Polyhydroxyalkanoate biosynthesis in *Pseudomonas pseudoalcaligenes* YS1

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**Abstract**

*Pseudomonas pseudoalcaligenes* strain YS1 isolated from oil contaminated soil was able to produce polyhydroxybutyrate blended with medium-chain-length polyhydroxyalkanoates (mcl PHA). PHA synthesis genes were cloned from this strain. A *fadB* (gene for fatty acid degradation) deleted mutant *Escherichia coli* KM32B (*fadB*::*Tet*) was constructed to express the cloned PHA synthesis gene *phaC1Pp* or *phaC2Pp*. The *fadB* deleted mutant KM32B harboring *phaC1Pp* or *phaC2Pp* showed mcl PHA accumulation while the intact *E. coli* KM32 did not. The results demonstrated that *P. pseudoalcaligenes* YS1 possessed at least two PHA synthesis pathways; one of them was responsible for production of mcl PHA. 

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**Keywords:** *fadB* mutant; Polyhydroxyalkanoate; Polyhydroxybutyrate; *Escherichia coli*; *Pseudomonas pseudoalcaligenes*

1. Introduction

Polyhydroxyalkanoates (PHA), microbially synthesized polylsters, have attracted increasing attention from scientific and industrial communities because of several properties including biodegradability, biocompatibility and piezoelectricity [1]. PHA biosynthesis is a complex process controlled by several enzymes catalyzing different metabolic pathways [2].

Most bacterially accumulated PHA can be divided into short-chain-length (scl) PHA and medium-chain-length (mcl) PHA according to their monomer structures [2]. A few bacteria have also been found to produce blend PHA consisting of polyhydroxybutyrate (PHB) and mcl PHA when grown on related carbon sources, such as fatty acids [3,4]. Others, including *Pseudomonas* sp. 61-3 and *Pseudomonas* sp. A33, were reported to be able to synthesize the blend PHA when growth was in a related (fatty acids) or unrelated carbon source (glucose) [5–7]. Further molecular study on *Pseudomonas* sp. 61-3 showed that the strain possessed two PHA synthetic pathways, one responsible for PHB production and the other for mcl PHA accumulation [8].

So far no study has been conducted on the molecular biology of blend PHA synthesis from fatty acids only. *Pseudomonas pseudoalcaligenes* YS1 is a typical strain able to produce blend PHA only from related carbon sources such as octanoic and myristic acids, but not from unrelated carbon sources such as glucose or sucrose [4,9]. In order to investigate the PHA biosynthetic pathways in this strain, two PHA synthesis genes were cloned from *P. pseudoalcaligenes* YS1 and expressed by a *fadB* (gene for fatty acid degradation) deleted *Escherichia coli* KM32B (*fadB*::*Tet*). The type of PHA produced by the recombinants harboring these genes should reveal information related to the PHA metabolic pathways in this bacterium.

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2. Materials and methods

2.1. Bacterial strains, vectors and growth conditions

All bacterial strains and plasmids used in this study are listed in Table 1. *P. pseudoalcaligenes* YS1 was cultivated at 30°C in Luria–Bertani (LB) medium, while all *E. coli* strains were grown at 37°C in LB medium. Antibiotics were added to the medium when needed. In all cases, the cultures were incubated in 500-ml conical flasks containing 150 ml culture broth shaken at 200 rpm (NBS, Series 25D, New Brunswick, NJ, USA).

2.2. Cloning of type II PHA biosynthesis genes from *P. pseudoalcaligenes* YS1

The cloning of type II PHA biosynthetic genes from *P. pseudoalcaligenes* YS1 was done according to the PCR cloning strategy established previously [13]. The identification and sequence alignment of the presumed phaC1Pp, phaZPp and phaC2Pp with other *Pseudomonas* pha loci (GenBank accession Nos. AB014758, X66592, M58445, AF150670 and AF129396) were performed with BLASTP and CLUSTAL [8,14–17].

