

Indirect positive effects of a sigma factor RpoN deletion on the lactate-based polymer production in *Escherichia coli*

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The production of bacterial polyesters, polyhydroxyalkanoates (PHAs), has been improved by several rational approaches such as overexpression and/or engineering of the enzymes directly related to PHA biosynthetic pathways. In this study, a new approach at transcription level has been applied to a new category of the copolymer of lactate (LA) and 3-hydroxybutyrate (3HB), P(LA-co-3HB). When the 4 disrupting mutants of sigma factors in *Escherichia coli*, *rpoN*, *rpoS*, *fliA*, *fecI*, were used as platforms for production of P(LA-co-3HB), increases in the production level and LA fraction of the copolymer were observed for the mutant strain with *rpoN* disruption. These positive impacts on the polymer production were caused in an “indirect manner” via changes in the multiple genes governed by RpoN. A genome-wide engineering by sigma factors would be a versatile approach for the production of value-added products of interest and available for combination with the other beneficial tools.

Polyhydroxyalkanoate (PHA) is an attractive material that can be developed as a bio-based, biodegradable and biocompatible plastic applicable for environmental and medical applications.¹ Compared to the fermentation management of natural PHA producers, PHA can be more efficiently synthesized in the recombinant microbial system by installing biosynthetic pathways for the production of both natural and tailor-made PHAs.² The material properties of PHA should be governed by its monomeric

composition, molecular weight and copolymer microstructure.³

The poly[lactate (LA)-co-3-hydroxybutyrate (3HB)], a new type of LA-based polymer, was synthesized by a microbial system carrying an LA-polymerizing enzyme (LPE).^{4,5} 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. The increase of P(LA-co-3HB) production level and the control of LA fraction in copolymer is very important problem.

The system of bioplastics production using microorganisms has been evolved through the following process. First, researchers explored the optimization of cultivation conditions of bioplastic natural producing strains (first generation).^{6,7} Subsequently, the productivity of bioplastic was more increased by using a recombinant *Escherichia coli* system (second generation).^{8,9} Furthermore, the bioplastic production is enhanced by applying metabolic engineering and molecular alteration (enzyme evolution engineering) of the enzymes involved in the bioplastics production (third generation).¹⁰

So far, the P(LA-co-3HB) production was carried out by introducing bioplastics production enzymes in various *E. coli* species. However, the production level and the LA fraction of P(LA-co-3HB) varied depending on the species. This is most likely because of the difference of small genotype of various *E. coli* strain. In other words, it is likely that minor differences in the genes encoded by the *E. coli* genome have some effect on P(LA-co-3HB) production and LA fraction. These results show that the genes encoded by the *E. coli*

genome, individually or in group, are involved in P(LA-co-3HB) productivity and LA fraction.

In the present study, we focused on the sigma factors that globally control the transcription of genes. It has been reported that *E. coli* possesses 4 non-essential sigma factors, RpoN, RpoS, FliA and Fecl.¹¹⁻¹³ RpoS is induced at stationary phase,¹⁴ while RpoN is activated under the nitrogen starvation conditions.¹⁵ The function of FliA is the transcriptional regulator of flagellar and chemotaxis genes.¹⁶ Fecl is recognized as a member of the extracytoplasmic function subfamily of sigma factor.¹⁷ The disruption of these sigma factors was expectedly found to reduce or enhance the expression levels of a broad range of genes.¹³ Our plan is to observe the effects on the deletion of the sigma factor at P(LA-co-3HB) biosynthesis.

Result and Discussion

The sigma factor disrupted strains of *E. coli*, $\Delta rpoS$, $\Delta rpoN$, $\Delta fliA$ and $\Delta fecI$, were obtained from the Keio collection and used in this study.¹⁸ The *E. coli* BW25113 was used as the parent strain. A plasmid pTV118N $pctC1$ (STQK)AB which contains the P(LA-co-3HB) biosynthesis genes encoding propionyl-CoA transferase (PCT), *phaC1* (STQK), *phaA* and *phaB* was introduced into these mutant strains. LB medium containing 2% glucose and 10 mM calcium pantothenate was used for P(LA-co-3HB) production. Cultivation was performed at 30°C for 48 hours with shaking at 180 rpm.¹⁹

