J. Biochem. **133**, 139–145 (2003)
DOI: 10.1093/jb/mvg015

Enhanced Synthesis of Poly(3-hydroxybutyrate) in Recombinant *Escherichia coli* by Means of Error-Prone PCR Mutagenesis, Saturation Mutagenesis, and *In Vitro* Recombination of the Type II Polyhydroxyalkanoate Synthase Gene

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Received October 27, 2002; accepted November 5, 2002

Type II synthase ($PhaC1_{Ps}$) for polyhydroxyalkanoate (PHA) from *Pseudomonas* sp. 61-3 was subjected to an *in vitro* evolution system including PCR-mediated mutagenesis in order to improve the function of $PhaC1_{Ps}$ in terms of its ability to produce poly(3-hydroxybutyrate) [P(3HB)] in recombinant *Escherichia coli*. Based on our established *in vivo* assay system, two positions (Ser325 and Gln481) where mutations provided remarkable increases in P(3HB) synthesis were identified. Saturation mutagenesis at these positions was carried out to explore whether there might be more beneficial sequences for P(3HB) synthesis than those identified in the point mutation library. As a result, five single mutants [S325C (T) and Q481M (K, R)] gave rise to highly enhanced P(3HB) synthesis. Drastically enhanced P(3HB) synthesis (up to 340- to 400-fold the amount of that of the wild type) was further achieved by generation of all five variants of the double mutants combining the codons for residues 325/481. It is feasible that the replacement of Ser (specific for type II synthase) by Thr (specific for type I synthase) at position 325 resulted in acquiring greater P(3HB) synthesis ability as exhibited by type I synthases. The other hot spot, 481, that positively contributes to enhanced P(3HB) synthesis is located adjacent to a His479, a residue that forms a putative catalytic diad that can be inferred by sequence alignment.

Key words: *in vitro* enzyme evolution, P(3HB) synthesis, *Pseudomonas* sp. 61-3 PHA synthase, recombination, saturation mutagenesis.

Abbreviations: P(3HB), poly(3-hydroxybutyrate); PHA, polyhydroxyalkanoate.

A wide variety of bacteria synthesize optically active homopolymers and copolymers of (*R*)-3-hydroxyalkanoates (PHA) ranging from 4 to 14 carbon atoms as an intracellular storage material of carbon and energy (*1*). Biodegradable thermoplastic PHA has received much attention as a new environmentally compatible material. In our current studies, artificial alteration of the key enzyme for PHA synthesis, PHA synthase, has been demonstrated to provide various custom-made enzymes applicable for more expanded practical uses. In fact, type I synthases derived from *Ralstonia eutropha* and *Aeromonas caviae* (at present termed *Aeromonas punctata*) were successfully improved with respect to enhancement of PHA synthesis or change in the monomer compositions of PHA copolymers (*2–5*), by means of our developed *in vitro* evolution system (*6*).

Previously, we identified two types of PHA biosynthesis gene loci (*phb* and *pha*) on the genome of *Pseudomonas* sp. 61-3, which produces a blend of P(3HB) homopolymer and a random copolymer, P(3HB-*co*-3HA), consisting of 3HB and 3HA units of 6 to 12 carbon atoms from sugars and alkanoic acids (*7*). The formations of two types of polymers are individually managed by two distinct types of PHA synthases [$PhbC_{Ps}$ for P(3HB) and $PhaC1_{Ps}$ for PHA copolymers] (*7*). Among type II PHA synthases, $PhaC1_{Ps}$ has the characteristic of synthesizing P(3HB-*co*-3HA) copolymers because of its broad substrate specificity ranging from C4 to C12. However, $PhaC1_{Ps}$ possesses extremely weak *in vitro* substrate specificity toward 3HB-CoA as compared with other longer monomer substrates (3HV-CoA to 3HD-CoA) (*8*). The enhancement of substrate specificity toward 3HB-CoA in $PhaC1_{Ps}$ is an attractive project to expand the utility of this enzyme for synthesizing 3HB-based copolymers in recombinant bacteria.

In the present study, we tried to obtain $PhaC1_{Ps}$ mutant enzymes with enhanced ability to synthesize P(3HB), based on an *in vitro* evolution program consisting of error-prone PCR mutagenesis, site-specific saturation mutagenesis at hot spots, and recombination of beneficial mutations. Two positions that contribute posi-

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Table 1. **Oligonucleotide sequences.**

Oligonucleotide linkers	
*Csp*45I-*Xba*I-*Eco*RI-*Bgl*II-*Pst*I linker	5′-CGAATCTAGAGAATTCAGATCTCTGCA-3′
	3′-TTAGATCTCTTAAGTCTAGAG-5′
Primers for amplification of $phaC1_{Ps}$	
C1NdeI	5′-GAGCGTCGCATATGAGTAACAAGAATAGCG-3′
C1BamHI	5′-GACTTCTAGACTGGATCCAACTTAACGTTC-3′
Primers for random mutagenesis	
T7P	5′-CCCAACGCTGCCCGAGATCTCGATCCCGCG-3′
T7T	5′-AGCTTCCTTTCGGGCTTTGTTAGCAGCCGG-3′
Primers for site-specific saturation mutagenesis at position Ser325	
S325X[a]	5′-CCCTTTTGGTCAGCGTGCTCGACACCACCC-3′
S325rev	5′-TCAGGGCATTGACCTTCTTCTCGCCG-3′
at position Gln481	
Q481X[a]	5′-AGTGGGCATATCCAGAGCATTCTGAACCC-3′
Q481rev	5′-GCTGGACAGCACGAATTCGACCTTGCC-3′

