For enhancing the lactate (LA) fraction of poly(lactate-co-3-hydroxybutyrate)s [P(LA-co-3HB)s], an exogenous d-lactate dehydrogenase gene (ldhD) was introduced into Escherichia coli. Recombinant strains of E. coli DH5α, LS5218, and XL1-Blue harboring the ldhD gene from Lactobacillus acetotolerans HT, together with polyhydroxyalkanoate (PHA)-biosynthetic genes containing a lactate-polymerizing enzyme (modified PHA synthase) gene, accumulated the P(LA-co-3HB) copolymer from glucose under microaerobic conditions (100 strokes/min). The LA fraction of copolymers synthesized in the strains of DH5α, LS5218, and XL1-Blue were 19.8, 15.7, and 28.5 mol%, respectively, which were higher than those of the strains without the ldhD gene (<6.7 mol% of LA units). Introduction of the exogenous ldhD gene into E. coli strains resulted in an enhanced LA fraction in P(LA-co-3HB)s.

Key Words: biodegradable plastics; lactate dehydrogenase; polyhydroxyalkanoate; poly(lactic acid) (PLA)
Table 1. Strains and plasmids used in this study.

| Strains or plasmids          | Characteristics                                      | Reference or source                      |
|-----------------------------|------------------------------------------------------|------------------------------------------|
| Bacterial strains           |                                                      |                                          |
| *Lactobacillus acetotolerans* HT | Wild strain                                          | TaKaRa (2015)                            |
| E. coli DH5α                 | *deoR*, *endA1*, *gyrA96*, *hsdR17* (rmA1, relA1, supE44, thi-1, *D(lacZYA-argF)V169*), *F contain* | Clontech (K+), Stratagene               |
| E. coli XL1-Blue             | *deoR*, *endA1*, *compatibility vector*, *Ap*        | TaKaRa (2015)                            |
| E. coli LS5218               |                                                      | Clontech (K+), Stratagene               |
| Plasmids                    |                                                      |                                          |
| pBluescript II KS            | E. coli cloning vector, *Ap*                         | TaKaRa (2015)                            |
| pMB181                       | Km, broad host range, *plac*, *phoA*, *Tn10*, *lacZ*, *D(M15)* | Goto et al. (2018)                       |
| pBBR1MCS-2                   | *Pst I*, broad host range, *PphT*, *phiB*, *lacZ*   | Spratt et al. (1981)                     |
| pTV118NpctC1(STQK)AB         | *PphT*, *lphA*                                       | This study                               |
| pTV118NpctC1(STQK)ldhDABdPRe | *PphT*, *lphA*                                       | This study                               |
| pTV118NpctC1(STQK)pctC1(STQK)ABdPRe | *PphT*, *lphA*                                     | This study                               |
| pTV118NpctC1(STQK)ldhDABdPRe | *PphT*, *lphA*                                       | This study                               |

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Trast, under aerobic conditions, the production of acetyl-CoA is increased, which is able to serve as a CoA donor for the generation of d-LA-CoA (Matsumoto and Taguchi, 2013). In addition, LPE can recognize d-LA-CoA as a substrate and incorporate the LA unit into the polymer chain only when (R)-3HB-CoA is present. Therefore, we introduced an exogenous d-LDH gene (*ldhD*) into *E. coli* in order to make d-LA constitutively produced even under aerobic conditions as well as anaerobic conditions.

In our previous study, we cloned and identified the *ldhD* gene of *Lactobacillus acetotolerans* HT (Goto et al., 2018). The recombinant *E. coli* DH5α with the introduced *ldhD* gene from *Lb. acetotolerans* HT produced a higher amount of d-LA than the control under a shaking culture. Hence, the aim of the present study was to enhance the LA fraction in P(LA-co-3HB) synthesized via the *ldhD*-introducing recombinant *E. coli*.

The bacterial strains and plasmids used in this study are listed in Table 1. To construct pRKmAX-ldhD, pBS-ldhD was digested with *Apa*I and *Xho*I, and the DNA fragment containing the SD sequence was ligated with the *Apa*I and *Xho*I-digested pBBR1MCS-2. The plasmid pRKmAX-ldhD was introduced into *E. coli* DH5α together with pTV118NpctC1(STQK)AB, which harbors the genes of propionyl-CoA transferase (Pct) from *Megasphaera elsdenii*, the Ser235Thr/Gln481Lys mutated PHA synthase from *Pseudomonas* sp. 61-3 [*PhaC1(STQK)*] as a LPE, and the 3HB monomer synthesizing enzymes [β-ketothiolase (PhaA) and NADPH-dependent acetoacetyl-CoA reductase (PhaB)] from *Ralstonia eutropha*.