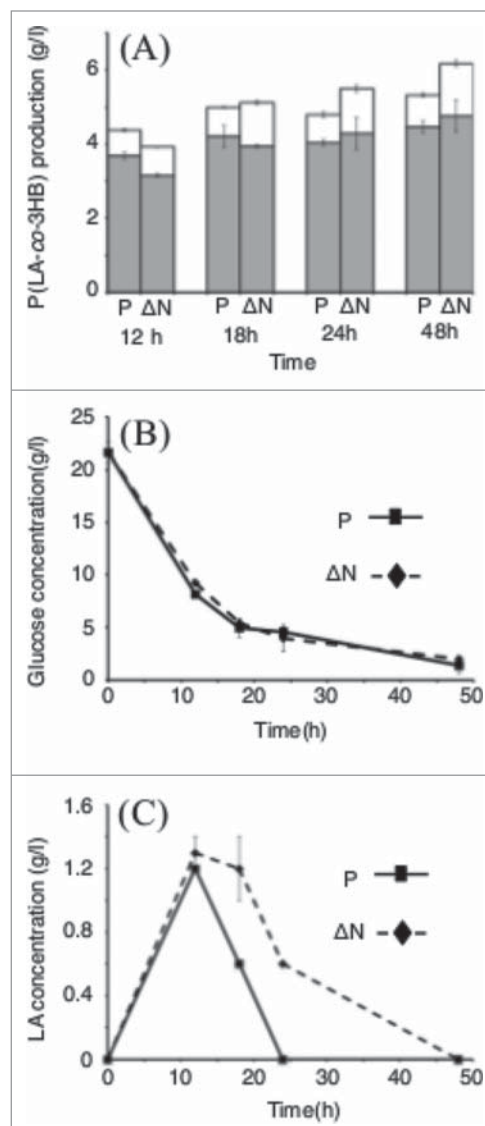


Figure 1. Time course profiles of the P(LA-co-3HB) production (A), amount for glucose (B) and lactic acid (C) in *E. coli* BW25113 (parent strain) and $\Delta rpoN$ (JW3169), respectively. (A) Gray, 3HB units in the copolymers; white, LA units in the copolymers. P: BW25113 (parent strain) and N: $\Delta rpoN$ (JW3169). (B) and (C) square and solid line, BW25113; diamond and broken line, $\Delta rpoN$ (JW3169).

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

Strain	Genotype	Cell dry weight (g/l)	Polymer production (g/l)			Polymer content (wt%)	LA fraction (mol%)
			Total	LA	3HB		
BW25113	Parent	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	$\Delta fliA$	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	$\Delta rpoN$	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	$\Delta fecI$	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	$\Delta rpoS$	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	$\Delta rpoN$ / Plac	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> ^a	$\Delta rpoN$ / Plac:: <i>rpoN</i>	7.2 ± 0.5	5.0 ± 0.1	0.7 ± 0.1	4.3 ± 0.0	70.0 ± 4.3	15.8 ± 1.4

pCA24N: empty vector of ASKA clone.

JW3169 / pCA24N: empty vector introduced into JW3169 (*rpoN* deletion strain).

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

JW3169 / pCA24N-*rpoN*: *rpoN* overexpression plasmid introduced into JW3169 (*rpoN* deletion strain).

^a100 μM of IPTG added.

Table 1 shows the production of P(LA-co-3HB) in the 4 sigma factor disrupted strains. In terms of the yield and LA fraction of P(LA-co-3HB), varied values were observed among the 4 mutant strains. It should be noted that the $\Delta rpoN$ showed the increases in both the yield and LA fraction of P(LA-co-3HB). The two mutants, the $\Delta rpoS$ and $\Delta fecI$ showed the increased P(LA-co-3HB) yield, although the LA fraction was decreased.

To clarify the effect of $rpoN$ disruption on P(LA-co-3HB) production, complementary experiment was performed. An expression vector of $rpoN$, pCA24N- $rpoN$ which was extracted from JW3169 of ASKA clone collection,²⁰ was introduced into the $\Delta rpoN$ strain. The P(LA-co-3HB) production level of the strain complemented with pCA24N- $rpoN$ showed almost the same with that of the parent strain (**Table 1**). This result indicated the positive effect on P(LA-co-3HB) production by the $rpoN$ disruption.

