**Enhanced cellular content and lactate fraction of the
P(lactate-co-3-hydroxybutyrate) polyester produced in recombinant
Escherichia coli by the deletion of σ factor RpoN**

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Abstract

A new approach at a transcription level was applied to lactate-based polyester production. Four σ factor disruptants, $\Delta rpoN$, $\Delta rpoS$, $\Delta fliA$ and $\Delta fecI$, of *Escherichia coli* were used as hosts for P(lactate-co-3-hydroxybutyrate) production from glucose. Among them, $\Delta rpoN$ caused dual positive effects of polymer production, enhanced cellular content and lactate fraction.

Keywords: polyhydroxyalkanoate, polyhydroxybutyrate, poly(lactic acid), biobased plastic

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To date, biosynthesis of bacterial polyesters polyhydroxyalkanoates (PHAs) have been improved by various conventional approaches such as gene dosage effects (1, 2), pathway engineering (3, 4) and enzyme engineering (5-8) of the PHA synthesis-related targets. In this study, a new approach was applied to this purpose based on the disruption of σ factors that globally govern the transcription of corresponding genes. This approach was aimed at giving the indirect positive effect on beneficial performances, increased production and altered monomer composition of the polymers of interest.

In our first trial was conducted towards a new type of lactate (LA)-based polymer (9) as a target. As a representative polymer, P[LA-*co*-3-hydroxybutyrate (3HB)], has been synthesized by microbial system carrying an LA-polymerizing enzyme (LPE) (9). An interesting feature of the copolymer over PLA is that the variation of the LA/3HB ratio in P(LA-*co*-3HB) has demonstrated the generation of polymers with different properties. P(LA-*co*-3HB) was processed into a semitransparent and flexible plastic material depending on the LA fraction that differed from poly(lactic acid) and P(3HB) homopolymers with rigid property (10), and thus, the copolymer has a potential to be used in the wider range of applications. The LA fraction has been controlled in the range of 0 to nearly 100 mol%, but the copolymers with >60 mol% LA was obtained relatively low productivity. Therefore, flexible regulation methodology of the lactate fraction is required for the case of P(LA-*co*-3HB).

We first attempted to examine the disruption effects of σ factors on the biosynthesis of P(LA-*co*-3HB) in terms of polymer production and LA fraction. It has been reported that *E. coli* possesses four non-essential σ factors, RpoS, RpoN, FliA

and FecI. The disruptions of the σ factors expectedly reduce or enhance the expression levels of broad range of genes (11).

The σ factor disrupted strains of *E. coli*, $\Delta rpoS$, $\Delta rpoN$, $\Delta fliA$ and $\Delta fecI$, were obtained from Keio collection (12). The parent strain *E. coli* BW25113 was used as a control. The cells were transformed using pTV118NpctCI(STQK)AB bearing propionyl-CoA transferase (PCT), *phaCI* (STQK), *phaA* and *phaB* genes. Pre-cultured samples were prepared using LB medium (2ml) at 30 °C for 12 h with reciprocal shaking at 180 rpm. The main cultivation was performed using the LB medium (1.7 ml) supplemented with 2% glucose and 10 mM calcium pantothenate at 30 °C for 48 h with reciprocal shaking at 180 rpm. The antibiotics (100 mg/l ampicillin, 25 mg/l kanamycin and 25 mg/l chloramphenicol) were added when needed. The polymer content and composition were determined using HPLC as described (13). The lactic acid concentration in medium was measured by capillary electrophoresis (Agilent 7100: Agilent Technologies) using running buffer α -AFQ109 (Otsuka Electronics CO. LTD). The data shown are averages $\pm$ standard deviations of three independent trials.

