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Short Communication

Production of two monomer structures containing medium-chain-length polyhydroxyalkanoates by β-oxidation-impaired mutant of *Pseudomonas putida* KT2442

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ABSTRACT

Pseudomonas putida KT2442 produces medium-chain-length (MCL) polyhydroxyalkanoates (PHA) from fatty acids. When gene encoding 3-hydroxyacyl-CoA dehydrogenase which catalyzes long-chain-3-hydroxyacyl-CoA to 3-ketoacyl-CoA, was partially or completely deleted in *P. putida* KTOY08, the PHA accumulated was shown to contain only two different monomer structures dominated by a monomer of the same chain length as that of the fatty acids fed and another monomer two carbon atoms shorter. Among the PHA copolymers, P(44% 3HD-*co*-3HDD) containing 44% 3HD and 56% 3HDD was demonstrated to have a melting temperature T_m, an apparent heat of fusion $\triangle H_m$ and a Young's modulus E of 75 °C, 51 J g^{-1} and 2.0 MPa, respectively, the highest among all the MCL PHA studied.

1. Introduction

Polyhydroxyalkanoates (PHA) are versatile polyesters produced by bacteria as intracellular energy and carbon storage materials (Reddy et al., 2003; Shen et al., 2009). PHA is generally divided into short-chain-length (SCL) PHA, medium-chain-length (MCL) PHA and copolymers of SCL and MCL PHA. MCL PHA copolymers are poorly manageable due to their 2+ multiple monomer structures that are difficult to control. Copolymers of SCL and MCL PHA have been found to be more suitable for applications in view of their better physical properties (Chen and Wu, 2005; Li et al., 2007).

MCL PHA produced by bacteria normally contains 3-hydroxyhexanoate (3HHx), 3-hydroxyoctanoate (3HO), 3-hydroxydecanoate (3HD) and 3-hydroxydodecanoate (3HDD). Ouyang et al. (2007a) achieved to produce MCL PHA consisting of mainly 3HDD using *Pseudomonas putida* KT2442 mutant with a weakened β-oxidation. MCL PHA biosynthesis in *Pseudomonas* spp. is closely linked to fatty acid β-oxidation (Cai et al., 2009), therefore, metabolic engineering of the β-oxidation pathway has been commonly adopted to increase PHA accumulation. A β-oxidation-impaired mutant of *P. putida* KT2442 was shown to reduce consumption of fatty acids, leading to increasing accumulation of monomers with similar chain length to the fatty acid substrate (Ouyang et al., 2007a,b). In this study, we intended to produce and characterize homo- or copolymers of C10 and C12 using mutants of *P. putida* KT2442 with multiple deletions in its key enzymes of β-oxidation.

2. Methods

2.1. Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. These strains were routinely cultivated in Luria–Bertani (LB) medium (Ouyang et al., 2007a).

2.2. Plasmid construction

All plasmids used in this study are listed in Table 1. A DNA fragment containing partial gene of *PP2047* and partial gene of *PP2048* of *P. putida* KT2442 were amplified by polymerase chain reaction. To obtain the 2.3 kb DNA fragment containing partial gene *PP2047* and partial gene *PP2048*, the primers PP2047F and PP2047R were used. PP2047F and PP2047R introduced flanking *Hind*III and *Xba*I sites to the 2.3 kb amplified DNA fragment. By using these sites, the 2.3 kb amplified DNA fragment was cloned into *Xba*I- and *Hind*III-digested pK18mobsacB, resulting in plasmid

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Table 1
Bacterial strains and plasmids used in this work.

Strains or plasmids	Relevant characteristics	References
Strains		
E. coli S17-1	*rec*A; harbors the *tra* genes of plasmid RP4 in the chromosome; *proA*, *thi-1*	Simon et al. (1983)
P. putida KT2442	MCL PHA producing strain with ampicillin resistance	Ouyang et al. (2007a)
P. putida KTOY08	*P. putida* KT2442 mutant (△fadB2x△fadAx△fadB△fadA)	Ouyang et al. (2007a)
P. putida KT2047P	*P. putida* KTOY08 mutant (half of PP2047 and half of PP2048 was deleted)	This work
P. putida KT2047A	*P. putida* KT2047P mutant (half of PP2046 and partial PP2047 was deleted)	This work
Plasmids		
pK18mobsacB	a mobilizable multi-purpose cloning vector with kanamycin resistance	Schäfer et al. (1994)
pKXZ01	pK18mobsacB carrying approximately 2.3 kb chromosomal fragment of *P. putida* KT2442	This work
pKSL01	PKXZ01 completely digested by restriction enzymes *pst*I, and then the large fragment was self-ligated	This work
pKXZ02	pK18mobsacB carrying approximately 2.4 kb chromosomal fragment of *P. putida* KT2047P	This work
pKSL02	PKXZ02 completely digested by restriction enzymes *pst*I, and then the large fragment was self-ligated	This work
DNA primers		
PP2047F	(CTGAAGCTTGAGGCCGACCTGGTGATTGA)	This work
PP2047R	(AGCTCTAGACATTGGCAGCCCTGACGT)	This work
PP2046F	(CTCAAGCTTAACCGTTGCAGCCACTGCT)	This work

pKXZ01. Similarly, a 2.4 kb DNA fragment from the *P. putida* KT2047P genome containing partial *PP2048* and partial *PP2046* was amplified by PCR using the primers PP2046F and PP2047R. Accordingly, PP2046F and PP2047R introduced *Hind*III and *Xba*I sites to the 2.4 kb DNA fragment. Plasmid pKXZ02 was originated from a *Hind*III–*Xba*I ligation of this 2.4 kb amplified DNA fragment in the pK18mobsacB. Plasmids pKXZ01 and pKXZ02 were then completely digested by *pst*I, then the large fragments were self-ligated to form pKSL01 and pKSL02, respectively.

