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Synthesis of Medium-chain-length Polyhydroxyalkanoate (mcl PHA) in Type I PHA Synthase Negative Mutant of *Aeromonas hydrophila*

中长链聚羟基脂肪酸酯(mcl PHA)在嗜水气单胞菌 I 型 PHA 合酶缺失突变株中的合成

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摘 要 拥有Ⅰ型聚羟基脂肪酸酯(PHA)合酶基因的嗜水气单胞菌 CGMCC 0911 株可利用月桂酸而不能利用葡萄糖作为碳源积累 PHBHHx。将氯霉素抗性基因(*Cm*)插入到该基因中,获得带有 I 型 PHA 合酶断裂基因(*phaC*∷*Cm*)的自杀质粒 pFH10。自杀质粒 pFH10 通过接合作用转入嗜水气单胞菌 CGMCC 0911 株中并发生体内同源重组,*Cm* 被整合到基因组上,获得Ⅰ型 PHA 合酶缺失突变株。DNA 序列测定证明了这一结果。GC 分析表明,突变株不再产生 PHBHHx,但却可利用月桂酸或葡萄糖积累中长链 PHA,明显表明野生型嗜水气单胞菌基因组中存在另一个编码Ⅱ型 PHA 合酶的基因,且只有Ⅰ型 PHA 合酶被钝化后,这个功能被隐藏的Ⅱ型 PHA 合酶才可在细胞中发挥作用。

关键词 嗜水气单胞菌,聚羟基脂肪酸酯(PHA)合酶,中长链 PHA,同源重组

中图分类号 R392.11 **文献标识码** A **文章编号** 1000-3061(2005)04-0524-06

Abstract *Aeromonas hydrophila* CGMCC 0911 possessing type I polyhydroxyalkanoate (PHA) synthase gene (*phaC*) only accumulate copolyesters consisting of 3-hydroxybutyrate (3HB) and 3-hydroxyhexanoate (3HHx), abbreviated as PHBHHx, from lauric acid as sole carbon source but not from glucose. The gene encoding type I PHA synthase was interrupted by insertion of a chloramphenicol resistance gene (*Cm*). Conjugation of suicide plasmid pFH10 transformed *A*. *hydrophila* CGMCC 0911 into a recombinant organism with the disrupted type I PHA synthase gene (*phaC*∷*Cm*), through an *in vivo* homologous recombination process, type I *phaC* of *A*. *hydrophila* genome was replaced by the disrupted *phaC*, and *Cm* gene was integrated

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into the genome of *A. hydrophila*, resulting in type I *phaC*-negative mutant, which was proved by DNA sequencing. Results of GC analysis showed that this mutant could not accumulate PHBHHx again but accumulate medium-chain-length (mcl) PHA from lauric acid or glucose as carbon source, clearly indicating the existence of another type Ⅱ PHA synthase in the wild type *A. hydrophila*. It will play its function and accumulate mcl PHA only when type I PHA synthase was inactivated.

Key words *Aeromona hydrophila*, PHA synthase, medium chain length PHA, homologous recombination

Polyhydroxyalkanoate (PHA) comprise a large family of bacterial storage polyesters that were accumulated by many genera of bacteria and members of the family Halobacteriaceas of the Archaea when there was a lack of an essential nutrient and/or when a carbon source is available in excess[1-2]. The water insoluble PHA exhibits high molecular weight, thermoplastic and/ or elastomeric features as well as other interesting physical and material properties[3]. Therefore, they have attracted increasing attention from scientific and industrial communities due to their interesting properties including biodegradability, biocompatibility and piezoelectricity[4].