Next, we measured the time course profile of the glucose consumption and P(LA-co-3HB) production. As shown in **Figure 1**, an early stage of the cultivation at 12 h, the parent strain showed the higher P(LA-co-3HB) production than the $\Delta rpoN$ strain. At 18 h, both strain produced comparable level of P(LA-co-3HB) (**Fig. 1A**). Here, it should be noted that the LA fraction in P(LA-co-3HB) was higher than that of the parent strain. The accumulation of the LA unit into P(LA-co-3HB) was accelerated during the early stage of cultivation, while the accumulation of 3HB unit of P(LA-co-3HB) was increased rather than middle to late stage of cultivation. This P(LA-co-3HB) production profile was clearly different from the both strains. After 48 hours cultivation, glucose consumption was same level in both strains (**Fig. 1B**). The carbon yield (Y_{pol/C}) in P(LA-co-3HB) synthesis from glucose was calculated to be 0.31 g/g for the $\Delta rpoN$ strain which is higher than that for the parent strain 0.26 g/g. The production of lactic acid concentration in the medium in the $\Delta rpoN$ strain was higher than that of the parent strain (**Fig. 1C**). The increased LA unit in P(LA-co-3HB) seemed to be correlated to the lactic acid production level.

To interpret the increase in lactic acid production by $rpoN$ deletion, we searched a direct link between $rpoN$ and lactic acid production. RpoN was proposed to regulated 74 genes based on the transcriptional assay of $rpoN$ deleted and overexpressed strains.²¹ In addition, we added 45 genes, which are regulated in the same operon of the 74 genes based on the operon database “DOOR2”, to the candidates.²² As the result, no gene was found to be related to lactic acid production according to the COGs category (<http://www.ncbi.nlm.nih.gov/COG/>) (**Table 2**) and KEGG pathway (<http://www.genome.jp/>). Therefore, $rpoN$ deletion presumably influenced lactic acid production in an indirect manner.

In order to study the change of expression level of enzymes involved in bioplastic synthesis in the $rpoN$ strain, intracellular protein levels of PCT, PhaA, PhaB and lactate dehydrogenase (LDH) in P(LA-co-3HB) production stage at 18 h were compared between the $\Delta rpoN$ strain and the parent strain by immunoblotting. The expression levels of these proteins were comparable in both strains (**Fig. 2**). These results indicated that the increased production level and LA fraction of the P(LA-co-3HB) in

the $\Delta rpoN$ strain did not depend on the expression level of P(LA-co-3HB) biosynthetic enzymes.

In other experiment, the overexpression of sigma factor SigE in *Synechocystis* sp. PCC 6803 is known to increase the production of P(3HB) under the condition of nitrogen starvation.²³ The *sigE* overexpression elevates the levels of proteins implicated in glycogen catabolism, the oxidative pentose phosphate pathway, and polyhydroxybutyrate (PHB) biosynthesis. We think that the change of the transcriptional network by disruption of sigma factor RpoN has given a positive indirect effect to the polymer production. The alterations in the expression of the genes governed by the RpoN seem to directly and/or indirectly modulate the carbon fluxes related to the polymer biosynthesis.

In our previous studies, xylose was found to contribute to the increased LA fraction compared to glucose as a carbon source.^{19,24} Therefore, in this study we attempted to examine the synergistic effect of xylose utilization also on the present mutant strain carrying $\Delta rpoN$. The LA fraction of P(LA-co-3HB) in the $\Delta rpoN$ strain using xylose as a carbon source was 33.9 mol% (**Table 3**).

Table 2. The number of genes controlled by RpoN

COG category	Gene number
C: Energy production and conversion	24 / 286
E: Amino acid transport and metabolism	23 / 362
F: Nucleotide transport and metabolism	2 / 98
G: Carbohydrate transport and metabolism	5 / 378
H: Coenzyme metabolism	1 / 157
I: Lipid metabolism	4 / 100
J: Translation, ribosomal structure and biogenesis	1 / 185
K: Transcription	8 / 311
L: DNA replication, recombination and repair	1 / 234
M: Cell envelope biogenesis, outer membrane	1 / 236
N: Cell motility and secretion	4 / 114
O: Posttranslational modification, protein turnover, chaperones	11 / 139
P: Inorganic Ion Transport and Metabolism	11 / 221
Q: Secondary metabolites biosynthesis, transport and catabolism	2 / 64
R: General function prediction only	11 / 408
T: Signal transduction mechanisms	2 / 180
U: Intracellular trafficking and secretion	1 / 130
V: Defense mechanisms	1 / 49
S: Function unknown	5 / 324
No COG assignment	12 / 614

Genes are classified into COG categories. The numbers represent the number of RpoN-controlled genes to the number of genes belonging to each of COG category.

