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Citation	Journal of Biotechnology, 156(3), 214-217 https://doi.org/10.1016/j.jbiotec.2011.07.040
Issue Date	2011-12-10
Doc URL	http://hdl.handle.net/2115/47658
Type	article (author version)
File Information	JoB156-3_214-217.pdf



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**Biosynthesis of glycolate-based polyesters containing
medium-chain-length 3-hydroxyalkanoates in recombinant *Escherichia
coli* expressing engineered polyhydroxyalkanoate synthase**

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ABSTRACT

Glycolate(GL)-based polyesters were for the first time produced in the recombinant *Escherichia coli* fatty acid β -oxidation pathway reinforcing mutant LS5218, using extracellularly added GL as a monomer precursor. Cells expressing a Ser325Thr/Gln481Lys mutant of polyhydroxyalkanoate synthase (PhaC1STQK) from *Pseudomonas* sp. 61-3, propionyl-CoA transferase from *Megasphaera elsdenii* and enoyl-CoA hydratase from *Pseudomonas aeruginosa* grown on GL and dodecanoate were found to produce novel copolymers of GL with 3-hydroxyalkanoates (3HAs) (C₄-C₁₂), P(GL-*co*-3HA), with a weight-average molecular weight of 34 000. The ¹H NMR analysis of the copolymer revealed the incorporation of GL units into the polymer chain. This result demonstrates that PhaC1STQK polymerized glycolyl-CoA as a monomer substrate. Additionally, the novel lactate(LA)-based polyester P(LA-*co*-3HA) was produced by substituting GL with LA, indicating that the method is versatile and allows the production of a variety of biopolymers.

Keywords:

poly(glycolide)

poly(lactide)

lactate-polymerizing enzyme

biobased plastic

polyhydroxyalkanoate

1. Introduction

Glycolate(GL)-based polyesters are biobased polyesters with superior

biodegradability and biocompatibility. There are many reports for GL-based polymers, such as P[lactate(LA)-*co*-GL], that are used in medical applications as a scaffold for tissue culture experiments (Huang et al., 2010). In addition, poly(glycolic acid) (PGA), which has the property of a high gas barrier, is considered to be useful in the production of plastic beverage bottles. Currently, the GL-based polyesters are chemically synthesized from a cyclic glycolide by ring-opening polymerization using heavy metal catalysts, most commonly tin. However, the use of potentially harmful catalysts has been considered a weak point in medical and food-related applications. To date, the biosynthesis of GL-based polyester has not been reported.

The aim of this study is to biosynthesize GL-based polyesters by means of a recombinant bacterium. To this end, we focused on the bacterial polyhydroxyalkanoates (PHAs), which are also a representative biobased plastic (Doi, 1990). The microbial process for the synthesis of natural polyesters can be performed using renewable carbon sources without using heavy metal catalysts. PHAs typically consist of 3-hydroxyalkanoates (3HAs) that are intracellularly produced by PHA synthase from the corresponding CoA monomer (3HA-CoA). To date, more than 60 PHA synthases have been isolated and their substrate specificities characterized (Rehm, 2003). These enzymes polymerize monomers with hydroxy group at position 3 or more, but none of them reportedly polymerize GL. If the GL monomer glycolyl-CoA were to be polymerized by PHA synthase, GL-based polyesters would be produced in the microbial cells.

Our group has worked with the engineering of PHA synthase to expand its substrate specificity, and generated numerous mutants with alterations in both its substrate specificity and activity. Among them, a Ser325Thr/Gln481Lys (ST/QK) mutant of the *phaC*_{Ps} (PhaC1STQK) gene from *Pseudomonas* sp. 61-3 was discovered

to have gained exceptional substrate specificity for incorporating LA units into the polymer chain (Taguchi et al., 2008), as well as short-chain-length (SCL, C₄ and C₅) and medium-chain-length (MCL, C₆-C₁₄) 3HAs. Because of the structural similarity between the two 2-hydroxyacids GL and LA, PhaC1STQK was also a potent candidate as a GL-polymerizing PHA synthase. Thus, in this study, we investigated the activity of this mutant toward the GL monomer for the purpose of the biosynthesis of GL-based polyesters.

