecular weight analysis of PHAs, E130D/S477R (the highest P(3HB-*co*-3HA) content), S325C/S477R/Q481G (the highest 3HB fraction in copolymer), and S325C/S477K/Q481L (the highest 3HB content in copolymer) (Table 1). The S325C/S477R/Q481G mutant exhibited relatively low molecular weight compared to the wild-type PHA synthase. Other two mutants did not change the molecular weight largely.

In addition, we carried out immunoblot analysis of PhaA_{Re}, PhaB_{Re}, and PhaC1_{Ps} for wild-type and three focusing mutants as described above to investigate a possibility that the enhanced polymer production and/or 3HB fraction was due to an alteration in expression levels of these enzymes. However, mutated PHA synthases were produced at levels similar to the wild-type enzyme (Fig. 5). Co-expressed monomer supplying enzymes also did not alter their production levels largely (Fig. 5). Therefore, the alterations in polymer production should be mainly due to the altered enzymatic characters of mutated PhaC1_{Ps} rather than the expression level of the enzyme.

Discussion

We performed mutation scrambling of PhaC1_{Ps} at four useful sites to increase 3HB fraction in P(3HB-*co*-3HA) without losing productivity. As summarized in Fig. 6, the mutants were categorized into three groups based on their patterns of P(3HB) and P(3HB-*co*-3HA) production. The evolution of the enzyme was driven by selective pressure for increasing P(3HB) content. As a result, many mutants acquired an enhanced ability to produce P(3HB) compared to the original PhaC1_{Ps} (0.1 wt%). A group of mutations (Group 1) increased both P(3HB) content and P(3HB-*co*-3HA) content, as well as 3HB fraction in the copolymer. However, those increases were small. In contrast, mutants in Group 2 increased both P(3HB-*co*-3HA) content and 3HB fraction in the copolymers compared to those of the wild-type PhaC1_{Ps} (Fig. 6). They further expanded the range of 3HB fraction (achieving up to 70 mol%) and their polymer contents (5 to 18 wt%) were greater than that of S477R/Q481R mutant (0.2 wt% with 62 mol% 3HB). Thus, they are suitable for 3HB-based P(3HB-*co*-3HA) production in terms of both increased polymer content and 3HB fraction. In addition, all mutants in Group 2 were S325C derivatives, suggesting that S325C serves as a promising template upon secondary mutation to generate desired mutants synthesizing P(3HB-*co*-3HA) with a high 3HB fraction.

In contrast, the other group of mutants (Group 3) exhibited very high P(3HB) content up to 68 wt%, which is comparable to that of class I PHA synthases. However, their productivity of P(3HB-*co*-3HA) was greatly decreased. The results suggest that substrate specificities of Group 3 mutants were largely shifted toward the 3HB monomer, and that 3HA-CoA negatively affected production of P(3HB-*co*-3HA). Therefore, for mutants in Group 3, MCL 3HA-CoAs may act as a competitive inhibitor for incorporation of 3HB units. Interestingly, a similar phenomenon was observed for class I PHA synthase from *R. eutropha* (PhaC_{Re}), which produced 67 wt% P(3HB)

in *E. coli* only when 3HB-CoA is supplied, but produced much less P(3HB) (15 wt%) when MCL 3HA-CoAs coexisted (Antonio et al. 2000). Because many mutants in Group 3 included S477G mutation, G477 should contribute to the shift in substrate specificity of PHA synthase toward 3HB-CoA.

Most of PhaC1_{PS} mutants obtained through saturation mutagenesis at sites 477 and 481 increased 3HB fraction in the copolymers. The results agreed with the hypothesis that residues at sites 477 and 481 are responsible for substrate specificity of the PhaC1_{PS} (Matsumoto et al. 2006). In addition, a clear pattern emerged for the selection of amino acids, *i.e.*, all selected mutants included S477G, S477R, Q481L, Q481M, or Q481R. Therefore, these residues at these sites are favored for conferring high P(3HB) production. Among them, the Q481L mutation was not determined to be an effective substitution for P(3HB) production by single saturation mutagenesis (Takase et al. 2003). Nevertheless, the S325C/S477K/Q481L mutant exhibited the greatest 3HB content in P(3HB-*co*-3HA) among the mutants tested (Fig. 3). Therefore, pairwise saturation mutagenesis was essential and effective for obtaining the beneficial mutant.