PHA can be divided into two groups, depending on the number of carbon atoms in monomer units[5]. One group of bacteria containing type I PHA synthase including *Wautersia eutropha* (formely *Ralstonia eutropha* or *Alcaligenes eutrophus*) and *Alcaligenes latus*, produces short-chain-length (scl) PHA consisting of C_3 to C_5 monomer units. Another group containing type Ⅱ PHA synthase including *Pesudomonas oleovorans* and *P. putida*, synthesizes medium-chain-length (mcl) PHA consisting of C_6 to C_{14} monomer units. Some bacteria can synthesize copolymers consisting of both scl- and mcl-monomer units, including *Rhodospirillum rubrum*[6], *Rhodocyclus gelatinosus*[7] and *Rhodococcus rubber*[8], produce terpolymers consisting of C_4, C_5 and C_6 3-hydroxyalkanoate (3HA) from hexanoate. Some pseudomonad strains accumulate PHA consisting of C_4 to C_{12} 3HA units[9-11]. Wild type *Aeromonas hydrophila* CGMCC 0911 containing type I PHA synthase (PhaC)[12] only accumulate copolyesters of 3-hydroxybutyrate (3HB) and 3-hydroxyhexanoate (3HHx), abbreviated as PHBHHx, from lauric acid as sole carbon source[12]. PhaC was encoded by type I PHA synthase gene (*phaC*)[12].

In this paper, for the first time, we inactivated type I PHA synthase in *A. hydrophila* CGMCC 0911 and found that the existence of another type Ⅱ PHA synthase in the wild type *A. hydrophila*.

1 MATERIAL AND METHODS

1.1 Bacterial strains, plasmids and culture conditions

Bacterial strains, plasmids and primers used in this study are listed in Table 1. *A. hydrophila* strains were cultivated at 30°C on Luria-Bertani (LB) medium, while *Escherichia coli* was grown at 37℃ on LB medium. Antibiotics were added to the medium when needed. In all cases, the cultures were incubated in conical flask at 200 r/min (NBS, Series 25D, New Brunswick, USA). Flask cultures were all conducted in 100 mL mineral salt medium supplemented with lauric acid or glucose, respectively.

1.2 DNA manipulation and RT-PCR procedure

Extraction of bacterial genomic DNA, isolation of plasmids, digestion of restriction endonucleases, agar gel electrophoresis, DNA ligation and polymerase chain reaction (Eppendorf Mastercycler and Mastercycler Gradient, Eppendorf, Germany) were performed according to standard procedures[15] or as recommended by the manufacturers. DNA restriction fragments and PCR fragments were isolated from agarose gels using a GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech, USA). All other DNA-manipulating enzymes were used as recommended by the manufacturers. All restriction endonucleases were purchased from Biolab (New England Biolabs, USA). *EX Taq* DNA polymerase was purchased from TaKaRa Biotechnology Company (Dalian, China). T4 DNA ligase was purchased from Promega (USA). Table 1 showed the primers used in this study. All primers were synthesized by Bioasia (Shanghai, China).

To confirm that type I PHA synthase was surely inactivated in the obtained mutant, reverse transcriptase-PCR (RT-PCR) was applied using the primers 4 ~ 5 described in Table 1. For RT-PCR study, *A. hydrophila* cells were lysed by lysozyme treatment and the lysates were subjected to purification by the bacterial total RNA miniprep super kit (SNBC, Shanghai, China). Total RNA free of DNA was obtained after on-column DNase I treatment and elution as described by the manufacturer (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 first strand cDNA synthesis kit (SNBC, Shanghai, China). One step RT-PCR was conducted according to standard procedures.

1.3 Plasmid transformation

Transformation of *E. coli* was carried out by standard procedures[15].

Transconjugation of *A. hydrophila* CGMCC 0911 with *E. coli* S17-1 harboring suicide plasmid was performed as described by Friedrich *et al*[16].

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Table 1 Bacterial strains, plasmids and primers used in this study