[a]X indicates the 19 amino acid residues other than the original one. Underlines indicate *Nde*I and *Bam*HI sites in C1NdeI and C1BamHI, respectively. Double underlines indicate the substitution codons for Ser325 (AGC) and Gln481 (CAG) in S325X and Q481X, respectively. Codons to perform site-specific saturation mutagenesis; Ala (GCC), Cys (TGC), Asp (GAC), Glu (GAA), Phe (TTC), Gly (GGC), His (CAC), Ile (ATC), Lys (AAG), Leu (CTG), Met (ATG), Asn (AAC), Pro (CCG), Gln (CAG), Arg (CGC), Ser (AGC), Thr (ACC), Val (GTC), Trp (TGG) and Tyr (TAC).

tively to increased P(3HB) accumulation in recombinant *Escherichia coli* were identified, and five double mutants carrying combined beneficial amino acids at these positions exhibited greatly enhanced synthesis of the P(3HB) homopolymer.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions—*Escherichia coli* JM109 (*9*) was used for all standard genetic engineering procedures and used as the host strain for screening mutants of *Pseudomonas* sp. 61-3 PHA synthase ($PhaC1_{Ps}$) and for P(3HB) accumulation. For P(3HB) accumulation, recombinant JM109 strains were grown on Luria-Bertani (LB) medium containing 2% glucose for 96 h at 30°C. When needed, ampicillin (50 μg/ml) was added to the medium.

DNA Manipulation and Plasmid Construction—Standard recombinant DNA manipulation (*10*) was used for the isolation of plasmid DNA. All restriction endonucleases and modification enzymes for genetic engineering were purchased from TaKaRa Shuzo and used under conditions recommended by the supplier. All other chemicals were of analytical grade for biochemical use and were used without further purification. The plasmid vector, pGEM″phaC1AB, was constructed for the biosynthesis of P(3HB) in *E. coli* JM109 strain, as illustrated in Fig. 1. First, the plasmid vector pGEM′phbCAB$_{Re}$ (*11*) carrying the *Ralstonia eutropha* P(3HB) operon with its promoter and terminator region was digested by *Csp*45I and *Pst*I, and the resulting 6.1 kb DNA fragment was ligated with a synthetic *Csp*45I-*Xba*I-*Eco*RI-*Bgl*II-*Pst*I linker. The resultant vector was named pGEM″ABex. Next, we constructed a plasmid for the random mutagenesis of the $phaC1_{Ps}$ gene by PCR-operated addition of restriction sites, *Nde*I and *Bam*HI, within an initiation codon and downstream of a stop codon, respectively. The $phaC1_{Ps}$ gene (approximately 1.7 kb) was amplified with primers C1NdeI and C1BamHI (underlined sequences show an *Nde*I site in C1NdeI and a *Bam*HI site in C1BamHI in Table 1) and plasmid pBSEX22 (*11*) as a template. The PCR product was purified and digested with *Nde*I and *Bam*HI, then subcloned into pET-23a(+) (Novagen). The resulting plasmid was termed pETphaC1$_{Ps}$, and the DNA sequence of the $phaC1_{Ps}$ gene region of pETphaC1$_{Ps}$ was confirmed by DNA sequencing. Finally, pETphaC1$_{Ps}$ was digested with *Xba*I and *Bam*HI. The 1.7 kb fragment (containing the $phaC1_{Ps}$ gene and the Shine-Dalgarno sequence of pET vector) was purified and ligated with *Xba*I and *Bgl*II digested pGEM″ABex. The resulting plasmid, pGEM″phaC1AB, was used for P(3HB) accumulation. The oligonucleotide linker and primers used in this study are summarized in Table 1.

DNA Sequencing—DNA sequencing to confirm the new plasmid constructs and to analyze the mutation points were carried out by the dideoxy chain termination method with a Prism 377 DNA sequencer (Applied Biosystems) and a CEQ2000XL DNA Analysis System (Beckman Coulter) using a BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems) and Dye Terminator Cycle Sequencing with a Quick Start Kit (Beckman Coulter), respectively. Nucleotide sequence data, deduced amino acid sequences and investigation of codon usage for site-specific saturation mutagenesis of the $PhaC1_{Ps}$ were analyzed with GENETYX-MAC software (Software Development, Tokyo).

Random Mutagenesis by Error-Prone PCR—Random mutagenesis of an entire region in the $phaC1_{Ps}$ gene was carried out by error-prone PCR. The forward primer (T7P) and reverse primer (T7T) were designed based on the pET vector sequence. Error-prone PCR was performed in 100 μl of reaction solution containing 25 ng pETphaC1$_{Ps}$ as template, 0.025 U of *Taq* DNA polymerase, 0.1 μM each of two primers, 0.2 mM each deoxynucleotide Triphosphate, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, with the addition of 5 mM $MgCl_2$ and 10% dim-