The molecular weight of the polymer is a very important factor that can influence properties of the polymer such as tensile strength (Kusaka et al. 1998). The result of this study demonstrated that a selected mutant (S325C/S477R/Q481G) decreased the molecular weights of P(3HB-*co*-3HA) produced. The alteration in the molecular weights is likely due to the enzymatic properties of the engineered PHA synthase, since the expression levels of PHA synthases and monomer supplying enzymes were not largely changed. Interestingly, mutations at A510 in PHA synthase of *R. eutropha* and A505 in PHA synthase of *Aeromonas caviae*, which correspond to Q481 in PhaC1_{PS}, also altered molecular weight of PHAs (Tsuge et al. 2004; Tsuge et al. 2007). These results suggested that residues at the beneficial sites affect molecular weight of the polymers, and that engineered PHA synthases may be used to regulate the molecular weight of

PHAs. However, mechanism for determining molecular weight of PHA has not been understood. Further research will be necessary to fully interpret the phenomena.

In summary, pairwise saturation mutagenesis at sites 477 and 481, combined with beneficial mutations at site 130 and 325 of PhaC1_{Ps} achieved a shift in substrate specificity toward 3HB units without reducing polymer productivity. Most of the PhaC1_{Ps} mutants, which were selected and functionally effective in *E. coli*, were also highly active in other hosts, such as *R. eutropha* and even *Arabidopsis* (Tsuge et al. 2005; Nomura et al. 2007; Matsumoto et al. 2009). Therefore, the new engineered PHA synthases should be useful in various cell platforms for efficient production of 3HB-based copolyesters with high 3HB fractions.

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Figure legends

Fig. 1 Three-dimensional model of α/β hydrolase fold region of PHA synthase from *Pseudomonas* sp. 61-3 (PhaC1_{PS}). Red residue represented as van der Waals spheres indicates Cys296, which is reported to be an active center of the enzyme. Other spherical residues indicated beneficial mutation sites. Blue: Ser325, purple: Ser477, yellow: Gln481.

Fig. 2 Construction of mutants and plasmid vector pGEM⁺C1AB used for synthesizing P(3HB) from glucose. The *phaA*_{Re} and *phaB*_{Re} genes derived from *R. eutropha* encode β -ketothiolase and NADPH-dependent acetoacetyl-CoA reductase, respectively. P_{Re} and T_{Re} are the promoter and terminator regions of the *phaCAB*_{Re} operon in *R. eutropha*, respectively. The *phaC1*_{PS} gene encodes PHA synthase from *Pseudomonas* sp. 61-3.

Fig. 3 Accumulation of P(3HB) in recombinant *E. coli* JM109 harboring the wild-type and mutated PHA synthase genes. The letters in the left column show the mutation in the PHA synthase; a hyphen indicates no mutation at the point. For example, “D – R M” means E130D/S477R/Q481M triple mutant. The white bars in the right column indicate P(3HB) content produced by PHA synthase used as a template for S477X/Q481X saturation mutagenesis. The black bars indicate P(3HB) content produced by the mutated PHA synthases newly obtained in this study. All cells were grown on LB medium containing 2% glucose for 48 h at 30 °C. P(3HB) contents are an average of three independent experiments.

