J Ind Microbiol Biotechnol (2003) 30: 322–326
DOI 10.1007/s10295-003-0059-8

ORIGINAL PAPER

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Biosynthesis of medium-chain-length poly(hydroxyalkanoates) with altered composition by mutant hybrid PHA synthases

Received: 2 December 2002 / Accepted: 1 April 2003 / Published online: 22 May 2003

Abstract *Pseudomonas resinovorans* harbors two isogenic poly(hydroxyalkanoates) (PHAs) synthase genes (*phaC1*$_{Pre}$, *phaC2*$_{Pre}$) responsible for the production of intracellular medium-chain-length (mcl-)PHAs. Sequence analysis showed that the putative gene-products of these genes contain a conserved α/β-hydrolase fold in the carboxy-terminal half of the proteins. Hybrid genes *pha7* and *pha8* were constructed by exchanging the α/β-hydrolase-fold coding portions of *phaC1*$_{Pre}$ and *phaC2*$_{Pre}$ at the 3′ terminal. When grown with decanoate as carbon source, the *pha7*- or *pha8*-transformed *Escherichia coli* LS1298 produced PHAs containing 73–75% β-hydroxydecanoate (β-HD) and 25–27% β-hydroxyoctanoate (β-HO). Deletion mutants, Δ*pha7* and Δ*pha8*, were isolated during the PCR-based construction of *pha7* and *pha8*, respectively. Cells harboring these mutants produced PHAs containing 55–60 mol% β-HD and 40–45 mol% β-HO. These results demonstrate the feasibility of generating active hybrid mcl-PHA synthase genes and their mutants with the potential of producing polymers having a varied repeat-unit composition.

Keywords α/β-Hydrolase fold · Hybrid gene · Poly(hydroxyalkanoate) synthases

Introduction

Poly(hydroxyalkanoates) (PHAs) are biopolyesters found in many microorganisms [2, 21]. These polymers are accumulated by cells as a form of carbon and energy storage. Because of its biodegradability property, PHA has been the subject of many studies aimed at developing the biomaterials into ecologically friendly substitutes of the petroleum-based polymers that degrade only slowly. Based on the length of the carbon chain of its monomers and the PHA synthase enzymes responsible for its synthesis, PHA can be grouped into two major classes [22]. The polymers that contain repeat-unit monomers with 3–5 carbon atoms are called short-chain-length (scl-)PHAs. They are synthesized by the type I (single-polypeptide) or type III (two-heterosubunit) PHA synthases. The medium-chain-length (mcl-)PHAs, in contrast, are composed of monomers with at least 6 carbon atoms in their chain length. The enzymes responsible for the mcl-PHA synthesis are termed type II PHA synthases. In vivo gene transfer and expression studies [3, 12] and in vitro enzymatic characterization [15, 25] suggested that the various PHA synthases are highly specific in terms of their preference for the monomer precursor. In this report, an approach for generating hybrid type II PHA synthase genes and their deletion mutants is presented. Their expression in an *Escherichia coli* host resulted in the synthesis of mcl-PHA with varied repeat-unit compositions.

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Materials and methods

Bacteria, plasmids and growth conditions

Table 1 lists the bacterial strains and plasmids used in this study. Cultures of *E. coli* and *Pseudomonas resinovorans* were grown in Luria-Bertani (LB; 1% w/v Bacto-tryptone, 0.5% w/v Bacto-yeast extract, 0.5% w/v NaCl) or tryptic soy broth (Difco, Detroit, Mich.) at 37 °C (for *E. coli*) or 30 °C (for *P. resinovorans*) with 200–250 rpm shaking. Kanamycin (Km; 30–50 μg/ml) and carbenicillin (Cb; 50 μg/ml) were added as needed.

