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Genetic Characterization of the Poly(hydroxyalkanoate) Synthases of Various *Pseudomonas oleovorans* Strains

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Abstract. We identified the poly(hydroxyalkanoate) synthase (PHAS) genes of three strains of *Pseudomonas oleovorans* by using polymerase chain reaction (PCR)-based detection methods. *P. oleovorans* NRRL B-14682 contains Class I PHA synthase gene *(phaC)*, NRRL B-14683 harbors Class II *phaC1* and *phaC2* genes, and NRRL B-778 contain both the Class I and II PHA synthase genes. Inverse-PCR and chromosomal walking techniques were employed to obtain the complete sequences of the Class I *phaC*s of NRRL B-778 ($phbC_{778}$; 1698 bps) and B-14682 ($phbC_{14682}$; 1899 bps). BLAST search indicated that these genes are new and had not been previously cloned. The gene product of $phbC_{778}$ (i.e., PhbC_{778}; 566 amino acid residues) is homologous to the Class I PHA synthases *of Pseudomonas* sp. HJ-2 and *Pseudomonas* sp. strain 61-3, and that of $phbC_{14682}$ (PhbC_{14682}; 632 amino acids) is homologous to PHAS *of Delftia acidovorans.* The PhbC_{14682} contains an extra sequence of 33 amino acids in its conserved α/β-hydrolase domain, making it only the second Class I PHA synthase found to contain this cellular proteolytic sequence. Consistent with their *Pseudomonas* origin, the codon-usage profiles of PhbC_{778} and PhbC_{14682} are similar to those of *Pseudomonas* Class II PHASs. These new *Pseudomonas* Class I *phbC* genes provide valuable addition to the gene pool for the construction of novel PHASs through gene shuffling.

Poly(hydroxyalkanoates) (PHAs) are biodegradable polyesters produced and sequestered as intracellular granular lipid storage materials by many microorganisms [15]. Because their materials properties range from thermoplastic-like to elastomeric, PHAs are extensively studied for use in a large number of applications [1, 21]. PHAs are grouped into three classes based on the carbon-chain length of their repeat-unit monomers. The short-chain-length (scl-) PHAs contain repeat-unit monomers with 3 to 5 carbon atoms, the medium-chain-length (mcl-) PHAs are composed of repeat-unit monomers having 6 to 14 carbon atoms, and the scl-*co*-mcl-PHAs have repeat-unit monomers containing 3–14 carbon atoms. PHA synthases (PHASs) are enzymes responsible for the polymerization of hydroxyacyl-CoA monomer precursors into the PHA polymers. Based on their structural properties and substrate specificity, these enzymes are classified into Class I, II, III, and IV [see ref. 9]. The gene clusters that code for the synthesis of each class of the PHASs have a different genetic organization [9].

Pseudomonas oleovorans is a versatile bacterium capable of carrying out industrially important biocatalytic reactions such as oxidative assimilation of alkanes [19], epoxidation of alkanes and fatty acids [5], and the production of mcl-PHA [7]. We have developed PCR screening methods to identify and characterize mcl-PHA-producing bacteria, including the three strains of *P. oleovorans* described here, to expand the diversity of microbial metabolic backgrounds potentially useful for the production of PHA [12, 14]. We subsequently showed that *P. oleovorans* NRRL B-14682 produces only scl-PHA, and strain NRRL B-778 synthesizes a mixture of scl- and mcl-PHA [3, 4]. In this communication, we re-

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port the cloning and characterization of the PHAS genes responsible for PHA biosynthesis in *P. oleovorans* strains NRRL B-778 and NRRL B-14682. The information not only serves to differentiate strains classified as *P. oleovorans*, but also provides important groundwork for the genetic manipulation of PHASs to generate enzymes with new catalytic specificity and activity.

Materials and Methods

Microorganisms. *Pseudomonas oleovorans* NRRL B-778, B-14682, and B-14683 (ATCC 29347/GPo1/TF4-1L) were from NCAUR-ARS-U.S. Department of Agriculture (Peoria, IL). *Escherichia coli* DH5α used as the host cell for recombinant DNAs was from Invitrogen (Carlsbad, CA).

PCR detection and characterization of *pha* genes. Detection of Class I *phaC* on the chromosomal DNA of *Pseudomonas* was performed as described by Sheu et al. [11], and the Class II *phaC1* and *phaC2* genes were individually amplified by a semi-nested PCR method [12]. Sequence determination was performed using a Perkin-Elmer ABI Prism 3730 DNA Analyzer. Sequence analysis was carried out using the web-based BLAST [2] and BLAST2 [17] programs of National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/), and CLUSTALW algorithm [6]. The partial sequences of *phaC1* (accession number AF318049) and *phaC2* (AF318050) of *P. olevorans* NRRL B-778 were deposited in GenBank.

