

PHA synthase engineering toward superbiocatalysts for custom-made biopolymers

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Abstract Poly-3-hydroxyalkanoates [P(3HA)s] are biologically produced polyesters that have attracted much attention as biodegradable polymers that can be produced from biorenewable resources. These polymers have many attractive properties for use as bulk commodity plastics, fishing lines, and medical uses that are dependent on the repeating unit structures. Despite the readily apparent benefits of using P(3HA)s as replacements for petrochemical-derived plastics, the use and distribution of P(3HA)s have been limited by their cost of production. This problem is currently being addressed by the engineering of enzymes involved in the production of P(3HA)s. Polyhydroxyalkanoate (PHA) synthase (PhaC) enzymes, which catalyze the polymerization of 3-hydroxyacyl-CoA monomers to P(3HA)s, were subjected to various forms of protein engineering to improve the enzyme activity or substrate specificity. This review covers the recent history of PHA synthase engineering and also summarizes studies that have utilized engineered PHA synthases.

Keywords Polyhydroxyalkanoates · PHA synthase · Genetic engineering · In vitro evolution · *Escherichia coli* · *Ralstonia eutropha* · *Arabidopsis thaliana* · PHA monomer-supplying enzymes

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Introduction

Poly-3-hydroxyalkanoates [P(3HA)s] are biodegradable polyesters that are produced as intracellular inclusion bodies by some bacteria grown under nutrient limitation in the presence of excess carbon, serving as carbon and energy storage materials in the cells (Lee 1996; Sudesh et al. 2000). P(3HA)s have been studied intensely because the physical properties of these biopolyesters can be similar to petrochemical-derived plastics such as polypropylene (Doi 1990). P(3HA) polymers can be divided into three main classes with properties that are dependent on their monomer composition. Short-chain-length P(3HA)s [SCL-P(3HA)s] have monomers consisting of three to five carbons, are thermoplastic in nature, and generally lack toughness. Medium-chain-length P(3HA)s [MCL-P(3HA)s] have monomers consisting of 6–14 carbons and these polymers are elastomeric in nature. The third main class of P(3HA)s are the SCL/MCL-P(3HA)s, which are copolymers made up of SCL and MCL-3HA monomers consisting of 3–14 carbons. SCL/MCL-P(3HA)s have properties in between the SCL-P(3HA)s and MCL-P(3HA)s dependent on the mole ratio of SCL to MCL monomers, and therefore have a wide range of physical and thermal properties. Table 1 lists typical bacterial strains capable of producing P(3HA) polymers, the types of polyhydroxyalkanoate (PHA) synthases associated with those strains and the type of P(3HA) polymers produced by those bacteria.

P(3HA) biopolymers have many potential uses, including use as bulk commodity plastics, fishing lines, woven materials, and potential biomedical applications. However, unlike their petrochemical-derived counterparts, P(3HA)s can be produced from biorenewable resources such as sugars, plant oils, and even CO₂ (Sudesh et al. 2000), and when used as a bulk commodity plastic, P(3HA)s can be

Table 1 Classes of PHA synthases and varieties of [P(3HA)s]

Substrate specificity ^a	Class ^b	Subunit(s) ^c	Microorganism ^d	Polymers produced ^e
SCL-3HA-CoA (C3–C5)	I	PhaC	<i>Ralstonia eutropha</i>	P(3HB), P(3HB-co-3HV)
	III	PhaC, PhaE	<i>Allochromatium vinosum</i>	
	IV	PhaC, PhaR	<i>Bacillus megaterium</i>	
MCL-3HA-CoA (C6–C14)	II	PhaC	<i>Pseudomonas oleovorans</i>	P(3HA)
			<i>Pseudomonas putida</i>	
			<i>Pseudomonas aeruginosa</i>	
SCL-MCL-P(3HA)-CoA (C3–C14)	I	PhaC	<i>Aeromonas caviae</i> FA440	P(3HB-co-3HA)
	II	PhaC	<i>Pseudomonas</i> sp. 61-3	

^a Substrates preferred by the PHA synthase. SCL-3HA-CoA (C3-C5), short-chain-length-3-hydroxyacyl-coenzyme A (3-5 carbons in length); MCL-3HA-CoA (C6-C14), medium-chain-length-3-hydroxyacyl-coenzyme A (6-14 carbons in length); SCL-MCL-3HA-CoA (C3-C14) short-chain-length-3-hydroxyacyl-coenzyme A (3-14 carbons in length).

^b Class of PHA synthase.

^c Name of PHA synthase subunit or subunits if the enzyme consists of more than PhaC.

^d Native microorganism where the PHA synthase and polymer are found.