Molecular biology procedures

Oligonucleotide primers used for the amplification of various genes and DNA segments are shown in Table 1. These oligonucleotides were custom-ordered from Invitrogen (Carlsbad, Calif.), Sigma-Genosys Fisher (Pittsburgh, Pa.), or MWG-Biotech (High Point, N.C.). Platinum *Pfx* DNA polymerase and Elongase enzyme mix

Table 1 Bacterial strains, plasmids and PCR primers. In the descriptions, only pertinent characteristics are listed

Strains, plasmids and primers	Description	Reference/source
Pseudomonas resinovorans NRRL B-2649		NCAUR/ARS/USDA
Escherichia coli DH5α		Invitrogen
E. coli LS1298	C600, *fadB*::Kan	DiRusso [6]
E. coli NovaBlue		Novagen
pETBlue-1		Novagen
pETC1b, pETC2b	pETBlue-1 containing *P. resinovorans* $phaC1_{Pre}$ and $phaC2_{Pre}$, respectively	Solaiman et al. [20]
pETCP7b, pETCP8b	pETBlue-1 containing hybrid genes *pha7* and *pha8*, respectively	Solaiman et al. [20]
pETΔCP7b, pETΔCP8b	pETBlue-1 containing Δ*pha7* and Δ*pha8*, respectively	This study
CLPR-III-63R	5′-TCA TCG CTC GTG CAC ATA GGT GCC-3′	
CLPR-IV-92L1	5′-ATG AGC AAC AAG AAC AAT GAA GAC CTG CAG CGC C-3′	
CLPR-IV-92L2	5′-ATG CGA GAA AAA CAA ATC CCG GGC ACC TTG CC-3′	
CLPR-IV-92R2	5′-TCA GCG CAC GTG TAC ATA GGT GCC GGG CGC-3′	
CL-VI-119A	5′-GAC TGG GCA TGC CGC CGT TGT TCA CC-3′	
CL-VI-119B	5′-ACG GCG GCA TGC CCA GTC AGG TCA GCA AG-3′	

(both from Life Technologies, Foster City, Calif.) were interchangeably used for PCR under the reaction conditions suggested by the supplier. Total genomic DNA that served as PCR template was isolated using the Wizard genomic DNA purification kit (Promega, Madison, Wis.) or the DNeasy tissue kit (Qiagen, Valencia, Calif.). Amplified PCR fragments were isolated by agarose gel electrophoresis, followed by elution using the GeneClean II kit (Qbiogene, Carlsbad, Calif.). For cloning into the *Eco*RV site of the pETBlue-1 expression vector (Novagen, Madison, Wis.), the PCR fragments were first subjected to an end-conversion reaction using the End-Conversion kit (Novagen). *E. coli* DH5α (Invitrogen) or NovaBlue (Novagen) served as the screening host cells for the recombinant plasmids. The desired plasmids were introduced into the expression host, *E. coli* LS1298 [6], by an electroporation protocol described by Sambrook and Russell [18]. *E. coli* LS1298 is a *fadB* strain organism required for the phenotypic expression of mcl-PHA synthesis [12]. DNA-sequencing reactions were performed using the BigDye (ver 3) terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, Calif.). Sequence determination was carried out in an ABI Prism 310 genetic analyzer or an ABI Prism 3700 DNA analyzer (Applied Biosystems). The database search and the sequence analysis and comparison were performed using the interactive, Web-based BLAST programs [1, 23] of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). All other routine molecular biology procedures were carried out according to the methods described by Sambrook et al. [17].

PHA characterization

An overnight culture of the recombinant *E. coli* to be tested was used to inoculate 200 ml of LB medium (in a 500-ml baffled Erlenmeyer flask) containing Km (50 μg/ml), Cb (50 μg/ml) and sodium decanoate (0.25% w/v; stock solution in 5% Brij 58 at 20% w/v). Cell culturing was performed for 48 h at 37 °C and 250 rpm shaking. Cells were harvested by centrifugation, washed once with cold de-ionized water and lyophilized to complete dryness. The weight of the lyophilized cells constituted the cell dry weight (CDW). To determine the repeat-unit composition of PHA, the lyophilized cells (5–15 mg) were subjected to acid-catalyzed methanolysis and silylation reactions, as detailed elsewhere [4, 24]. The resultant samples were analyzed by GC/MS, according to the protocol of Ashby et al. [4]. Large-scale fermentation was performed to obtain cell and PHA yields. To this end, cells were grown in LB medium (10 l) containing Km (50 μg/ml), Cb (50 μg/ml) and sodium decanoate (0.25% w/v), using a BioFlo3000 fermentor (New Brunswick Scientific, Edison, N.J.) operated at 37 °C, 300 rpm and 0.5 l airflow/min. After 48 h of growth, cells were harvested, washed and lyophilized; and the CDW was determined as described earlier in this paragraph. The PHA in the lyophilized cells was extracted with chloroform and subsequently purified by precipitation in methanol [4]. The PHA content was calculated as the weight-percentage of the precipitated PHA in the total CDW.