2. Results

In designing synthetic strategy for GL-based polyesters, the addition of GL into the culture medium was useful procedure, because the construction of *de novo* monomer-synthesizing pathways can thus be omitted. For the incorporation of the extracellular precursor into the polymer chain, the following three steps were essential (Fig. 1); (i) uptake of the precursor into the cells, (ii) activation of the precursor into the CoA form as catalyzed by PCT, and (iii) polymerization of the monomer into the polyester catalyzed by PhaC1STQK. We have demonstrated that propionyl-CoA transferase from *Megasphaera elsdenii* (PCT_{Me}) was efficiently expressed in *E. coli* so as to generate LA-CoA for polymer synthesis (Taguchi et al., 2008). The enzyme was reported to also catalyze the CoA transfer reaction also toward GL using acetyl-CoA as a CoA donor (Tung and Wood, 1975). Thus, PCT_{Me} was expected to meet our requirements. In addition to the aforementioned conditions, a supply of 3HA-CoAs is essential to the polymer production, because it has been shown that PhaC1STQK intrinsically requires preferable 3HA monomers for the incorporation of unnatural LA units into the polymer chain, and thus, the enzyme synthesizes only copolymers, but

hardly produces PLA homopolymer (Taguchi, 2010). The 3HA monomers presumably act as an initiator of the polymerization. Based on the analogy with the reaction mechanism, the 3HA monomers may be needed at the initial step for the polymerization of GL.

To examine the steps, the recombinant *E. coli* JM109, harboring pTV118NpctC1STQKAB bearing the *phaC1STQK* and *pct* genes along with the *phaA* and *phaB* genes from *Ralstonia eutropha*, was grown on glucose as described previously (Taguchi et al., 2008) using supplements of GL [0.25, 0.5 and 1.0% (w/v)] as well as LA, which was tested as an assured substrate of PhaC1STQK. However, the addition of LA did not increase the LA fraction in the polymer, although the cells produced P(6 mol% LA-co-3HB), the LA fraction of which was supplied from glucose via intrinsic LA dehydrogenase (Taguchi et al., 2008). The addition of GL resulted in the production of similar polymer, and no GL-incorporated polymer was produced (data not shown). These results suggested that uptake of the precursors [step (i)] was not efficient under the conditions employed. This result may be due to the catabolite repression by glucose.

Therefore, we next used the *E. coli* mutant LS5218 [*FadR*, *atoC*(Con)] (Spratt et al., 1981), which constitutively expresses the fatty acid β -oxidation pathway, and is capable of efficiently utilizing fatty acids as a sole carbon source (Fig. 1). In addition, the β -oxidation of dodecanoate generates an abundant amount of acetyl-CoA, which is likely used as a main CoA donor for the PCT reaction. Furthermore, MCL 3HA-CoAs may be supplied from the β -oxidation pathway by the introduction of the enoyl-CoA hydratase (*phaJ4*) gene from *Pseudomonas aeruginosa* (Tsuge et al., 2003). Thus, the system should meet the required conditions mentioned above. To investigate this strategy, recombinant *E. coli* LS5218 harboring pTVpctC1(ST/QK)J4 (Shozui et al.,

2010) was grown on M9 medium containing 0.3% (w/v) dodecanoate and various concentrations of LA. The polymer was extracted from cells with chloroform and subjected to gas chromatography/mass spectroscopy (GC/MS) analysis as described previously (Taguchi et al., 2008) using chemically synthesized P(LA-*co*-GL) (SIGMA) as a reference. As a result, the strain successfully produced up to 6 wt% polymers consisting of LA and 3HA monomers (Table 1). The LA units in the polymer were not detected when LA was not added to the medium, indicating that the incorporated LA was derived from the extracellular LA. This is the first demonstration of the biosynthesis of LA-based polyesters copolymerized with multiple components of MCL 3HA monomers.