^e Polymers produced. P(3HB), poly-3-hydroxybutyrate; P(3HB-co-3HV), poly-3-hydroxybutyrate-co-3-hydroxyvalerate; P(3HA), poly-3-hydroxyalkanoate; P(3HB-co-3HA), poly-3-hydroxybutyrate-co-3-hydroxyalkanoate.

degraded by several naturally occurring bacterial strains, alleviating long-term disposal problems associated with most petrochemical-derived plastics. Despite the many potential benefits derived from using P(3HA)s as substitutes for petroleum-derived polymers, two main issues prevent its widespread uses: the cost of production relative to petroleum-based polymers with similar properties and the inability to produce P(3HA) high performance polymers in substantial amounts. Optimization of both of these factors would allow for more widespread uses of these environmentally friendly polymers, thus, it is important to develop new methods to solve these problems. One potential strategy to address these issues is the subject of this review: the engineering of the PHA synthase for more effective P(3HA) production. The PHA synthase is the central enzyme for the polymerization of various *R*-3-hydroxyacyl-CoA precursors, which are derived from various metabolic pathways to produce P(3HA) polymer (Nomura and Doi 2006). The performance of PHA synthase is closely related to the properties of the polymers generated, cellular P(3HA) content, molecular weight, and monomer composition upon copolymerization. It is possible that P(3HA) content and monomeric composition could be correlated with the specific activity and substrate specificity of PHA synthase, respectively. Extensive attempts at molecular evolution were recently performed for several PHA synthases without any tertiary structure information (Taguchi and Doi 2004). In this review, we will discuss the history of enzyme engineering of PHA synthases and recent application of engineered PHA synthases for the production of PHAs in various recombinant organisms, and give some future prospects for enzyme engineering within the PHA research field.

Engineering of PHA synthases

There are four classes of PHA synthases that can be categorized based on their nucleotide and predicted amino acid sequences (Rehm 2003), but in this review we have classified the enzymes based on their substrate specificity and subunit composition (Table 1). An alignment of primary sequences of these PHA synthases shows an overall identity of 8–96% with only eight strictly conserved amino acid residues. Different threading algorithms and biochemical studies suggest that PHA synthases belong to the α/β -hydrolase superfamily with a conserved cysteine residue as a catalytic nucleophile (Rehm 2003; Jia et al. 2001). Most of the microbes capable of producing P(3HA)s synthesize SCL-P(3HA)s or MCL-P(3HA)s consisting of 3-hydroxyoctanoate (3HO) and 3-hydroxydecanoate (3HD) (Lee 1996; Madison and Huisman 1999). The PHA synthase enzymes from *Aeromonas caviae* and *Pseudomonas* sp. 61-3 are the only known native enzymes that are capable of producing SCL/MCL-P(3HA) copolymers and these enzymes are members of the class I and class II PHA synthase families, respectively. In addition, the most widely studied PHA synthase, PhaC from *Ralstonia eutropha*, belongs to class I. Class I and class II PHA synthases consist of a single subunit as opposed to the multisubunit PHA synthases from class III and class IV, and thus represented the logical choices for initiating genetic engineering projects. Applications of genetic engineering to improve PHA synthases are summarized in Table 2. While structural models allow for the “rational” design of enzyme, the lack of a suitable structural model for any PHA synthase has limited attempts to improve the activity, substrate specificity, and stability of these enzymes to

Table 2 Studies on PHA synthase enhancement through enzyme engineering

Year	Enzyme source ^a	Method	Polyester ^b	Changed enzyme property	Reference
2001	<i>Ralstonia eutropha</i>	Random mutagenesis	P(3HB)	Activity	Taguchi et al. (2001)
2002	<i>Ralstonia eutropha</i>	Random mutagenesis	P(3HB)	Activity, Thermostability	Taguchi et al. (2002)
	<i>Aeromonas caviae</i> (<i>punctata</i>)	Random mutagenesis	P(3HB- <i>co</i> -3HHx)	Activity, Substrate specificity	Kichise et al. (2002)
2002	<i>Ralstonia eutropha</i>	Intragenic suppression mutagenesis	P(3HA)	Activity, Substrate specificity	Taguchi et al. (2002)
	<i>Ralstonia eutropha</i> and <i>Pseudomonas aeruginosa</i>	Gene shuffling	P(3HA)	Activity, Substrate specificity	Rehm et al. (2002)
	<i>Aeromonas caviae</i> (<i>punctata</i>)	Random mutagenesis (in vivo)	P(3HB- <i>co</i> -3HHx)	Activity, Substrate specificity	Amara et al. (2002)
2003	<i>Pseudomonas</i> sp. 61-3	Random mutagenesis, Site-specific saturation mutagenesis, Recombination	P(3HB- <i>co</i> -3HA)	Activity, Substrate specificity	Takase et al. (2003)
	<i>Pseudomonas</i> <i>resinovorans</i>	Site-specific chimeragenesis ^b	P(3HA)	Substrate specificity	Solaiman (2003)
2004	<i>Pseudomonas</i> sp. 61-3	Random mutagenesis, Site-specific saturation mutagenesis, Recombination	P(3HB- <i>co</i> -3HA)	Activity, Substrate specificity	Takase et al. (2004)
	<i>Pseudomonas putida</i> GPo1	Localized semi-random mutagenesis	P(3HA)	Activity, Substrate specificity	Sheu and Lee (2004)
	<i>Pseudomonas oleovorans</i>	PCR-mediated random chimeragenesis ^b	P(3HA)	Activity, Substrate specificity	Niamsiri et al. (2004)
	<i>Ralstonia eutropha</i>	Site-specific saturation mutagenesis	P(3HB), P(3HB- <i>co</i> -3HA)	Activity, Substrate specificity	Tsuge et al. (2004b)
2005	<i>Ralstonia eutropha</i>	Intragenic suppression mutagenesis, Site-specific saturation mutagenesis	P(3HB)	Protein expression	Normi et al. (2005a)
	<i>Ralstonia eutropha</i>	Recombination	P(3HB)	Activity	Normi et al. (2005b)
	<i>Pseudomonas</i> sp. 61-3	Site-specific saturation mutagenesis, Recombination	P(3HB)	Activity, Substrate specificity	Matsumoto et al. (2005b)

^a Strain of bacteria from which the PHA synthase was derived.

