Polymer Journal (2015) 47, 767–770
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NOTE

Behavior of different polyhydroxyalkanoate synthases in response to the ethanol level in *Escherichia coli* cultures

Ayaka Hiroe[1], Masaya Shiraishi[1], Kouhei Mizuno[2] and Takeharu Tsuge[1]

Polymer Journal (2015) **47,** 767–770; doi:10.1038/pj.2015.53; published online 15 July 2015

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are aliphatic polyesters that are synthesized by a wide range of bacteria as an intracellular carbon and energy storage material.[1,2] Because PHAs are biodegradable and renewable, unlike petroleum-based plastic, PHAs have received considerable attention as eco-friendly plastics.[3] Among PHAs, poly[(*R*)-3-hydroxybutyrate] [P(3HB)] is most commonly produced by native PHA producers, the most well characterized of which is *Ralstonia eutropha*. Although *Escherichia coli* is a non-native PHA producer, P(3HB) can be produced in *E. coli* by expressing PHA biosynthesis genes. Interestingly, *E. coli* sometimes produces high molecular weight P(3HB) with number-average molecular weight (M_n) exceeding 2×10^6 g mol^{-1}.[4] Since the molecular weight of polymers influences their mechanical properties and melt processability, its regulation during PHA biosynthesis is important. For *in vivo* analysis of factors affecting the molecular weight of PHA, *E. coli* is a suitable host because of the absence of PHA degradation enzymes and its well-developed genetic background.

Previously, it was found that ethanol is a key compound in molecular weight regulation of PHA in *E. coli*. Ethanol induces a chain transfer (CT) reaction, where the elongating PHA polymer chain is transferred from PHA synthase to ethanol as a CT agent,[5] resulting in chain termination. Therefore, the presence of CT agents leads to decreased molecular weight of PHA. Not only ethanol but also various alcohols such as polyethylene glycol induce the CT reaction of PHA synthases.[6] However, in previous studies,[6] PHA synthase from *Aeromonas caviae* (PhaC$_{Ac}$) showed different behavior in response to CT agents than that of other PHA synthases. PhaC$_{Ac}$ produced P(3HB) with a relatively low molecular weight ($M_n=6.4\times10^4$ g mol^{-1}) in *E. coli* JM109 and produced PHA with a slightly decreased molecular weight when the culture medium was supplemented with polyethylene glycols.[7] These observations prompted us to survey the behavior of PhaC$_{Ac}$ in response to ethanol levels in *E. coli* cultures.

Recently, we found that PhaCs from *Halomonas* strains have an atypical amino-acid sequence in their active sites.[8] Most PhaCs have a lipase box-like sequence of G-X-C-X-G in the active site, and the Cys residue has an important role in PHA polymerization.[2] On the other hand, PhaCs from *Halomonas* have the unique sequence of S-X-C-X-G, where the first Gly in the lipase box-like sequence is replaced with Ser.[8] This finding indicates that *Halomonas* PhaC may have acquired a novel property during the course of evolution. Therefore, it is of fundamental interest to understand how the *Halomonas* PhaC responds to ethanol during PHA polymerization *in vivo*, rather than *in vitro* behavior. This is because *in vitro* kinetics of PhaC is not directly liked to *in vivo* polymer production as demonstrated previously.[9]

In this study, to understand the behavior of PHA synthases in response to ethanol, molecular weights of P(3HB) produced by PhaC$_{Ac}$ and *Halomonas elongata* DSM2581 PHA synthase (PhaC$_{He}$) were compared over a range of ethanol concentrations (0–5 g l^{-1}) in culture. PHA synthase from *R. eutropha* (PhaC$_{Re}$) was used as a reference enzyme.

EXPERIMENTAL PROCEDURE

Bacterial strain, plasmids and culture conditions

For PhaC$_{Re}$, PhaC$_{Ac}$ and PhaC$_{He}$, the plasmids pGEM-3aSD-*phaC$_{Re}$AB*,[5] pGEM"*phaC$_{Ac}$AB*,[10] and pGEM"*phaC$_{He}$AB*,[8] respectively, were expressed in *E. coli* XL1-Blue (Stratagene Inc., La Jolla, CA, USA). Each plasmid carries *R. eutropha phaAB* genes for 3HB monomer provision. Transformants were cultured at 37 °C for 72 h using a reciprocal shaker (130 strokes per minute) in 500-ml shake flasks containing 100 ml of LB medium (10 g l^{-1} NaCl, 10 g l^{-1} tryptone, 5 g l^{-1} yeast extract) supplemented with 20 g l^{-1} glucose, 50 mg l^{-1} carbenicillin, and 0–5 g l^{-1} ethanol. After cultivation, the collected cells were washed with water, frozen and lyophilized for 3 days.