Results and discussion

Construction and characterization of hybrid mutant genes

The nucleotide sequences of $phaC1_{Pre}$ and $phaC2_{Pre}$ genes of *P. resinovorans* NRRL B-2649 have been reported previously [19]. Also, the PCR cloning of these two genes and the construction of the hybrid *pha7* and *pha8* genes has been described [20]. Briefly, $phaC1_{Pre}$ and $phaC2_{Pre}$ were amplified from the chromosome of *P. resinovorans* by PCR, using the primer pairs CLPR-IV-92L1/CLPR-III-63R and CLPR-IV-92L2/CLPR-IV-92R2, respectively. These genes were cloned into the expression vector pETBlue-1 (Novagen) at the unique *Eco*RV site. Recombinant plasmids pETC1b and pETC2b (Fig. 1) were isolated, in which an *E. coli* promoter of the pETBlue-1 directs the transcription of the cloned gene. The hybrid gene, *pha7*, containing the 5′ terminal segment of $phaC2_{Pre}$ and the 3′ end portion of $phaC1_{Pre}$, was constructed in situ from pETC2b by a restriction-fragment replacement procedure. For this purpose, both pETC1b and pETC2b were first subjected to *Bgl*II/*Sph*I double-digestion. The 1.2-kb and 9.3-kb restriction fragments of pETC1b and pETC2b, respectively, were isolated and ligated to form pETCP7b. In this plasmid, the hybrid gene *pha7* (GenBank accession number AF491939) was formed. This approach could not be used to construct *pha8*, which consists of the 5′ end of $phaC1_{Pre}$ and the 3′ end of $phaC2_{Pre}$, because of the lack of appropriate restriction sites. Consequently, the *pha8* hybrid gene (GenBank accession number AF491940) was constructed by a splicing-by-overlap-extension procedure [8]. A 514-bp 5′ terminal portion of $phaC1_{Pre}$ and a 1,169-bp 3′ terminal fragment of $phaC2_{Pre}$ were separately amplified from the

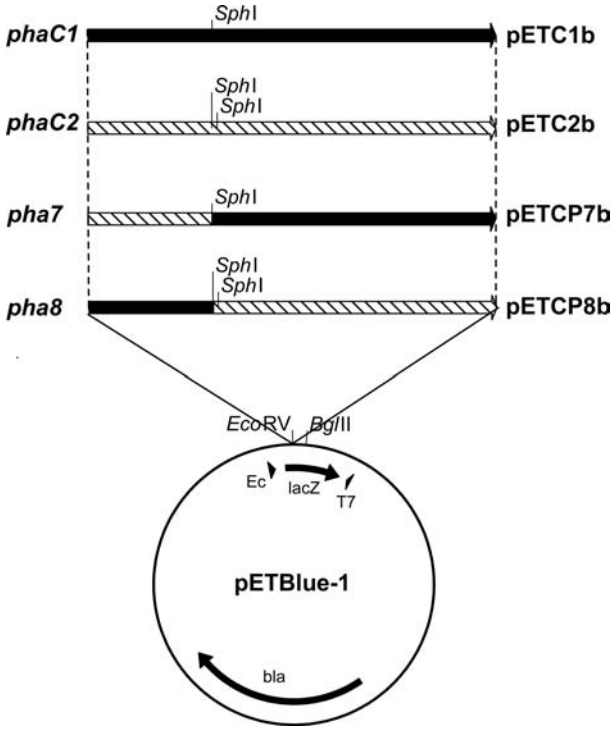

Fig. 1 Plasmid maps. The $phaC1_{Pre}$ (1,680 bp) and $phaC2_{Pre}$ (1,683 bp) genes of *Pseudomonas resinovorans* and the synthetic hybrid genes, *pha7* and *pha8*, were inserted into the expression vector, pETBlue-1, at an *Eco*RV restriction site downstream from an *Escherichia coli* promoter sequence in the plasmid. *bla* The *β*-lactamase gene, *Ec* and *T7*, the *E. coli* and T7 promoters, respectively, *lacZ* the α-complement of the *lacZ* gene