2.3. Mutation procedure

The transconjugants were prepared after integration of pKSL01 or pKSL02 into the chromosome by homologous recombination. Transconjugants were selected on LB agar + 50 μg km/ml and 100 μg Amp/ml. To select double cross-over events, a single colony was grown for 24 h in non-selective LB medium at 30 °C. The culture medium was diluted with sterile water to 100 times, then 100 μl diluted culture medium were plated onto LB agar containing 10% sucrose + 100 μg Amp/ml and incubated for 48 h at 30 °C. The resulting colonies were sensitive to km, indicating the excision of the plasmid from the chromosome by a second cross-over event (Schäfer et al., 1994; Ouyang et al. 2007b).

2.4. Shake-flask culture

Shake-flask cultivation was conducted in LB medium for the first 9 h for cell growth, and then fatty acid including sodium octanoate, decanoate or dodecanoate was added into the broth to a final concentration of 12 g L^{-1}. The total culture time was 48 h (Ouyang et al., 2007a).

2.5. PHA extraction, purification, composition analysis and NMR study

PHA extraction, purification, composition analysis and NMR study were performed as described by Liu and Chen (2007).

2.6. PHA physical characterization

The weight–average molar mass (M_w) and number–average molar mass (M_n) of MCL PHA were determined by gel permeation chromatography (GPC) (Liu and Chen, 2007). Thermogravimetry analysis (TGA), differential scanning calorimetry study and mechanical property study were performed as described by Liu and Chen (2007).

3. Results and discussions

3.1. Construction of P. putida KT2442 mutant strains KT2047P and KT2047A

If β-oxidation in *P. putida* KT2442 was completely disrupted, MCL PHA homopolymers containing one monomer structure should be obtained. In our previous study, key genes involved in β-oxidation of *P. putida* KT2442 including *fadB2x*, *fadAx*, *fadB* and *fadA* were deleted, leading to a mutant termed *P. putida* KTOY08 (Ouyang et al., 2007a). These deletions only weakened the β-oxidation process, leading to increasing accumulation of MCL PHA consisting of more monomers of the same chain length to the fatty acid substrate (Ouyang et al., 2007a), yet not homo- or two monomer copolymer MCL PHA was formed. It was found that another gene encoding 3-hydroxyacyl-CoA dehydrogenase involves in β-oxidation of long-chain fatty acids. The locus tag of this gene is recorded as *PP2047* in the NCBI database, two adjacent genes to *PP2047* are coded as *PP2046* and *PP2048* (Fig. 1 A), which putatively encode *LysR* family transcriptional regulator and acyl-CoA dehydrogenase, respectively. *PP2046* and *PP2048* appear not to be indispensable genes for bacterial survival, they were disrupted in this study. A part of *PP2047* (Fig. 1 A) were deleted in *P. putida* KTOY08 using cloning vector pKSL01. Whole of *PP2047* (Fig. 1 B) were deleted in *P. putida* KT2047P, using cloning vector pKSL02 (Schäfer et al., 1994; Ouyang et al., 2007a). The deletion of approximately half of *PP2047* and half of *PP2048* in *P. putida* KTOY08 led to *P. putida* KT2047P (Fig. 1 A and B; Table 1). Similarly, deletion of about half of *PP2046* and partial *PP2047* in *P. putida* KT2047P resulted in *P. putida* KT2047A (Fig. 1 B and C; Table 1). When grown on 12 g L^{-1} dodecanoate as the only substrate, *P. putida* strain KTOY08 and KT2047P showed similar cell dry weight (CDW) and PHA content of approximately 1.5 g L^{-1} and 15%, respectively, demonstrating that the deletion of *PP2047* and *PP2048* did not much affect the cell growth and PHA accumulation (data not shown). However, with the more extensive deletion of multiple genes in the β-oxidation pathway of *P. putida* KT2047P, 3-hydroxydodecanoate (3HDD) containing homopolymer PHDD was not produced by *P. putida* KT2047P grown on dodecanoate as carbon source, instead, the strain produced a copolymer consisting of 3HD and 3HDD (Fig. 1D). This phenomenon indicates that the β-oxidation pathway was not completely inhibited although key enzymes were all disrupted. Two possible reasons for the formation of PHA copolymer by *P. putida* KT2047P were suggested: Firstly, the residual DNA fragment of gene *PP2047* could still encode a truncated protein which contains some enzyme activity. Secondly, there are some unknown isozymes or epimerases. To test these