^b Method for generation of chimeric genes used in this study (see text).

^c Polymer produced by the study. P(3HB), poly-3-hydroxybutyrate; P(3HB-*co*-3HHx), poly-3-hydroxybutyrate-*co*-3-hydroxyhexanoate; P(3HA), poly-3-hydroxyalkanoate; P(3HB-*co*-3HA), poly-3-hydroxybutyrate-*co*-3-hydroxyalkanoate.

“irrational” approaches, such as random mutagenesis and gene shuffling. Once residues were identified by these irrational approaches, site-specific saturation mutagenesis of residues known to affect the activity of the enzyme, and the recombination of beneficial mutations can be used to improve the enzymes (Taguchi and Doi 2004).

Engineering of the class I *R. eutropha* PHA synthase

The first study that established methods for genetically engineering PHA synthase took place in 2001 using the best biochemically studied enzyme at the time, the *R. eutropha* PHA synthase (Taguchi et al. 2001). This initial study involved the establishment of the “in vivo evolution-

ary technique” and used an error-prone PCR method coupled to two convenient screening methods to generate a “fitness landscape” representative of mutations that had similar, slightly lower, and much lower activity compared to the wild-type enzyme (Taguchi et al. 2001). Once the mutant enzymes are identified by primary mutation analysis, a secondary round of mutation is used to evolve these enzymes to proteins with better characteristics than the wild-type enzyme (Fig. 1). One of the mutants (Ser80Pro, abbreviated S80P) identified in this study was more thermostable than the wild-type enzyme but possessed only 27% of the wild-type enzyme activity. To identify other residues that may affect enzyme activity, the E-11 mutant harboring the S80P mutation was coupled to a secondary round of mutagenesis. This second round of mutagenesis

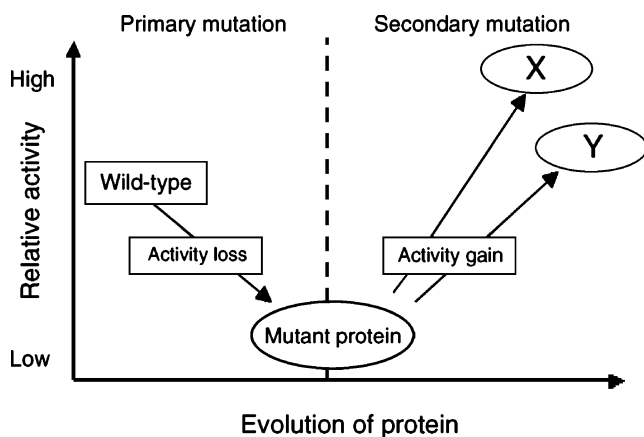


Fig. 1 Strategy for intragenic suppression-type mutagenesis. The genes encoding the protein of interest are mutated and the proteins are screened for a loss of activity (primary mutation). The mutant proteins are then subjected to a second round of mutagenesis (secondary mutation) to look for suppressors of the loss of phenotype resulting in the creation of proteins (X and Y) with higher activity (evolution) than the original protein (wild-type)

generated the E11S12 mutant, which had up to 79% of the activity of the wild-type PHA synthase level of P(3HB) production (Taguchi et al. 2002). Uncoupling of this mutation revealed the Phe420Ser (F420S) mutation that was independent of the primary mutation (S80P), and when a PHA synthase harboring only the F420S substitution was examined, increased the specific activity toward 3HB-CoA by 2.4-fold compared to the wild-type enzyme. This was the first study to demonstrate increased activity via “intragenic suppression-type mutagenesis” (Taguchi et al. 1998) in a type I PHA synthase (Taguchi et al. 2002). After a third round of in vitro evolution on the S80P mutant, the E11-4 mutant was found, which could produce twofold higher P(3HB) compared to the E11(S80P) harboring *Escherichia coli* strain. The E11-4 mutant was dissected and three mutations were found that could individually enhance P(3HB) accumulation compared to the E11 mutant. Of the three mutations, the Gly4Asp (G4D) mutant exhibited higher levels of protein accumulation and P(3HB) production compared to a recombinant *E. coli* strain harboring the wild-type PHA synthase (Normi et al. 2005a). Site-specific saturation mutagenesis is a powerful tool for genetic engineering and further examination of potentially beneficial sequences in PHA synthases, as demonstrated for other types of enzymes (Taguchi et al. 2000). Site-specific saturation mutagenesis was performed on the codon encoding the G4 residue of the *R. eutropha* PHA synthase and many substitutions resulted in much higher P(3HB) content and higher molecular weights of the polymers. These substitutions were subsequently combined with the F420S mutation and resulted in increased polymer yields but lower molecular weights for the P(3HB) polymers produced (Normi et al. 2005b).