The successful incorporation of extracellular LA into the polymer chain prompted us to apply the same strategy to GL. Similarly, the recombinant *E. coli* LS5218 harboring pTVpctC1(ST/QK)J4 was grown on dodecanoate with the GL supplement (0.25, 0.5 and 1.0%). The cells produced the polyesters consisting of GL and the 3HA units (Table 1). The GL fraction was 17 mol% when 0.25% GL was added, while cell growth was inhibited at higher concentrations of GL (0.5% and 1.0%, data not shown). This result strongly suggests that both PCT_{Me} and PhaC1STQK, respectively, recognized GL and its CoA form as a substrate.

The molecular weight of the polymers was determined by gel permeation chromatography as described (Taguchi et al., 2008). P(GL-*co*-3HA) (Sample No. 5 in Table 1) was eluted as a unimodal peak having an weight-average molecular weight of 34 000 with a polydispersity of 1.6, suggesting that the sample was a high-molecular-weight polymer containing GL. The weight-average molecular weight of P(LA-*co*-3HA) (Sample No. 3 in Table 1) was 27 000, with a polydispersity of 1.9.

To evaluate the polymer structure, P(GL-*co*-3HA) (Sample No. 5 in Table 1) was

subjected to ^1H NMR analysis. The sample exhibited four resonance patterns in the range of δ 4.55 - 4.85 (Fig. 2). The chemical shift of these peaks was different from that of the methine proton of monomeric glycolic acid dissolved in CDCl_3 (δ 4.26, data not shown), and close to that of chemically synthesized P(LA-*co*-GL) (δ 4.82) (Wang et al., 2010). Therefore, these peaks were ascribed to the GL units in the polymer. The GL fraction determined by ^1H NMR was 21 mol%. Based on the analogy with the ^1H NMR signals from P(LA-*co*-3HB) (Matsumoto and Taguchi, 2010), these split resonances should correspond to the four triad sequences in the copolymers. Considering the GL fraction in the copolymer, the major resonance at δ 4.59 was presumably ascribed to the 3HA-GL*-3HA sequence. The other signals at δ 4.66, δ 4.73 and δ 4.83 may be ascribed to either the GL-GL*-3HA, 3HA-GL*-GL or GL-GL*-GL triads, although these peaks were not fully ascribable, because PGA was not dissolved in chloroform. Additionally, a characteristic low-field shift was observed for the resonance of the methine proton of the 3HA units (δ 5.15-5.40), which was also observed for the methine proton of the 3HB units in P(LA-*co*-3HB), indicating a linkage between GL and the 3HA units. Furthermore, the resonance of methine carbon was observed at δ 60.4-60.8 by ^{13}C NMR (Supplementary Fig. S1), which was different from that of glycolic acid (δ 60.07). The results clearly indicate that the sample was a novel copolymer containing GL units, not P(3HA) mixed with glycolic acid molecules.

3. Discussion

The exploration of PHA synthase with polymerizing activity toward 2-hydroxy monomers, especially LA, has attracted considerable research interest (Valentin and Steinbüchel, 1994; Yuan et al., 2001). PhaC1STQK was originally created through the

