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Biosynthesis of polyhydroxyalkanoate copolyesters by *Aeromonas hydrophila* mutant expressing a low-substrate-specificity PHA synthase $PhaC2_{Ps}$

Ling-Fang Qin, Xue Gao, Qian Liu, Qiong Wu, Guo-Qiang Chen *

Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing 100084, China

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Abstract

A polyhydroxyalkanoate (PHA) synthase negative mutant termed *Aeromonas hydrophila* CQ4 was constructed from its wild type strain *A. hydrophila* 4AK4. Heterologous expression of a low-substrate-specificity PHA synthase $PhaC2_{Ps}$ cloned from *Pseudomonas stutzeri* 1317 in *A. hydrophila* CQ4 could copolymerize short-chain-length (SCL) 3-hydroxybutyrate (3HB) and medium-chain-length (MCL) 3-hydroxyalkanoates (3HA). Co-expressing (R)-specific enoyl-CoA hydratase and PHA synthase $PhaC2_{Ps}$ in *A. hydrophila* CQ4 ($phaJ_{Ah}$, $phaC2_{Ps}$) led to accumulation of 20.86 wt% PHA copolyesters containing 59 mol% 3HB, 37 mol% 3-hydroxyhexanoate (3HHx) and 4 mol% of other MCL 3HA in shake flask culture. When grown in fermentor, cellular dry weight (CDW) and PHA content were 22.75 g L^{-1} and 20.08 wt%, respectively, with 72.38 mol% 3HB, 25.18 mol% 3HHx and 2.45 mol% other MCL 3HA in the copolymer. If beta-ketothiolase and acetoacetyl-CoA reductase of *Ralstonia eutropha* were co-expressed with $PhaC2_{Ps}$, *A. hydrophila* CQ4 ($phaC2_{Ps}$, $phbA_{Re}$, $phbB_{Re}$) accumulated PHA copolyesters containing enhanced 3HB content up to 79.95 mol% and reduced 3HHx fraction of 8.55 mol%. When grown in fermentor it produced 16.28 g L^{-1} CDW containing 11.64 wt% PHA consisting of 87.38 mol% 3HB, 9.75 mol% 3HHx and 2.87 mol% other MCL 3HA. These results further demonstrate that *A. hydrophila* is a good candidate for metabolic engineering for diverse PHA production.

Keywords: Polyhydroxyalkanoates (PHA); *Aeromonas hydrophila*; Fermentation; Metabolic engineering; PHA synthase

1. Introduction

Polyhydroxyalkanoates (PHA) are a family of intracellular biopolyesters which are accumulated by various bacteria as carbon and energy storage material under nutrient-limitation conditions in the presence of excess carbon source [1]. These bacterial PHAs have attracted increasing attention from scientific and industrial communities due to their interesting properties including biodegradability, biocompatibility and piezoelectricity [2,3].

Bacterial PHA can be divided into two groups according to the number of carbon atoms in the monomeric units: short-chain-length (SCL) and medium-chain-length (MCL) PHA [4]. Industrial scale production of three types of PHA have been reported, namely, poly-3-hydroxybutyrate (PHB) [5], copolyesters of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) (PHBV) [6], and copolyesters of 3-hydroxyhexanoate (3HHx) and 3HB (PHBHHx) [7]. *Aeromonas hydrophila* 4AK4 shows some advantages for production of PHBHHx, including robust growth and simple substrate requirements, which make it a potential strain for industrial production [8–11]. The strain was employed for large scale production of PHBHHx [7]. Zhang et al. (2002) studied the PHBHHx synthesis genetics. They found that PHBHHx biosynthesis genes of *A. hydrophila* 4AK4 consisted of *phaP*, *phaC* and *phaJ* genes, encoding phasin, PHA synthase and (R)-specific enoyl-CoA hydratase, respectively [8].

PHA synthases are the key enzymes of PHA biosynthesis. They catalyze the conversion of 3-hydroxyacyl-CoA (3HA-CoA) substrates into PHA with the concomitant release of CoA [12]. To a considerable extent, the substrate specificity of PHA synthase determines the composition of the accumulated PHA. Biosynthesis of P(3HB-co-3HA) is possible due to PHA syn-

* Corresponding author. Present address: Multidisciplinary Research Center, Shantou University, Shantou 515063, Guangdong, China.
Tel.: +86 754 2901186; fax: +86 754 2901175.
E-mail address: chengq@stu.edu.cn (G.-Q. Chen).

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thases exhibiting extraordinarily broad substrate ranges [13]. It was also reported that the whole PHA synthesis gene locus of *Pseudomonas stutzeri* 1317 strain containing PHA synthase genes *phaC1*$_{Ps}$, *phaC2*$_{Ps}$ and PHA depolymerase gene *phaZ*$_{Ps}$ was cloned using a PCR cloning strategy [14]. The results demonstrated that PhaC1$_{Ps}$ and PhaC2$_{Ps}$ of *P. stutzeri* 1317 had different substrate specificities: PhaC2$_{Ps}$ had low substrate specificity that could incorporate both SCL 3HB-CoA monomer and MCL 3HA-CoA monomer into PHA, while PhaC1$_{Ps}$ only favored MCL-3HA-CoA for polymerization [14–16].

