Engineering of Chimeric Class II Polyhydroxyalkanoate Synthases

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PHA synthase is a key enzyme involved in the biosynthesis of polyhydroxyalkanoates (PHAs). Using a combinatorial genetic strategy to create unique chimeric class II PHA synthases, we have obtained a number of novel chimeras which display improved catalytic properties. To engineer the chimeric PHA synthases, we constructed a synthetic *phaC* gene from *Pseudomonas oleovorans* (*phaC*_{1Po}) that was devoid of an internal 540-bp fragment. Randomly amplified PCR products (created with primers based on conserved *phaC* sequences flanking the deleted internal fragment) were generated using genomic DNA isolated from soil and were substituted for the 540-bp internal region. The chimeric genes were expressed in a PHA-negative strain of *Ralstonia eutropha*, PHB[−]4 (DSM 541). Out of 1,478 recombinant clones screened for PHA production, we obtained five different chimeric *phaC*_{1Po} genes that produced more PHA than the native *phaC*_{1Po}. Chimeras S1-71, S4-8, S5-58, S3-69, and S3-44 exhibited 1.3-, 1.4-, 2.0-, 2.1-, and 3.0-fold-increased levels of *in vivo* activity, respectively. All of the mutants mediated the synthesis of PHAs with a slightly increased molar fraction of 3-hydroxyoctanoate; however, the weight-average molecular weights (*M*_w) of the PHAs in all cases remained almost the same. Based upon DNA sequence analyses, the various *phaC* fragments appear to have originated from *Pseudomonas fluorescens* and *Pseudomonas aureofaciens*. The amino acid sequence analyses showed that the chimeric proteins had 17 to 20 amino acid differences from the wild-type *phaC*_{1Po}, and these differences were clustered in the same positions in the five chimeric clones. A threading model of *PhaC*_{1Po}, developed based on homology of the enzyme to the *Burkholderia glumae* lipase, suggested that the amino acid substitutions found in the active chimeras were located mostly on the protein model surface. Thus, our combinatorial genetic engineering strategy proved to be broadly useful for improving the catalytic activities of PHA synthase enzymes.

Polyhydroxyalkanoates (PHAs) are polyesters of various hydroxyalkanoates that are synthesized by numerous microorganisms as an energy reserve material. Cells accumulate PHA when an essential nutrient, such as nitrogen or phosphorus, is limiting in their environment while at the same time there is an excess of carbon present (29). To date, ~130 different types of PHAs have been reported, and their biosynthetic genes have largely been identified (15). PHAs are biodegradable, and they possess material properties similar to those of various synthetic thermoplastics and elastomers, such as polypropylene and synthetic rubbers. Since PHAs are of biological origin, they are also capable of being completely broken down to water and carbon dioxide by microorganisms found in a wide range of environments, such as soil, water, and sewage. Moreover, PHAs can be synthesized from renewable carbon sources derived from agricultural or even industrial wastes (1). At present, the scale of industrial production of biodegradable plastics from PHA is relatively small, and it takes place largely via bacterial fermentation processes. The major obstacle to large-scale commercial production of PHAs is the high cost of a sustainable closed-cycle biotechnological process (24). There are several important factors that influence cost, including the product yield, the process of final polymer product separation, and the capital equipment costs. To improve the product yield, an efficient recombinant production system for PHA is needed.

To date, attempts to improve microbial PHA production have been carried out by exploring the heterologous expression of combinations of PHA biosynthetic genes in recombinants (9) or by increasing the number of copies of key PHA biosynthetic genes, such as the PHA synthase gene (*phaC*) (23). However, recent studies have demonstrated that when the number of PHA biosynthetic genes is artificially increased, the resulting increase in PHA production is often marginal (23).

A total of 54 PHA synthase genes have been characterized and sequenced from 44 different bacteria (15). Nucleotide sequence comparisons provide important clues for predicting important amino acid residues that might influence the activities and structures of enzymes. Until now, mechanistic studies of polymerization and substrate specificity have mainly focused on PHA synthases from classes I and III (5, 6, 12, 33). For example, there have been several reports of random mutagenesis of PHA synthase from *Ralstonia eutropha* (*PhaC*_{Re}) to probe its structure and function and also to obtain enzymes with higher activity levels (16, 25, 26, 27). In contrast, the class II PHA synthases, which can produce elastomeric PHA polymers (i.e., medium-chain-length 3-hydroxyalkanoic acid [3HA_{MCL}] with C₆ to C₁₄), have not been extensively studied. PHAs comprising 3HA_{MCL} units typically exhibit material characteristics superior to those of PHAs of short-chain-length 3HA (a lower melting point and higher flexibility). To date, no high-resolution structural information has been reported for any class of PHA synthase protein. Consequently, it has been a great challenge to engineer variant PHA synthases displaying improved functionalities. In the present study, we report the first attempt to engineer the putative catalytic regions of a class

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TABLE 1. Bacterial strains, plasmids, and oligonucleotides used in the study