engineering of PHA synthase in order to enhance the activity toward 3HB-CoA (Taguchi and Doi, 2004; Takase et al., 2003). The activity of PhaC1STQK toward LA-CoA was found by means of an *in vitro* chemo-enzymatic synthesizing system and formally designated LPE (Taguchi et al., 2008; Tajima et al., 2009). The result of the present study demonstrated that PhaC1STQK also recognized glycolyl-CoA as a monomer substrate. In addition, we detected the incorporation of 2-hydroxybutyrate (2HB) units into the polymer chain by PhaC1STQK (manuscript in preparation). These findings suggest that PhaC1STQK possesses an extraordinary capacity for polymerizing a broad range of 2-hydroxyalkanoate (2HA) monomer substrates, and thus can also be designated a “2HA-polymerizing enzyme”. Furthermore, Han et al. recently reported that PHA synthase from *R. eutropha* polymerizes 2HB-CoA using an improved *in vitro* system (Han et al., 2011). This was the first case of a wild-type enzyme with 2-hydroxy monomer-polymerizing activity. These results suggest that a wide range of PHA synthases might be capable of polymerizing various 2-hydroxy monomers. Therefore, reevaluation of the substrate specificity of the PHA synthases will be required to clarify this issue. The current findings serve as a reminder to reconsider the substrate capacity of the wild-type or engineered PHA synthases for the production of new polymers (Taguchi, 2010).

It should be noted that the GL- and LA-based copolymers contain MCL 3HA units. The usefulness of the MCL units has been demonstrated by the P(3HB-*co*-MCL 3HA) copolymers possessing flexible properties compared to P(3HB), which enables a utility in a wider range of applications (Matsusaki et al., 2000). Recently, LA-based polyester P(LA-*co*-3HB)s were shown to be flexible and semitransparent material depending on their LA fractions (Yamada et al., 2011). Because the MCL units with 8-12 carbons should have a more potent effect of material softening than 3HB units, the physical

properties of P(GL-*co*-3HA) and P(LA-*co*-3HA) comprise an interesting research target.

Acknowledgements We thank Dr. Toshihiko Ooi (Hokkaido University) for critical reading of the manuscript. The study was partly supported by grant-in-aid for scientific research (No. 23681015) to K.M. from Ministry of Education, Culture, Sports, Science and Technology-Japan, and funding from Japan Bioindustry Association, and the Association for the Progress of New Chemistry (Japan). Pacific Edit reviewed the manuscript prior to submission.

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FIGURE LEGENDS

Fig. 1. Proposed pathways in recombinant *E. coli* LS5218 for the production of glycolate and lactate-based polyesters. PCT, propionyl-CoA transferase; PhaC1STQK, S325T/Q481K mutant of polyhydroxyalkanoate synthase; PhaJ4, enoyl-CoA hydratase; 3HA, 3-hydroxyalkanoate. Acetyl-CoA presumably acts as a main CoA donor for the PCT-catalyzed reaction (indicated by dashed line).

Fig. 2. ^1H NMR analysis of P(glycolate-*co*-3-hydroxyalkanoates). The spectrum of the polymer dissolved in CDCl_3 was obtained using a Bruker MSL400 spectrometer (400 MHz), and the chemical shifts are reported in ppm with tetramethylsilane as an internal reference.

Table 1

Content and monomer composition of glycolate- and lactate-based polyesters produced in recombinant *E. coli* LS5218.^a

Sample No.	Precursor	Cell dry weight (g/L)	Polymer content (wt%) ^b	Monomer composition (mol%) ^c						
				GL	LA	3HB	3HHx	3HO	3HD	3HDD
1	-	0.52±0.18	7.4±6.6	-	-	16	17	36	16	15
2	0.25% LA	0.73±0.08	3.4±2.2	-	4	26	19	31	12	8
3	0.5% LA	0.80±0.12	6.3±2.5	-	17	15	18	30	10	10
4	1.0% LA	0.64±0.11	Trace							
5	0.25% GL	0.64±0.11	4.8±3.8	17	-	19	22	26	8	8

^a Cells harboring pTVpctC1(ST/QK)J4 was grown on 100 mL M9 medium containing sodium dodecanoate (0.3% w/v), Brij-35 (0.4% v/v), 100 µg/l ampicilin, and indicated concentrations of glycolate (GL) or lactate (LA) (w/v) at 37 °C for 72 h. ^b Polymer content was determined based on the weight of extracted polymer and cell dry weight. Data is an average of at least three trials. ^c 3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate; 3HD, 3-hydroxydecanoate; 3HDD, 3-hydroxydodecanoate.