Analyses

P(3HB) content in the cells was determined by gas chromatography after methanolysis of approximately 15 mg of lyophilized cells in the presence of

[1]Department of Innovative and Engineered Materials, Tokyo Institute of Technology, Yokohama, Japan and [2]Department of Materials Science and Chemical Engineering, Kitakyushu National College of Technology, Kitakyushu, Fukuoka, Japan
Correspondence: Dr A Hiroe or Dr T Tsuge, Department of Innovative and Engineered Materials, Graduate School of Science and Engineering, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8502, Japan.
E-mail: hiroe.a.aa@m.titech.ac.jp or tsuge.t.aa@m.titech.ac.jp
Received 20 April 2015; revised 10 June 2015; accepted 11 June 2015; published online 15 July 2015

15% (v/v) sulfuric acid, as described previously.[11] P(3HB) was extracted from cells by stirring with chloroform for 72 h at room temperature. The cells were removed by filtration, and the filtrate was dropped into methanol to obtain pure P(3HB) samples.

The molecular weight of P(3HB) was determined by gel permeation chromatography (GPC) using two Shodex K-806M joint columns and a refractive index detector or multi-angle laser light scattering photometer (GPC-MALLS, DAWN HEEOS 8+; Wyatt Technology Co., Santa Barbara, CA, USA). The relative molecular weight of P(3HB) was determined based on a refractive index detector peak from a calibration curve of polystyrene standards (peak molecular weight $M_p = 1.3\times10^3$ to $7.3\times10^6\,g\,mol^{-1}$). Absolute molecular weight was measured by multi-angle laser light scattering (MALLS) with dn/dc = 0.023 at 658 nm.

Ethanol concentration in culture broth was measured enzymatically using the F-kit (Roche Diagnostics, Basel, Switzerland) as described previously.[5]

RESULTS AND DISCUSSION

Effect of ethanol on P(3HB) production

To understand the behavior of PHA synthases in response to ethanol, P(3HB) production by $PhaC_{Re}$, $PhaC_{Ac}$ and $PhaC_{He}$ was compared over a range of ethanol supplementation concentrations ($0–5\,g\,l^{-1}$) during culture. To minimize the effect of host-produced ethanol, *E. coli* XL1-Blue was employed as a low ethanol producer.[5] The results obtained are shown in Tables 1–3.

For the $PhaC_{Re}$-expressing strain, P(3HB) accumulation was over 80 wt% with ethanol supplementation up to $5\,g\,l^{-1}$, showing no effect on the production of P(3HB) (Table 1). On the other hand, both $PhaC_{Ac}$- and $PhaC_{He}$-expressing strains showed decreased P(3HB) accumulation with ethanol supplementation (Tables 2 and 3). The two strains showed somewhat different behaviors. The $PhaC_{Ac}$-expressing strain exhibited a greater decrease in P(3HB) accumulation (from 26 wt% to 17 wt%) than that seen in the $PhaC_{He}$-expressing strain (from 42 wt% to 38 wt%), particularly at low ethanol supplementation ($0.5\,g\,l^{-1}$).

Effect of ethanol on P(3HB) molecular weight

The molecular weight of purified P(3HB) was determined by GPC, and the values are given in Tables 1–3. The ethanol dependency of M_n is shown in Figure 1. In all the strains tested, P(3HB) molecular weights decreased with increasing ethanol supplementation. For the $PhaC_{Re}$-expressing strain, the molecular weight considerably decreased from $1.37\times10^6\,g\,mol^{-1}$ to $0.63\times10^6\,g\,mol^{-1}$ (a 54% decrease) on increasing the ethanol supplementation from 0 to $5\,g\,l^{-1}$.

For the $PhaC_{Ac}$-expressing strain, ethanol supplementation at $0.5\,g\,l^{-1}$ resulted in a remarkable reduction of 67% in M_n. It further decreased to 82% at $5\,g\,l^{-1}$ ethanol supplementation. This indicates that $PhaC_{Ac}$ is highly sensitive to the effect of ethanol, which has not been recognized previously. This high sensitivity could result in $PhaC_{Ac}$ behaving differently from other PHA synthases. As mentioned earlier, $PhaC_{Ac}$-expressing *E. coli* JM109 produced relatively low molecular weight P(3HB) and showed only a slight decrease in molecular weight on polyethylene glycol supplementation.[7] Taking the sensitivity to ethanol into consideration, it is thought that the molecular weight decrease was already induced by host-produced ethanol being favored over polyethylene glycol.