To remove the promoter (P<sub>Re</sub>) of *R. eutropha* from pTV118NpctC1(STQK)AB, inverse PCR was performed using primers phaC1(STQK)(UP)-f1 (5'-GAATCTAGAAATAATTGGTTTAACCTTAA-3') and pct(DS)-r1 (5'-CCCGGGATCCGGTTATTTTTT-CAGTCCCAT-3'). The amplified DNA fragment was phosphorylated using the T4 Polynucleotide Kinase (Toyobo), followed by self-ligation to yield pTV118NpctC1(STQK)ABdP<sub>Re</sub>, in which all inserted genes were expressed under control of the lac promoter (P<sub>lac</sub>). The *ldhD* gene was amplified by PCR from the genomic DNA of *Lb. acetotolerans* HT with primers ldhD-UP(*Pst*I)-f1 (5'-AACGCGAAGGCTGCATCTCATTATTGCTGAGG-3') and ldhD-*Pst*I(TAA)-r1 (5'-AATTGAAGAAAATATGCTAATCC-3') underlined sequences indicate *Pst*I site). The PCR product was cloned into the pMD20-T vector. The 1.0-kb *Pst*I fragment containing the *ldhD* gene from the pMD20-T derivative was introduced into pTV118NpctC1(STQK)ABdP<sub>Re</sub> at the *Pst*I site to give pTV118NpctC1(STQK)ABdDABdP<sub>Re</sub>. The plasmid pTV118NpctC1(STQK)ABdDABdP<sub>Re</sub> or pTV118NpctC1(STQK)ABdDABdP<sub>Re</sub> was introduced into *E. coli* DH5α, XL1-Blue and LS5218, respectively.

For P(LA-co-3HB) production, the recombinant strains of *E. coli* were grown in 1.5 mL lysogeny broth (LB) medium (Sambrook et al., 1989) at 30°C for 15 h. When needed, ampicillin (100 mg/L) and kanamycin (50 mg/L) were added to the medium. Following this, 1 mL of culture broth was transferred to 300-mL conical flasks with 100 mL LB medium. Cells were cultivated at 30°C in a
Cells were cultivated at 30°C for 48 h in a 300-ml conical flask containing 100 ml of LB medium. 2% (w/v) glucose was added to the medium after 8 h of cultivation.

Table 2. P(LA-co-3HB) production by the recombinant strains of E. coli DH5α.

| Plasmid                                      | Shaking speed (strokes/min) | Dry cell weight (g/L) | Polymer content (wt%) | Polymer composition (mol%) | pH |
|----------------------------------------------|----------------------------|-----------------------|-----------------------|---------------------------|----|
| pTV118NpctC1(STQK)AB                       | 0                          | 0.35±0.02             | 32.5±0.5             | 29.5±0.9                  | 7.0±0.9 | 4.77±0.09 |
|                                              | 60                         | 0.55±0.17             | 31.7±0.4             | 30.0±0.9                  | 7.0±0.9 | 4.80±0.08 |
|                                              | 100                        | 1.32±0.05             | 46.9±2.3             | 9.8±0.9                   | 9.0±0.9 | 4.94±0.06 |
| pTV118NpctC1(STQK)AB and pRKmAX-ldhD       | 0                          | 0.39±0.03             | 33.5±2.1             | 36.6±1.3                  | 6.3±2.1 | 4.70±0.08 |
|                                              | 60                         | 0.77±0.06             | 40.5±2.3             | 36.4±2.0                  | 6.3±2.0 | 4.74±0.08 |
|                                              | 100                        | 1.0±0.11              | 45.3±0.6             | 19.8±2.0                  | 8.0±2.0 | 4.87±0.05 |

Fig. 1. P(LA-co-3HB) production by the recombinant strains of E. coli DH5α.

Fig. 2. Relationship between the γ-LA concentration in the culture supernatant and LA molar fraction of P(LA-co-3HB)s accumulated in the cells.

White diamond, DH5α/pTV118NpctC1(STQK)AB; black diamond, DH5α/pTV118NpctC1(STQK)AB and pRKmAX-ldhD.

Fig. 3. P(LA-co-3HB) production by the recombinant strains of E. coli.