Fig. 4 PHA contents and monomer compositions of P(3HB-*co*-3HA) in the recombinant *E. coli* LS5218 harboring the wild-type and mutated PHA synthase genes. The letters in the middle

indicate mutation of the PHA synthase; a hyphen indicates no mutation at the point. For example, “D, T, G, F” at the right edge means the E130D/S325T/S477G/Q481F quadruple mutant. The upper figure shows P(3HB-*co*-3HA) content. Black and white bars indicate the weight ratio of short-chain-length (SCL, C₄) and medium-chain-length (MCL, C₆ to C₁₂) fractions in P(3HB-*co*-3HA), respectively. The lower figure shows the molar composition of P(3HB-*co*-3HA). E130D, S325C, S325T, E130D/S325T, E130D/S325C data were determined previously (Matsumoto et al. 2005). 3HB: 3-hydroxybutyrate, 3HHx: 3-hydroxyhexanoate, 3HO: 3-hydroxyoctanoate, 3HD: 3-hydroxydecanoate, 3-HDD: 3-hydroxydodecanoate. All cells were grown on M9 medium containing 0.3% dodecanoate for 72 h at 37 °C. P(3HB-*co*-3HA) contents and monomer compositions are presented as an average of three independent experiments.

Fig. 5 Immunoblot analysis of wild-type and mutated PhaC1_{PS} and monomer-supplying enzymes (PhaA_{Re} and PhaB_{Re}) expressed in recombinant *E. coli* JM109. M: Size marker, Lane 1: Recombinant *E. coli* JM109 harboring pGEM”AB_{ex}, lane 2: pGEM”C1(wild-type)AB, lane 3: pGEM”C1(E130D/S477R)AB, lane 4: pGEM”C1(S325C/S477R/Q481G)AB, and lane 5: pGEM”C1(S325C/S477K/Q481L)AB.

Fig. 6 Functional shift of PHA synthase by evolutionary engineering. Each bar corresponds to PhaC1_{PS} mutant. Red bar indicates the wild-type enzyme. Other bars indicate mutated PHA synthases; green: E130D-derived mutants, purple: S325T-derived mutants, pink: S325C-derived mutants, sky blue: E130D/S325C-derived mutants, blue: E130D/S325T-derived mutants, asterisk indicates mutants including S477G.

Table 1 Molecular weight of P(3HB-*co*-3HA) copolymers produced by engineered PHA synthases.

PHA synthase	Molecular weights ^a		
	M_n (10^4)	M_w (10^4)	M_w/M_n
wild-type	9.1	14.6	1.6
E130D/S477R	11.5	17.4	1.5
S325C/S477R/Q481G	3.3	4.6	1.4
S325C/S477K/Q481L	10.5	12.9	1.2

^a M_n : number-average molecular weight; M_w : weight-average molecular weight; M_w/M_n : polydispersity.

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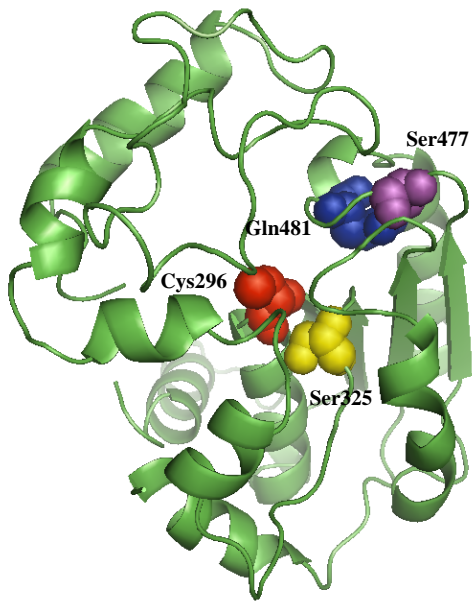