P. resinovorans genome by PCR, using CLPR-IV-92L1/CL-VI-119A and CL-VI-119B/CLPR-IV-92R2 (Table 1), respectively, as the primer pairs. The resultant PCR products were subsequently fused into the *pha8* hybrid gene by PCR using CLPR-IV-92L1 and CLPR-IV-92R2 as the primer pair. The *pha8* was cloned into the *Eco*RV site of pETBlue-1 and the recombinant plasmid pETCP8b was isolated, in which an *E. coli* promoter of the vector mediates the expression of the cloned gene. The sequences of all the recombinant expression plasmids were determined to confirm the intactness of the gene inserts. In the process of sequence determination, a few clones containing various forms of deletion were identified and studied further. Notable among these deletion mutants are pETΔCP7b and pETΔCP8b, which mediated the production of mcl-PHA with a varied repeat-unit composition.

Sequence analysis of the deletion mutant of *pha7* (i.e., Δ*pha7*) in pETΔCP7b showed that the altered gene is missing the first nucleotide, nucleotides 15–17 and the last four nucleotides of its parental gene. The deletion at the 5′ end abolished the original ATG start-codon of the hybrid gene. Nevertheless, the residue T preceding the original ATG start-codon could essentially interject to form a new TTG start-codon [5, 10]. This still places the AGGA ribosomal binding site at an effective distance of six nucleotides away from the TTG codon. As a result of these deletions at the 5′ terminal end, the first six amino acid residues (a.a.; i.e., MREKQI) of the wild-type Pha7 protein were changed to LREKH in the mutant ΔPha7 gene-product. The four-nucleotide deletion at the 3′ end of the parental *pha7* removed the TGA stop-codon, and added an additional 5 a.a. (i.e., SISPS) to the gene-product of Δ*pha7*. The net result is a modified hybrid synthase enzyme containing 563 a.a. (Fig. 2). In comparison, intact *pha7* encodes a putative gene-product (Pha7) of 559 a.a. Sequence determination of the Δ*pha8* gene in the pETΔCP8b plasmid showed that the first 12 nucleotides of the wild-type *pha8* were deleted in the altered gene. The deletion linked the AT nucleotide sequence immediately preceding the truncated gene to the first residue (G) of Δ*pha8* to form the start-codon that is in-frame with the remaining Pha8 gene-product. This deletion resulted in a ΔPha8 gene-product, in which the amino-terminal end is devoid of a.a. 2–4 (i.e., SNK) of the wild-type Pha8 enzyme. The results of the sequence determination further showed the occurrence of five base substitutions that summarily resulted in three changes of amino acid residues (i.e., L217P, F228Y, I376V) and an early termination of the translation process at a.a. 521. Aside from the conserved substitution of I376V, the catalytically significant α/*β*-hydrolase fold of the gene-product was not disturbed. All the mutations in the Δ*pha8* gene resulted in a putative gene-product, ΔPha8, of 518 a.a. The results of the analysis of pETΔCP7b and pETΔCP8b thus summarily showed that the deletions in these constructs had not drastically disturbed the catalytically important α/*β*-hydrolase fold of the *P. resinovorans* PHA synthases [16, 20].

Characterization of PHA

The results of the repeat-unit composition analysis of PHAs produced by the various *E. coli* LS1298 transformants described in this study are summarized in Table 2. When grown with decanoate as a carbon source, the recombinant strains containing the expression vector pETC1b, pETC2b, pETCP7b, or pETCP8b accumulated PHA with *β*-hydroxydecanoate (73-78 mol%) and *β*-hydroxyoctanoate (23–27 mol%) as

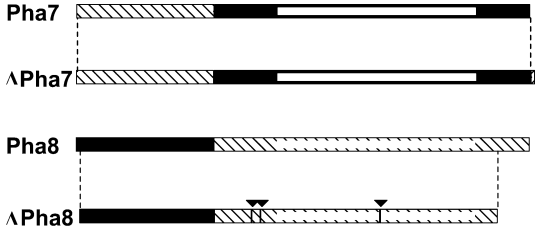

Fig. 2 Comparison of gene-products Pha7, Pha8 and their mutants. *Right-slanted cross-hatched box* $PhaC2_{Pre}$ sequence, *dark-filled box* $PhaC1_{Pre}$ sequence, *white-filled box* α/*β*-hydrolase fold region, *left-slanted cross-hatched box* the additional carboxy-end fusion sequence (i.e., SISPS) of the ΔPha7 gene-product, *arrows* the locations of amino acid substitutions in the ΔPha8 gene-product