Cloning of *pha* genes. An inverse PCR technique [10] and the DNA Walking Speedup Premix Kit (Seegene, Rockville, MD) were variously employed to amplify and clone the complete sequences of the Class I *pha* genes of *P.oleovorans* NRRL B-778 and B-14682. For the inverse-PCR experiments, chromosomal DNA was digested with *Eco*RI restriction enzyme for 16 h, and the restriction fragments were ligated in situ using T4 DNA ligase (Invitrogen). The mixture of ligation products provided the DNA templates in the inverse-PCR reactions. The sequences of the PCR products obtained from chromosome-walking experiments were determined as described in the preceding text. The complete sequences of the Class I *phaC* genes of NRRL B-778 (Accession Number AF422801) and NRRL B-14682 (AF422800) were deposited in GenBank.

Results and Discussion

The genetic systems for the biosynthesis of PHA in three strains of *P. oleovorans* (i.e., NRRL B-778, NRRL B-14682, and NRRL B-14683) were studied. We had previously used PCR methods to show the presence of Class II PHA synthase genes in *P. oleovorans* strains NRRL B-778 and NRRL B-14683 [12, 14]. The PCR method yielded Class II *phaC1/C2* gene fragments of approximately 0.54 kb. The sequences of the PCR-amplified *phaC1* and *phaC2* fragments (termed $\Delta phaC1_{14863}$ and $\Delta phaC2_{14863}$, respectively) of *P. oleovorans* NRRL B-14683 were expected to be identical to those of *P. oleovorans* strain GPo1 [8] and were, therefore, not determined [12]. The sequences of the two *phaC* PCR fragments (i.e., $\Delta phaC1_{778}$ and $\Delta phaC2_{778}$) from NRRL B-778 were determined (GenBank AF318049 and AF318050). As reported earlier [12], BLAST2 nucleotide sequence comparison of $\Delta phaC1_{778}$ and $\Delta phaC1_{14863}$ showed 83% homology. The $\Delta phaC2_{778}$ and $\Delta \text{phaC2}_{14863}$, on the other hand, are 79% homologous to each other. On the amino acid sequence level, BLASTX analysis of $\Delta phaC1_{14863}$ and $\Delta phaC2_{14863}$ showed that these NRRL B-14683 genes are highly homologous (97–100%) to the similar regions of *phaC1* and *phaC2*, respectively, of both the *P. putida* strains BMO1 (GenBank AF042276) and KT2440 (GenBank NP747107). These results agree with the recent proposal to reclassify *P. oleovorans* GPol as *P. putida* [20]. The amino acid sequences of $\Delta phaC1_{778}$ and $\Delta phaC2_{778}$, on the other hand, are highly homologous to the corresponding region of the *phaC1* of *P. pseudoalcaligenes* (98%; GenBank AY043314) and identical to the similar fragment of the *phaC2* of *P. nitroreducens* (AF336849) and *P. pseudoalcaligenes*, respectively.

As expected with cells containing Class II PHAS genes, we subsequently demonstrated that NRRL B-778 produced mcl-PHA when grown on glucose or fatty acid having an even-number carbon chain, but only as a mixture with scl-PHB (poly(β-hydroxybutyrate)) [3]. The same study also showed that B-778 synthesized only poly(β-hydroxybutyrate-*co*-β-hydroxyvalerate) copolymer when cultured on fatty acid containing an odd-number carbon chain. We also showed in a separate study that *P. oleovorans* NRRL B-14682 synthesizes only the scl-PHA [4]. These results suggest that NRRL B-778 and NRRL B-14682 contain Class I PHAS and its associated genetic systems. Accordingly, we attempted the detection of Class I PHAS genes in NRRL B-778 and B-14682 by performing a semi-nested PCR procedure [11], which is non-specific and able to detect PHAS genes of all classes. Our results showed that the first-round PCR yielded an amplified gene fragment of approximately 0.5 kb only with the B-778 sample, but the second-round (semi-nested) PCR resulted in the amplification of a 0.4-kb gene fragment from all three strains of *P. oleovorans*. Subcloning, sequence determination, and BLASTX analysis of the PCR products showed that the 0.5-kb gene fragment of B-778 (termed $\Delta phbC_{778}$) was homologous to the corresponding region of scl-PHAS of *Pseudomonas* sp. 63-1 (75%, GenBank AB014757), and the PCR product of B-14682 ($\Delta phbC_{14682}$) is homologous to a similar region of the scl-*phaC* of *Delftia acidovorans* (previously *Comamonas acidovorans*; GenBank AB009273). As expected of a non-specific PCR procedure, some of the cloned PCR products from B-778 and B-14683 were fragments of the Class II PHAS genes.

