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**Enhanced cellular content and lactate fraction of the
poly(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 the transcriptional 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 poly(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, σ factor

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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 the corresponding genes was developed. This approach was aimed at achieving indirect positive effects on beneficial performance, increased production, and altered monomer composition of the polymers of interest.

Our first trial was conducted to synthesize a new type of lactate (LA)-based polymer (9) as a target. As a representative polymer, poly[LA-co-3-hydroxybutyrate (3HB)], was synthesized by a microbial system carrying an LA-polymerizing enzyme (LPE) (9). An interesting feature of the copolymer over poly(lactic acid) is that the variation of the LA/3HB ratio in P(LA-co-3HB) facilitates 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. This copolymer differs from PLA and P(3HB) homopolymers, which possess rigid property (10), and can thus, the copolymer has a potential to be used in the wider range of applications. Although the LA fraction was controlled within the range of 0 to nearly 100 mol%, >60 mol% LA copolymers were generated with a relatively low productivity. Therefore, it was essential to regulate the lactate fraction for the efficient synthesis of P(LA-co-3HB).

We first examined the effects of the disruption of the σ factors on the biosynthesis of P(LA-co-3HB) in terms of the polymer production and LA fraction. *Escherichia coli* is known to possess four non-essential σ factors, namely, RpoS, RpoN, FliA and FecI. The disruption of these σ factors was expectedly found to reduce or

enhance the expression levels of a 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 the Keio collection (12). The parent strain *E. coli* BW25113 was used as the control. The cells were transformed using the pTV118NpctC1(STQK)AB plasmid bearing the propionyl-CoA transferase (PCT), *phaC1* (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, ampicillin (100 mg/l), kanamycin (25 mg/l) and chloramphenicol (25 mg/l) were added as required. The polymer content and composition were determined using HPLC as previously described (13). The lactic acid concentration in the medium was measured by capillary electrophoresis (Agilent 7100: Agilent Technologies) using running buffer α -AFQ109 (Otsuka Electronics Co. Ltd). The data are represented as the averages $\pm$ standard deviations of three independent trials.

The four σ factor deletion mutant and the parent strain harboring pTV118NpctC1(STQK)AB were cultured for P(LA-co-3HB) production. As shown in Table 1, compared to the parent strain, $\Delta rpoN$ exhibited the remarkably enhanced polymer production [5.3 g/l (cellular content, 58.3 wt%) vs 6.2 g/l (75.1 wt%), respectively]. However, the accumulation of 3HB units in the copolymer was not significantly changed. Thus, the increase in the polymer production must be due to an increase in LA units. Indeed, the LA fraction in the copolymer was found to increase up to 26.2 mol% (compared to 18.6 mol% in the parent strain). The $\Delta fliA$ strain showed a slight decrease in the polymer production. Although the $\Delta fecI$ strain exhibited increased polymer production, its LA fraction was drastically reduced. The

$\Delta rpoS$ strain showed increased polymer production without any change in the LA fraction. Overall, the $\Delta rpoN$ mutation was found to be the most beneficial, with respect to enhancing both the polymer content and LA fraction; consequently this strain was subjected to further comprehensive analyses.

Next, a complement experiment of the *rpoN* gene was performed. The plasmid pCA24N-*rpoN* harboring the *rpoN* gene obtained from ASKA clone (JW3169) (14), was introduced into the $\Delta rpoN$ strain. The complemented cells produced P(LA-co-3HB) with the content and LA fraction similar to that as the parent strain (Table 1), demonstrating that the *rpoN* disruption contributes to an increase in the polymer content and LA fraction.

In order to monitor the detailed behavior of the *rpoN* deletion mutant, the time course profile of its glucose consumption and polymer production was analyzed (Figure 1). At 12 h, the parent strain consumed moderately greater amount of glucose and produced more polymer than the $\Delta rpoN$ strain. However, at 18 h, both the strains consumed almost the same amount of glucose and accumulated comparable levels of the polymer. It is important to note that the LA fraction in the copolymer was higher for the $\Delta rpoN$ strain at the given time point. Thus, the accumulation of the LA units in the $\Delta rpoN$ strain was promoted during the early stage of cultivation, while in the middle and late stages, the amount of the accumulated 3HB units was comparable to that in the parent strain. In addition, the production of lactic acid in the $\Delta rpoN$ strain was higher than that in the parent strain, leading to the increased LA fraction in the polymer. These results indicated that the $\Delta rpoN$ strain achieved a higher carbon yield ($Y_{pol/C} = 0.31$ g/g) than the parent strain (0.26 g/g).