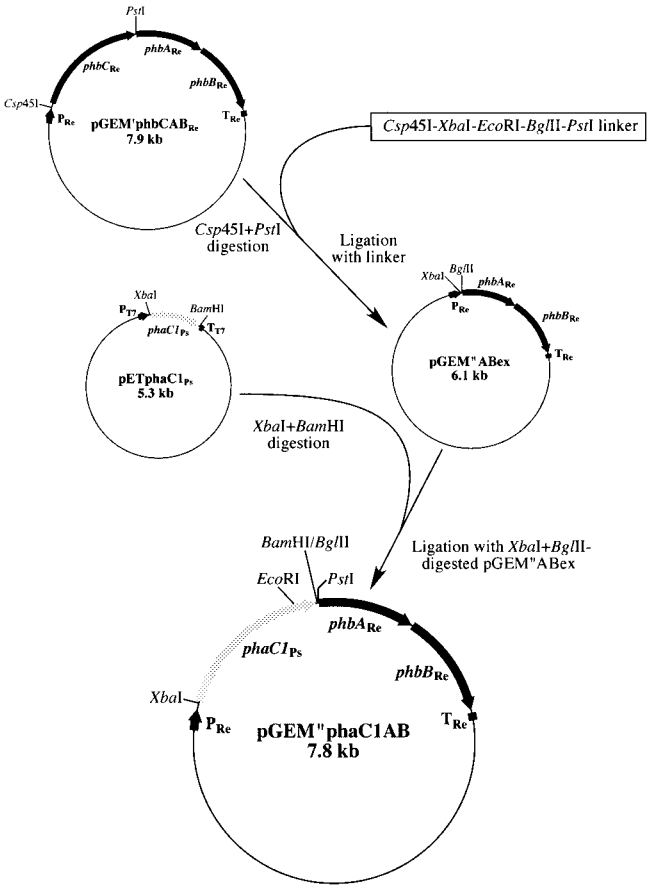

Fig. 1. **Construction strategy of the plasmid vector, pGEM″phaC1AB, used for synthesizing the P(3HB) homopolyester from glucose in recombinant JM109 strain *Escherichia coli*.** *phbC*$_{Re}$, *phbA*$_{Re}$, and *phbB*$_{Re}$ encode for the P(3HB) synthase, β-ketothiolase, and NADPH-dependent acetoacetyl-CoA reductase genes, respectively, derived from *Ralstonia eutropha*; P_{Re} and T_{Re}, the promoter and terminator regions of the *phbCAB*$_{Re}$ operon in *R. eutropha,* respectively; P_{T7} and T_{T7}, the T7 promoter and T7 terminator regions of pET vector, respectively; *phaC1*$_{Ps}$ encodes for the PHA synthase gene derived from *Pseudomonas* sp. 61-3.

ethyl sulfoxide (*12*). The PCR program consisted of 25 cycles of 96°C for 1 min, 55°C for 30 s, and 72°C for 2 min with Gene Amp PCR system 9700 (Perkin-Elmer Applied Biosystems).

Screening Mutants Leading to Enhanced P(3HB) Accumulation—Screening of P(3HB) accumulating mutants of PhaC1$_{Ps}$ was carried out based on a previously established *in vivo* screening system (*6*). PCR mutagenesis of an entire region in the *phaC1*$_{Ps}$ gene was carried out by error-prone PCR. After amplification, a mixture of randomly mutagenized *phaC1*$_{Ps}$ genes was purified, digested with *Xba*I and *Bam*HI and ligated with *Xba*I and *Bgl*II digested pGEM″ABex to generate a mutant library. The recombinants harboring *phaC1*$_{Ps}$ mutant genes were grown on LB plates supplemented with 2% glucose, 0.5 μg/ml Nile red, and 50 μg/ml ampicillin. The change in P(3HB) accumulation resulting from the introduction of mutations into the *phaC1*$_{Ps}$ gene was judged on the basis of the intensity of the pinkish pigmentation of the cells caused by Nile red staining (*13*). For precise quantification of cellular P(3HB) accumulation, mutants were cultivated in LB medium with 2% glucose at 30°C for 96 h. The cellular P(3HB) content was determined by analytical high-performance liquid chromatography (HPLC) after the cellular P(3HB) was converted to crotonic acid by treatment with hot concentrated sulfuric acid (H_2SO_4) (*14*).

Site-Specific Saturation Mutagenesis at Ser325 and Gln481—Ser325 and Gln481 substituted mutants of PhaC1$_{Ps}$ were made by oligonucleotide-directed mutagenesis. For Ser325 substitution, twenty 5′-phosphorylated primers (nineteen forward primers and one reverse primer) were designed and synthesized. Forward and reverse primers were designed as a from of tail-to-tail ligation. For example, substitution of Ser at position 325 with Ala was carried out using a pair of primers, S325A and S325rev. The PCR mixtures (50 μl) contained 0.3 μM of each phosphorylated primer, 0.3 mM of each dNTPs, 10 ng of pGEM″phaC1AB, 1 mM of $MgSO_4$, 2.5 U of PLATINUM *Pfx* DNA polymerase (Invitrogen) and 5 μl of 10× *Pfx* amplification buffer. The program for the PCR was one cycle at 94°C for 5 min, followed by 30 cycles consisting of 96°C for 1 min, 58°C for 30 s and 68°C for 8 min. Other site-specific Ser325 mutagenesis was performed by the same method using the corresponding primers to the amino acid being used as the replacement at position 325. PCR products (approximately 7.8 kb) were purified, self-ligated and transformed into *E. coli* JM109. Nineteen kinds of Ser325 mutagenized *phaC1*$_{Ps}$ genes were confirmed by DNA sequencing, subsequently digested with *Xba*I and *Pst*I, and ligated into the same restriction site of pGEM″ABex to generate Ser325 saturated mutants. Gln481 saturated mutants were constructed by the same strategy using Q481X and Q481rev primers. The oligonucleotide primers used for mutagenesis experiments are summarized in Table 1.

Construction of Ser325/Gln481 Double Mutants—Five double mutants, S325C/Q481K(M, R) and S325T/Q481K(M, R), were constructed by combining 285-bp *Eco*RI–*Pst*I fragments containing mutations for Q481K, M or R with the corresponding region of pGEM″phaC1AB having S325C or T mutation.

Western Blotting—Rabbit antisera against PhaC1$_{Ps}$ was prepared by injection of a synthetic 17-mer oligo-peptide (CSGKLKKSPTSLGNKAY: near the COOH terminus of PhaC1$_{Ps}$). A whole-cell extract of the recombinant *E. coli* was prepared by sonication (*6*). In addition, each soluble fraction was obtained by centrifugation (18,000 ×*g*, 4°C, 10 min). The concentration of total cellular proteins was determined using a Bio-Rad Protein Assay Kit with bovine serum albumin as the standard. Ten micrograms of each soluble protein was subjected to SDS-PAGE on a 12.5% gel and electroblotted to a PVDF membrane using a Criterion™ Blotter (Bio-Rad). Western blotting was performed as described previously (*15*), and protein bands were visualized using goat anti-rabbit IgG conjugated to alkaline phosphatase as a secondary antibody.