Based on the homology between *R. eutropha* PHA synthase and *Burkholderia glumae* lipase whose tertiary structure was resolved by X-ray analysis, a threading model of the PhaC enzyme was proposed by Rehm et al. (2002) and was used to map mutations generated by a single gene shuffling of four mutants (GS1[Ala334Val, Gly376Ser], GS2[Ala57Val, Ile357Val], GS3[Lys139Arg, Arg386Cys], and G4[Gly206Ser, Pro230Ser, Glu337Lys]) that had reduced in vivo activity compared to the wild-type PHA synthase (Rehm et al. 2002). Although functional mapping of mutant enzymes diagrammatized on this threading model may predict which amino acid residues are responsible for enzymatic properties such as activity or dimer formation, the results achieved by Taguchi et al. (2002) that resulted in increased activity could not be predicted by a structural model.

Engineering of the class I *A. caviae* PHA synthase

It was shown previously that P(3HB-co-3HA) random copolymers with a ratio of 95mol% 3HB to 5mol% 3HA had properties similar to low-density polyethylene (Matsusaki et al. 2000; Abe and Doi 2002). Because these properties are desirable for the use of P(3HA)s as bulk, commodity plastics, development of methods to efficiently produce P(3HA) copolymers has become an important research topic. The *A. caviae* (*punctata*) PHA synthase is unique among class I PHA synthases because it can synthesize not only P(3HB) homopolymer but also random copolyesters of 3HB and 3-hydroxyhexanoate (3HHx) (Doi et al. 1995). Kichise et al. (2002) performed the first successful in vitro molecular evolution experiments on PhaC from *A. caviae* by applying the in vitro evolutionary technique to a limited region of the *phaC* gene and coexpressing the mutants with the monomer-supplying enzyme genes, *phaAB* [encoding β -ketothiolase (PhaA) and NADPH-dependent acetoacetyl-CoA reductase (PhaB)] from *R. eutropha* and *phaJ* [encoding (*R*)-specific enoyl-CoA hydratase] from *A. caviae* to supply monomers from glucose or dodecanoate, respectively. Two evolvants were isolated (E2-50 and T3-11), which had increased activity toward 3HB-CoA of 56 and 21%, respectively, compared to the wild-type enzyme in in vitro assays. These mutations led to enhanced accumulation (up to 6.5-fold higher than the wild-type PhaC) of P(3HB-co-3HHx) and increases in the 3HHx mole fraction (16–18mol% compared to 10mol% of the wild type PHA synthase) in recombinant *E. coli* LS5218 strains grown on dodecanoate. The single mutations responsible for these phenotypes [Asn149Ser (N149S) in the E2-50 strain and Asp171Gly (D171G) for T3-11] are not highly conserved among PHA synthases. These successful studies prompted the use of in vitro evolutionary

technique to the entire *A. caviae phaC* gene to find other beneficial mutations.

Although the study by Kichise et al. (2002) utilized the in vitro evolutionary technique to generate mutations in the PHA synthase gene sequence, a subsequent study by Amara et al. (2002) used the *E. coli* XL1-Red mutator strain, which has a 5,000-fold higher mutation rate than wild-type *E. coli*, for the in vivo random mutagenesis of the *A. punctata phaC* gene. Mutant plasmids harboring the *phaC* gene were cotransformed with plasmid pBHR69 harboring the *phaAB* P(3HB) monomer supplying enzyme genes into *E. coli* JM109, after which, five mutants were isolated out of ~200,000 transformant screen on agar plates containing Nile red. The mutants were divided into groups with only single amino acid substitutions (mutants M1 and M2), two amino acid substitutions (mutants M4 and M5), and quadruple amino acid substitution mutant (mutant M3). Of the five mutants, four (M1, M2, M4, and M5) were able to accumulate higher levels of P(3HB) (~1.1- to 1.5-fold) compared to the wild-type PHA synthase, while the quadruple mutant M3 accumulated about half as much P(3HB) compared to the wild type enzyme.

Engineering of the class II *Pseudomonas* sp. PHA synthases

Unlike class I PHA synthases, class II PHA synthases typically have substrate specificity toward MCL-3HA-CoA substrates but relatively poor substrate specificity toward SCL-3HA-CoA substrates like 3HB-CoA, with the only class II PHA synthase with substrate specificity toward the 3HB-CoA being the *Pseudomonas* sp. 61-3 PHA synthase (Table 1). In the landmark study by Takase et al. (2003), the in vitro evolutionary technique was applied to the PhaC1 PHA synthase from *Pseudomonas* sp. 61-3 to increase the activity toward 3HB-CoA monomers (Takase et al. 2003). Substitutions at two amino acid residues, Ser325 and Gln481, were found to dramatically affect the production of P(3HB) homopolymer in recombinant *E. coli* with glucose as a carbon source. The codons for these amino acids were subjected to saturation site-specific mutagenesis and several individual substitutions [Ser325Cys (S325C), Ser325Thr (S325T), Gln481Lys (Q481K), Gln481Met (Q481M), and Gln481Arg (Q481R)] were found that could dramatically increase the level of P(3HB) production. These mutations were combined as double mutants to further increase the level of P(3HB) production (340- to 400-fold higher than the wild-type enzyme) (Takase et al. 2003). In a subsequent study, it was shown that these engineered PhaC1 synthases were able to accumulate higher amounts of SCL/MCL-P(3HA)s compared to the wild-type PhaC1 synthase (Takase et al. 2004). In addition, there was a shift

in the monomer composition toward 3HB of the P(3HB-co-3HA) copolymer produced by the mutant synthases compared to the wild-type PHA synthase (Takase et al. 2004). The changes in the in vivo produced P(3HB-co-3HA) copolymer mole compositions correlated well with the in vitro biochemical data of the substrate specificity and activity of the enzymes and represents one of the most well-rounded studies to date (Takase et al. 2004).