Strain, plasmids ^a , and oligonucleotides	Relevant characteristics ^b	Source or reference
Bacterial strains		
<i>E. coli</i> TOP10F'	F' ⁺ { <i>lacI</i> ^q Tn10(Tet ^R)} <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>deoR recA1 araD139</i> Δ(<i>ara-leu</i>)7697 <i>galU galK rpsL endA1 nupG</i>	Invitrogen
<i>R. eutropha</i> DSM 541 (PHB ⁻ 4)		10
Plasmids^a		
pUC19-CAB3	PUC19 derivation; <i>lacZ</i> promoter, <i>P. oleovorans phaC1</i> gene, <i>P. aeruginosa phaA</i> gene, <i>R. eutropha phaB</i> gene	This study
pBBR1MCS-2	Kanamycin resistant; cloning vehicle	Procter & Gamble
pBBR1MCS-2 <i>phaC1</i> _{P_o}	pBBR1MCS-2 derivation; <i>R. eutropha phb</i> promoter, <i>P. oleovorans phaC1</i> gene	This study
pBBR1MCS-2 <i>phaC1</i> _{P_o} -I	pBBR1MCS-2 derivation; <i>R. eutropha phb</i> promoter, <i>P. oleovorans phaC1</i> gene; I225L, N399A, D400T	This study
pBBR1MCS-2 <i>phaC1</i> _{P_o} -II	pBBR1MCS-2 derivation; <i>R. eutropha phb</i> promoter, <i>P. oleovorans phaC1</i> gene; I225L, D399A, T401S	This study
pBBR1MCS-2 <i>phaC1</i> _{P_o} -III	pBBR1MCS-2 derivation; <i>R. eutropha phb</i> promoter, <i>P. oleovorans phaC1</i> gene; I225L	This study
pBBR1MCS-2 <i>phaC1</i> _{P_o} I225L-linker	pBBR1MCS-2 derivation; <i>R. eutropha phb</i> promoter, deleted <i>P. oleovorans phaC1</i> gene	This study
Oligonucleotides		
For constructing pBBR1MCS-2 <i>phaC1</i> _{P_o} I225L-linker		
phaC FS-A (nucleotide location 219–240)	5'-AAGCGACGACCGTCGCTTCAAT-3'	
phaC FS-B (nucleotide location 664–685)	5'-AGAACTTGTTCAATTGCGGCGG-3'	
phaC FS-C (nucleotide location 1185–1214)	5'-GTTCTGGAACAACGACACGCGTCTGCC-3'	
phaC FS-D (nucleotide location 1478–1497)	5'-ATCGGCACCGGTCATGAAGC-3'	
Linker sequence	5'-CAATTGAACCATCGATGGGAACGCGT-3'	
For amplification of various <i>phaC</i> genes		
For-MfeI-general <i>phaC</i>	5'-CCGCAATTGAACAAGTTCTACGT-3'	
Rev-MluI-general <i>phaC</i>	5'-CGGGAGACGCGTGGTGTCTGTTG-3'	
For sequencing the isolated <i>phaC</i> genes		
F _{sequencing} - <i>phaC</i>	5'-CAGTGAAGGCGCCGTGGTGTA-3'	
R _{sequencing} - <i>phaC</i>	5'-GATCAGGTCGCCGTGGAAGG-3'	

^a PUC19-CAB3, pBBR1MCS-2*phaC1*_{P_o}-I, and pBBR1MCS-2*phaC1*_{P_o}-II constructs were created previously in our laboratory (unpublished data).

^b CAATTG and ACGCGT are restriction sites for MfeI and MluI, respectively.

II PHA synthase from *P. oleovorans* (PhaC_{1P_o}) in order to improve its enzymatic characteristics. In addition, we introduce a novel combinatorial method of molecular breeding to generate libraries of evolved mutants with enhanced in vivo activities toward PHA biosynthesis.

MATERIALS AND METHODS

Soil sampling and DNA extraction from soil. Clay-loam soil samples were collected from various areas around Cornell University in Ithaca, N.Y. The pH of each soil sample was measured using a Corning pH Meter 220 after allowing it to equilibrate with distilled water (11). All soil samples were stored at 4°C prior to use. DNA was extracted from soil samples using an UltraClean Soil DNA Extraction kit (MoBio Inc., Solana, Calif.).

Bacterial strains and culture conditions. All bacterial strains and plasmids used in this study are listed in Table 1. One Shot cells, *Escherichia coli* TOP10F' (Invitrogen, Carlsbad, Calif.), were used as the first host strain for the transformation of pools of mutants. The PHA-negative strain of *R. eutropha*, PHB⁻4 (DSM 541), was used for heterologous expression of the chimeric PHA synthase genes. Bacteria were cultured in Luria-Bertani (LB) medium (Difco) or mineral salt (MS) medium (8) containing the appropriate carbon source and supplements. Organic acids were provided as sodium salts. Bacteria were cultured at 30°C (for *R. eutropha*) and 37°C (for *E. coli*). Kanamycin (50 mg/liter for *E. coli* and 200 mg/liter for *R. eutropha* strains; Sigma Chemical, St. Louis, Mo.) was added to the medium when needed.

Plasmid construction. All recombinant DNA techniques were performed as described by Sambrook et al. (18). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Beverly, Mass.). The oligonucleotide primers used in this study were synthesized at the BioResource Center, Cornell

University. The pBBR1MCS-2 vector (5.52 kb) was linearized at the EcoRI/BamHI site. *phaC1* from *Pseudomonas oleovorans* was excised from pUC19-CAB3 (Table 1) by EcoRI/BamHI digestion and ligated into the EcoRI/BamHI-digested pBBR1MCS-2 vector, resulting in the 7.22-kb plasmid pBBR1MCS-2*phaC*_{P_o}. The vectors pBBR1MCS-2*phaC*_{P_o}-I and pBBR1MCS-2*phaC*_{P_o}-II were constructed previously in our laboratory (unpublished data). The vector pBBR1MCS-2*phaC*_{P_o}-III was constructed as follows. First, two rounds of site-directed mutagenesis were performed on *phaC1*_{P_o} to introduce two unique restriction sites for MfeI and MluI at nucleotides 673 (Ile225) and 1207 (Arg403), respectively. These two unique restriction sites were designed to flank the most conserved catalytic regions found in all PHA synthases (Fig. 1). A megaprimer containing an MfeI site was generated by PCR using the primers PhaC FS-A and FS-B. The PCR products were purified using a QIAGEN PCR purification kit, and then a second PCR was carried out using 50 ng of megaprimer as the forward primer. An initial five cycles of PCR were carried out with only the megaprimer, and then the reverse primer (phaC FS-D) was added after the fifth cycle. The annealing temperature was 60°C. An MluI site was introduced into the gene in a similar manner by first generating a megaprimer containing an MluI site using the primer pair phaC FS-C and phaC FS-D. The megaprimer was purified and then used as a reverse primer in the second PCR, with the forward primer (phaC FS-A) added after five cycles. The primers phaC FS-A and phaC FS-D contained a PshAI and a DraIII site, respectively, and therefore the site-directed mutagenized 1.2-kb *phaC1*_{P_o} containing MfeI and MluI sites was cloned into the PshAI/DraIII-digested pBBR1MCS-2*phaC*_{P_o}, resulting in the 7.22-kb pBBR1MCS-2*phaC*_{P_o}-III plasmid (pBBR1MCS-2*phaC*_{P_o}I225L). To construct pBBR1MCS-2*phaC*_{P_o}I225L-linker, we first used MfeI and MluI to remove a central 540-bp portion of the *phaC1*_{P_o} gene. A 20-bp nucleotide linker flanked by MfeI and MluI restriction sites was digested with MfeI and MluI and ligated into pBBR1MCS-2*phaC*_{P_o}I225L, resulting in the 6.7-kb plasmid pBBR1MCS-