RESULTS

Isolation of Mutants Showing Enhanced P(3HB) Production—Approximately 130,000 clones were screened by colony formation on selection plates containing glucose

Table 2. **Accumulation of P(3HB) in *E. coli* JM109 recombinants and substitutions of PhaC1$_{Ps}$ mutants.**

Clone no.	P(3HB) content (wt%)[a]	Substitution	
		Nucleotide	Amino acid
Wild type	0.1		
ep(1-1)	0.4	CGT → CAT	Arg27 → His
		AGT → AGA	Ser477 → Arg
ep(1-6)	0.2	GAA → GAT	Glu130 → Asp
		CTC → TTC	Leu327 → Phe
ep(1-7)	**1.0**	CAG → CGG	**Gln481** → Arg
		CAA → CAG	–[b]
ep(1-8)	**1.0**	AAC → ACC	Asn16 → Thr
		ATG → GTG	Met292 → Val
		AGC → ACC	**Ser325** → Thr
ep(1-16)	**0.8**	AGC → TGC	**Ser325** → Cys
ep(2-1)	0.2	CAA → CGA	Gln12 → Arg
		GCG → GCT	–
		ATG → TTG	Met362 → Leu
		AGC → GGC	Ser497 → Gly
ep(3-1)	0.3	GCG → GTG	Ala304 → Val
		ATG → TTG	Met369 → Leu
ep(4-2)	**0.9**	AGC → TGC	**Ser325** → Cys
		CAC → TAC	His350 → Tyr
ep(5-1)	**0.6**	GAA → AAA	Glu115 → Lys
		AGC → TGC	**Ser325** → Cys
ep(5-2)	**1.3**	GCC → GCT	–
		CAG → CGG	**Gln481** → Arg
ep(5-3)	0.3	AGT → AGG	Ser477 → Arg
		TCA → ACA	Ser547 → Thr
ep(7-24)	0.2	GAT → AAT	Asp30 → Asn
		CAT → CAC	–
		CGT → CGC	–
		AAC → TAC	Asn247 → Tyr
ep(10-3)	**0.8**	CAG → CGG	**Gln481** → Arg
ep(10-4)	**0.8**	CTT → CCT	Leu20 → Pro
		CAG → CGG	**Gln481** → Arg
ep(10-5)	**1.5**	AAT → GAT	Asn5 → Asp
		GGT → GGC	–
		CAG → AAG	**Gln481** → Lys
ep(10-6)	0.2	AAC → AAT	–
		AGT → AGA	Ser477 → Arg
ep(10-7)	0.4	GAA → GAT	Glu130 → Asp
		TTG → TTA	–
ep(10-8)	**0.9**	GCA → GCT	–
		CAG → CGG	**Gln481** → Arg

[a]P(3HB) content in dry cells is presented as an average of three or more independent experiments. [b]– in the column of substituted amino acid indicates silent mutation. Bold letters indicate the names of mutants showing 5-fold or more P(3HB) contents of wild type and its amino acid substitution positions.

and Nile red. Out these, only 121 clones exhibited light pinkish pigmentation that indicates the accumulation of P(3HB) within cells. These positive clones were subjected to HPLC analysis, allowing further precise estimation of the P(3HB) content. As a result, 18 clones showed two times or more greater P(3HB) content than that (0.1 wt%) within recombinant *E. coli* cells harboring the wild type *phaC1*$_{Ps}$. All mutants were subjected to DNA sequencing analysis to determine the mutation points. Table 2 summarizes the P(3HB) contents, mutation points and amino acid substitutions of 18 positive

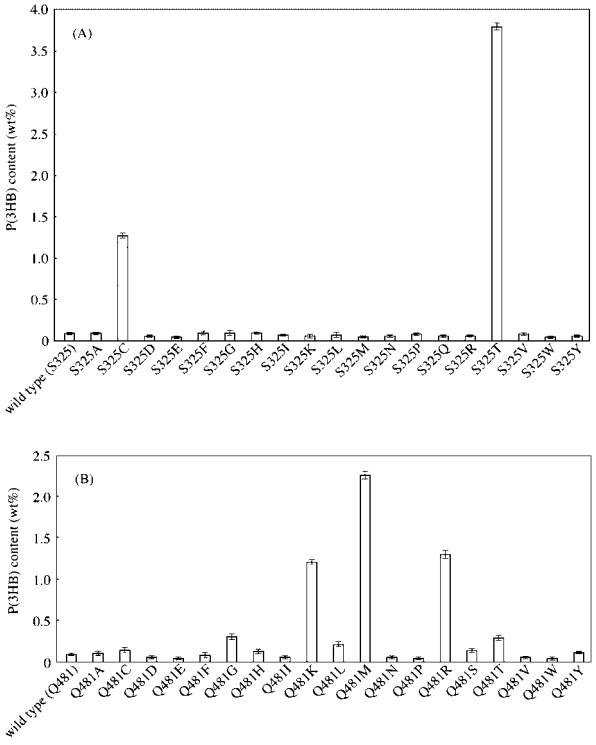

Fig. 2. **Intercellular P(3HB) accumulation levels in recombinant JM109 strain *E. coli* harboring site-specific saturated mutants of *Pseudomonas* sp. 61–3 PHA synthase.** All cells were cultivated on LB medium containing 2% (w/v) glucose for 96 h at 30°C. The cellular P(3HB) content was determined by analytical HPLC after cellular P(3HB) was converted to crotonic acid by treatment with hot concentrated sulfuric acid. (A) P(3HB) content in dry cells of Ser325 mutants. (B) P(3HB) content in dry cells of Gln481 mutants.

mutants. DNA sequencing revealed 7 single mutants, 9 double mutants and 2 triple mutants. The even distribution of mutation could be seen for the coding region (ranging from Asn5 to Ser547) of the 18 positive clones.