The host strain used here is XL1-Blue, which is a low ethanol producer, thereby enabling P(3HB) production by $PhaC_{Ac}$ with higher molecular weight ($M_n = 1.26\times10^6\,g\,mol^{-1}$) than other strains ($M_n = 0.38–0.64\times10^6\,g\,mol^{-1}$)[7,9] in the absence of ethanol. $PhaC_{Ac}$

Table 1 Effect of ethanol on the molecular weight of P(3HB) produced in recombinant *E. coli* XL1-Blue expressing PHA synthase from *R. eutropha* ($PhaC_{Re}$)

			Molecular weight (GPC)		
Ethanol ($g\,l^{-1}$)	*Dry cell wt. ($g\,l^{-1}$)*	*P(3HB) content (wt%)*	*M_n ($\times10^6\,g\,mol^{-1}$)*	*M_w/M_n*	*M_n reduction (%)*
0	7.74±0.21	85±4	1.37±0.19	1.8	0
0.5	7.63±0.18	81±3	1.04±0.10	1.8	24
1	8.10±0.04	83±2	0.89±0.10	1.9	35
2	7.86±0.17	84±2	0.80±0.07	1.9	42
5	7.84±0.52	80±3	0.63±0.02	1.8	54

Abbreviations: GPC, gel permeation chromatography; P(3HB), poly[(*R*)-3-hydroxybutyrate]; PHA, polyhydroxyalkanoate.
Cells were cultured in LB medium with glucose ($20\,g\,l^{-1}$), carbenicillin ($50\,mg\,l^{-1}$) and ethanol ($0–5\,g\,l^{-1}$) at 37 °C for 72 h. The results are the averages±standard errors from three independent experiments.

Table 2 Effect of ethanol on the molecular weight of P(3HB) produced in recombinant *E. coli* XL1-Blue expressing PHA synthase from *A. caviae* ($PhaC_{Ac}$)

			Molecular weight (GPC)		
Ethanol ($g\,l^{-1}$)	*Dry cell wt. ($g\,l^{-1}$)*	*P(3HB) content (wt%)*	*M_n ($\times10^6\,g\,mol^{-1}$)*	*M_w/M_n*	*M_n reduction (%)*
0	1.27±0.10	26±3	1.26±0.08	2.5	0
0.5	1.12±0.05	17±1	0.41±0.11	3.7	67
1	1.15±0.08	14±2	0.29±0.05	4.0	77
2	1.09±0.04	11±1	0.27±0.01	3.1	79
5	1.13±0.03	8±1	0.23±0.02	2.8	82

Abbreviations: GPC, gel permeation chromatography; P(3HB), poly[(*R*)-3-hydroxybutyrate]; PHA, polyhydroxyalkanoate.
Cells were cultured in LB medium with glucose ($20\,g\,l^{-1}$), carbenicillin ($50\,mg\,l^{-1}$) and ethanol ($0–5\,g\,l^{-1}$) at 37 °C for 72 h. The results are the averages±standard errors from three independent experiments.

Table 3 Effect of ethanol on the molecular weight of P(3HB) produced in recombinant *E. coli* XL1-Blue expressing PHA synthase form *H. elongata* ($PhaC_{He}$)

			Molecular weight (GPC)		
Ethanol (g l^{-1})	*Dry cell wt. (g l^{-1})*	*P(3HB) content (wt%)*	M_n ($\times 10^6$ g mol^{-1})	M_w/M_n	M_n *reduction (%)*
0	2.76±0.20	42±2	5.54±0.33	1.6	0
0.5	1.94±0.13	38±2	3.69±0.25	1.7	33
1	1.68±0.11	29±1	2.61±0.21	1.5	53
2	1.50±0.12	30±3	2.68±0.15	1.5	52
5	1.06±0.07	14±1	2.24±0.31	1.4	60

Abbreviations: GPC, gel permeation chromatography; P(3HB), poly[(*R*)-3-hydroxybutyrate]; PHA, polyhydroxyalkanoate.
Cells were cultured in LB medium with glucose (20 g l^{-1}), carbenicillin (50 mg l^{-1}) and ethanol (0–5 g l^{-1}) at 37 °C for 72 h. The results are the averages ± standard errors from three independent experiments.