Table 1. BLAST2 sequence comparison of $PhbC_{778}$, $PhbC_{14682}$, and selected Class I and II PHA synthases[a]

	$PhbC_{Weut}$ (%)	$PhbC_{778}$ (%)	$PhbC_{14682}$ (%)
Entire Sequence			
$PhbC_{Weut}$	—	53	55
$PhaC1_{Pr}$	37	36	36
$PhaC2_{Pr}$	39	37	34
$PhaC1_{Po}$	39	38	34
$PhaC2_{Po}$	40	37	36
α/β-hydrolase domain			
Phb_{Weut}	—	61	56
$PhaC1_{Pr}$	39	34	34
$PhaC2_{Pr}$	39	35	32
$PhaC1_{Po}$	39	37	32
$PhaC2_{Po}$	38	35	32

[a]Values presented are the degree of amino acid sequence identities obtained from BLAST2 analysis.

$PhbC_{Weut}$, gene-product of Class I *phbC* gene of *W. eutropha* (GenBank accession number J05003); $PhbC_{778}$ and $PhbC_{14682}$, gene products of Class I $phbC_{778}$ and $phbC_{14682}$ genes; $PhaC1_{Pr}$ and $PhaC2_{Pr}$, gene products of Class II *phaC1* and *phaC2* genes of *P. resinovorans* NRRL B-2649; $PhaC1_{Po}$ and $PhaC2_{Po}$, gene products of Class II *phaC1* and *phaC2* genes of *P. oleovorans* GPol (GenBank M58445).

Using the sequence information of $\Delta phbC_{778}$ and $\Delta phbC_{14682}$, we variously applied inverse-PCR and chromosomal walking techniques (see Materials and Methods) to eventually obtain the complete sequences of the Class I PHAS genes of *P. oleovorans* strains NRRL B-778 and NRRL-B-14682. These sequences were labeled $phbC_{778}$ (1698 bps) and $phbC_{14682}$ (1899 bps) appropriately. A BLASTX analysis of these sequences showed the presence of the ubiquitous α/β-hydrolase fold of PHAS in the putative translation products $PhbC_{778}$ (amino acid residues 251–500) and $PhbC_{14682}$ (a.a. 280–561). A sequence alignment study using a CLUSTALW program was performed to compare $PhbC_{778}$ and $PhbC_{14682}$ to the best studied Class I PHAS, i.e., that of *Wautersia eutropha*. The results (Fig. 1) showed that the three sequences were 42–53% homologous, with the alignment of *W. eutropha* $PhbC_{778}$ and $PhbC_{14682}$ having the highest homology score. Based on the CLUSTALW alignment, the signature lipase box-like sequence (GXCXG) of PHAS was identified between a.a. 298–302 for $PhbC_{778}$ and a.a. 327–331 for $PhbC_{14682}$ in the highly conserved α/β-hydrolase domain. Within this domain, the catalytic triad comprising cysteine (C), histidine (H), and aspartate (D) residues is located [see ref. 9]. In $PhbC_{778}$, these catalytically important residues are found in a.a. 300 (C), 459 (D), and 487 (H). In $PhbC_{14682}$, they are located at a.a. 329 (C), 520 (D), and 548 (H). Among the three sequences shown in Fig. 1, $PhbC_{14682}$ (632 total a.a. residues) is the longest and $PhbC_{778}$ (566 residues) is the shortest polypeptide. The amino-terminal region of $PhbC_{778}$ has considerably fewer amino acid residues than the other two PhbCs, as evidenced by the presence of gaps in the CLUSTALW alignment (data not shown). Most notable is the 33-a.a-length extra sequence found in the presumably highly conserved α/β-hydrolase domain of $PhbC_{14682}$ (a.a. 351–383). The $PhbC_{14682}$ is only the second reported Class I PHAS that contains a lengthened α/β-hydrolase domain; the first being the PHAS of *Delftia acidovorans* (previously *Comamonas acidovorans*) [16]. Tsuge et al. [18] showed that the removal of this extra sequence from *D. acidovorans* PHAS dramatically lowers the specific activity of the enzyme. The same study also suggested that the extra sequence provides the cleavage sites at the junctions for the cellular proteolysis of PHAS. Sequence comparison between the extra sequences of $PhbC_{14682}$ and *D. acidovorans* PHAS showed that they are not homologous except at the two terminal areas. It is thus possible that the extra sequence of $PhbC_{14682}$ serves a similar function of providing cleavage sites for the cellular degradation of the PHAS in *P. oleovorans* NRRL B-14682.