FIG. 1. (A) Construction of plasmids used in this study. (B) PCR detection and amplification of putative *phaC* gene fragments from soil DNA extracts using a set of generic primers. Lane 1, no DNA; lanes 2 to 6, PCR products from soil DNA extracts 1 (S1), S2, S3, S4, and S5, respectively; lane 7, *phaC* amplified from *B. megaterium* cell lysate as a control. (C) Fluorescence measurements of Nile blue A dye screening assay for functional chimeric PHA synthases; shown are active chimeric S3-44 and S3-69, wild-type *phaCI*_{P₀} (W1), deleted *phaCI*_{P₀}I225L-linker (D1), and one point mutation I225L*phaCI*_{P₀} (W2).

2*phaC*_{P₀}I225L-linker. The *phaCI*_{P₀}I225L-linker gene was expressed under the control of the *phb*_R *eutropha* promoter.

PCR detection and amplification of putative *phaC* gene fragments from soil DNA extracts. A set of generic primers (For-MfeI-general *phaC* and Rev-MluI-general *phaC*) was designed to amplify an ~540-bp fragment of the *phaC* gene (Table 1). The conserved region was identified by aligning all *phaC* sequences from different organisms, including *R. eutrophus*, *Acinetobacter* sp., *Aeromonas caviae*, *Alcaligenes* sp., *Pseudomonas aeruginosa*, *P. oleovorans*, *Paracoccus denitrificans*, *Rhodobacter sphaeroides*, *Bacillus megaterium*, and *Methylobacterium extorquens*. The forward and reverse generic primers contained at their termini MfeI and MluI restriction sites, respectively. They were designed to clone the amplified fragments in frame into the pBBR1MCS2*phaC*_{P₀}I225L-linker expression vector. The PCR products were detected by electrophoresis in a 1.5% (wt/vol) agarose gel in Tris-borate-EDTA buffer. The gels were stained in an aqueous solution of 0.4 mg of ethidium bromide/liter, destained in distilled water, and photographed with a Sony Video Graphic UP-860 camera under a UV source (Dual Intensity UV Transilluminator; Labnet).

Cloning of putative *phaC* gene fragments in the linker site of pBBR1MCS-2*phaCI*_{P₀}I225L-linker. The *phaC* fragments amplified from each soil were purified (PCR purification kit; QIAGEN) and digested with the MfeI and MluI enzymes. They were ligated into the MfeI/MluI-digested pBBR1MCS-2*phaCI*_{P₀}I225L-linker. The ligation mixture was transformed into *E. coli* TOP10F'. Transformants were selected on LB agar containing kanamycin (100 µg/ml) and incubated at 37°C for 24 h. All colonies were scraped from the plates and subsequently grown in 50 ml of LB medium supplemented with kanamycin (100 µg/ml) at 37°C with shaking at 250 rpm for 12 h. From each culture, 10 ml was taken and plasmid DNA purified using a QIA prep Spin Miniprep kit (QIAGEN). The plasmid pools were transformed into the PHA-negative strain of *R. eutropha*, PHB⁻4 (DSM 541), by electroporation as described previously (13). Transformants were selected on LB agar medium containing kanamycin (200 µg/ml).

Screening for functional chimeric PhaC enzymes using viable-colony staining method. For routine analysis, the viable-colony staining method developed by Spiekermann et al. (20) was slightly modified and used for detection of the presence of PHAs. Briefly, Nile blue A was added to the sterilized MS medium (8) to a final concentration of 20 µg/liter. Each *R. eutropha* transformant was transferred from LB agar to Nile blue A-MS agar plates containing 20 mM

octanoic acid. The plates were incubated at 30°C for 5 days. The agar plates were observed under UV light (312-nm wavelength), and bacterial clones producing PHA fluoresced bright orange (Fig. 1). To confirm PHA production, the cells were stained with Nile blue A and examined by fluorescence microscopy (Nikon phase-contrast attachment Ph for OPTIPHOT-2, LABOPHOT-2A, or LABOPHOT-2). Stained PHA granules appear as bright reddish to orange fluorescent spots inside the cells. The stain was kept as a stock solution in ethanol at 10 µg/ml and diluted to 0.10 µg/ml with ethanol just before use.