To examine the possibility that the expression of all the polymer biosynthetic enzymes contributes to the enhanced synthesis of P(LA-co-3HB), the protein levels of

PCT, PhaA, PhaB, and lactate dehydrogenase (LDH) in the $\Delta rpoN$ strain were compared to those in the parent strain by immunoblot analysis. A crude protein extract was prepared from the cells using the previously described methods (15). The antibody to LDH was developed using His-tagged protein expressed in *E. coli*. Other antibodies were prepared in the previous works (1). The total protein concentration was normalized using Bradford's method and confirmed by SDS-PAGE (Fig. 2-A). The expression level of the enzymes in the $\Delta rpoN$ strain was not increased, compared to that in the parent strain (Figure 2-B), indicating that the enhanced production of P(LA-co-3HB) by the $\Delta rpoN$ strain is not caused by the increased expression levels of the polymer biosynthetic enzymes and LDH.

To date, multiple strategies have been used to increase the production of P(LA-co-3HB) (1-8). These strategies were designed to directly reinforce the PHA production. On the other hand, in our study, the effect of the disruption of *rpoN* indirectly contributed to increased P(LA-co-3HB) production. A combination of these two different approaches may provide synergetic effects towards improving the biosynthetic system for polymer production. In addition, the overexpression of σ factor SigE in *Synechocystis* sp. PCC 6803 is known to increase the production of 3HB homopolymers under the condition of nitrogen starvation (16). Such alterations in the expression of the genes governed by the factors of interest seem to directly and/or indirectly modulate the carbon fluxes related to 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 regulates at least 120 genes in the *E. coli* genome, and deletion of the *rpoN* gene is known to reduce their expression levels (17). Therefore, the enhanced polymer production by the $\Delta rpoN$ strain may be attributed to the effect of the individual RpoN-regulated genes

and/or their combinations. To test this, the gene(s) closely related to the beneficial effects should be evaluated using a set of the single gene deletion mutant of the Keio collection (12).

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References

1. **Jo, Sung-Jin., Matsumoto, K., Leong, C. R., Ooi, T., and Taguchi, S.:** Improvement of poly(3-hydroxybutyrate) [P(3HB)] production in *Corynebacterium glutamicum* by codon optimization, point mutation and gene dosage of P(3HB) biosynthetic genes, *J. Biosci. Bioeng.*, 104, 457-463 (2007).

2. **Maehara, A., Ikai, K., Ueda, S., and Yamane, T.:** Gene dosage effects on polyhydroxyalkanoates synthesis from n-alcohols in *Paracoccus denitrificans*. *Biotechnol. Bioeng.*, 60, 61-69 (1998).

3. **Matsumoto, K. and Taguchi, S.:** Enzymatic and whole-cell synthesis of lactate-containing polyesters: toward the complete biological production of polylactate, *Appl. Microbiol. Biotechnol.*, 85, 921-932 (2010).

4. **Aldor, IS. and Keasling, JD.:** Process design for microbial plastic factories: metabolic engineering of polyhydroxyalkanoates, *Curr. Opin. Biotechnol.*, 14, 475-483

(2003).

5. **Yamada, M., Matsumoto, K., Shimizu, K., Uramoto, S., Nakai, T., Shozui, F., and Taguchi, S.:** Adjustable mutations in lactate (LA)-polymerizing enzyme for the microbial production of LA-based polyesters with tailor-made monomer composition, *Biomacromolecules*, 11, 815-819 (2010).

6. **Shozui, F., SUN, J., Song, Y., Yamada, M., Sakai, K., Matsumoto, K. Takase, K and Taguchi, S.:** A new beneficial mutation in *Pseudomonas* sp. 61-3 polyhydroxyalkanoate (PHA) synthase for enhanced cellular content of 3-hydroxybutyrate-based PHA explored using its enzyme homolog as a mutation template, *Biosci. Biotechnol. Biochem.*, 74, 1710-1712 (2010).

7. **Taguchi, S. and Doi, Y.:** Evolution of polyhydroxyalkanoate (PHA) production system by “enzyme evolution”: successful case studies of directed evolution, *Macromol. Biosci.*, 4, 146-156 (2004).