BLASTP analysis was carried out on $PhbC_{778}$ and $PhbC_{14682}$ sequences to find the closest homologs of these gene products. The results showed that $PhbC_{778}$ has the highest homology to the PHAS's of *Pseudomonas* sp. HJ-2 (82%; GenBank AAQ72537) and *Pseudomonas* sp. strain 61-3 (69%; GenBank T44363). As expected with the presence of an extra sequence in the α/β-hydrolase domain of $PhbC_{14682}$, this gene product showed the highest homology with the PHAS of *D. acidovorans* (71%; GenBank AB009273). The next closest homolog of $PhbC_{14682}$ is the PHAS of *Alcaligenes* sp. SH-69, which lacks the extra sequence in α/β-hydrolase domain (62%; GenBank U78047). These results indicate that the two newly characterized genes, i.e., $phbC_{778}$ and $phbC_{14682}$, code for putative Class I PHASs that are unique and have only low homology to known enzymes belonging to this class.

We have previously constructed chimeric PHA synthases producing mcl-PHA with changed monomer compositions [13]. Attempts to construct chimeric PHASs composed of Class I *W. eutropha* and Class II *P. resinovorans* enzymes, however, did not yield active PHA synthases. We, thus, performed a detailed sequence comparison study to evaluate the potential superiority of the newly isolated *Pseudomonas* Class I PHASs over the *W. eutropha* PHAS in the construction of chimeric enzymes with the Class II PHASs of *P. oleovorans* NRRL B-14683 (ATCC 29347/GPo1/TF4-1L) and *P. resinovorans*. Table 1 presents the results of BLAST2 alignment of the whole sequences and of the conserved