Sequencing analysis of *phaC* gene fragments. The *phaC* gene fragments were sequenced using oligonucleotides flanking the linker sites of the pBBR1MCS-2*phaCI*_{P₀}I225L-linker vector (F_{sequencing}-*phaC* and R_{sequencing}-*phaC*) (Table 1). Sequence assembly and analysis were performed by using Lasergene software (DNASTar, Inc.). The identification of each *phaC* gene fragment resulted from the best matches with the GenBank database (BLASTn and BLASTx searches) (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Cell extracts and Western immunoblotting. Selected recombinant *R. eutropha* strains were grown overnight in 5 ml of MS medium supplemented with 200 µg of kanamycin/ml and 20 mM octanoic acid. Approximately 0.5 to 1.0 g (wet weight) of cells was suspended in 100 µl of phosphate-buffered saline (PBS) buffer (0.14 M NaCl, 10 mM Na₂HPO₄, 3 mM KCl, and 1.8 mM KH₂PO₄, pH 7.4) and disrupted by sonication at power level 2 to 3 at 20 to 30% duty for 8 to 10 bursts with a Sonifier 250 (Branson Ultrasonics Corp., Danbury, Conn.). After centrifugation at 14,000 × g for 10 min at 4°C, soluble and insoluble cell fractions were obtained. The insoluble cell fraction was resuspended in 100 µl of PBS buffer, pH 7.4, with 1× NuPAGE lithium dodecyl sulfate sample buffer (Invitrogen) and disrupted with a second round of sonication as described above. Mercaptoethanol (5% [vol/vol]) was added to the samples. The samples were subsequently heated at 70°C for 10 min prior to electrophoresis. Lithium dodecyl sulfate and mercaptoethanol-denatured proteins were separated in 10% NuPAGE Bis-Tris Gel with MOPS (morpholinepropanesulfonic acid) running buffer (Invitrogen) and stained with SimplyBlue SafeStain (Invitrogen). Western blots used rabbit polyclonal anti-*phaCI*_{P₀} antiserum, and detection was performed using a WesternBreeze Chromogenic Kit-Anti-Rabbit (Invitrogen).

PHA extraction. PHA production by *R. eutropha* was quantified in shake flask experiments. A stationary-phase culture (5 to 10 ml) was inoculated into 500 ml of MS medium in a 2-liter Erlenmeyer flask supplemented with 20 mM sodium octanoic acid and kanamycin (200 µg/ml). The cultures were incubated at 30°C

TABLE 2. In vivo activities of site-specific *PhaC*_{Po} mutants heterologously expressed in *R. eutropha*

Construct	Mutation(s)	No. of point mutations	PHA content (% of CDW) ^a	Relative PHA content (% of wild-type PHA accumulation)
Native <i>PhaC</i> _{Po}		0	4.2 ± 0.16	100
<i>PhaC</i> _{Po} -I	I225L, N399A, D400T	3	ND	0
<i>PhaC</i> _{Po} -II	I225L, D400A, T401S	3	ND	0
<i>PhaC</i> _{Po} -III	I225L	1	4.2 ± 0.25	100
<i>PhaC</i> _{Po} I225L-linker	I225 to R403 deleted	0	ND	0
S1-7	I225L, V230E, E236D, Y242F, S246G, Q247N, Q248V, I253V, A261E, D272E, A280V, L282T, L290V, M331L, N333S, Q334D, D340N, A346S, S360R, E361D	20	5.3 ± 0.5	126
S3-44	I225L, Y242F, S246N, Q247N, Q248V, I253V, A261E, D272E, A280V, L282T, A283S, D289N, L290V, V306L, L312I, M331L, N333S, Q334D, S360R, E361D	20	12.4 ± 1.5	295
S3-69	I225L, Y242F, S246N, Q247N, Q248M, I253V, A261E, D272E, A280V, L282T, L290V, V306L, L312I, M331L, N332S, Q334D, H350Q, S360R, E361D	19	9.0 ± 1.1	214
S4-8	I225L, E236D, Y242F, S246N, Q247N, Q248V, I253V, D272E, A280V, L282T, L290V, V306L, L312I, N315K, M331L, N332S, Q334D, A346T, S360K, E361D	20	5.7 ± 0.4	135
S5-58	I225L, Y242F, Q248V, I253V, D272E, E276D, A280V, L282M, L290I, V306L, N315Q, M331L, N333S, Q334D, A346S, S360R, E361D	17	8.4 ± 0.3	200

^a ND, not detectable. The PHA content was measured in triplicate. Mean values and standard deviations are listed. Recombinant *R. eutropha* DSM 541 was cultivated in MS medium containing 20 mM sodium octanoate as the carbon source and 200 µg of kanamycin/ml for 72 h at 30°C. CDW, cell dry weight.

on a shaker at 250 rpm for 72 h. Bacterial growth was monitored by measuring the optical density at 600 nm. The cells were harvested by centrifugation (7,000 × g; 30 min; 4°C), washed twice with PBS buffer (pH 7.4), lyophilized, and weighed to quantify the cell dry weight. PHAs were extracted as described previously (3).

Analyses of PHAs. The PHA composition was analyzed by gas chromatography (GC)-mass spectrometry. Purified polymer was dissolved in chloroform at a concentration of ~5 mg/ml and subjected to methanolysis in the presence of 15% (vol/vol) sulfuric acid in methanol. The resulting methyl esters were assayed by GC according to the method of Timm and Steinbüchel (28). GC analyses were performed with a Hewlett-Packard GCD model gas chromatograph equipped with a 0.32-µm-inner-diameter Rtx5-MS capillary column 30 m in length (Restek, Bellefonte, Pa.). The weight-average molecular weight (*M_w*) and number-average molecular weight (*M_n*) of the purified PHA were determined by gel permeation chromatography. Briefly, the PHA was dissolved in tetrahydrofuran and separated at 40°C. A Waters 2410 refractive index detector and a 1-ml/min tetrahydrofuran flow rate were used. Calibration was performed with narrow molecular weight polystyrene standards (PSS Polymer Standards Service, Silver Spring, Md.). The data were collected and analyzed with PSS WINGPC software scientific version 6.20. Differential scanning calorimetry (DSC) measurements were carried out using the DSC 220C oscillating differential scanning calorimeter (Seiko Instruments Inc., Chiba, Japan) as previously described (3). The sample was first heated to ~30°C above the estimated melting temperature (*T_m*) and held for 3 min to eradicate any vestiges of crystallinity. The sample was tested from -80 to +100°C in a nitrogen atmosphere at a heating rate of 10°C/min. A step-like change in the DSC trace was observed. The midpoint of the step increase was taken as the glass transition (*T_g*). For *T_m* information, the endothermic peak was observed upon heating, and the temperature at the peak was taken as the *T_m*.

