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SHORT COMMUNICATION

Inactivation of type I polyhydroxyalkanoate synthase in *Aeromonas hydrophila* resulted in discovery of another potential PHA synthase

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Abstract *Aeromonas hydrophila* CGMCC 0911 possessing type I polyhydroxyalkanoate (PHA) synthase (PhaC) produced only PHBHHx from lauric acid but not from glucose. Medium-chain-length (mcl) PHA was produced from lauric acid or glucose only when PhaC of *A. hydrophila* was inactivated, indicating the existence of another PHA synthase in the wild type. Using PCR cloning strategy, the potential PHA synthase gene ($phaC_{mcl}$) was obtained from genomic DNA of the wild type and exhibited strong homology to type II PHA synthase genes of *Pseudomonas* strains. The $phaC_{mcl}$ gene was PCR subcloned into plasmid pBBR1MCS2 and expressed in a PHA-negative mutant of *Pseudomonas putida*. Recombinant *P. putida* synthesized mcl PHA from gluconate or octanoate. This result proved that wild type *A. hydrophila* possessed another type II PHA synthase, which was responsible for the synthesis of mcl PHA, besides type I PHA synthase.

Keywords *Aeromonas hydrophila* · PHA synthase · *Pseudomonas putida*

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Introduction

Polyhydroxyalkanoates (PHAs) comprise a large family of bacterial storage polyesters that are accumulated by many genera of bacteria and members of the family Halobacteriaceae of the Archaea under nutrient-limitation conditions in the presence of excess carbon source [3]. The water insoluble PHAs exhibit high molecular weight, thermoplastic and/or elastomeric features as well as other interesting physical and material properties [3, 11, 12]. They have attracted increasing attention from scientific and industrial communities due to their interesting properties including biodegradability, biocompatibility and piezoelectricity [5, 26].

Depending on the number of carbon atoms in monomer units, PHA can be divided into two groups: short-chain-length (scl) PHA with C_3 to C_5 monomer units and medium-chain-length (mcl) PHA with C_6 to C_{14} monomer units [22]. Only a few reports are available of bacteria, for example, *Rhodospirillum rubrum* [2], *Rhodocyclus gelatinosus* [16], *Aeromonas hydrophila* [19], *Rhodococcus rubber* [8] and some *Pseudomonad* strains [4, 12, 22, 24], which produce PHA consisting of both scl and mcl PHA.

Polyhydroxyalkanoate synthases are the key enzymes of PHA biosynthesis [20]. With respect to the primary structures deduced from the nucleotide sequences and substrate specificity of the enzymes, three major types of PHA synthases can be distinguished [20]. *A. hydrophila* CGMCC 0911 possessed type I PHA synthase (PhaC) and produced only PHBHHx from lauric acid. Growth of the strain was inhibited when octanoate was used as carbon source, while grew on glucose or gluconate only traces of PHA were detected in the cells [17]. In this paper we report, for

the first time, that inactivation of PhaC resulted in synthesis of mcl PHA in *A. hydrophila*. The potential PHA synthase gene, which was responsible for the synthesis of mcl PHA in PhaC-negative mutant of *A. hydrophila*, was cloned from genomic DNA of the wild type using PCR cloning strategy.

Materials and methods

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *A. hydrophila* and *Pseudomonas putida* strains were cultivated at 30°C in Luria-Bertani (LB) medium, while *Escherichia coli* was cultivated at 37°C in the same medium. Antibiotics were added to the medium when needed. All liquid cultures were incubated in conical flasks at 200 rpm (NSB, Series 25D, New Brunswick, USA).

DNA manipulation and RT-PCR procedure

All molecular manipulations were carried out by standard procedures [21] or as recommended by the manufacturers. All restriction endonucleases were purchased from Biolab (New England Biolabs, USA), *EX Taq* DNA polymerase from TaKaRa Biotechnology Company (Dalian, China) and T_4 DNA ligase from Promega (USA). All primers were synthesized by TaKaRa (Dalian, China). DNA was sequenced by TaKaRa (Dalian, China).

Reverse transcriptase-PCR (RT-PCR) was applied to analyze transcripts of type I and type II PHA synthase genes in *A. hydrophila* strains. Total RNA free of DNA was purified as recommended by the manufacturers (SNBC, Shanghai, China). RNA was analyzed by agarose gel electrophoresis and the concentration was determined by measuring the absorbance at 260 nm in a spectrophotometer. cDNA was synthesized using a first strand cDNA synthesis kit (SNBC, Shanghai, China). One step RT-PCR was conducted by standard procedures.