Strains, plasmids or primers	Important features or primer sequences*	Sources or ref.
Strains		
A. hydrophila CGMCC 0911	Wild type.	[12]
A. hydrophila WA	Type I *phaC*-negative mutant of *A. hydrophila*	This study
E. coli JM109	General strain for plasmid maintenance	TaKaRa
E. coli S17-1	For conjugation	TaKaRa
Plasmids		
pBBR1MCS1	Source of chloromycetin resistance gene (*Cm*). Cm^r	[13]
pGEM-4590-1C	Source of type I *phaC*. Amp^r	[12]
pUC18	Cloning vector. Amp^r	TaKaRa
pFH4	pUC18 derivative, type I *phaC* from pGEM-4590-1C. Amp^r	This study
pFH5	pFH4 derivative, disrupted *phaC*. Amp^r. Cm^r.	This study
pSUP202	Suicide plasmid. Tet^r. Cm^r. Amp^r. mob	[14]
pSUP202A	pSUP202 derivative. Tet^r. mob	This study
pFH10	pSUP202A derivative. Cm^r. mob. Disrupted *phaC*	This study
Primers		
Cm 1	5′-TCTCTCGAGCCAGCTTTTGTTCCCTTTAGTG-3′	
Cm 2	5′-TATCTCGAGCTGACGGAACAGCGGGAAGT-3′	
Primer 1	5′-TTGGATCCTGGAGACCGATGATGAATATG-3′	
Primer 2	5′-AATAAGCTTGGCCTTGGCCGTGCTCTTC-3′	
Primer 3	5′-ATGAATTCTTAAGGCAGCTTGACCACGG-3′	
Primer 4[a]	5′-GCAGCAGGCTGTTGTGGGTCTT-3′	
Primer 5	5′-TGACCGACGAATCCGCCTTC-3′	
Primer 6[b]	5′-TCAGCCACACTGGAACTGAGACAC-3′	
Primer 7	5′-GCTAAACCTCCGACACAGGAAC -3′	

* Restriction enzyme sequences are underlined;

[a] Primer 4 (470 bp ~ 492 bp) and 5 (1074 bp ~ 1093 bp) were designed from the central of type I *phaC* gene.

[b] Primer 6 (301 bp ~ 324 bp) and 7 (817 bp ~ 838 bp) were designed from the central of 16S rRNA.

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1.4 Construction of suicide plasmid

The 2.6 kb fragment harboring type I *phaC* was cloned into the plasmid pUC18 in *Eco*RI site, resulting in plasmid pFH4. Cm 1 and Cm 2 used as primers, chloramphenicol resistance gene (*Cm*) amplified from plasmid pBBR1MCS[1] was cloned into plasmid pFH4 in *Xho*I site, resulting in plasmid pFH5 harboring the disrupted gene (*phaC*∷*Cm*). The disrupted gene was cloned into the plasmid pSUP202A in *Sal*I site, resulting in suicide plasmid pFH10 (Fig. 1). The plasmid pSUP202A is a derivative of suicide plasmid pSUP202, of which ampicillin resistance gene (*Amp*) and *Cm* were deleted.

1.5 DNA sequence analysis

DNA sequences were determined by dideoxy chain termination modified as described by Sanger *et al*[17] with an automatic LICOR 4000L sequencer (MWG-Biotech, Ebersberg, Germany).

1.6 Production and analysis of PHA

A. hydrophila strains were cultivated in 500 mL conical flasks containing 100 mL mineral salt medium[12] supplemented with 10 g/L laurica acid or 20 g/L glucose and shaken at 30℃ 200 r/min (NBS, Series 25D, New Brunswick, USA). Cells were harvested and lyophilized after 48 h of cultivation. The lyophilized cells were subjected to methanolysis[18]. Gas chromatographic analysis of intracellular PHA content and PHA composition (Hewlett-Packard model 6890, Palo Alto, CA) was performed as described previously[19].

2 RESULTS AND DISCUSSION

2.1 Construction of suicide plasmid pFH10

A suicide plasmid pSUP202 was chosen as a delivery system for *A. hydrophila* CGMCC 0911. The suicide plasmid pSUP202 that can replicate in *E. coli* does not replicate in the host. To disrupt type I *phaC* of *A. hydrophila* CGMCC 0911 genome by homologous recombinant, the 2.6 kb fragment harboring type I *phaC* was cloned into plasmid pUC18. Subsequently, chloramphenicol resistance gene (*Cm*) was inserted into the central region of type I *phaC* in *Xho*I site, resulting in plasmid pFH5 harboring the disrupterd gene (*phaC*∷*Cm*). The disrupted gene was cloned into a derivative of suicide plasmid pSUP202 in *Sal*I site, resulting in suicide plasmid