RESULTS

Activity of chimeric *phaC* gene in pBBR1MCS-2*phaC*_{Po}-I, pBBR1MCS-2*phaC*_{Po}-II, and pBBR1MCS-2*phaC*_{Po}-III. Two unique restriction sites, MfeI and MluI, flanking a 540-bp region were introduced so that they included the catalytic site of the PHA synthases. The effects of these mutant hybrid *phaC* genes on PHA production were examined. The construct pBBR1MCS-2*phaC*_{Po}-I (Table 1) had three mutations resulting from the restriction sites introduced: I225L at the MfeI site

and N399A and D400T at the MluI site. The construct pBBR1MCS-2*phaC*_{Po}-II (Table 1) also had three mutations introduced during construction: I225L at the MfeI site and D399A and T401S at the MluI site. Recombinant strains of *R. eutropha* carrying either of the two *phaC*_{Po} mutants did not produce PHA, although the mutant enzymes were expressed, as determined by the appearance of an immunoreactive protein of the appropriate molecular weight (data not shown). The mutations I225L, N399A, D400T, D400A, and T401S alone or in combination resulted in a loss of PHA synthase activity. pBBR1MCS-2*phaC*_{Po}-III (Fig. 1 and Table 1) was constructed so that it had only the I225L mutation at the MfeI site. The level of PHA production observed via Nile blue A staining of *R. eutropha* expressing the mutant I225L *phaC*_{Po} construct was similar to that of the *R. eutropha* strain expressing wild-type *phaC*_{Po} (data not shown). The levels of PHA accumulation in these two *R. eutropha* strains were quantified by extracting the PHA (Table 2). The recombinant *R. eutrophus* expressing either wild-type *phaC*_{Po} or mutant I225L *phaC*_{Po} produced 4.2 weight percent PHA content per cell (dry weight). The PHAs produced by the I225L *PhaC*_{Po} mutant consisted mainly of 3-hydroxyoctanoate (3HO) (86 mol%) and 3-hydroxyhexanoate (3HHx) (12 mol%), with a partial molar fraction of 3-hydroxybutyrate (3HB) (2 mol%) and traces of 3-hydroxydecanoate and 3-hydroxydodecanoate (Table 3). The composition was similar to that of the PHA from wild-type *PhaC*_{Po}. DSC showed that both polymers are copolymers and not blends, since both have single (sharp) *T_g* and *T_m* values (*T_g* and *T_m* were -31 and 54°C, respectively, for PHA from the wild-type *PhaC*_{Po} and -30 and 55°C, respectively, for PHA from the I225L *PhaC*_{Po}) (Table 3).

Combinatorial generation of *phaC* gene fragments from soil to produce active chimeras. *phaC* fragments from each soil isolate were amplified using a set of generic primers, For-MfeI-

TABLE 3. Characterization of PHAs isolated from *R. eutropha* DSM 541 recombinants

Wild-type and mutant PhaC1 _{Po}	Composition of PHA (mol%) ^a					<i>M_n</i> (10 ⁻³ g mol ⁻¹)	<i>M_w</i> (10 ⁻³ g mol ⁻¹)	PDI	<i>T_m</i> (°C)	<i>T_g</i> (°C)
	3HB	3HHx	3HO	3HD	3HDD					
Wild-type PhaC1 _{Po}	1.5 ± 1.0	11.7 ± 0.6	85.9 ± 1.6	0.6 ± 0.2	0.3 ± 0.1	37.0	67.1	1.8	54	-31
I225LPhaC1 _{Po}	1.4 ± 0.4	12.0 ± 0.9	85.8 ± 0.8	0.6 ± 0.5	0.2 ± 0.0	32.1	60.0	1.9	55	-30
S1-7	0.7 ± 0.4	5.1 ± 0.9	93.6 ± 0.3	0.6 ± 0.4	0.1 ± 0.1	25.9	48.2	1.9	50	-34
S3-44	1.0 ± 0.8	5.7 ± 0.5	92.5 ± 1.5	0.6 ± 0.3	0.2 ± 0.0	32.5	61.0	1.9	51	-35
S3-69	0.7 ± 0.4	5.4 ± 1.4	93.4 ± 1.9	0.5 ± 0.4	0.2 ± 0.1	34.3	63.4	1.8		
S4-8	1.7 ± 0.2	4.1 ± 0.1	93.0 ± 0.1	1.0 ± 0.0	0.3 ± 0.0	28.8	52.9	1.8		
S5-58	1.5 ± 0.0	7.2 ± 1.0	90.6 ± 1.6	0.5 ± 0.2	0.1 ± 0.0	30.4	57.4	1.9	52	-33

^a PHA composition was determined by GC analysis in triplicate. Mean values and standard deviations are listed. Recombinant *R. eutropha* DSM 541 was cultivated in MS medium containing 20 mM sodium octanoate as the carbon source and 200 µg of kanamycin/ml for 72 h at 30°C. 3HD, 3-hydroxydecanoate; 3HDD, 3-hydroxydecanoate.