The four disruptants and parent harboring pTV118NpctCI(STQK)AB were cultured for P(LA-co-3HB) production. As shown in Table 1, among the disruptants tested, $\Delta rpoN$ exhibited the remarkably enhanced polymer production from 5.3 g/l (cellular content, 58.3 wt%) to 6.2 g/l (75.1 wt%). The accumulation of 3HB units in the copolymer was not significantly changed. Thus, the increase in polymer production was mostly due to the increase in LA units. Consequently, the LA fraction in the copolymer was also increased up to 26.2 mol% from 18.6 mol%. The $\Delta fliA$ disruption slightly decreased the polymer production. The $\Delta fecI$ increased the polymer production, but the LA fraction was drastically decreased. The $\Delta rpoS$ increased polymer production without changing LA fraction. Overall, the $\Delta rpoN$ mutation was found to

be the most beneficial for enhancing both of polymer content and LA fraction, and thus the strain was subjected to detailed analyses.

In the next, the complement experiment of the *rpoN* gene was performed. The plasmid pCA24N-*rpoN* harboring the *rpoN* gene, which is obtained from ASKA clone (JW3169) (14), was introduced into $\Delta rpoN$ strain. The complemented cell produced P(LA-co-3HB) with the similar content and LA fraction compared to the parent strain (Table 1). Thus, it was concluded that the *rpoN* disruption contributed to the increase in the polymer content and LA fraction.

In order to monitor the detailed behavior of the *rpoN* disruptant, the time course profile of the glucose consumption and polymer production was analyzed (Figure 1). At 12 h, the parent strain consumed slightly greater amount of glucose and production more polymer than $\Delta rpoN$ strain. At 18 h however, both strains consumed almost the same amount of glucose and accumulated comparable level of polymer. Here, it should be noted that LA fraction in the copolymer was higher for $\Delta rpoN$ strain at the time period. Thus, the accumulation of LA units in $\Delta rpoN$ strain was promoted at early stage of cultivation. In the middle and late stages of cultivation, eventually the accumulated the comparable amount of 3HB units to the parent. The other point, the production of lactic acid in $\Delta rpoN$ strain is higher than the parent strain. Therefore, the $\Delta rpoN$ strain was increased LA fraction in the polymer. Overall, the $\Delta rpoN$ strain achieved the higher carbon yield ($Y_{pol/C} = 0.31$ g/g) than the parent (0.26 g/g).

Considering the mechanism for the enhanced synthesis of the P(LA-co-3HB), the expression levels of all the polymer biosynthetic enzymes might contribute to the effect. To examine this possibility, the expression levels of polymer biosynthesis enzymes (PCT, PhaA and PhaB) and LDH were compared $\Delta rpoN$ with parent strain by immunoblot analysis. The crude extract was prepared from the cells (15). The antibody

to LDH was developed using His-tagged protein expressed in *E. coli*. Other antibodies were used, as carried out in the previous works (1). Total protein concentration was normalized using Bradford's method and confirmed by SDS-PAGE (Figure 2-A). As the result, the expression level of all enzymes at $\Delta rpoN$ strain was not increased compared to the wild type (Figure 2-B). Therefore, the enhanced production of P(LA-co-3HB) in the $\Delta rpoN$ strain was not caused by the increased expression levels of the polymer biosynthesis enzymes and LDH.

To increase the production of P(LA-co-3HB), many approaches were devised in the past experiments (1-8). These strategies were designed to directly reinforce PHA production. On the other hand, the effect of $\Delta rpoN$ was contributed to the indirect positive effects towards the P(LA-co-3HB) production. These two different approaches would give us synergetic effects in improving the biosynthetic system for the polymer production. As another study, overexpression of the σ factor SigE in *Synechocystis* sp. PCC 6803 increased the production of 3HB homopolymer during nitrogen starvation (16). These repressed expression and accelerated expression of the genes governed by the factors of interest would change the carbon fluxes directly and/or indirectly related to the polymer biosynthesis.

At the present, the relationship between the RpoN deletion and its beneficial effects on the P(LA-co-3HB) production remains to be elucidated. RpoN is known to regulate at least 120 genes in *E. coli* genome, and the RpoN-encoding gene deletion expectedly reduced the expression levels of these genes (17). Therefore, the enhanced polymer production by $\Delta rpoN$ should be attributed to the individual RpoN-regulated genes and/or their combinations. The details would be explored in future work.