Production and analysis of PHA

A. hydrophila and *P. putida* strains were cultivated in 500 ml conical flasks containing 100 ml mineral salt medium [17] supplemented with 10 g l^{-1} lauric acid, 20 g l^{-1} glucose , 20 g l^{-1} gluconate or 10 g l^{-1} octanoate and shaken at 30°C and 200 rpm. Kanamycin (50 μg ml^{-1}) was added to medium when needed. Isopropyl-β-D-thiogalactopyranoside (IPTG) (0.2 mmol l^{-1}) was added to medium after 6 h of cultivation when needed. Cells were harvested and lyophilized after 48 h of cultivation. The lyophilized cells were subjected to methanolysis [1]. Gas chromatography–mass spectrometry (GC–MS) (Perkin-Elmer Autosystem XL GC-Turbomass, USA) was used to determine the composition of the polymers produced. Gas chromatographic analysis of intracellular PHA content and PHA composition (Hewlett-Packard model 6890, Palo Alto, CA, USA) was performed as described previously [19].

Table 1 Bacterial strains and plasmids used in this study

Strains and plasmids	Important feature	Source or reference
Strains		
Aeromonas hydrophila CGMCC 0911	Wild type; source of $phaC_{mcl}$	This study
A. hydrophila WH	PhaC-negative mutant of *A. hydrophila*	This study
Pseudomonas putida GPp104	PHA-negative mutant of *P. putida* KT2442	[10]
Escherichia coli JM109	General strain for plasmid maintenance	TaKaRa
E. coli S17-1	For conjugation	TaKaRa
Plasmids		
pBBR1MCS	Source of chloramphenicol resistance gene; Cm^r	[14]
pUC18	Suicide plasmid. Amp^r	TaKaRa
pFH4	pUC18 derivative, *phaC* from *A. hydrophila*; Amp^r	This study
pFH5	pFH4 derivative, disrupted *phaC*::*Cm*; Amp^r; Cm^r	This study
pMD18-T	Cloning vector; Amp^r	TaKaRa
pFH12	pMD18-T derivative, $phaC_{mcl}$ from *A. hydrophila*; Amp^r	This study
pBBR1MCS2	Broad host range; expression vector; *lacPOZ'*; Kan^r	[14]
pFH16	PBBR1MCS2 derivative, $phaC_{mcl}$ from pFH12; *lacPOZ'*; Kan^r	This study

Results

Inactivation of *phaC* in *A. hydrophila*

Plasmid pUC18 (TaKaRa) that can replicate in *E. coli* but not in *A. hydrophila* was chosen as a delivery system for this host. For inactivation of type I PHA synthase gene (*phaC*) of *A. hydrophila*, the 2.6 kb fragment harboring *phaC* was cloned into *Eco*RI site of plasmid pUC18, resulting in the new plasmid pFH4. A unique *Xho*I cleavage site downstream of the *phaC* start codon, within the conserved region of the gene, was used to insert a *Xho*I–*Xho*I fragment harboring the chloramphenicol resistance gene from pBBR1MCS [14]. The resultant plasmid pFH5 haboring *phaC*::*Cm* was introduced into *A. hydrophila* by electroporation [6]. The resistant clones were confirmed for double crossover events by PCR method. Primers P1 (5′-TTGGATCCTGGAGACCGATGATGAATATG-3′), P2 (5′-AATAAGCTTGGCCTTGGCCGTGCTCTTC-3′) and P3 (5′-ATGAATTCTTAAGGCAGCTTGACCACGG-3′) were designed according to the organization of type I PHA synthesis genes in *A. hydrophila* [17]. PCR products, amplified from genomic DNA of resistant clones and the wild type, were sequenced and analyzed (data not shown). The results showed that 2 out of 12 resistant clones had lost the *phaC* wild-type gene as a result of a double crossover event, indicating the successful chromosomal integration of *phaC*::*Cm*. Therefore, PhaC-negative mutants of *A. hydrophila* were obtained as homologous recombinants.

PHA accumulation in *A. hydrophila* strains

To evaluate the phenotypic effect of the *phaC* inactivation, the accumulation of PHA was analyzed in the mutant strain and the wild type. Interestingly, the mutant produced mcl PHA consisting of 2.3 wt% 3-hydroxyhexanoate (3HHx), 51 wt% 3-hydroxyoctanoate (3HO), 29 wt% 3-hydroxydecanoate (3HD) and 17 wt% 3-hydroxydodecanoate (3HDD) from lauric acid (Table 2). When grown in glucose, the wild type produced only 0.7 g l^{-1} CDW with not detectable PHA. However, the mutant grew well in glucose and produced 18% mcl PHA in the CDW. These results showed that there was another PHA synthase in the wild type, which was able to compensate for the missing activity of the gene product of *phaC* in the mutant.