The findings obtained from the study by Takase et al. (2004) for the type II PHA synthase would be very useful for evaluating a similar evolution strategy to the other types of PHA synthases based on the amino acid sequence alignment of the PHA synthases. For example, position 481 in PhaC1 PHA synthase from *Pseudomonas* sp. 61-3 was found to be one of the residues determining substrate specificity of the enzyme, as described above. It is interesting to note that the amino acid residues corresponding to the position of this enzyme are conserved within each type of PHA synthases: Ala for type I, Gln for type II, Gly for type III, and Ser for type IV enzymes. Thus, the effects of mutating the highly conserved alanine (Ala510) of the *R. eutropha* PHA synthase (corresponding to the position 481 in *Pseudomonas* sp. 61-3 PhaC1) were analyzed via site-specific saturation mutagenesis. Mutations Ala510Met/Gln/Cys at Ala510 were found to affect the substrate specificity of the *R. eutropha* PHA synthase, allowing slightly higher 3HA incorporation compared to the wild-type PHA synthase in *R. eutropha* PHB⁻⁴ [P(3HB) negative mutant], while other mutations lowered or completely eliminated the amount of 3HA that could be incorporated into the polymer compared to the wild-type enzyme when grown on dodecanoate (Tsuge et al. 2004a).

Glu130 was also identified during the in vitro evolution screening as a positive mutation. The Glu130Asp (E130D) mutant was able to accumulate tenfold higher P(3HB) from glucose compared to the wild-type PhaC1 enzyme (Matsumoto et al. 2005b). This study also showed that polymers produced by PHA synthases with mutations at E130 had higher molecular weights than polymers from other enzymes. Finally, a mutation at the S477 residue of PhaC1 further changes the substrate specificity toward SCL-3HA-CoA monomers, but when combined with a S325C or S325T mutation, exhibits a synergistic enhancement of PHA production in addition to altered substrate specificity (Matsumoto et al. 2006a,b). “Mutation scrambling” among four beneficial positions (130, 325, 477, and 481) for activity increase, change in substrate specificity, and regulation of polymer molecular weight would further create new super-enzymes. Recently, we have proposed a possible mechanistic model for PHA polymerization on the basis of the accumulated evolutionary studies (Matsumoto et al. 2006a,b).

Other engineering studies were performed using type II PHA synthases. A study by Solaiman (2003) used two

isogenic PHA synthase genes (*phaC1* and *phaC2*) from *Pseudomonas resinovorans* by exchanging the α/β -hydrolase-fold-coding regions to develop the hybrid genes *pha7* and *pha8*. The gene products of *pha7* and *pha8* produced polymers with repeating unit compositions similar to the wild-type enzymes when grown on decanoate as a carbon source (23–27mol% C8 and 73–78mol% C10) (Solaiman 2003). However, two deletion mutants were identified during the construction of the *pha7* and *pha8* hybrid genes (Δ *pha7* and Δ *pha8*, respectively) produced polymers with different repeating unit concentrations (40–45mol% C8 and 55–60mol% C10) compared to the wild-type PHA synthases and *pha7* and *pha8* genes (Solaiman 2003). The substrate specificity of PHA synthase 1 from *Pseudomonas putida* GPo1 was altered by localized semirandom mutagenesis, whereby the enzyme was evolved by using PCR-based gene fragmentation with degenerate primers followed by reassembly of the chimeric genes (Sheu and Lee 2004). Based on multiple sequence alignments of PHA synthases, six conserved regions located in the predicted α/β -hydrolase-fold were used to design degenerate primers corresponding to 23 amino acids distributed across the six regions. The fragments were mixed and primerless reassembly PCR was used to generate the recombined *phaC* genes and used generate 20,000 clones in *E. coli*. These plasmids were screened by transformation into *P. putida* GPP104 and narrowed down to 13 candidates based on the opaqueness of 10,000 transformants. Of these 13 transformants, 6 candidates were chosen to transform into *R. eutropha* PHB-4 based on the mole percent composition of the polymers they produced in *P. putida* GPP104. One of these candidates, PS-E1, had the highest accumulation of PHA content and an increased 3HB mole percent composition compared to the copolymer produced by the wild-type PhaC1. PS-E1 has several mutations from the semirandom mutagenesis that likely contribute to the changed substrate specificity and activity (F231L, M292T, A295V, L404M, S482G, L484P, S524G, and E534K) (Sheu and Lee 2004). The authors of this study also mutated the locus for Q481A, but this had little effect on the PHA composition because this was a specifically designed point mutation for all of the semirandom generated mutants and based on the previous data for the *Pseudomonas* sp. 61-3 saturation mutagenesis at Q481 (Takase et al. 2003). A comparison of the amino acid sequences of the PhaC1 proteins from *Pseudomonas* sp. 61-3 and *Pseudomonas oleovorans* GPo1 is shown in Fig. 2. Because there is a high degree of homology (83.7%) between the PhaC1 enzymes from *Pseudomonas* sp. 61-3 and *P. oleovorans* GPo1, it will be interesting to see if the recombination of mutants generated from the in vitro evolutionary technique and semirandom recombinant PCR have synergistic effects on PHA production.