The mutant ep(10-5), which exhibited the highest P(3HB) content (15-fold over wild type), had two amino acid substitutions, Asn5 to Asp and Gln481 to Lys, and one silent mutation. Comparing amino acid substitutions of mutants that showed 5-fold or more greater P(3HB) content than wild type PhaC1$_{Ps}$ among the 18 obtained mutants, two possibly beneficial substitutions were found in two or more mutants (Table 2, indicated in bold). Ser325 to Cys or Thr substitutions were found in mutants ep(1-8), ep(1-16), ep(4-2), and ep(5-1), and Gln481 to Arg or Lys was found in mutants ep(1-7), ep(5-2), ep(10-3), ep(10-4), ep(10-5), and ep(10-8). Since, among 18 clones, these mutations were found in 10 mutants, and an enhancement of P(3HB) homopolyester synthesis was observed by amino acid substitutions at two positions (S325C or T and Q481R or K substitutions), we assumed that these two positions ("hot spots") would have the potential for enzyme improvement by saturation mutagenesis.

Site-Specific Saturation Mutagenesis at Hot Spots for Enhanced P(3HB) Synthesis—From the results shown in Table 2, various amino acid substitutions at Ser325 or

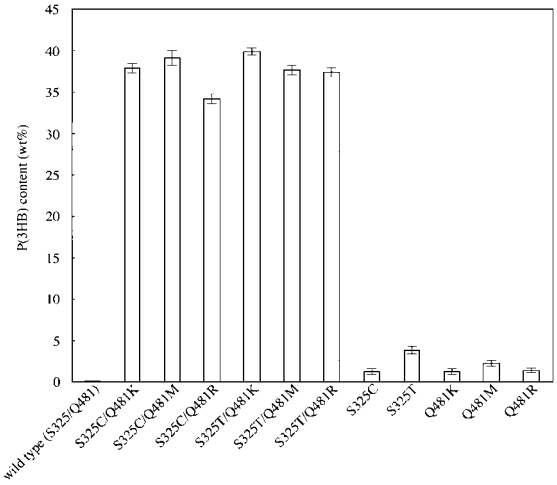

Fig. 3. **Intercellular P(3HB) accumulation levels in recombinant JM109 strain *E. coli* harboring single or double mutants of *Pseudomonas* sp. 61-3 PHA synthase.** All cells were cultivated on LB medium containing 2% (w/v) glucose for 96 h at 30°C. The cellular P(3HB) content was determined by analytical HPLC after the cellular P(3HB) was converted to crotonic acid by treatment with hot concentrated sulfuric acid.

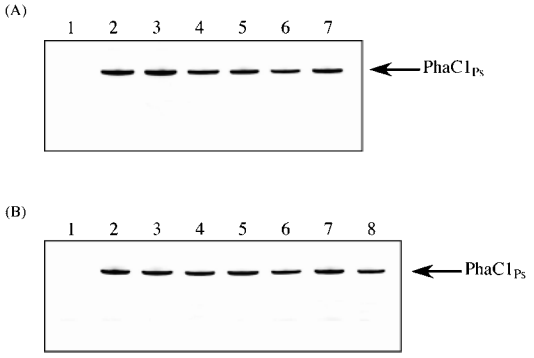

Fig. 4. **Western blot analysis of the soluble fractions of recombinant JM109 strain *E. coli* harboring wild type and mutant *phaC1*$_{Ps}$ genes by anti-PhaC1$_{Ps}$ antiserum.** (A) Single mutants. Control plasmid (pGEM″ABex) (lane 1), wild type PhaC1$_{Ps}$ (lane 2), S325C mutant (lane 3), S325T mutant (lane 4), Q481K mutant (lane 5), Q481M mutant (lane 6), and Q481R mutant (lane 7). (B) Double mutants. Control plasmid (pGEM"ABex) (lane 1), wild type PhaC1$_{Ps}$ (lane 2), S325C/Q481K mutant (lane 3), S325C/Q481M mutant (lane 4), S325C/Q481R mutant (lane 5), S325T/Q481K mutant (lane 6), S325T/Q481M mutant (lane 7), S325T/Q481R mutant (lane 8).

Gln481 were expected to produce an enhanced accumulation of P(3HB), although the effect of silent mutations can not be disregarded. Then, we applied site-specific saturation mutagenesis to Ser325 and Gln481. Figure 2 shows the P(3HB) contents in S325 or Q481 saturated mutants (Fig. 2, A or B, respectively). As shown in Fig. 2, two amino acid substitutions (Cys and Thr) were very effective [13- and 38-fold higher P(3HB) content, respectively] at position 325. The P(3HB) contents in the other amino acid-replaced mutants were nearly the same as or less than in the wild type (Fig. 2A). On the other hand, following site-specific saturation mutagenesis at G481, 11 mutants showed increased P(3HB) contents. Especially, Q481M, Q481R, and Q481K exhibited 22-, 13-, and 12-fold higher P(3HB) contents than the wild type, respectively. Other amino acid substitutions were ineffective in increasing P(3HB) accumulation (Fig. 2B).

In Vitro Recombination of Beneficial Mutations for Further Evolution—Through site-specific saturation mutagenesis, we could identify 5 beneficial mutations, S325C, S325T, Q481K, Q481M, and Q481R, in terms of producing an increase in P(3HB) accumulation. Next, we produced combinations (a total of 6 double mutants) of the two beneficial mutations obtained at Ser325 and the three beneficial mutations obtained at Gln481 by genetic engineering, and determined the P(3HB) contents in the 6 double mutants. Figure 3 shows the dry cell P(3HB) contents of the wild type, 6 double mutants and 5 single mutants of PhaC1$_{Ps}$. The P(3HB) contents of all the double mutants were greatly elevated, as much as 340- to 400-fold higher than in the wild type. Western blot analysis of soluble fractions of recombinant *E. coli* JM109 cells revealed that the expression level was indistinguishable among the wild type and mutant enzymes, five single mutants [S325C(T) and Q481M(K,R)] and six double mutants [S325C(T)/Q481M(K,R)] (Fig. 4).