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Figure captions

Figure 1. Time course profiles of carbon amount for glucose (A & B), lactic acid (C & D) and the P(LA-co-3HB) produced (E & F) in *E. coli* BW25113 and $\Delta rpoN$ (JW3169). Cells harboring pTV118NpctCI(STQK)AB was grown on 1.7 ml media containing 20 g/l glucose at 30 °C for 48 h with reciprocal shaking at 180 rpm. The data are the average $\pm$ standard deviation of three independent trials. Gray, 3HB units in the polymers; white, LA units in the polymers.

Figure 2. Western blotting analysis of the polymer synthetic enzymes and LDH. Total protein was extracted from the BW25113 (wild type) and $\Delta rpoN$ mutant grown in LB containing 2% glucose and 10 mM calcium pantothenate liquid culture.

(A) Total protein concentration was normalized by SDS-PAGE. MM: molecular marker.

(B) The expression levels of polymer biosynthesis enzymes (PCT, PhaA and PhaB) and LDH were compared $\Delta rpoN$ with parent strain by immunoblot analysis.

Figure 1

Figure 2

Table 1. P(LA-co-3HB) production by *E. coli* BW25113 and σ factor deleted strains of Keio collection.

Strain	Genotype	Specific growth rate (h ⁻¹)	Cell dry weight (g/l)	Polymer production (g/l)			Polymer content (wt%)	LA fraction (mol%)
				Total	LA	3HB		
BW25113	WT	1.4 ± 0.2	9.1 ± 0.4	5.3 ± 0.2	0.9 ± 0.0	4.5 ± 0.2	58.3 ± 2.2	18.6 ± 0.9
JW1907	$\Delta fliA$	1.2 ± 0.1	8.6 ± 1.3	4.9 ± 0.6	0.8 ± 0.1	4.2 ± 0.7	57.2 ± 2.0	18.4 ± 4.2
JW3169	$\Delta rpoN$	1.2 ± 0.1	8.2 ± 0.8	6.2 ± 0.4	1.4 ± 0.1	4.8 ± 0.4	75.1 ± 3.3	26.2 ± 2.5
JW4253	$\Delta fecI$	1.4 ± 0.1	10.7 ± 0.3	5.7 ± 0.2	0.2 ± 0.1	5.6 ± 0.2	53.5 ± 2.8	3.2 ± 1.4
JW5437	$\Delta rpoS$	1.1 ± 0.1	10.0 ± 0.1	5.8 ± 0.1	0.6 ± 0.1	5.2 ± 0.0	57.6 ± 0.9	12.3 ± 1.4
JW3169 / pCA24N ^a	$\Delta rpoN$ / <i>Plac</i>	1.1 ± 0.1	8.0 ± 0.2	6.2 ± 0.3	1.3 ± 0.1	4.9 ± 0.2	77.7 ± 2.2	24.4 ± 1.2
JW3169 / pCA24N- <i>rpoN</i> ^b	$\Delta rpoN$ / <i>Plac::rpoN</i>	1.1 ± 0.1	7.2 ± 0.5	5.0 ± 0.1	0.7 ± 0.1	4.3 ± 0.0	70.0 ± 4.3	15.8 ± 1.4

Each σ factor deletion mutants were grown on 1.7 ml LB medium containing 20 g/l of glucose at 30 °C for 48 h with reciprocal shaking at 180 rpm. The data are the average ± standard deviation of three independent trials.

pCA24N: empty vector of ASKA clone.

JW3169 / pCA24N: empty vector cloned in JW3169 (*rpoN* deletion strain).

pCA24N-*rpoN*: *rpoN* gene was cloned downstream *Plac* in pCA24N.

JW3169 / pCA24N-*rpoN*: *rpoN* overexpression plasmid was cloned in JW3169 (*rpoN* deletion strain).

^{a, b} : 100 mM IPTG added