Another study aimed to improve PHA synthases by a combinatorial strategy using the *P. oleovorans phaC1* gene as a scaffold from which to generate chimeras (Niamsiri et al. 2004). PCR products corresponding to the putative catalytic regions of PHA synthase enzymes were generated from soil DNA extracts and cloned into a synthetic *P. oleovorans phaC1* gene with a linker replacing the catalytic region of the enzyme. Transformants were screened with Nile blue A dye and out of 1,478 clones, 5 were isolated that could produce more PHA than the native PhaC1 enzyme. Sequence analysis revealed that the active synthases all contained sequences corresponding to different species of *Pseudomonas* and contained 17 to 20 amino acid differences from the *P. oleovorans* PhaC1. On the other hand, inactive chimeras contained sequences from type I synthases, indicating that this region could not be interchanged between the two types (I and II) of synthases (Niamsiri et al. 2004).

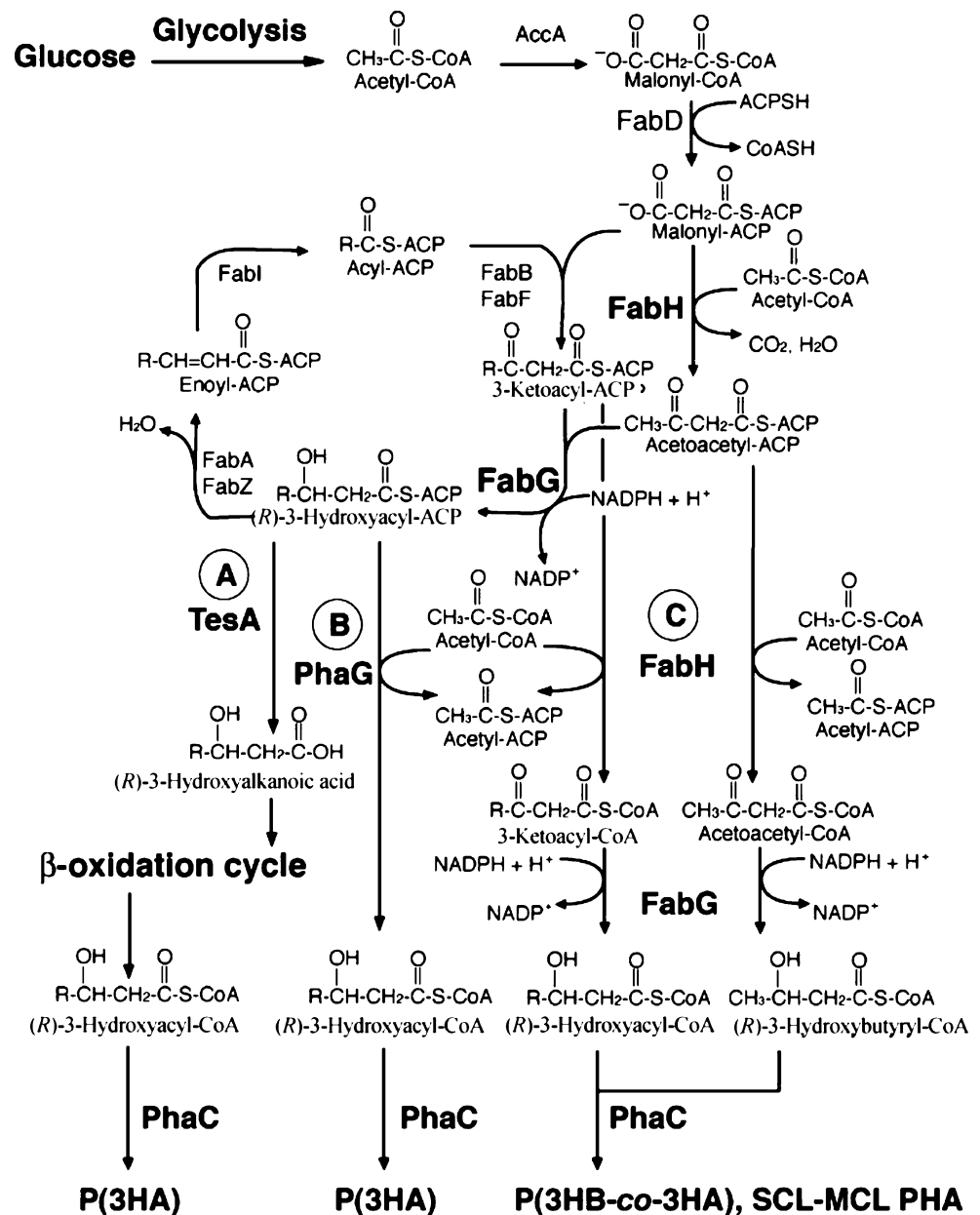
Application of engineered PHA synthases in recombinant organisms

Recombinant bacteria as “minimal but powerful factories”