8. **Matsumoto, K., Tanaka, Y., Watanabe, T., Motohashi, R., Ikeda, K., Tobitani, K., Yao, M., Tanaka, I., and Taguchi, S.:** Directed evolution and structural analysis of NADPH-dependent Acetoacetyl Coenzyme A (Acetoacetyl-CoA) reductase from *Ralstonia eutropha* reveals two mutations responsible for enhanced kinetics, *Appl. Environ. Microbiol.*, 79, 6134-6139 (2013).

9. **Taguchi, S., Yamada, M., Matsumoto, K., Tajima, K., Satoh, Y., Munekata, M., Ohno, K., Kohda, K., Shimamura, T., Kambe, H., and Obata, S.:** A microbial

176 factory for lactate-based polyesters using a lactate-polymerizing enzyme, *Proc. Natl.*
177 *Acad. Sci. USA*, 105, 17323-17327 (2008).

178

179 10. **Yamada, M., Matsumoto, K., Uramoto, S., Motohashi, R., Abe, H., and**
180 **Taguchi, S.:** Lactate fraction dependent mechanical properties of semitransparent
181 poly(lactate-co-3-hydroxybutyrate)s produced by control of lactyl-CoA monomer
182 fluxes in recombinant *Escherichia coli*, *J. Biotechnol.* 154: 255–260 (2011).

183

184 11. **Wösten, M. M.:** Eubacterial sigma-factors, *FEMS Microbiol. Rev.*, 22, 127-150
185 (1998).

186

187 12. **Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., and Baba, M.:**
188 Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the
189 Keio collection, *Mol. Syst. Biol.*, 2, (2006).

190

191 13. **Yamada, M., Matsumoto, K., Nakai, T., and Taguchi, S.:** Microbial production
192 of lactate-enriched poly[(R)-lactate-co-(R)-3-hydroxybutyrate] with novel thermal
193 properties, *Biomacromolecules*, 10, 677-681 (2009).

194

195 14. **Kitagawa, M., Ara, T., Arifuzzaman, M., Ioka-Nakamichi, T., Inamoto, E.,**
196 **Toyonaga, H., and Mori, H.:** Complete set of ORF clones of *Escherichia coli* ASKA
197 library (a complete set of *E. coli* K-12 ORF archive): unique resources for biological
198 research, *DNA Res.*, 12, 291-299 (2005).

199

200 15. **Laemmli, U.K.:** Cleavage of structural proteins during the assembly of the head of

bacteriophage T4, *Nature*, 227, 680-685 (1970).

16. **Osanai, T., Numata, K., Oikawa, A., Kuwahara, A., Iijima, H., Doi, Y., Tanaka, K., Saito, K., and Hirai, M. Y.:** Increased bioplastic production with an RNA polymerase sigma factor SigE during nitrogen starvation in *Synechocystis* sp. PCC 6803. *DNA Res.*, 20, 525-535 (2013).

17. **Zhao, K., Liu, M., and Burgess, R. R.:** Promoter and regulon analysis of nitrogen assimilation factor, σ^{54} , reveal alternative strategy for *E. coli* MG1655 flagellar biosynthesis, *Nucleic Acids Res.*, 38, 1273-1283 (2010).

Figure Captions

Figure 1. Time course profiles of 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), respectively. Cells harboring pTV118NpctC1(STQK)AB were grown on 1.7 ml of LB media 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. Immunoblot analysis of the polymer synthetic enzymes and LDH.

Total protein was extracted from the BW25113 (parent strain) and $\Delta rpoN$ mutant strain grown on 1.7 ml of LB media.

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

226 (B) Immnoblots analysis of the polymer biosynthesis enzymes (PCT, PhaA and PhaB)
227 and LDH in parent and $\Delta rpoN$ strain.

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

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	parent	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> ^a	$\Delta rpoN$ / <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 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).

^a, 100 μ M of IPTG added.