DISCUSSION

In vitro evolution can be defined as a methodology that generates new biological functions by the intended use of molecular evolutionary mechanisms regardless of the molecular structural basis of the enzyme of interest (*16*). Our initial attempts to obtain evolved PHA synthases with enhanced activity or changed substrate specificity are one such case, as has been demonstrated for type I PHA synthases from *Ralstonia eutropha* (*2–4*) and *Aeromonas caviae* (*5*). Subsequent similar work was recently reported with the same *A. caviae* PHA synthase by Amara *et al*. (*17*). Intrinsically, type II PhaC1$_{Ps}$ has a very weak ability to incorporate 3HB units, which is technically advantageous in that it allows us to obtain, in a positive selection manner, mutant PHA synthases with improved P(3HB) synthesis from a huge mutant library (*3*, *18–21*). Actually, it was not such a labor-consuming task to pick 18 positive mutants showing enhanced P(3HB) accumulation among 130,000 clones. Four of the 18 mutants had amino acid substitutions at position 325, and a mutation at position 481 was commonly shared by 6 mutants (Table 2). Usually not many amino acids can be reached by a single nucleotide substitution, *i.e.* at position 325, Ser(AGC) can change to 6 amino acids, Arg, Asn, Cys, Gly, Ile, and Thr. In this context, a complete amino acid substitution at all positions that are possible hot spots for the desired properties evolutionary engineering would be very useful (*22*, *23*). In fact, the variant with the most enhanced P(3HB) synthesis, Q481M, was newly obtained by site-specific amino acid substitution at position 481. For mutation at position 325, it might be imagined that the 6 possible amino acid substitutions would be exhaustively tried during the process to create artificial point mutations.

Figure 5 shows the partial alignment of the amino acid sequences of PhaC1$_{Ps}$ with other PHA synthases. Mutational studies revealed that *R. eutropha* PHA synthase possesses an essential catalytic diad (Cys and His resi-

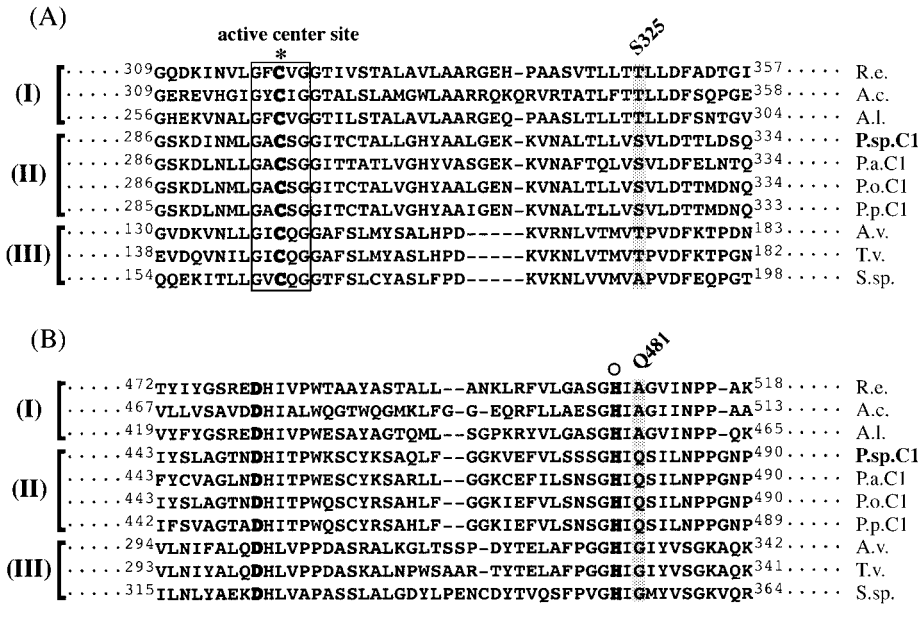

Fig. 5. **Partial alignment of the amino acid sequences of PHA synthase (PhaC) from *Pseudomonas* sp. 61-3 (PhaC1) (P.sp.C1) with those from *R. eutropha* (R.e.) (*26–28*), *A. caviae* (A.c.) (*29*), *A. latus* (A.l.) (*30*), *P. aeruginosa* (PhaC1) (P.a.C1) (*31*), *P. oleovorans* (PhaC1) (P.o.C1) (*32*), *P. putida* (PhaC1) (P.p.C1) (*32*), *A. vinosum* (A.v.) (*33*), *T. violaceae* (T.v.) (*34*) and *Synecocystis* sp. PCC6803 (S.sp.) (*35*, *36*).** I, II, and III indicate classification of PHA synthases. The asterisk indicates the conserved Cys residue in the active center of PHA synthases. The open box indicates a lipase box-like sequence. The open circle indicates the conserved His residue of PHA synthases. Amino acid residues at positions 325 and 481 are shaded. (A) A limited region containing the active center site and S325 of $PhaC1_{Ps}$ is presented. (B) A limited region containing the conserved His residue and Q481 of $PhaC1_{Ps}$ is presented.