For maximum flexibility and economic feasibility, it is important to develop metabolic pathways in recombinant organisms that use nonrelated carbon sources to produce P(3HA)s. Current MCL-3HA-CoA monomer supplying pathways from nonrelated carbon sources are shown in Fig. 3. All of the engineered pathways shown in Fig. 3 derive precursors from the fatty acid biosynthesis pathway. The thioesterase (Tes)-mediated monomer supply pathway (Fig. 3a) required the deletion of genes in the host strain encoding enzymes involved in the β -oxidation pathway (*fadR* and *fadB*) to be effective (Klinke et al. 1999). The PhaG-mediated monomer supply pathway (Fig. 3b) required the presence of the fatty acid biosynthesis inhibitor triclosan to be effective (Rehm et al. 2001). Although both of the TesA and PhaG monomer supply pathways were capable of producing substrates for MCL-PHA production, neither of these pathways was capable of producing SCL/MCL-PHA. On the other hand, the FabH/FabG-mediated monomer supply pathway (Fig. 3c) was capable of producing both SCL-3HA-CoA and MCL-3HA-CoA substrates for the production of SCL/MCL-P(3HA) copolymer without inhibitors or chromosomal modifications of the host strain (Nomura et al. 2004a, 2005).

E. coli is the most convenient bacterium to study these pathways because of its fast growth, ease of transformation, known genetic background, and lack of any native P(3HA)-producing genes. We had previously developed a metabolic pathway to produce SCL and MCL monomer precursors

Fig. 3 Metabolic pathways for MCL-3HA-CoA monomer supply from an unrelated carbon source. **a** TesA-mediated MCL-PHA monomer supply (via the β -oxidation pathway). **b** 3-Hydroxyacyl-ACP:CoA transacetylase (PhaG) mediated MCL-PHA monomer supply. **c** FabH and FabG mediated SCL/MCL-PHA monomer supply



is relatively stable and high-cell density fermentation is applicable to this recombinants. In fact, heterologous expression of the PhaC1 gene from *Pseudomonas* sp. 61-3 in *R. eutropha* PHB⁻4 recombinant led to the synthesis of random copolymers of P(3HB-co-3HA) with very high 3HB compositions from glucose (Matsusaki et al. 2000). However, limitations on the productivity of P(3HA)s and the variation of monomers incorporated into P(3HA) copolymers still remain. Recombinants of *R. eutropha* PHB⁻4 carrying the genes encoding the evolved *Pseudomonas* sp. 61-3 PhaC1s (S325C/T and/or Q481K/M/R) produced significantly increased amounts of P(3HA) (55–68wt%) compared with the one harboring the wild-type gene (49wt%). Particularly, those evolved PhaC1 mutants having multiple amino acid substitutions

showed higher activities for P(3HA) synthesis. It was also confirmed that amino acid substitution at position 481 in PhaC1 led to an increasing molecular weight, as revealed by the Q481K mutant that conferred 4.6-fold higher production of the wild-type enzyme (Tsuge et al. 2005).

Similarly, P(3HA) production by recombinant *R. eutropha* PHB⁻4 harboring mutants of PHA synthase gene from *A. caviae* (Kichise et al. 2002) was investigated. The strain harboring wild-type phaC gene produced a P(3HB-co-3HHx) with 3.5mol% of 3HHx fraction from soybean oil. When the beneficial PhaC mutants containing N149S and D171G (Kichise et al. 2002) were applied to this production system, 3HHx fraction in copolymers was varied in the ranges of 0–5.1mol%. Thus, the regulation of compositions

Table 3 SCL/MCL-P(3HA) production from unrelated carbon sources in recombinant *E. coli*

Relevant genes	P(3HA) content (wt% of CDW) ^a	P(3HA) composition (mol%) ^b					Reference
		3HB (C4)	3HHx (C6)	3HO (C8)	3HD (C10)	3HDD (C12)	
<i>fabH</i> (F87T), <i>phaC</i> _{Ac}	2	97	3	–	–	–	Nomura et al. (2004a)
<i>fabH</i> (F87T), <i>phaC</i> _{Ps}	1	77	18	4	1	–	Nomura et al. (2004a)
<i>fabH</i> (F87T), <i>phaC</i> _{Ps} (STQK)	1	91	6	2	1	1	Nomura et al. (2005)
<i>fabH</i> (F87T), <i>phaC</i> _{Ps} (SCQM)	1	89	8	2	0.5	0.5	Nomura et al. (2005)
<i>fabH</i> (F87T), <i>phaC</i> _{Ps} (STQK), <i>fabG</i> _{Ec}	5	94	5	1	–	–	Nomura et al. (2005)
<i>fabH</i> (F87T), <i>phaC</i> _{Ps} (SCQM), <i>fabG</i> _{Ec}	2	91	7	1	1	–	Nomura et al. (2005)
<i>fabH</i> (F87T), <i>phaC</i> _{Ps} (STQK), <i>fabG</i> _{Ps}	1	96	4	–	–	–	Nomura et al. (2005)
<i>fabH</i> (F87T), <i>phaC</i> _{Ps} (SCQM), <i>fabG</i> _{Ps}	1	95	5	–	–	–	Nomura et al. (2005)
<i>fabH</i> (F87T), <i>phaC</i> _{Ps} (STQK), <i>phaAB</i>	23	99.9	0.1	–	–	–	Nomura et al. (2004a)
<i>fabH</i> (F87T), <i>phaC</i> _{Ps} (SCQM), <i>phaAB</i>	15	99.3	0.7	–	–	–	Nomura et al. (2004a)

3HB 3-Hydroxybutyrate, 3HHx 3-hydroxyhexanoate, 3HO 3-hydroxyoctanoate, 3HD 3-hydroxydecanoate, and 3HDD 3-hydroxydodecanoate

^a Grams of PHA accumulated per 100 g of dry cells

^b Composition of PHA as determined by gas chromatography analysis after methanolysis of lyophilized cells in the presence of 15% sulfuric acid

in P(3HA) copolymer related to polymer properties was also achieved by the use of another type of evolved PHA synthases (Tsuge et al. 2004a). These copolymers melt during the drawing of polymer material at a low temperature while avoiding thermal degradation, suggesting the usefulness of these type copolymers for commercial application.