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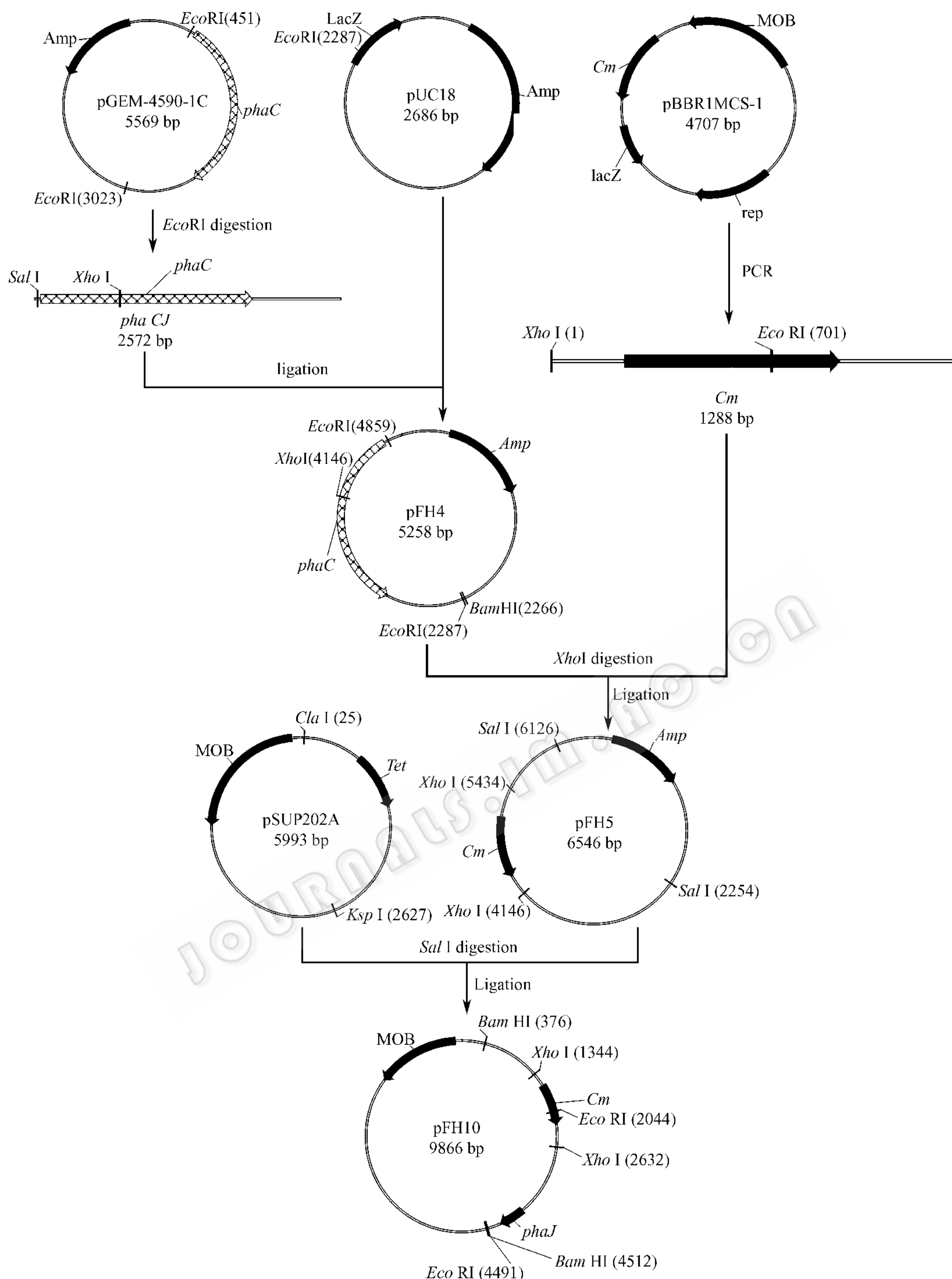

Fig. 1 Construction of suicide plasmid pFH10 for homologous recombination

pFH10 (Fig. 1).