Fig. 1

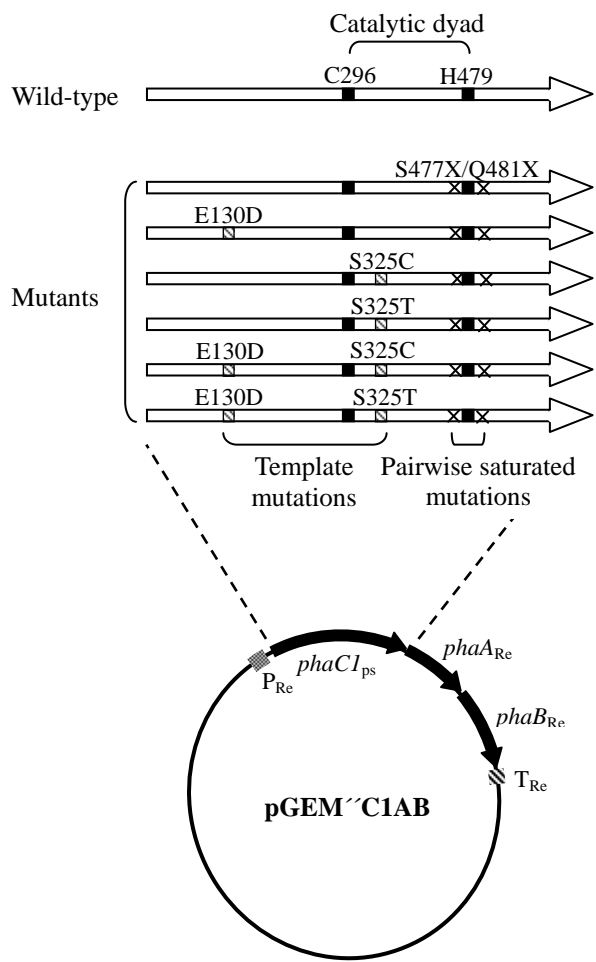


Fig. 2

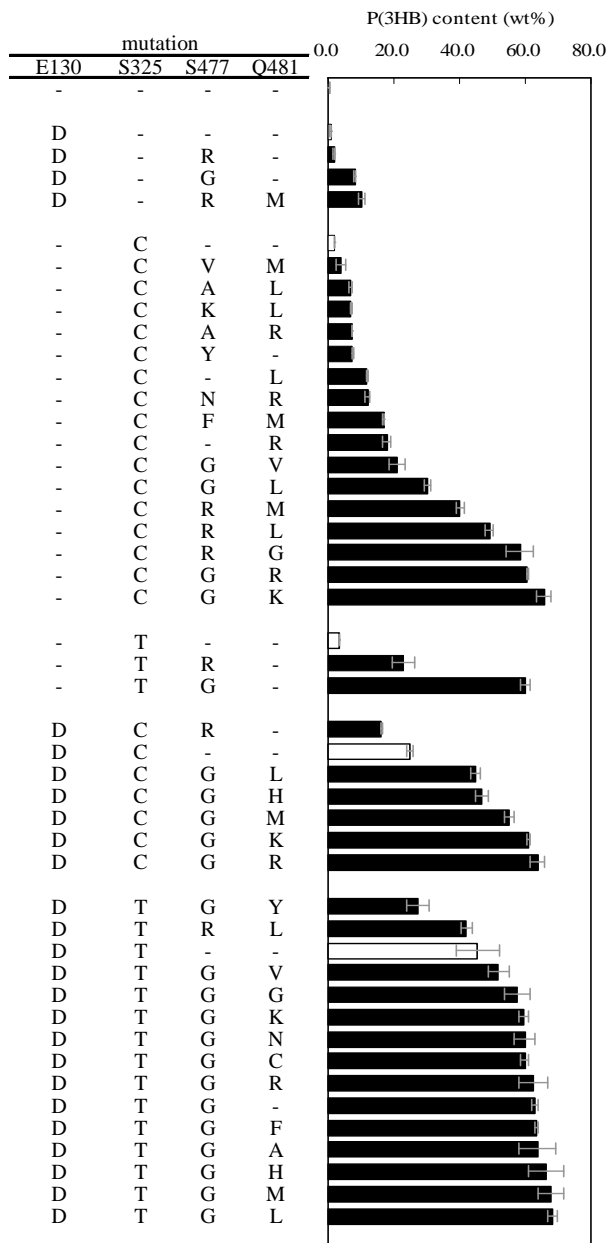


Fig. 3