The physical properties of PHA depend on the monomer composition and molecular weight [2]. PHBHHx has a much lower degree of crystallization, it has higher elasticity than that of PHB, with a high elongation to break ranging from 100% to 800%, depending on 3HHx content. However, their tensile strength is low [17]. A number of studies showed that SCL-MCL PHA [P(3HB-co-3HA)] copolymers possessed the suitable physical properties among other members of the PHA family, and they were flexible materials with adjustable toughness and tensile strength [15,18–20]. Recombinant strains of *Pseudomonas sp.* 61-3 and *Ralstonia eutropha* were reported to produce a random copolymer of P(3HB-co-3HA) with high 3HB content from glucose. However, the final cell concentration and PHA productivity were too low and they had not been examined in a fermentor, although random copolyesters P(3HB-co-3HA) had been considered to be copolyesters with suitable properties for application compared with their corresponding homo- or copolymers [7,15,18–20].

In this study, PhaC$_{Ah}$ in *A. hydrophila* 4AK4 was inactivated to achieve a PHA synthase negative mutant *A. hydrophila* CQ4, into which the low-substrate-specificity PHA synthase gene *phaC2*$_{Ps}$ of *P. stutzeri* 1317 was introduced. The ability of recombinant *A. hydrophila* CQ4 to produce random copolyesters of P(3HB-co-3HA) with different monomer compositions was studied in an attempt to obtain this material on a large scale via its growth in a fermentor.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

A. hydrophila 4AK4 and *Escherichia coli* S17-1 [21] were cultivated in Luria-Bertani (LB) medium at 30 °C and 37 °C, respectively. When necessary, kanamycin (50 mgL^{-1}) or ampicillin (100 mgL^{-1}) or gentamicin (15 mgL^{-1}) was added to the medium to maintain the stability of plasmids. *A. hydrophila* CQ4 (this study), which was the PHA synthase negative mutant of *A. hydrophila* 4AK4, was cultured under the same condition as that of *A. hydrophila* 4AK4. All plasmids listed in Table 1, except pKnockout-G [25], were derived from pBBR1MCS-2 [22] by inserting DNA fragment of *phaC*$_{Ah}$, *phaC2*$_{Ps}$, *phaJ*$_{Ah}$, *phaG*$_{Pp}$, and/or *phbAB*$_{Re}$ genes.

All DNA manipulations were carried out using standard procedures [23]. The recombinant plasmids were first introduced into *E. coli* S17-1 by electroporation. Subsequently they were conjugationally introduced into *A. hydrophila* CQ4. All restriction endonucleases and T4 DNA ligase were purchased from TaKaRa (Japan). All the enzymes were used as recommended by manufacturers. Primers were synthesized by Augct Co. Ltd. (Beijing, China).

2.2. Construction of suicide plasmid and expression plasmids

The pKnockout-G is an approximate 6.0 kb vector containing the ColE1 origin of replication which restricts its host range to

Table 1
Plasmids and primers used in this study

Name	Relevant characteristics	Source
Plasmids		
pKnockout-G	Suicide plasmids, Amp^r, Gm^r	[25]
pQLF-KGC[a]	pKnockout-G derivative, *phaC′*[a] from *A. hydrophila* 4AK4	This study
pBBR1MCS2	Cloning vector, Lac promoter, Km^r	[22]
pAH02	pBBR1MCS2 derivative, *phaC* from *A. hydrophila* 4AK4	[16]
pAH05	pBBR1MCS2 derivative, *phaCJ* from *A. hydrophila* 4AK4	[16]
pCJY08	pBBR1MCS2 derivative, *phaC2* from *P. stutzeri* 1317	[16]
pCJY02	pCJY08 derivative, *phbAB* from *R. eutropha* and *phaC2*	[10]
pQLF-C2J	pCJY08 derivative, *phaJ* and *phaC2*	This study
pQLF-C2G	pCJY08 derivative, *phaG* from *P. putida* KT2442 and phaC2	This study
pQLF-C2JG	pCJY08 derivative, *phaC2*, *phaG* and *phaJ*	This study
Primers[b]		
CF-EcoRI	5′-AGAGGAATTCTGCTGCAGACCAATCTGGACGAT-3′	This study
CR-XbaI	5′-GAGATCTAGACATGATGCCCTTGGCCTCATTTT-3′	This study
JF-EcoRI	5′-ATAGAATTCAGGACGCCGCATGAGC-3′	This study
JR-XbaI	5′-GTCTCTAGATTAAGGCAGCTTGACCACGG-3′	This study
GF-XbaI	5′-AGATCTAGAAGGAGTCGATGACATGAGGCC-3′	This study
GR-SacI	5′-AGGGAGCTCTCAGATGGCAAATGCAT-3′	This study
MF	5′-TTCCATTCGCCATTCAGGCTG-3′	This study
MR	5′-TTGGTGCTGTCGCTGTTCCA-3′	This study

[a] *phaC′* was a 0.9-kb fragment of truncated *phaC*$_{Ah}$ gene from *A. hydrophila* 4AK4.
[b] The restriction enzyme sites are underlined.