dues indicated by the asterisk and open circle, respectively, in Fig. 5) in which the Cys residue is involved in covalent catalysis and the His residue serves as a general base catalyst (*24*). These two residues are conserved in all PHA synthases (*25*). Ser325 is located near downstream of Cys296, the active center of $PhaC1_{Ps}$. Interestingly, throughout the sequence alignment of all PHA synthases (type I to type III), Ser is conserved at position 325, being specific for type II synthases. On the other hand, Thr is conserved at the aligned positions of types I and III synthases, both of which prefer short chain length 3HB substrates (Fig. 5A). This is in good agreement with the fact that the S325T mutant exhibited higher P(3HB) accumulation compared to wild type $PhaC1_{Ps}$. This suggests a change in substrate specificity toward monomer units (shifting from 3HA to 3HB) for $PhaC1_{Ps}$. The other hot spot, 481, that contributes positively to enhanced P(3HB) synthesis is located adjacent to His479, forming a putative catalytic diad which can be inferred by sequence alignment. The amino acids at this position are more diverse (Gln for type II, Ala for type I and Gly for type III) (Fig. 5B) than those at position 325. The P(3HB) content of the Q481A mutant (type I type) was equivalent to that of wild type, while the Q481G mutant (type III type) exhibited a 3-fold higher P(3HB) content than the wild type (Fig. 2B).

Finally, we succeeded in achieving the further evolution of $PhaC1_{Ps}$ by recombination of beneficial mutations [five single mutants: S325C(T) and Q481M(K,R)] as searched by point mutations and saturation mutagenesis. This strongly suggests that positions 325 and 481 do not interfere with each other in the tertiary structure of $PhaC1_{Ps}$, and that amino acid substitutions at these positions contribute cooperatively to enhanced P(3HB) synthesis. Most type II PHA synthases from pseudomonads do not possess the ability to incorporate the 3HB unit at all. In such a sense, $PhaC1_{Ps}$ can be considered to be an intermediate enzyme between type I and type II PHA synthases in substrate specificity. Further accumulation of beneficial mutations for improved P(3HB) synthesis will provide useful clues for achieving the conversion of type II PHA synthase to its type I counterpart or the opposite conversion.

The authors thank Ms. Y. Ichikawa and Ms. R. Nakazawa for DNA sequencing (Bioarchitect Research Group, RIKEN Institute).

This work was supported in part by a grant for Ecomolecular Science Research to the RIKEN Institute and a President's Special Research Grant (to S. Taguchi) funded by the RIKEN Institute.

REFERENCES

1. Sudesh, K., Abe, H., and Doi, Y. (2000) Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Prog. Polym. Sci.* **25**, 1503–1555
2. Taguchi, S., Maehara, A., Takase, K., Nakahara, M., Nakamura, H., and Doi, Y. (2001) In vivo assay system of the polymerase as a key enzyme for PHA biosynthesis. *RIKEN Rev.* **42**, 3–6
3. Taguchi, S., Nakamura, H., Hiraishi, T., Yamato, I., and Doi, Y. (2002) In vitro evolution of a polyhydroxybutyrate synthase by intragenic suppression-type mutagenesis. *J. Biochem.* **131**, 801–806
4. Taguchi, S., Matsusaki, H., Matsumoto, K., Takase, K., Taguchi, K., and Doi, Y. (2002) Biosynthsis of biodegradable polyesters from renewable carbon sources by recombinant bacteria. *Polymer Intern.* **51**, 899–906
5. Kichise, T., Taguchi, S., and Doi, Y. (2002) Enhanced accumulation and changed monomer composition in polyhydroxyalkanoate (PHA) copolyester by in vitro evolution of *Aeromonas caviae* PHA synthase. *Appl. Environ. Microbiol.* **68**, 2411–2419
6. Taguchi, S., Maehara, A., Takase, K., Nakahara, M., Nakamura, H., and Doi, Y. (2001) Analysis of mutational effects of polyhydroxybutylate (PHB) polymerase on bacterial PHB accumulation using an in vivo assay system. *FEMS Microbiol. Lett.* **198**, 65–71
7. Matsusaki, H., Manji, K., Taguchi, K., Kato, M., Fukui, T., and Doi, Y. (1998) Cloning and molecular analysis of the poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-*co*-3-hydroxyalkanoate) biosynthesis genes in *Pseudomonas* sp. strain 61-3. *J. Bacteriol.* **180**, 6459–6467