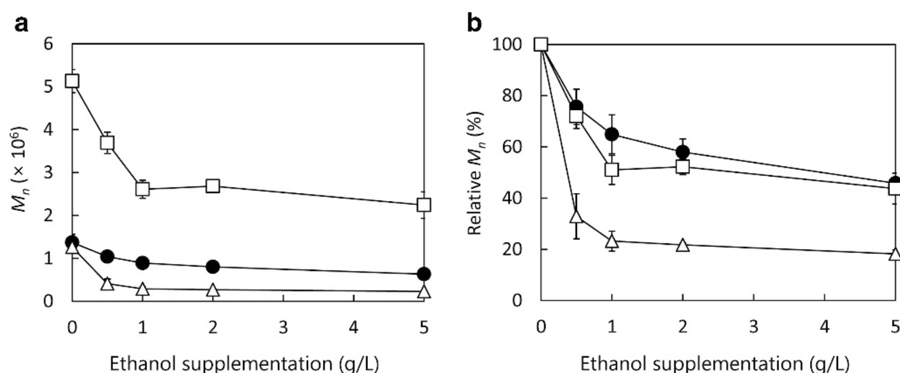

Figure 1 Change in the molecular weight of poly[(*R*)-3-hydroxybutyrate] (P(3HB)) in response to ethanol supplementation. (**a**) Number-average molecular weight (M_n) determined by GPC. (**b**) M_n relative to that without ethanol supplementation. Closed circle: P(3HB) from XL1-Blue expressing *R. eutropha* polyhydroxyalkanoate (PHA) synthase ($PhaC_{Re}$); open triangle: P(3HB) from XL1-Blue expressing *A. caviae* PHA synthase ($PhaC_{Ac}$); open square: P(3HB) from XL1-Blue expressing *H. elongata* PHA synthase ($PhaC_{He}$).

is one of the PHA synthases producing high-performance PHAs such as P(3HB-*co*-3-hydroxyhexanoate) and P(3HB-*co*-3-hydroxy-4-methylvalerate).[12] Our findings may be useful in regulating the molecular weight of these PHA copolymers.

P(3HB) produced by $PhaC_{Ac}$ showed a larger molecular weight distribution (M_w/M_n) than those from other PhaCs. Furthermore, ethanol supplementation increased the M_w/M_n values from 2.5 to 2.8–4.0. The detailed mechanism is not clear, but it seems that the random termination of P(3HB) polymerization was increased by the presence of ethanol.

For the $PhaC_{He}$-expressing strain, the M_n reduction was almost the same as that observed for the $PhaC_{Re}$-expressing strain up to 5 g l^{-1} ethanol. However, P(3HB) molecular weights remained as high as 2.24–3.69 × 10^6 g mol^{-1}, even in the presence of ethanol. These P(3HB)s were further analyzed by MALLS as discussed below.

Molecular weight analysis by MALLS

GPC is a convenient method for evaluating molecular weight of given polymers. However, it is difficult to determine the exact molecular weights of high molecular weight polymers by conventional GPC than by MALLS. High molecular weight polymers often increase the eluent pressure of GPC due to their high viscosity, thereby influencing elution profile. Additionally, GPC analysis provides molecular weights relative to those of standard materials. As demonstrated for P(3HB) by Kusaka *et al.*,[13] the relative molecular weight determined by GPC ($M_{w(\mathrm{GPC})}$) tends to be higher than the absolute molecular weight determined by MALLS ($M_{w(\mathrm{MALLS})}$).

To determine the absolute average molecular weight of P(3HB) produced by $PhaC_{He}$, GPC-MALLS analysis was performed. The molecular weight determined by GPC-MALLS was as high as 8.0 × 10^6 g mol^{-1} in $M_{w(\mathrm{MALLS})}$ (Figure 2). Additionally, on comparison with P(3HB) produced by $PhaC_{Re}$ ($M_{w(\mathrm{MALLS})} = 2.1 \times 10^6$) and $PhaC_{Ac}$ ($M_{w(\mathrm{MALLS})} = 3.1 \times 10^6$), MALLS analysis provided convincing evidence that $PhaC_{He}$ has the potential to produce very high molecular weight P(3HB).