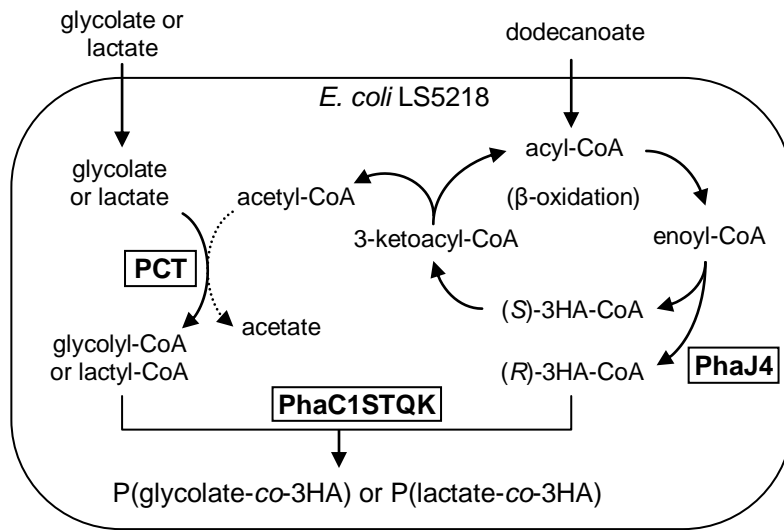


Figure 1

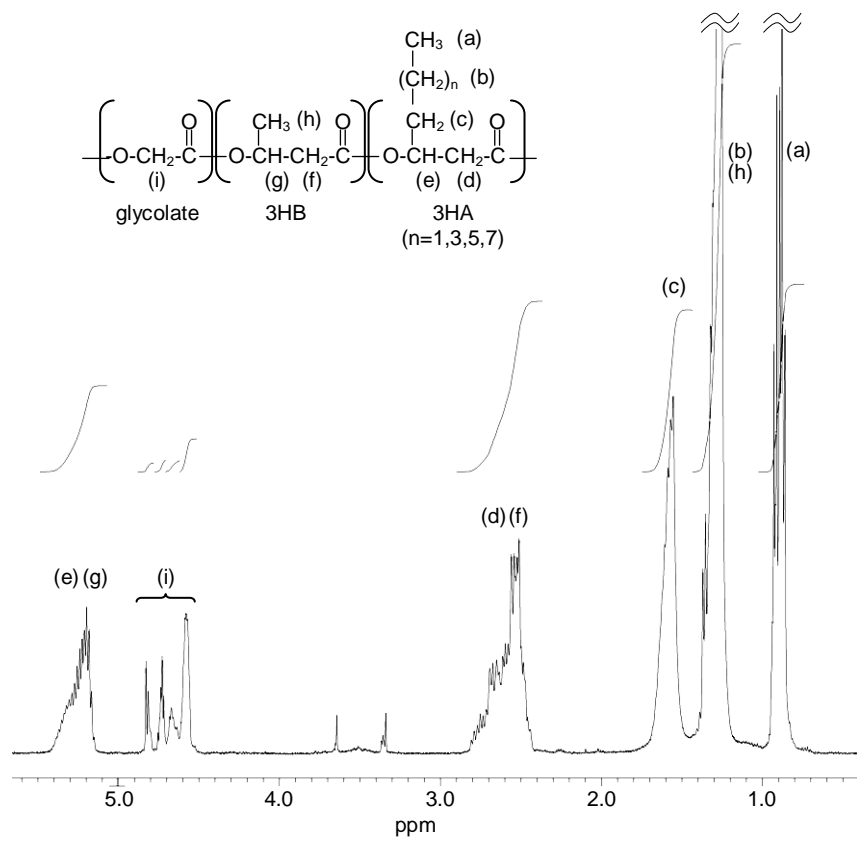


Figure 2