general *phaC* and Rev-MluI-general *phaC*. Fragments of the expected size (540 bp) were recovered (Fig. 1). The PCR products amplified from each soil DNA extract were purified and cloned into the pBBR1MCS-2*phaC*_{1Po}I225L-linker vector. They were first transformed into *E. coli* TOP10F' as an initial host and then transformed into *R. eutropha* PHB⁻4 (DSM 541). Approximately 1,500 *R. eutropha* recombinant transformants from different soil DNA extracts were screened for PHA production by Nile blue A staining of the colonies. Five clones (S1-71, S3-8, S3-69, S4-8, and S5-58) that produced either similar or higher levels of PHA than the native PhaC1_{Po} enzyme were selected and further analyzed. Table 2 shows the amount of PHA produced by *R. eutropha* carrying the wild-type PhaC1_{Po} and the chimeric PhaC1_{Po} constructs. All five chimeric clones produced more PHA than the wild-type PhaC1_{Po}. Recombinant *R. eutropha* strains carrying chimeras S1-71, S4-8, S5-58, S3-69, and S3-44 produced 1.3-, 1.4-, 2.0-, 2.1-, and 3.0-fold higher levels of PHA than the wild type, respectively. Table 3 shows the monomer compositions, molecular weights, and *T_m* and *T_g* analyses of the PHAs produced from the wild-type and chimeric strains. The PHA compositions of all of the chimeras were similar to each other. Their PHAs consisted mainly of 3HO (at least 90 mol%) and 3HHx (up to 10 mol%). The 3HO molar fractions in the PHA copolyester is higher for all active chimeras (93.6, 92.5, 93.4, 93, and 90.6 mol% 3HO for S1-71, S3-44, S3-69, S4-8, and S5-58, respectively) than those of the wild-type PhaC1_{Po} (85 mol% 3HO). The 3HHx molar fractions were lower in all chimeras (6 mol%) than that of the wild type (12 mol%). Similar small amounts of the 3HB unit (1 to 2 mol%) and a trace of the long-chain 3HA unit (including C₁₀ or C₁₂) were detected in both the wild-type and the active chimeras. The DSC scans showed a single depression of *T_m* and *T_g* at ranges of 50 to 55°C and -30 to -35°C, respectively, for both PHAs produced from the wild type and the active chimeras. This observation suggested that the PHA was a copolymer rather than a blend of PHAs. The average molecular weights (*M_w*) of all PHA copolyesters (from the wild type and all active chimeras) were determined to be 4.82 × 10⁴ to 6.71 × 10⁴ g/mol by gel permeation chromatography analysis. The polydispersity indexes (PDIs) were determined to be ~1.8 to 1.9.

Sequence analysis of *phaC* gene fragments. Nucleotide sequence analysis (BLASTn) revealed that all five *phaC* gene fragments that restored activity in the recovered chimeric PHA synthases were highly homologous to sequences found in dif-

ferent species of *Pseudomonas*. The active chimeras could be divided into two groups based upon sequence analysis. The internal region in chimeras S1-71, S3-44, and S3-69 had homology to *Pseudomonas fluorescens* PhaC with 89, 87, and 88% nucleotide matches, respectively. The other chimera group, S4-8 and S5-58, had homology to *Pseudomonas aureofaciens* PhaC with 87 and 88% nucleotide matches, respectively. The results from alignments of their amino acid sequences (Fig. 2 and Table 2) revealed at least seven amino acid differences among them. Each of the active chimeric PHA synthases contained 17 to 20 amino acid differences from the wild-type PhaC1_{Po}.

The chimeric PHA synthases in recombinant *R. eutropha* strains that failed to accumulate PHA were further examined. Twenty negative chimeric transformants from five different soil DNA extracts were randomly selected and sequenced. All the *phaC* fragments were inserted in frame relative to the *phaC*_{1Po} gene of pBBR1MCS-2*phaC*_{1Po}I225L-linker. A BLASTn search revealed that the *phaC* fragments in the inactive chimeras N1, N2, N3, N4, N9, N11, N13, and N14 were homologous to those of *Methylobacterium extorquens*, *R. eutropha*, *Rhodobacter sphaeroides*, *Azorhizobium caulinodans*, *Rhodospirillum rubrum*, *Pseudomonas* sp. strain 61-3, *Pseudomonas pseudoalcaligenes*, and *Paracoccus denitrificans*, respectively. The ClustalW method (MegAlign; DNASTar, Inc., Madison, Wis.) was employed to construct a phylogenetic tree based on the amino acid sequence data obtained for some of the inactive chimeras, all of the active chimeras, the wild-type PhaC1_{Po}, and certain related bacterial PHA synthases (Fig. 3). As expected, the resulting tree revealed a tight clustering of the active chimeras, which was located a distinct distance from the other inactive chimeras. Western analysis of selected clones revealed that each of the chimeric PhaC enzymes was expressed as an immunoreactive protein of ~65 kDa (data not shown).

Development of a threading model of the class II *P. oleovorans* PHA synthase 1. The PhaC1_{Po} sequence was threaded onto known bacterial lipase structures using MegAlign and the SWISS-MODEL facility (19; <http://us.expasy.org/>). The alignments showed ~19% similarity between PhaC1_{Po} and the lipase from *Burkholderia glumae*, whose crystal structure is known (Fig. 4). The *B. glumae* structure was then used to generate a threading model (residues P208 to K513). Gaps were inserted into both sequences where needed based upon the alignment (Fig. 4). Three deletions (F264 to L267, M331 to K348, and A407 to F408) within the PhaC1_{Po} sequence were

QLNKFYVFDLSPEKSLARFCLRNWQTFIV Majority																						
10 20 30																						
1	Q	I	N	K	F	Y	V	F	D	L	S	P	E	K	S	L	A	R	F	C	L	R
1	Q	I	N	K	F	Y	V	F	D	L	S	P	E	K	S	L	A	R	F	C	L	R
1	Q	I	N	K	F	Y	V	F	D	L	S	P	E	K	S	L	A	R	F	C	L	R
1	Q	I	N	K	F	Y	V	F	D	L	S	P	E	K	S	L	A	R	F	C	L	R
1	Q	I	N	K	F	Y	V	F	D	L	S	P	E	K	S	L	A	R	F	C	L	R
1	Q	I	N	K	F	Y	V	F	D	L	S	P	E	K	S	L	A	R	F	C	L	R
1	Q	I	N	K	F	Y	V	F	D	L	S	P	E	K	S	L	A	R	F	C	L	R
1	C	I	N	K	Y	V	I	L	D	L	Q	P	E	S	S	L	V	R	H	V	V	E
Pseudomonas oleovorans PHA synthase 1																						
31-71																						
34-8																						
35-58																						
33-69																						
33-44																						
Ralstonia eutrophus PHA synthase																						
3WRNPTKAQREWGLSTYIE - ALKEAVDVVT Majority																						
40 50 60																						
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
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31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
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31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
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31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
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31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31	3	W	R	N	P	T	K	A	Q	R	E	W	G	L	S	T	Y	I	E	-	A	L
31</