2.3. Construction of expression plasmids

Oligonucleotides (N1: AGCGAATTCTATGAGTGAAAGAACAAC; C1: TCTAAGCTTCGGAATACGAGTT; N2: GTAGAATTCCATGCGAGACAAGTCGAAAC; C2: TAAAAGCTTCGTAGCGACATCTCTCAT) were supplied from BioAsia Company (Shanghai, China) for subcloning (N1 and C1 for phaC1Pp, and N2 and C2 for phaC2Pp). The PCR products were double-digested by EcoRI and HindIII and then ligated into the vector pKKH. The resulting recombinant plasmids were named pHXM12 and pHXM22 (Fig. 1). All molecular manipulations were carried out by standard procedures [18] or as recommended by the manufacturers.

2.4. Construction of fadB deleted mutant *E. coli* KM32B

pBHR91 was used as PCR template for amplifying the Tet gene fragment [11]. Oligonucleotides (L1: CATCGTTCATCCAGAGCGTGATTTC; B: GACGGGTGTGGTCGCCATGA; C: TCATGGCGACACACCCGTC; D: GCCAGCAGCGTACGCATTAAATGAG; E: GTGCTGCAAGGGCGATTAGTTGGG; F: GCCTTGTAGCCAATACCCGCAGGCC) were used as primers. PCR was carried out under the following conditions: 94°C 10 min, 94°C 1 min, 76°C 1 min, 72°C 1.5 min, 30 cycles, 72°C 10 min, hold in 4°C. The amplified fragment was assigned as TET. Preparation of electrocompetent cells and electroporation with linear DNA fragments were carried out by standard procedures [19]. 1 mM IPTG was added to induce the expression of Ptac-gam-red [10]. A 42°C heat shock for 15 min was recommended prior to centrifugation in order to help stabilize the Red functions. Electroporation was performed under 20 kV cm⁻¹ for 5 ms.

The deleted mutants were determined by PCR identification using six pairs of primers: A-D, A-C, B-D, E-F, B-F and C-E (A: CATCGTTCATCCAGAGCGTGATTTC; E: GTGCTGCAAGGGCGATTAGTTGGG; F: GCCTTGTAGCCAATACCCGCAGGCC). The resulting deletion mutant was termed *E. coli* KM32B.

2.5. Heterologous expression of phaC1Pp and phaC2Pp in *E. coli* strains

*E. coli* strains JM109, KM32 and KM32B harboring pHXM12 or pHXM22 were grown for 48 h in 150 ml MY₀.₅ medium (mineral salt medium supplemented with 0.5% (w/v) yeast extract), supplemented with sodium butyrate (0.5 g l⁻¹), sodium octanoate (2.5 g l⁻¹) or sodium decanoate (2.5 g l⁻¹), respectively. Ampicillin (100 mg ml⁻¹) and acrylic acid (0.2 mg ml⁻¹) were added at the beginning of growth to maintain plasmids and to block β-oxidation of fatty acid [20]. Cells were harvested and lyophilized after 48 h. Gas chromatographic analysis of intracellular PHA content and PHA composition were performed as described previously (Hewlett-Packard model 6890, Hewlett-Packard, Palo Alto, Ca, USA) [5].

Table 1

<table>
<thead>
<tr>
<th>Bacterial strains and plasmids used in this study</th>
<th>Important features</th>
<th>Sources or Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. pseudoalcaligenes</em> YS1</td>
<td>Source of phaC1 and phaC2</td>
<td>This study</td>
</tr>
<tr>
<td><em>E. coli</em> JM109</td>
<td>General strain for plasmid maintenance</td>
<td>TaKaRa</td>
</tr>
<tr>
<td><em>E. coli</em> KM32</td>
<td>ΔarcBCD: Ptac-gam-red, Cm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>[10]</td>
</tr>
<tr>
<td>pKKH</td>
<td>Trc promoter; Δtop; Amp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>[12]</td>
</tr>
<tr>
<td>pHXM12</td>
<td>Containing phaC1 from <em>B. caryophylli</em></td>
<td>This study</td>
</tr>
<tr>
<td>pHXM22</td>
<td>Containing phaC2 from <em>B. caryophylli</em></td>
<td>This study</td>
</tr>
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</table>
3. Results

3.1. PCR cloning of the pha locus from P. pseudoalcaligenes YS1

Purified genomic DNA of P. pseudoalcaligenes YS1 was used as template. The preferred fragments were approximately 3.0 kb and 2.8 kb for the phaC1 and phaC2 products, respectively. Subsequently, the PCR fragments were separately ligated to the cloning vector pGEM-T and the resulting plasmids were sequenced.