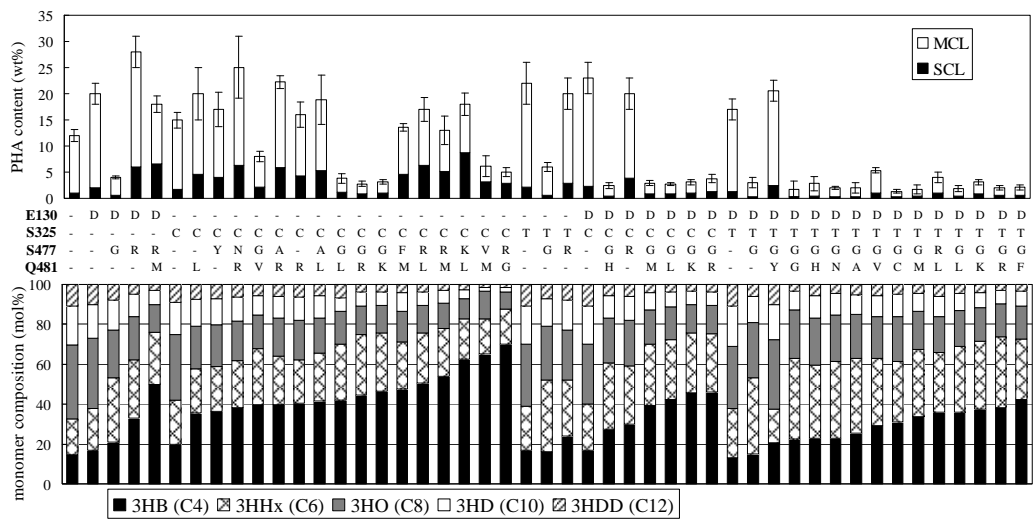


Fig. 4

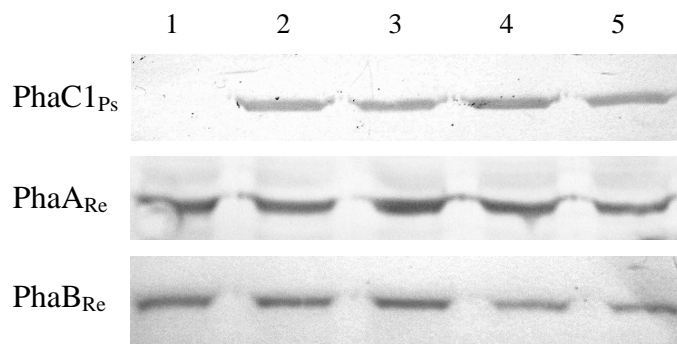


Fig. 5

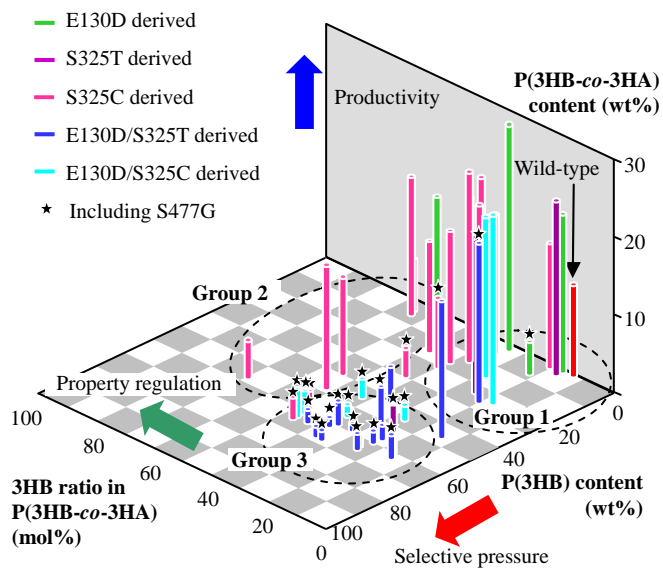


Fig. 6