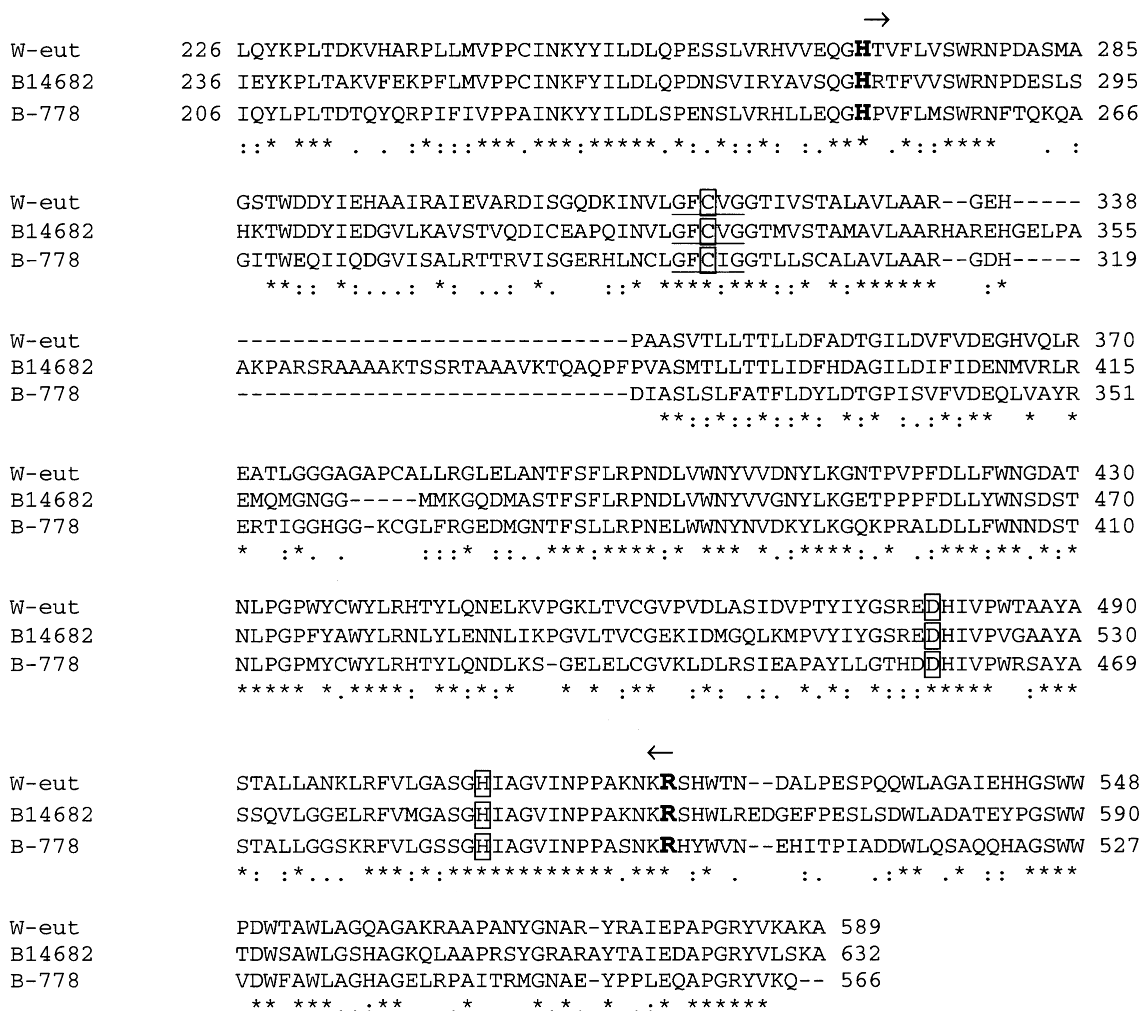

Fig. 1. CLUSTALW analysis of $PhbC_{778}$, $PhbC_{14682}$, and $PhbC_{Weut}$. The alignment of the amino-terminal regions is omitted; only the alignment of the sequence portions containing functional features is shown. Boxed residues, the catalytic triad consisting of histidine, aspartic acid, and cysteine residues. Underlined GxCxG sequence, the lipase-box-like sequence. Boldface H with a rightward arrow (→), the first residue of the α/β-hydrolase fold. Boldface R with a left-pointing arrow (←), the last residue of the α/β-hydrolase fold. W-eut, PhbC of *W. eutropha.* B14682, $PhbC_{14682}$. B-778, $PhbC_{778}$. *, identical residues in all sequences. :, conserved substitution of residues. Dot (.), semiconserved substitution of residues.

α/β-hydrolase domains of the Class I PHASs of *W. eutropha*, *P. oleovorans* NRRL B-778, and *P. oleovorans* NRRL B-14682, and the Class II PHASs of *P. oleovorans* GPo1 (=NRRL B-14683) and *P. resinovorans* NRRL B-2649 (GenBank AF 129396). In general, the Class I PHASs have a 53–55% homology among themselves. These values increased to 56–61% homology when only the α/β-hydrolase domains were compared, The homology between Class I and II PHASs ranged from 32–40% regardless of whether the entire sequences or only the α/β-hydrolase domains were compared. A separate alignment study using CLUSTALW yielded similar results (data not shown). Thus, there is no obvious advantage based on sequence homology analysis to use $PhbC_{778}$ and $PhbC_{14682}$ instead of $PhbC_{Weut}$ for the construction of hybrid enzymes with Class II PHASs. A codon-usage preference analysis of these sequences, however, showed that the two Class I *Pseudomonas* PhbCs are more compatible with the Class II *Pseudomanas* PhaC1/C2 than the $PhbC_{Weut}$ is (data not shown). Most notable is the codon-usage preference for the serine residue. In the $phbC_{Weut}$ gene, TCG codon (48%) was used predominantly for serine, while in the other 6 genes compared here (i.e., $phbC_{778}$, $phbC_{14682}$, $phaC1_{Pr}$, $phaC2_{Pr}$, $phaC1_{Po}$, and $phaC2_{Po}$) the AGC triad (35–76%) was the preferred codon.

In summary, we have characterized two complete sequences of Class I PHAS genes of *Pseudomonas* sp. The $phbC_{14682}$ was only the second PHAS gene char-

acterized that contained a cellular proteolytic sequence in the α/β-hydrolase domain. Although the two *Pseudomonas* Class I genes are similar to the well-known *phbC* of *W. eutropha* in terms of their amino-acid sequence homology to the Class II PHASs, codon-preference analysis clearly demonstrated that the Class I $phbC_{778}$ and $phbC_{14682}$ are more compatible with the Class II *phaC1* and *pha*C2 than the $phbC_{Weut}$ is. These results provide valuable resources for our ongoing efforts to construct chimeric PHA synthases with novel specificity via the gene-shuffling approach.

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