8. Matsumoto, K., Matsusaki, H., Taguchi, K., Seki, M., and Doi, Y. (2002) Isolation and characterization of polyhydroxyalkanoates inclusions and their associated proteins in *Pseudomonas* sp. 61-3. *Biomacromolecules* **3**, 787–792
9. Yanisch-Perron, C., Vieira, C., and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119
10. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed. (Laboratory, C.S.H., ed.) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
11. Matsusaki, H., Abe, H., Taguchi, K., Fukui, T., and Doi, Y. (2000) Biosynthesis of poly(3-hydroxybutyrate-*co*-3-hydroxyalkanoates) by recombinant bacteria expressing the PHA synthesis gene *phaC1* from *Pseudomonas* sp. 61–3. *Appl. Microbial. Biotechnol.* **53**, 401–409
12. Taguchi, S., Kuwasako, K., Suenaga, A., Okada, M., and Momose, H. (2000) Functional mapping against *Escherichia coli* for the broad-spectrum antimicrobial peptide, thanatin, based on an in vivo monitoring assay system. *J. Biochem.* **128**, 745–754
13. Spiekermann, P., Rehm, B.H.A., Kalscheuer, R., Baumeister, D., and Steinbüchel, A. (1999) A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. *Arch. Microbiol.* **171**, 73–80
14. Karr, D.B., Waters, J.K., and Emerich, D.W. (1983) Analysis of poly-β-hydroxybutyrate in *Rhizobium japonicum* bacteroids by ion-exclusion high-pressure liquid chromatography and UV detection. *Appl. Environ. Microbiol.* **46**, 1339–1344
15. Murata, T., Takase, K., Yamato, I., Igarashi, K., and Kakinuma, Y. (1997) Purification and reconstitution of Na^+-translocating vacuolar ATPase from *Enterococcus hirae*. *J. Biol. Chem.* **272**, 24885–24890
16. Kunchner, O., and Arnold, F.H. (1997) Directed evolution of enzyme catalysts. *Trends Biotechnol.* **15**, 523–530
17. Amara, A.A., Steinbüchel, A., and Rehm, B.H.A. (2002) In vivo evolution of the *Aeromonas punctata* polyhydroxyalkanoate (PHA) synthase: isolated and characterization of modified PHA synthases with enhanced activity. *Appl. Microbiol. Biotechnol.* **59**, 477–482
18. Tange, T., Taguchi, S., and Momose, H. (1994) Improvement of a useful enzyme (subtilisin BPN′) by an experimental evolution system. *Appl. Microbiol. Biotechnol.* **41**, 239–244
19. Kano, H., Taguchi, S., and Momose, H. (1997) Cold adaptation of a mesophilic serine protease, subtilisin, by in vitro random mutagenesis. *Appl. Microbiol. Biotechnol.* **47**, 46–51
20. Taguchi, S., Ozaki, A., and Momose, H. (1998) Engineering of a cold-adapted protease by sequential random mutagenesis and a screening system. *Appl. Environ. Microbiol.* **64**, 492–495
21. Taguchi, S., Ozaki, A., Nonaka, T., Mitsui, Y., and Momose, H. (1999) A cold-adapted protease engineered by experimental evolution system. *J. Biochem.* **126**, 689–693
22. Taguchi, S., Komada, S., and Momose, H. (2000) The complete amino acid substitutions at position 131 that are positively involved in cold adaptation of subtilisin BPN′. *Appl. Environ. Microbiol.* **66**, 1410–1415
23. Miyazaki, K. and Arnold, F.H. (1999) Exploring nonnatural evolutionary pathways by saturation mutagenesis: rapid improvement of protein function. *J. Mol. Evol.* **49**, 716–720
24. Jia, Y., Yuan, W., Wodzinska, J., Park, C., Sinskey, A.J., and Stubbe, J. (2001) Mechanistic studies on class I polyhydroxybutyrate (PHB) synthase from *Ralstonia eutropha*: class I and III synthases share a similar catalytic mechanism. *Biochemistry* **40**, 1011–1019
25. Steinbüchel, A. and Hein, S. (2001) Biochemical and molecular basis of microbial synthesis of polyhydroxyalkanoates in microorganisms. *Adv. Biochem. Eng. Biotechnol.* **71**, 81–123
26. Peoples, O.P. and Sinskey, A.J. (1989) Poly-β-hydroxybutyrate biosynthesis in *Alcaligenes eutrophus* H16. Identification and characterization of the PHB polymerase gene (*phbC*). *J. Biol. Chem.* **264**, 15298–15303
27. Schubert, P., Steinbüchel, A., and Schlegel, H.G. (1988) Cloning of the *Alcaligenes eutrophus* poly-β-hydroxybutyrate synthetic pathway and synthesis of PHB in *Escherichia coli*. *J. Bacteriol*. **170**, 5837–5847
28. Slater, S.C., Voige, W.H., and Dennis, D.E. (1988) Cloning and expression in *Escherichia coli* of the *Alcaligenes eutrophus* H16 poly-β-hydroxybutyrate biosynthetic pathway. *J. Bacteriol*. **170**, 4431–4436
29. Fukui, T. and Doi, Y. (1997) Cloning and analysis of the poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) biosynthesis genes of *Aeromonas caviae*. *J. Bacteriol*. **179**, 4821–4830
30. Choi, J.-I., Lee, S.Y., and Han, K. (1998) Cloning of the *Alcaligenes latus* polyhydroxyalkanoate biosynthesis genes and use of these genes for enhanced production of poly(3-hydroxybutyrate) in *Escherichia coli*. *Appl. Environ. Microbiol.* **64**, 4897–4903
31. Timm, A. and Steinbüchel, A. (1992) Cloning and molecular analysis of the poly(3-hydroxyalkanoic acid) gene locus of *Pseudomonas aeruginosa* PAO1. *Eur. J. Biochem.* **209**, 15–30
32. Huisman, G.W., Wonink, E.W., Meima, R., Kazemier, B., Terpstra, P., and Witholt, B. (1991) Metabolism of poly(3-hydroxyalkanoates) (PHAs) by *Pseudomonas oleovorans*. *J. Biol. Chem.* **266**, 2191–2198
33. Liebergesell, M. and Steinbüchel, A. (1992) Cloning and nucleotide sequences of genes relevant for biosynthesis of poly(3-hydroxybutyric acid) in *Chromatium vinosum* strain D. *Eur. J. Biochem.* **209**, 135–150
34. Liebergesell, M. and Steinbüchel, A. (1993) Cloning and molecular analysis of the poly(3-hydroxybutyric acid) biosynthetic genes of *Thiocystis violacea*. *Appl. Microbiol. Biotechnol*. **38**, 493–501
35. Kaneko, T., Sato, S., Kotani, H., Tanaka, A., Asamizu, E., Nakamura, Y., Miyajima, N., Hirosawa, M., Sugiura, M., Sasamoto, S., Kimura, T., Hosouchi, T., Matsuno, A., Muraki, A., Nakazaki, N., Naruo, K., Okumura, S., Shimpo, S., Takeuchi, C., Wada, T., Watanabe, A., Yamada, M., Yasuda, M., and Tabata, S. (1996) Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. *DNA Res.* **3**, 109–136
36. Hein, S., Tran, H., and Steinbüchel, A. (1998) *Synechocystis* sp. PCC6803 possesses a two-component polyhydroxyalkanoic acid synthase similar to that of anoxygenic purple sulfur bacteria. *Arch. Microbiol.* **170**, 162–170