Three complete open reading frames (ORFs) were found in the cloned pha locus; they were named phaC1Pp, phaZPp and phaC2Pp, respectively (Fig. 2). The identities of phaC1Pp, phaZPp and phaC2Pp were 61.5%, 65.5% and 55.7% compared with the corresponding pha loci of five known Pseudomonas strains [8,14–17]. The ORFs of the whole pha locus are shown in Fig. 2 and parts of the sequences have been deposited in GenBank under accession No. AY043314.

3.2. Construction of fadB deleted mutant E. coli KM32B

In order to amplify the proper TET fragment flanked by 45 bases upstream and downstream from fadB for gene replacement (Fig. 3), LA Taq (TaKaRa) was used. The resulting fragment was approximately 1.4 kb. Subsequently, the PCR fragment was electroporated into E. coli KM32 competent cells and screened by LB agar containing tetracycline. Thirty-two colonies were obtained and four of these were identified by PCR using the primers described in Fig. 3. The expected size of PCR products from both E. coli KM32 and the deleted mutant using different pairs of primers are listed in Table 2. The electrophoresis result showed that all PCR products had the sizes estimated previously (Fig. 4). Therefore, a fadB deleted mutant E. coli KM32B was successfully constructed.

3.3. Heterologous expression of phaC1Pp and phaC2Pp in E. coli strains

Growth on octanoate and decanoate led to over 1.6% and 1.0% PHA accumulation as a proportion of cell dry weight (CDW) of E. coli KM32B (pHXM12) harboring phaC1Pp (Table 3). 3-Hydroxyoctanoate (3HO) was the major PHA monomer when grown on octanoate while 3-hydroxydecanoate (3HD) was the major PHA monomer on decanoate. No PHA was synthesized when butyrate and docanoate were supplied as the sole carbon sources.

E. coli KM32B (pHXM22) harboring phaC2Pp produced over 1.8% of CDW as PHA when decanoate was used as substrate. Only 3HO and 3HD were produced from decanoate. Growth on octanoate produced a trace amount of PHA. No PHA was detectable when butyrate or dodecanoate were used as the sole substrate.

In comparison, recombinant E. coli JM109 or KM32 harboring plasmid pHXM12 or pHXM22 did not show any PHA accumulation (data not shown).

4. Discussion

It is well known that the intracellular accumulation of PHA is a complex physiological process controlled by different enzymes catalyzing many metabolic pathways [2]. The properties of PHA are greatly dependent on their monomer structures [21]. One of the factors known to affect the monomer structure is the PHA synthesis genes [2].

According to our previous studies, P. pseudoalcaligenes YS1 had the ability to produce PHB blended with mcl PHA only from related carbon sources such as octanoic and myristic acid [4]. Strain Pseudomonas sp. 61-3 possessing two PHA biosynthesis pathways was capable of producing blend PHA from both fatty acids and glucose [5,6,8]. To investigate the difference in PHA biosynthesis pathways in P. pseudoalcaligenes YS1, two type II PHA
synthesis genes were cloned from this strain. The homologies of the cloned genes with five other Pseudomonas genes [8,14–17] were high (identity of phaC1Pp was 61.5% and of phaC2Pp was 55.7%).

In order to express the cloned phaC genes from P. pseudoalcaligenes YS1, a fadB deleted mutant of E. coli was constructed using a gene replacement method [19]. A recBCD mutant E. coli KM32 was used to promote the gene replacement in E. coli [10]. The advantages of this method include no cloning procedure and no secondary PCR. Two 70-base primers are required only, each composed of 45 bases complementary to the N-terminal or C-terminal region of the E. coli target gene and 25 bases targeting the N-terminal or C-terminal region of a resistant marker. This allows almost any known gene replacement in E. coli.