FIG. 3. Phylogenetic tree analysis of different *phaC* fragments recovered from soils, which contribute to active (P1 to -5) and inactive (N1 to -14) chimeric PHA synthase genes. The analysis was done based on a comparison of amino acid sequence data using the DNASTar MegAlign program.

made to account for the lack of homology to structurally conserved regions of the *B. glumae* lipase. In addition, the deletions were located exclusively within nonconserved regions, according to the multiple alignments of the different PHA synthases (data not shown). There is significant homology between the active-site nucleophile of the lipase and residues L267 to V306 of the PhaC_{1P_o} enzyme (Fig. 4). A conserved residue, C296, of PhaC_{1P_o} aligns with the active-site residue (S87) of the lipase, and a conserved H479 of PhaC_{1P_o} aligns with the active-site H284 of the lipases. A protein threading model of PhaC_{1P_o} suggested that the active-site C296 and the conserved H479 and D451 form a catalytic triad within the active site (Fig. 5). The active-site C296 is conserved in the α/β hydrolase family and is located at the α - β elbow positioned between a β -strand and an α -helix.

DISCUSSION

There have been several reported efforts to genetically engineer PHA synthases to improve their enzymatic properties specifically to make them better suited for large-scale PHA production. The three-dimensional structure of PHA synthase,

however, has not yet been reported, impeding protein engineering. In 1992, Steinbüchel's group noted that all PHA synthases contain a putative lipase box (G-X-[S/C]-X-G) in their core structures (22). Several threading models of PHA synthases have been developed based on their homology to the prokaryotic lipases, whose structures have been resolved by X-ray analysis (5, 16). Jia et al. (5) reported the first mechanistic study of a class III PHA synthase from *Chromatium vinosum*, and their evidence suggested that PHA synthases and the lipases have similar folds in their catalytic domains. The catalytic residues C319 of PhaC_{Re} and C149 of PhaC_{Cv} have been demonstrated to be involved in covalent catalysis (12, 30). Site-directed mutagenesis of PhaC_{Cv} suggested that the conserved H331 is required to activate C149 for the nucleophilic attack on the substrates, and the conserved D302 is required to form the catalytic triad (5). The first threading model of a class I PHA synthase from *R. eutropha* was recently developed based on the known structure of lipase from *B. glumae*, and it was used for the mutational analysis of PhaC_{Re}, employing both site-specific- and random-mutagenesis protocols (16). Permissive PhaC_{Re} mutants that were generated by single-gene shuffling, however, exhibited reduced in vitro activity and con-

FIG. 4. Alignment of the *P. oleovorans* PHA synthase 1 (PhaC1_{Po}) with the *B. glumae* lipase (1THA_A). The PhaC1_{Po} sequence was threaded onto the bacterial lipase structures using SWISS-MODEL, and the alignment was performed using MegAlign. Identical residues in the sequences are shown as boldface letters, and conservative replacements are shown as light-gray letters. The open arrowheads indicate the catalytic cysteine of PhaC1_{Po}, the catalytic serine of the lipase, the conserved H479 of PhaC1_{Po}, the conserved H285 of the lipase, and the conserved D451 of PhaC1_{Po}. The lipase catalytic region is boxed. The two unique restriction sites (MfeI and MluI) are shown. The arrows indicate two highly conserved regions found in all PHA synthases that were used to design primer For-MfeI-general *phaC* and primer Rev-MluI-general *phaC*. The asterisks indicate residues where amino acid substitutions occurred in all five active chimeras.

tained mutations that mapped at various surface-exposed regions of the enzymes (16). Other studies employed PCR-mediated random mutagenesis and in vivo activity measurements to analyze the mutational effects of class I and III PHA synthases (7, 25). Taguchi et al. (25) reported an in vitro evolutionary experiment employing PCR-mediated random mutagenesis to study mutational effects on PhaC_{Re} synthase in recombinant strains of *E. coli*; however, none of mutants exhibited higher activity than the wild type. Another study with *Aeromonas caviae* PHA synthase (PhaC_{Ac}, a class III PHA synthase) expressed in recombinant *E. coli* accumulated from 3- to 6.5-fold-higher PHA levels than the wild type (7).

In this study, we established a combinatorial protein-engineering approach to create novel class II PHA synthases that produce elastomeric PHA copolyesters. Our final goal was to

obtain modified PHA synthases from *P. oleovorans* (PhaC1_{Po}) with improved enzymatic characteristics, i.e., enhanced PHA accumulation or modified copolymer composition. We first employed a genetic panning strategy for obtaining genes encoding PHA synthase (*phaC*) from soil based upon two observations. First, genes involved in PHA biosynthesis have significant DNA sequence homology, thus allowing a consensus set of PCR primers to be designed. Second, only a small fraction of all the microbes in the environment can be cultured, even using the vast array of microbiological media and conditions presently available to researchers. There may, therefore, be several unidentified species of PHA-producing bacteria living in soil that cannot be easily cultivated under normal laboratory conditions. As a result, DNA extracted from soil would be predicted to be a potential source for novel *phaC* genes which

FIG. 5. Threading model of PHA synthase 1 from *P. oleovorans*. (A) Localization of all point mutations inherent in the construction of hybrid PhaC1_{Po}. The genetically engineered internal 540-bp region is represented by blue color (I225 to R403). Inherent mutations are indicated by arrows. Catalytic residues are shown as stick side chains and indicated by red letters. The upper model represents one set of mutations. The lower model represents a second set of mutations with the PhaC1_{Po} model rotated by 180°. (B) Threading model of localization of amino acid residue substitutions of all five PhaC1_{Po} chimeras. Substituted amino acids are located in the colored regions. Blue indicates the amino acid substitutions found in all five active chimeras. Green indicates the amino acid substitutions found in some of active chimeras. Pink indicates the amino acid substitutions found only in the chimeric S3-44.