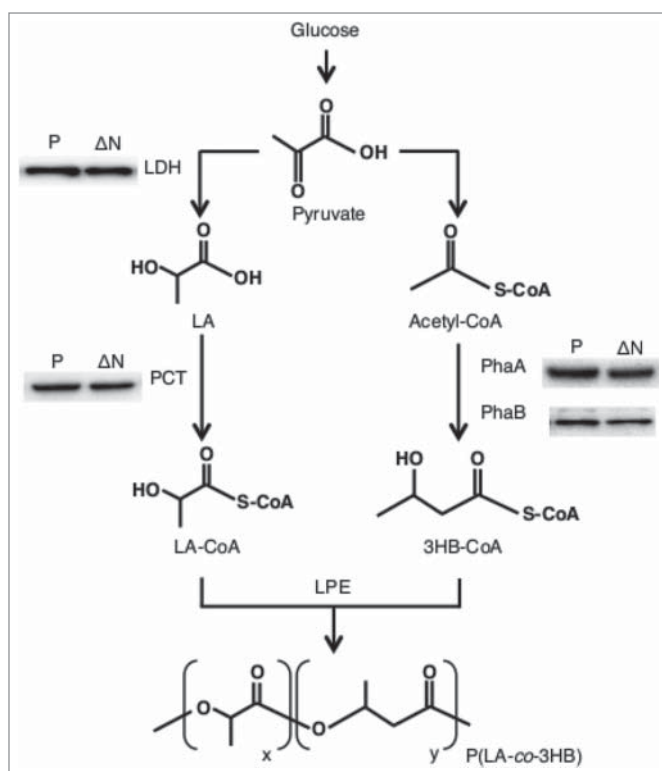


Figure 2. P(LA-co-3HB) synthesis pathway and immunoblot analysis of the polymer synthetic enzymes and LDH. Total protein was extracted from the BW25113 (parent strain) and $\Delta rpoN$ (JW3169) grown on 1.7 ml of LB media. Immunoblot analysis of the polymer biosynthesis enzymes (PCT, PhaA and PhaB) and LDH in the parent (P) and the $\Delta rpoN$ (ΔN) strain.

This value is higher than that of obtained the LA fraction of copolymer using glucose cultivation (26.2 mol%). However, this value was same as the LA fraction of the parent strain using xylose cultivation (35.9 mol%). These results suggested the positive effect on the LA fraction of LA-co-3HB, but the synergistic effect of xylose utilization and *rpoN* disruption was not observed.

Conclusion and Further Perspective

Our experimental results suggested that the disruption of *rpoN* indirectly

contributed to the increased in P(LA-co-3HB) production. The enhanced polymer production and LA fraction by the $\Delta rpoN$ strain may be attributed to the effect of the individual RpoN-regulated genes and/or their combinations. In order to resolve this issue, we plan to check the polymer production using the single gene deletion mutant collection (KEIO collection).

On the other hand, we have discovered that the gene relevant to high production of polymer exists besides *rpoN* disruptant. The deletion of *mtgA* may have some effect on the cell morphology and cell wall construction. Interestingly, the enlargement of the recombinant host cell took place in association with polymer

Table 3. The LA fraction of P(LA-co-3HB) in the $\Delta rpoN$ strain and the parent strain using xylose or glucose as a carbon source

Strain	Genotype	Carbon source	LA fraction of polymer (mol%)
JW3169	$\Delta rpoN$	Glucose	26.2
JW3169	$\Delta rpoN$	Xylose	33.9
BW25113	parent	Glucose	18.6
BW25113	parent	Xylose	35.9

accumulation. This changeable morphological property gives us good advantage in obtaining higher polymer productivity.²⁵ The next issue is to study the possibility of producing more bioplastics by combining the enzyme evolution, metabolic engineering, *mtgA* deletion (cell morphological change) and *rpoN* deletion (to suppress the expression of multiple genes).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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