The PHA accumulated in the fadB deleted mutant E. coli KM32 harboring phaC1Pp and phaC2Pp were found to be PHA polymers consisting of only mcl 3-hydroxyalkanoate (3HA) monomers (Table 3). This indicated that both of the two PHA synthesis genes cloned from P. pseudoalcaligenes YS1 could only incorporate mcl 3HA monomers, such as 3-hydroxyhexanoate (3HHx), 3HO and 3HD, into the PHA polymer. Another PHA synthesis gene must be responsible for PHB accumulation since P. pseudoalcaligenes YS1 had the ability to produce blends of PHB and mcl PHA.

PHA accumulation in the recombinant E. coli was also found to be dependent on the carbon sources provided. This may be attributed to the fact that PHA precursors supplied in E. coli come predominantly from fatty acid degradation pathways [20], only fatty acids with certain

Table 2
The expected size of PCR products identified by different pairs of primers

<table>
<thead>
<tr>
<th>Primers</th>
<th>E–F</th>
<th>B–F</th>
<th>C–E</th>
<th>A–C</th>
<th>B–D</th>
<th>A–D</th>
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</thead>
<tbody>
<tr>
<td>Expected size of deleted mutant (bp)</td>
<td>1381</td>
<td>338</td>
<td>1062</td>
<td>1207</td>
<td>468</td>
<td>1656</td>
</tr>
<tr>
<td>Expected size of KM32 (bp)</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>2460</td>
</tr>
</tbody>
</table>

For details, see Fig. 4.

Table 3
Growth and PHA accumulation by E. coli KM32B harboring pHXM12 or pHXM22 cultivated in various media

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Medium MY0.5</th>
<th>CDW (g l⁻¹) PHA/CDW (%)</th>
<th>PHA composition (%)</th>
<th>3HHx</th>
<th>3HO</th>
<th>3HD</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHXM12</td>
<td>Butyrate₀.₅</td>
<td>1.27 ± 0.04</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Octanoate₂.₅</td>
<td>0.96 ± 0.01</td>
<td>1.63 ± 0.08</td>
<td>13.7</td>
<td>66.8</td>
<td>19.5</td>
</tr>
<tr>
<td></td>
<td>Decanoate₂.₅</td>
<td>0.63 ± 0.03</td>
<td>1.05 ± 0.05</td>
<td>–</td>
<td>20.2</td>
<td>79.8</td>
</tr>
<tr>
<td></td>
<td>Dodecanoate₂.₅</td>
<td>0.68 ± 0.07</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>pHXM22</td>
<td>Butyrate₀.₅</td>
<td>0.38 ± 0.05</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Octanoate₂.₅</td>
<td>0.54 ± 0.02</td>
<td>trace</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Decanoate₂.₅</td>
<td>0.65 ± 0.01</td>
<td>1.85 ± 0.04</td>
<td>–</td>
<td>19.3</td>
<td>80.7</td>
</tr>
<tr>
<td></td>
<td>Dodecanoate₂.₅</td>
<td>0.54 ± 0.06</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Butyrate₀.₅ means 0.5 g l⁻¹ butyrate is used as the sole carbon source. Ampicillin (100 mg ml⁻¹) and acrylic acid (0.2 mg ml⁻¹) were added at the beginning of growth. Cells were harvested and lyophilized after 48 h. Gas chromatographic analyses of intracellular PHA content and PHA composition were performed. All above data were obtained from three parallel studies.

Fig. 4. Identification of a deleted mutant by PCR. Lanes 1–6 show the PCR product using KM32 as template. E–F, B–F, C–E, A–C, B–D and A–D were used as primers. Lane 7 is a 1-kb ladder (MBI Fermentas); lanes 8–13 show the PCR product using the recombinant as template. E–F, B–F, C–E, A–C, B–D and A–D were used as primers.
carbon length are suitable for both cell growth and PHA synthesis in *E. coli*.

The employment of the *E. coli* *fadB* mutant had a positive effect on PHA synthesis since there was no PHA accumulation observed in *fadB* intact *E. coli* JM109 or KM32 harboring the same plasmids, but the total yield of PHA in the recombinant *E. coli* KM32B was still low. This may be due to the lack of functional redirection from products of fatty acid degradation to PHA biosynthesis. Co-expression of the *phaC* with *phaJ* [22] or *phaG* [23] could help convert intermediates from fatty acid degradation to PHA synthesis, and thus may be helpful for significantly increasing PHA content in the recombinant cells.

Acknowledgements

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References