Table 2 Repeat-unit compositions of poly(hydroxyalkanoates) (PHAs) in transformed *E. coli* LS1298. The transformants were grown in 500-ml baffled-bottom Erlenmeyer flasks containing 200 ml LB medium supplemented with 50 μg carbenicillin (Cb)/ml, 50 μg kanamycin (Km)/ml and 0.25% (w/v) sodium decanoate (dissolved in 5% Brij 58; see Materials and methods). Incubation was performed at 37 °C, 250 rpm for 48 h. At least duplicate cultures of each transformant were used for the determination. For each culture, two samples were prepared for GC/MS measurement. Each value thus represents the average of at least four measurements, with a typical standard error of ≤ 10%

Plasmid	Composition (mol%)	
	β-Hydroxyoctanoate	β-Hydroxydecanoate
pETC1b	23	78
pETC2b	27	73
pETCP7b	27	73
pETCP8b	25	75
pETΔCP7b	45	55
pETΔCP8b	40	60

the two most abundant monomers (Table 2). Other researchers reported similar composition data from studies of octanoate-grown *E. coli* LS1298 transformants expressing the mcl-PHA synthase genes [12, 14]. The recombinant LS1298 cells containing plasmid pETΔCP7b or pETΔCP8b, however, accumulated mcl-PHAs with repeat-unit compositions different from those found with recombinants harboring the undeleted hybrid genes (Table 2). The polymers accumulated in the transformants harboring the deletion mutant genes contained β-hydroxydecanoate (55–60 mol%) and β-hydroxyoctanoate (40–45 mol%) as the major monomers. The ability of the deletion mutant genes to mediate mcl-PHA accumulation at all indicated that the putative truncated hybrid gene-products still retained PHA synthase activity. A series of large-scale (10 l) fermentation experiments was performed with selected recombinants to determine the effects of these mutations on cell growth and PHA yield. Table 3 shows that cells containing the native (i.e., pETC1b or pETC2b), hybrid (pETCP7b), or mutant (pETΔCP7b) gene yielded a CDW of 4.9–6.6 g. The PHA content of these cells range over 4–9% CDW. These results showed that genetic modification of the PHA synthase genes did not significantly affect cell growth and PHA yield. Rehm et al. [16] compiled an activity map using the existing data on the effects of various mutations on *Ralstonia eutropha* poly(3-hydroxybutyrate) synthase. A comparison of the deletion sites of Δ*pha7* and Δ*pha8* with this map shows that the genetic lesions of these hybrid mutant genes are located in sequence areas that are relatively insensitive to mutations. These mutations could conceivably affect the substrate or product-specificity of the enzymes without dramatically altering their activity. Huisman et al. [9] suggested that the substrate-specificity of synthase enzymes governs the composition of their PHA. Hein et al. [7] proposed that the physiological background of the PHA-producing cells plays an important role in determining the monomer incorporation by the synthases. Still other researchers [11, 13] argued that the level of polymerizing enzyme activity dictates the compositional makeup of the polymers. In view of the cell and PHA yield data presented in Table 3, the observed compositional change of PHA produced by Δ*pha7* and Δ*pha8* most likely resulted from a change in substrate or product-specificity. An in vitro enzymological study to characterize the Δ*pha7* and Δ*pha8* constructs is in progress to help delineate the basis for the compositional change of PHA resulting from the deletions.

Table 3 Cell and PHA yields of transformed *E. coli* LS1298. The transformants were grown in LB medium (10 l) supplemented with 50 μg Cb/ml, 50 μg Km/ml and 0.25% (w/v) sodium decanoate (dissolved in 5% Brij 58). Culturing was performed for 48 h with a BioFlo 3000 fermentor (New Brunswick Scientific, Edison, N.J.) operated at 37 °C, 300 rpm and 0.5 l airflow/min

Plasmid	Cell dry weight (g)	PHA content (% cell dry weight)
pETC1b	4.9	5
pETC2b	5.1	4
pETCP7b	6.4	9
pETΔCP7b	6.6	4

Acknowledgements The authors thank Dr. DiRusso for the kind gift of *E. coli* LS1298. The assistance of Drs. Richard D. Ashby and Alberto Nuñez in acquiring the compositional data is acknowledged. The author also thanks Nicole Cross and Marshall Reed for technical assistance.

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