could be amplified by PCR with a set of generic primers designed to flank a conserved region of the *phaC* genes. Thousands of chimeric PHA synthases were created by cloning the putative *phaC* fragments amplified from soil into the defective *phaC1_{Po}*. Three different *phaC1_{Po}* constructs were made by site-directed mutagenesis, introducing two unique restriction sites (MfeI and MluI) flanking the region of PhaC1_{Po} from I225 to R403. The resulting point mutations (I225L, N399A, D400A, D400T, and T401S), however, abolished the enzyme's activity (Table 2). pBBR1MCS-2*phaC1_{Po}*-III was constructed with only one point mutation, I225L, at the MfeI restriction site, and it exhibited 100% of the wild-type enzyme activity. The alignment of various PHA synthases showed that amino acids at positions 399, 400, and 401 are quite highly conserved, especially among PHA_{MCL} synthases (data not shown). Interestingly, all those amino acids (I225, N399, D400, and T401) are predicted to be located on the surface of the protein (Fig. 5). Therefore, these residues might not be required for catalytic activity but are required for maintaining the structural integrity of the enzyme.

We isolated a number of different *phaC* gene fragments from various PHA-producing soil bacteria that might not have been obtained via classical microbiological isolation. Figure 3 shows a phylogenetic tree of *phaC* fragments obtained by PCR and compared to known bacterial PHA synthases. Some of the fragments recovered showed high sequence homology to *phaC*

genes from the genera *Methylobacterium*, *Azorhizobium*, *Rhodobacter*, *Rhodospirillum*, etc. Most strains of the genus *Methylobacterium* grow slowly, and many of them do not grow at all on nutrient agar (4). Strains from the genus *Rhodobacter* and *Rhodospirillum* are very difficult to grow and must be cultivated on special medium under anaerobic conditions (2, 17). However, none of the recombinant *R. eutropha* transformants expressing the chimeric constructs carrying (in-frame) *phaC* fragments originating from those bacteria was able to produce PHAs. The amino acid sequence alignments of selected chimeric clones revealed a large number of amino acid differences between each inactive chimera and the wild-type PhaC1_{Po} (data not shown). Most of the amino acid differences between the inactive chimeric synthases and the wild-type PhaC1_{Po} occurred within highly conserved residues. As a result, we suspected that those chimeric constructs contain many mutations that abolish the in vivo activity of the enzyme.

Some chimeras were able to confer a higher degree of PHA accumulation on their *R. eutropha* host than the wild-type PhaC1_{Po} (Table 2). They contain 17 to 20 amino acid differences from the wild-type. These amino acids substitutions occur at similar positions among the five active chimeras, and none are conserved among class I, II, and III synthases. Most of the amino acid substitutions are conservative (i.e., E236D, Y242F, Q247N, I253V, D272E, A280V, and V306L), while others are not (i.e., Q248V, A261E, L282T, A283S, and

S260R). Our threading model showed that most amino acid substitutions were located on the surface (Fig. 5), suggesting that their roles are structural, involving protein-substrate or protein-protein interactions, e.g., in the dimerization of the PhaC subunits. Interestingly, all the active chimeras synthesized PHAs with an increase in the molar fraction of 3HO and a decrease in the molar fraction of 3HHx. The slight changes in substrate specificity indicated that certain of the respective amino acid substitutions found among the five active mutants may have affected amino acids that play a role in substrate specificity (Table 3). These amino acid replacements were Y242F, I253V, M331L, N333S, Q334D, and E361D. No significant differences in the weight average molecular weights of the PHA polymers synthesized by each of the active chimeras were observed, suggesting that none of the amino acid substitutions affected the processivity of the enzyme (Table 3).

In recent years, a number of PHA granule-associated proteins have been reported (14, 31, 32). PhaP, for example, belongs to a group called phasins that are predominately found on the surfaces of PHA granules synthesized by short-chain-length PHA producers. Several studies have shown that phasins affect the size and the number of PHA granules and positively affect PHA synthesis (14, 31, 32). Two models have therefore been proposed in which PhaP promotes PHB synthesis by regulating the ratio of surface area to volume of PHB granules by interacting with the PHA synthase. In medium-chain-length PHA producers, other types of granule-associated proteins, i.e., PhaF and PhaI, have been reported. The specific activities of type II *P. aeruginosa* PHA synthases were increased in vitro when the purified phasin from *R. eutropha* was added (21). In our study, the wild-type and the mutant PhaC_{Po} proteins were expressed in *R. eutropha* strains carrying the endogenous PhaP_{Re} enzyme. Thus, it is possible that the chimeras might have an enhanced activity through a better interaction with PhaP_{Re} or other granule-associated proteins. The higher in vivo activity level found in the chimeric S3-44 strain, possessing the most active chimeric synthase, contained two unique amino acid substitutions (A283S and D289N) that were not found in any of the other active chimeras. From our threading model, these residues reside on the accessible surface region of the protein, indicating that they are not directly involved in catalysis (Fig. 5). Therefore, it would appear plausible that such amino acid substitutions might promote enzyme activity by enhancing interactions between chimeric PhaC1_{Po} and other proteins, including PhaP_{Re}, or by the dimerization of the PhaC subunits.

In summary, through our combinatorial strategy of genetic engineering of class II PHA synthases, we succeeded in obtaining *P. oleovorans* synthase 1 chimeras which enhanced overall levels of PHA accumulation and which slightly altered the monomeric compositions of the resulting PHA polymer products. Further detailed information about the tertiary structures and polymerization mechanisms of PHA synthases is needed to allow us to find other reasonable explanations for the mechanistic effects of those beneficial mutations on synthase activity. In general, we expect that our genetic engineering scheme will be a very useful approach for improving the catalytic activities of other types of desirable enzymes as well.

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