PCR cloning and heterologous expression of $phaC_{mcl}$

To clone the potential PHA synthase gene, the degenerate primers H1 (5′-CCAC/TGACAGCGGCCTGTTCACCTG-3′) and H2 (5′-GTCGTCGTCA/GCCGGCCAGCACCAG-3′) were designed based on the highly conserved regions of type II *pha* loci of *Pseudomonas spp* [27]. Purified genomic DNA from the wild type was used as template and two-step and touchdown PCR technology were applied to obtain the preferred product. After the initial 1 min of denaturation at 95°C, the subsequent annealing elongation temperature was lowered from 72°C (5 cycles) to 70°C (5 cycles), then further to 68 °C (20 cycles). The last elongation step was 10 min at 72°C. A 2.9 kb fragment was obtained and cloned into vector pMD18-T (TaKaRa) and the resulting plasmid pFH12 was sequenced.

The sequences were analyzed and one whole open reading frame (ORF) was found. The identity of nucleotide sequence of the ORF was more than 90% with such known type II PHA synthases as the enzymes (GenBank accession number AF150670, AF286491, AY113181, AF394660) of *P. putida* and *Burkholderia*

Table 2 Cell growth and polyhydroxyalkanoate (PHA) accumulation

Strains	Carbon source	CDW (g l^{-1})	PHA/CDW (w/w %)	PHA composition (wt%)				
				3HB	3HHx	3HO	3HD	3HDD
A. hydrophila CGMCC 0911	Lauric acid	3.61 ± 0.11	28.64 ± 0.15	95.74	4.26	0	0	0
	Glucose	0.71 ± 0.14	0	0	0	0	0	0
A. hydrophila WH	Lauric acid	3.57 ± 0.26	35.74 ± 0.09	0	2.33	51.03	29.22	17.43
	Glucose	4.31 ± 0.34	18.05 ± 0.21	0	Trace[a]	27.54	60.96	11.50
P. putida GPp104	Gluconate	2.03 ± 0.14	0	0	0	0	0	0
	Octanoate	2.11 ± 0.29	0	0	0	0	0	0
P. putida GPp104 (pBBR1MCS2)	Gluconate	1.42 ± 0.18	0	0	0	0	0	0
	Octanoate	1.52 ± 0.31	0	0	0	0	0	0
P. putida GPp104 (pFH16)	Gluconate	1.37 ± 0.21	3.62 ± 0.11	0	ND	ND	23.78	76.22
	Octanoate	1.48 ± 0.22	15.32 ± 0.27	0	4.3	77.1	16.4	2.2

[a] Trace: 3HHx wt% < 1 wt%

CDW cell dry weight; *3HB* 3-hydroxybutyrate, *3HHx* 3-hydroxyhexanoate, *3HO* 3-hydroxyoctanoate, *3HD* 3-hydroxydecanoate, *3HDD* 3-hydroxydodecanoate; *nd* not detected

caryophylli. However, the homology was much lower with the *phaC* of *A. hydrophila*, indicating that there has been much interest in the two PHA synthases encoded by genes in different *pha* cluster. The ORF was concluded as a structural gene of type II PHA synthase in *A. hydrophila* and termed as $phaC_{mcl}$. The sequences of $phaC_{mcl}$ have been deposited in GenBank under accession No. AY786298.

The $phaC_{mcl}$ gene was PCR subcloned into plasmid pBBR1MCS2 using primers H3 (5′-CCGGAATTCGTCCTGAAGATATAGGGTT-3′) and H4 (5′-TTACTCGAGCTAACCGGTACTCGTCTCAG-3′) for expression in PHA-negative mutant of *P. putida* GPp104. The resultant plasmid pFH16 was transferred from *E. coli* S17-1 (TaKaRa) to *P. putida* GPp104 by conjugation [7]. The trans-conjugant was cultivated in a mineral salt medium containing gluconate or octanoate as carbon source. The recombinant *P. putida* containing plasmid pFH16 recovered the ability to synthesize mcl PHA when grown in gluconate or octanoate (Table 2).

Transcriptional analysis of *phaC* and $phaC_{mcl}$

Transcription of *phaC* and $phaC_{mcl}$ in *A. hydrophila* strains was studied using the above cells. RT-PCR was conducted using specific primers S1 (5′-TCAGCCAC ACTGGAACTGAGACAC-3′) and S2 (5′-GCTAA ACCTCCGACACAGGAAC-3′) for 16S rRNA, C1 (5′-GCAGCAGGCTGTTGTGGGTCTT-3′) and C2 (5′-TGACCGAC GAATCCGCCTTC-3′) for PhaC, M1 (5′-GTGCCGGCAACG CTGAAGAT-3′) and M2 (5′-ATCATCAGCTGGCGCAACCC-3′) for $PhaC_{mcl}$. RT-PCR products with sizes of 480, 723 and 593 bp were obtained and sequenced, respectively (Fig. 1). The same products of 16S rRNA were obtained, indicating that the wild type was identical to the mutant. The same products of the $phaC_{mcl}$ gene were obtained from the wild type and the mutant, which proved the existence of the $phaC_{mcl}$ gene. It simultaneously indicated that the $phaC_{mcl}$ gene could be transcribed in the wild type. However, the *phaC* gene was transcribed in the wild type and not in the mutant, resulting from gene inactivation.