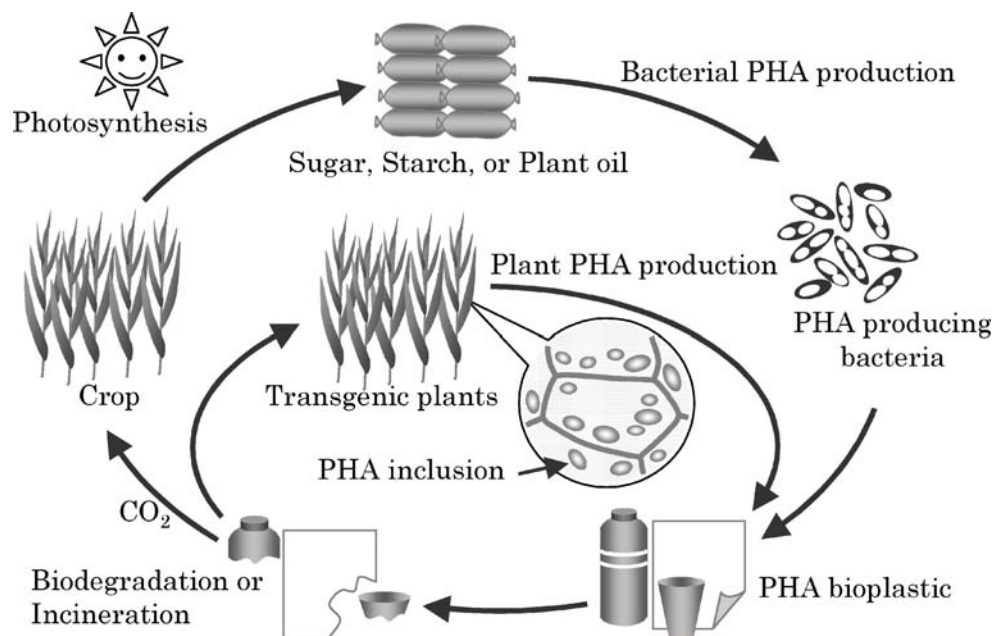
Recombinant plants for “polymer farming”

One of the limiting factors for commercial P(3HA) production is the cost of the carbon feedstocks used by the bacteria. Because organisms such as plants fix their carbon directly via photosynthesis, they have the potential to reduce the costs of P(3HA) production by converting atmospheric CO₂

directly into the biopolyester (Fig. 4). SCL-PHA production in plants was reported using a variety of transgenic plants (Arai et al. 2004; Bohmert et al. 2000; Houmiel et al. 1999; Nakashita et al. 2001; Nawrath et al. 1994; Poirier 1999, 2001; Poirier et al. 1995; Slater et al. 1999). But there were relatively few studies examining the incorporation of MCL-3HA-CoA monomers into P(3HA) in plants.

Recently, both type I and type II engineered PHA synthases were applied to plants for the production of P(3HA)s. For *A. caviae* type I PHA synthase, wild-type and highly active mutants (N149S and D171G) created by the in vitro evolutionary technique were introduced into *Arabidopsis thaliana* to produce P(3HB-co-3HV). It was shown that PHA production was increased up to eight-

Fig. 4 P(3HA) production in transgenic plants potentially decreases the cost of production. Microbial fermentation uses carbon sources such as oils and sugars derived from plants to produce P(3HA). By using plants, the carbon source would be CO₂, which could be directly fixed into biomaterial from the atmosphere, thus eliminating the costs of the carbon source



tenfold in the transgenic plants harboring the highly active mutant PHA synthases compared to plants harboring the wild-type synthase (Matsumoto et al. 2005a).

MCL-P(3HA) production in plants was also reported using *Arabidopsis* in which PHA synthases were targeted into peroxisome (Mittendorf et al. 1998, 1999; Moire et al. 2004). Recent plant studies were conducted using the evolved *Pseudomonas* sp. 61-3 PhaC1 carrying the S325T/Q481K double substitutions (STQK) in plants. It was observed that in the peroxisomes, the highly active mutant synthase, produced P(3HA)s in amounts similar to the wild-type PHA synthase, likely indicating that the amount of substrates available for P(3HA) production was rate-limiting (Matsumoto et al. 2006a,b). However, when the mutant synthase was targeted to plastids, there was a marked enhancement in P(3HA) production (K. Matsumoto, personal communication). Further developments in plant P(3HA) production will be facilitated by highly active, evolved PHA synthases, and may lead to economic production of P(3HA)s.

In the future, custom-made prominent PHA synthases generated through enzyme evolution will be utilized extensively to create high-performance P(3HA)s in various organisms from renewable carbon sources or through improved in vitro systems.

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