Ethanol production levels of strain XL1-Blue

For cultures of strain XL1-Blue not supplemented with ethanol, host-produced ethanol was assayed. Ethanol production at 24 h was 43, 24 and 31 mg l^{-1} for the $PhaC_{Re}$, $PhaC_{Ac}$ and $PhaC_{He}$-expressing strains, respectively; these levels of production were maintained until 72 h of cultivation (data not shown). These ethanol production levels of strain XL1-Blue were equal to that of the alcohol dehydrogenase gene deletion strain *E. coli* BW25113 *ΔadhE*,[5] suggesting that the ethanol-producing ability of strain XL1-Blue is quite low. In this study, by using strain XL1-Blue, the effect of host-produced ethanol on the molecular weight of PHAs was minimized. In addition, ethanol production by strain XL1-Blue was kept at very low levels regardless of the different PHA synthases expressed. In the case of $PhaC_{He}$-expressing XL1-Blue, particularly high molecular weight polymers ($M_{w(\mathrm{MALLS})} = 8.0 \times 10^6$ g mol^{-1}) were produced. These results showed

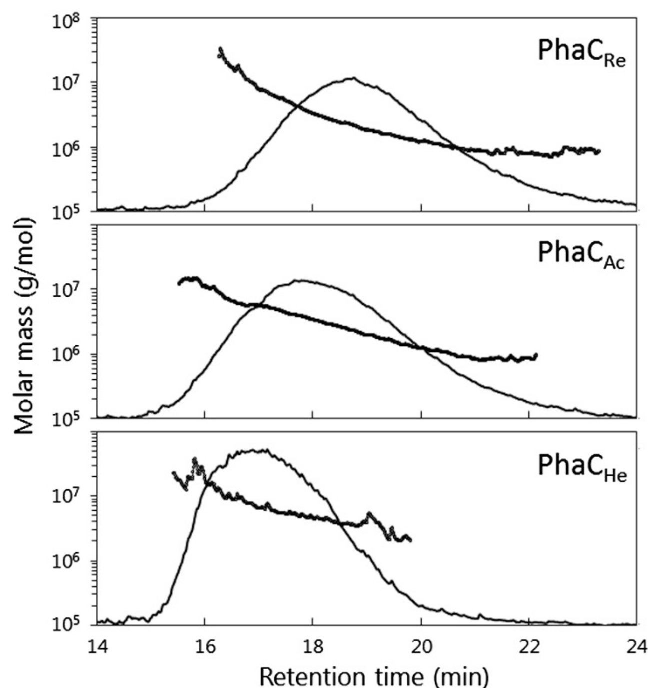

Figure 2 Plot of molar mass measured using multi-angle laser light scattering (MALLS) vs retention time of poly[(*R*)-3-hydroxybutyrate] (P(3HB)) samples from XL1-Blue expressing *R. eutropha* PHA synthase (PhaC$_{Re}$), *A. caviae* polyhydroxyalkanoate (PHA) synthase (PhaC$_{Ac}$) and *H. elongata* PHA synthase (PhaC$_{He}$). The refractive index detector (RID) signals are plotted as an overlay.

that the combination of low ethanol-producing host and PHA synthase with the ability to produce high molecular weight polymer leads to the production of high molecular weight PHA.[5,14,15]

CONCLUSIONS

E. coli is a non-native PHA producer but is an attractive alternative host for PHA production via genetic engineering. During PHA biosynthesis by *E. coli*, host-produced ethanol affects the molecular weight of PHAs due to chain termination of PhaCs. In this study, *E. coli* XL1-Blue expressing PhaC$_{Re}$, PhaC$_{Ac}$ or PhaC$_{He}$ was cultured to compare the molecular weight of P(3HB) in response to ethanol levels present in the culture broth (0–5 g l^{-1}). In all cases, the molecular weights decreased with increasing ethanol supplementation. However, at low ethanol concentration (0.5 g l^{-1}), PhaC$_{Ac}$ showed different behavior from the two other PhaCs. The molecular weight of P(3HB) decreased remarkably for the PhaC$_{Ac}$-expressing strain, but this decrease was not severe for other strains. This indicates that PhaC$_{Ac}$ might be more sensitive than other PhaCs to the effect of ethanol. On the other hand, PhaC$_{He}$ tended to produce higher molecular weight P(3HB) than other PhaCs, even though ethanol was present. These P(3HB)s were further characterized by MALLS, providing additional support for the molecular weight of *E. coli*-produced P(3HB)s. Strain XL1-Blue produced very small amounts of ethanol regardless of the PhaCs expressed. These findings will be useful in the regulation of the molecular weight of PHAs during biosynthesis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was partially supported by funding from JST, CREST.

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