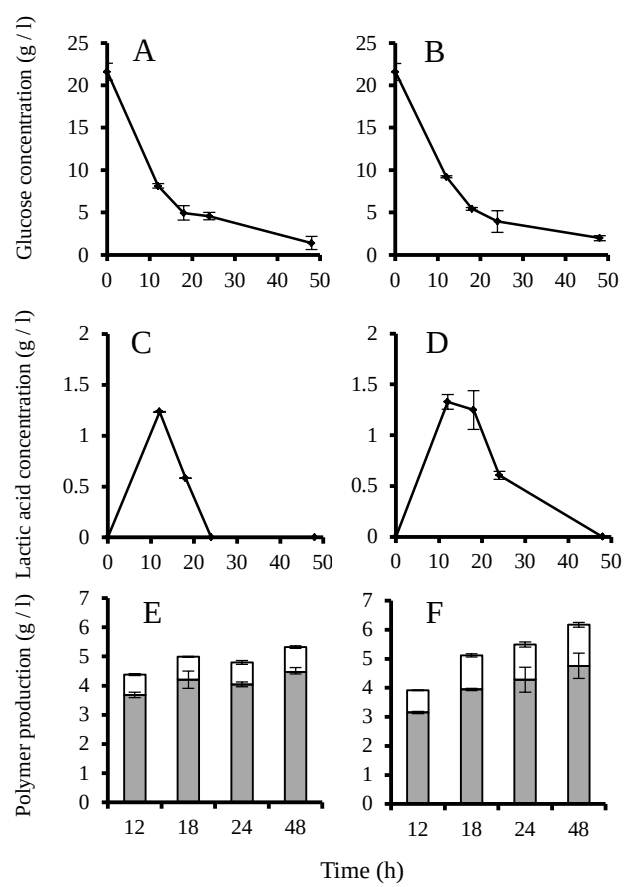


Figure 1

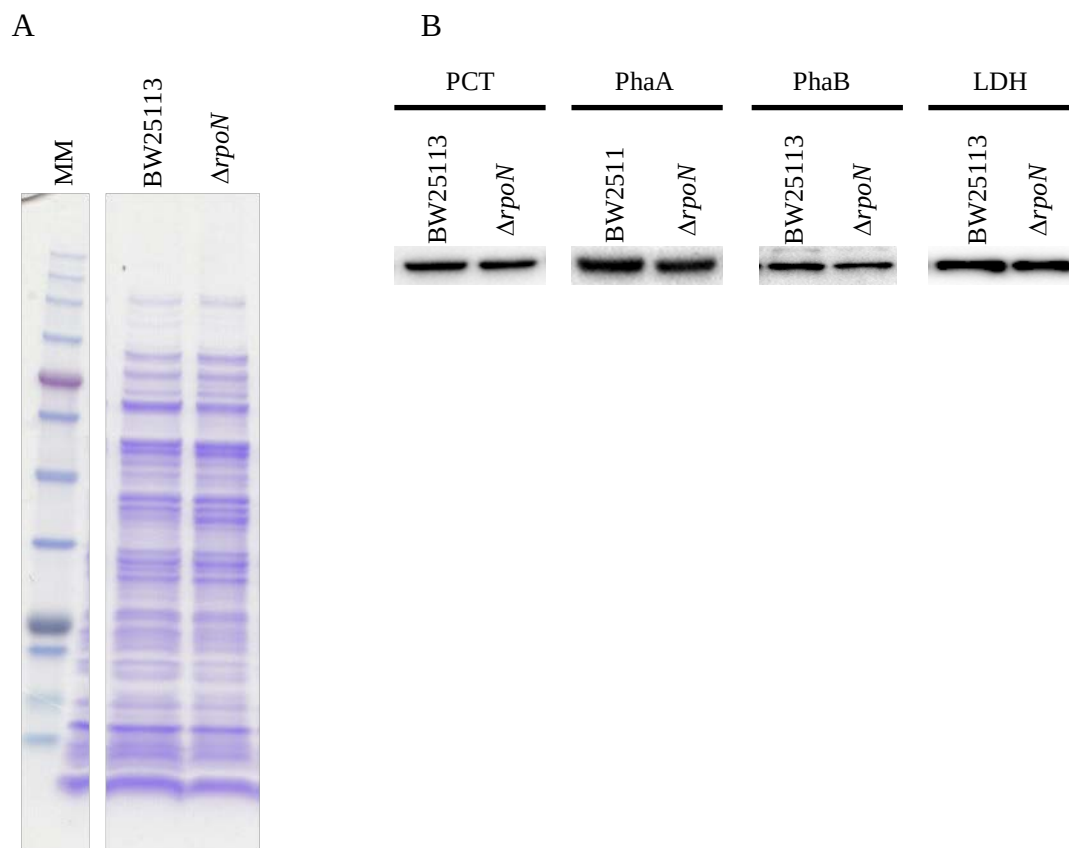


Figure 2