Discussion

Type II PHA synthase genes have been cloned and functionally expressed by various research groups from a large number of different bacteria. In this paper, for the first time, the new $phaC_{mcl}$ was directly cloned from the wild type genome using PCR strategy, showing that this method can be also used for cloning this gene from non-*Pseudomonas* strains [27] and may help to clone more PHA synthase genes from other microorganisms.

A. hydrophila possessing PhaC utilizes lauric acid to produce only PHBHHx through the beta-oxidation pathway. The wild type cannot produce PHA and grows poorly when high concentrations of glucose were used as carbon source [17]. However, after PhaC was inactivated, the resultant mutant produced mcl PHA and also grew well on lauric acid or high concentrations of glucose, showing that the mutant still had the ability to express active PHA synthase. The cloning and functional expression of the $phaC_{mcl}$ gene proved our hypothesis along with the RT-PCR results. Type II PHA synthase utilizes the precursor, provided by the transacylase (PhaG), to produce PHA through fatty acid de novo biosynthesis [9]. Our collaborator in Tsinghua Univeristy successfully cloned the PhaG gene from the wild type, indicating the existence of another pathway for PHA biosynthesis in the *A. hydrophila* strain (personal communication).

Mcl PHA was synthesized only when PhaC of *A. hydrophila* was inactivated, which indicated that synthesis of PHBHHx and mcl PHA was strictly regulated in *A. hydrophila*. It was well-known that regulation of PHA synthesis was reported to take place at the enzymatic and transcription level [13]. The PHA synthetic enzymes are activated by specific cell components or metabolic intermediates. In *Alcaligenes eutrophus*, PHB synthesis is stimulated by both high intracellular concentrations of NAD(P)H and high ratios of NAD(P)H/NAD(P). Citrate synthase activity

Fig. 1 Reverse transcriptase-PCR analysis of type I and type II polyhydroxyalkanoates (PHA) synthase gene in *Aeromonas hydrophila* strains. Total RNA free of DNA was isolated from the above cells cultivated in mineral salt medium containing 10 g l^{-1} lauric acid as sole carbon source. *Lanes 1–6* total RNA was purified from *A. hydrophila* WH. *Lanes 7–12* total RNA was purified from *A. hydrophila* CGMCC 0911. *Lanes 1, 2, 7* and *8* show products of type II PHA synthase gene ($phaC_{mcl}$) using M1 and M2 as primers; *lanes 3, 4, 9* and *10* show the products of type I PHA synthase gene (*phaC*) using C1 and C2 as primers; *lanes 5, 6, 11* and *12* show the products of 16S rRNA using S1 and S2 as primers

is significantly inhibited by NADH and NADPH, indicating that PHA accumulation is enhanced by facilitating the metabolic flux of acetyl-CoA to PHB synthetic pathway [15]. The known regulatory proteins which are involved in regulation of PHA synthesis at the transcription level are classified into the different regulator families, such as RpoN sigma factor in *P. aeruginosa* PAO1 [25] and $PhbR_{Ps}$ in *Pseudomonas* sp. 61-3 [18]. But not much is known about the regulation of PHA production in *A. hydrophila*. Glucose played an important role in cell growth and PHBHHx accumulation when lauric acid was used [17]. According to our results, glucose could not be utilized for the production of PHA by the wild type unless PhaC was inactivated, which indicated that regulation of PHA synthesis was closely related to glucose metabolism in *A. hydrophila*. Furthermore, some genes seem to exist in PHA accumulating bacteria, which regulate PHA synthesis and accumulation by affecting the flow of carbon in the cells [23]. The *phaC* gene of *A. hydrophila* may exhibit the similar function and play its role as the potential global regulator relating to glucose metabolism. It regulates PHA synthesis by affecting intracellular levels of 3HB-CoA and other CoA-derivates. In the presence of the functional *phaC*, 3HB-CoA and other CoA-derivates originating from beta-oxidation pathway are rapidly drawn to PHBHHx synthesis while PHA synthesis pathway from glucose may be repressed or inhibited due to the negative regulation of the functional *phaC*. When the *phaC* gene was inactivated, negative regulation was wiped off and PHA synthesis from the glucose pathway was reactivated. The PhaC-negative mutant of *A. hydrophila* utilized precursor provided by PhaG and synthesized mcl PHA from glucose.

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