2.2 Disruption of genomic type I *phaC* by homologous recombination

Conjugation of suicide plasmid pFH10 transformed *A. hydrophila* CGMCC 0911 into a recombinant organism with the disrupted gene, through an *in vivo* homologous recombination process, type I *phaC* of *A. hydrophila* CGMCC 0911 genome was replaced by the disrupted *phaC*, and *Cm* gene was integrated into the genome of *A. hydrophila*, resulting in type I *phaC*-negative mutant harboring *Cm* gene. To confirm this result, primers 1 ~ 3 (Table 1) were designed according to the organization of PHA synthesis genes in wild type *A. hydrophila*[12]. Primer 1 and 3 were used, an approximately 2.6 kb fragment was obtained from wild type, while the 3.9 kb fragment contained a 1.3 kb *Cm* gene, the result demonstrated clearly the successful insertion of *Cm* gene into genome of *A. hydrophila* CGMCC 0911 (Fig. 2). PCR products of this mutant were sequenced (data not shown), demonstrating that type I *phaC* was replaced by the disrupted gene, resulting in type I PHA

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synthase negative mutant of *A*. *hydrophila*.

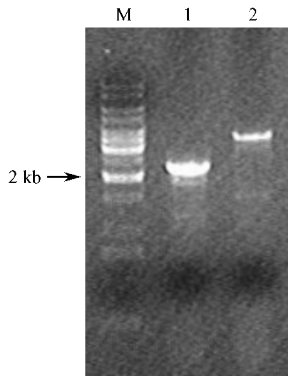

Fig.2 PCR confirmation of type I *phaC*-negative mutant of *A*. *hydrophila*

Genomic DNA of *A*. *hydrophila* strains were used as a template. Fragments with sizes of 2.6 kb (1) and 3.9 kb (2) were obtained using primer 1 and primer 3, respectively. M: 1 kb DNA marker

2.3 RT-PCR analysis of type I *phaC* in *A*. *hydrophila* strains

Transcription of type I *phaC* in *A*. *hydrophila* strains was studied using the above cells cultivated in mineral salt medium containing 10 g/L lauric acid. Primers were designed according to 16S rRNA and type I *phaC* of *A*. *hydrophila*, respectively. After isolation of total RNA from cells in the late logarithmic growth phase, RT-PCR was conducted using primers 6 and 7 for 16S rRNA, primers 4 and 5 for type I PHA synthase, two DNA fragments with sizes of approximately 0.5 kb and 0.6 kb, respectively, were obtained as RT-PCR products (Fig. 3). The same 16S rDNA product was obtained from wild type and the mutant. In wild type, the expectant RT-PCR product was obtained, however, RT-PCR results showed that transcription of type I *phaC* was not detected in the mutant, resulting from disruption of genomic type I *phaC*.

This result demonstrated that genomic type I *phaC* in wild type *A*. *hydrophila* was surely disrupted and resulted in type I *phaC*-negative mutant.

2.4 PHA accumulation by *A*. *hydrophila* strains

A. *hydrophila* strains were cultivated on lauric acid. Wild type accumulated approximately 29 % PHBHHx in the cell dry weight

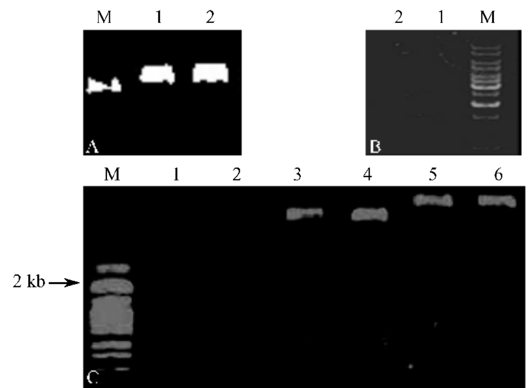

Fig.3 RT-PCR analysis of type I *phaC* in *A*. *hydrophila* strains

a) The obtained total RNA. Lanes 1 and showed the isolated total RNA from wild type and mutant, respectively. M: 1 kb DNA marker. b) PCR result of total RNA used as template using primer 4 and primer 5. Lanes 1 and 2 showed PCR product from wild type and mutant, respectively. M: 1 kb DNA marker. c) RT-PCR results. Lanes 1, 2 and 5: total RNA used as template for RT-PCR was isolated from type I PHA synthase negative mutant of *A*. *hydrophila*. Lanes 3, 4 and 6: total RNA used as template for RT-PCR was isolated from wild type *A*. *hydrophila*. Lanes 1, 2, 3 and 4 showed RT-PCT products of type I *phaC* using primer 4 and 5; Lanes 5 and 6 showed the RT-PCT products of 16S rDNA using primer 6 and 7. M: 1 kb DNA marker.

(Table 2). PHBHHx produced from lauric acid consisted of 96 wt% 3HB and 4 wt% 3HHx. Interestingly, type I *phaC*-negative mutant accumulated approximately 36% mcl PHA consisting of 2.3 wt% 3HHx, 51 wt% 3-hydroxyoctanoate (3HO), 29 wt% 3-hydroxydecanoate (3HD) and 17 wt% 3-hydroxydodecanoate (3HDD) from lauric acid. When grown in glucose, wild type produced only 0.7 g/L CDW with not detectable PHA. However, the mutant grew well in glucose, producing 18% mcl PHA in the CDW.

The mutant utilized glucose to accumulate mcl PHA, indicating that gene *phaC*, its transcriptional or translational product may related to the glycolysis regulation that negatively affected the glycolysis in wild type *A*. *hydrophila*. When type I *phaC* was disrupted, the glycolysis is reactivated, resulting in normal growth of the mutant and additional acetyl-CoA generated from the glycolysis led to the production of mcl PHA. Similar results were reported by Blencke *et*

Table 2 PHA accumulation in *A*. *hydrophila* strains

Strains	C-Source	PHA/CDW (*W*/*W* %)	PHA composition (wt %)				
			3HB	3HHx	3HO	3HD	3HDD
A. *hydrophila* CGMCC 0911	LA	28.64 ± 0.15	95.74	4.26	—	—	—
	Glucose	nd[a]	—	—	—	—	—
A. *hydrophila* WA[b]	LA	35.74 ± 0.09	nd	2.33	51.03	29.22	17.43
	Glucose	18.05 ± 0.21	nd	trace[c]	27.54	60.96	11.50

A. *hydrophila* strains were grown on mineral salt medium supplemented with 10 g/L lauric acid (LA) or 20 g/L glucose at 30℃ for 48 h. Cells were harvested and lyophilized after cultivation for 48 h. Gas chromatographic analysis of intracellular PHA content and PHA composition were performed as described[20]. Notes: [a] nd: not detected; [b] *A*. *hydrophila* WA: type I PHA synthase negative mutant of *A*. *hydrophila* CGMCC 0911; [c] trace: 3HHx wt% < 1 wt%.

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al[20].

According to our results, another type Ⅱ PHA synthase gene ($phaC_{mcl}$) was contained in wild type *A*. *hydrophila*. It indicated that synthesis of PHBHHx and mcl PHA was rigidly regulated in wild type *A*. *hydrophila*. It was known that regulation of PHA metabolism was reported to take place at different levels[21]. Mcl PHA was synthesized only when type I *phaC* was disrupted. It might show that synthesis of PHA was linked to glycolysis pathway of the central metabolic pathways. Type I *phaC*, its transcriptional or translational product may play its role as the possible global regulator in wild type. Due to negative regulation from the possible global regulator in wild type, PHA synthesis pathway from glucose may be repressed, deactivated or inhibited, leading to accumulation of only PHBHHx. When type I *phaC* was disrupted, negative regulation was removed, resulting in synthesis of mcl PHA.

In summary, the above results revealed that besides the type I PHA synthase that is responsible for producing PHBHHx in wild type *A*. *hydrophila*, a type Ⅱ PHA synthases similar to those in the pseudomonad that are responsible for the synthesis of mcl PHA, is also present in the *A*. *hydrophila* CGMCC 0911. Type Ⅱ PHA synthase gene was unable to produce mcl PHA only when type I PHA synthase was inactivated.

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