Cells were cultivated at 30°C for 48 h at a shaking speed of 100 strokes/min in a 300-ml conical flask containing 100 ml of LB medium. 2% (w/v) glucose was added to the medium after 8 h of cultivation. ldhD (−), the recombinant strains of E. coli harboring pTV118NpctC1(STQK)AB; ldhD (+), the recombinant strains of E. coli harboring pTV118NpctC1(STQK)AB and pRKmAX-ldhD. White bar, 3HB unit in the polymer; gray bar, γ-LA unit in the polymer.

With a shaking of 100 strokes/min, 0.13 g/L γ-LA was secreted from E. coli DH5α harboring pTV118NpctC1(STQK)AB and pRKmAX-ldhD into the medium, whereas the γ-LA concentration produced by E. coli DH5α harboring pTV118NpctC1(STQK)AB was very low (<0.01 g/L) (Fig. 1). Moreover, the LA fraction (19.8%...
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mol%) in the P(LA-co-3HB) obtained by E. coli DH5α harboring pTV118NpctC1(STQK)AB and pRKmAX-ldhD was higher than the strain harboring pTV118NpctC1(STQK)AB only (9.8 mol%), indicating that the high δ-LA concentration in the culture supernatant led to an increase in the LA fraction of P(LA-co-3HB) accumulated in the cells. In other words, the production of δ-LA would be increased by the expression of the exogenous ldhD gene, and, therefore, it would increase the LA fraction in P(LA-co-3HB).

Subsequently, cells were relatively anaerobically cultivated in a 60 strokes/min shaking culture and in a static culture. The relationship between the δ-LA concentration in the culture supernatant and the LA fraction of P(LA-co-3HB)s accumulated in the cells under all culture conditions is shown in Fig. 2. In both recombinant strains, the δ-LA concentrations in the static culture (1.93 and 2.07 g/L) were higher than those in the 60 strokes/min shaking culture (1.35 and 1.49 g/L); whereas the LA fractions (30 and 36 mol%) in P(LA-co-3HB)s synthesized were almost the same in the static culture and the 60 strokes/min shaking culture. The results indicated that the LA fraction in P(LA-co-3HB) could not be increased any more, even though the cells were cultivated in the static culture. In a previous study, under anaerobic conditions by nitrogen aeration, the pflA-deficient E. coli strain JW0885 harboring pTV118NpctC1(STQK)AB produced 5.7 g/L LA and accumulated P(47% LA-co-3HB), whereas the amount of LA in the polymer was decreased compared with the microaerobic culture (Yamada et al., 2011). This phenomenon has been interpreted as a decreased LA-CoA supply due to reduced acetyl-CoA, as CoA is the LA donor, under anaerobic conditions.

Table 2 summarizes the results of P(LA-co-3HB)s production in all culture conditions. DH5α/pTV118NpctC1(STQK)AB and pRKmAX-ldhD showed the highest LA fraction (36.6 mol%) in a static culture; however, the dry cell weight (0.39 g/L) was lower than those in a microaerobic culture (100 strokes/min). This result indicated that the LA produced inhibited cell growth. Although LA production increased in E. coli as the host under anaerobic conditions, the cell growth decreased as compared with the microaerobic condition (de Graef et al., 1999; Matsuoka and Kurata, 2017; Yamada et al., 2011). PhaC1(STQK) is capable of polymerizing the unusual substrate δ-LA-CoA into a polyester; however, no polymer containing LA units has been synthesized without a supply of the natural substrate (R)-3HB-CoA to the system (Matsumoto and Taguchi, 2013; Taguchi et al., 2008; Yamada et al., 2011). For these reasons, the exogenous ldhD-expressing recombinant strains would be suitable for synthesizing P(LA-co-3HB) with a relatively high LA fraction under microaerobic conditions since sufficient cell growth and the presence of (R)-3HB-CoA were observed.

To further investigate the effects of introducing ldhD gene in other E. coli strains, P(LA-co-3HB) copolymers were produced using E. coli XL1-Blue and LS5218 as hosts. pTV118NpctC1(STQK)ABdPRe or pTV118NpctC1(STQK)ldhDABdPRe, in which all genes were expressed under control of the lac promoter (Plac), was introduced into E. coli DH5α, XL1-Blue, and LS5218. All recombinant strains synthesized P(LA-co-3HB) copolymers under microaerobic conditions (100 strokes/min) (Fig. 3). Introduction of the ldhD gene into E. coli strains resulted in the synthesis of P(LA-co-3HB)s with relatively high LA fractions. That is, P(LA-co-3HB)s with an LA fraction of 14.3–28.5 mol% were synthesized by the recombinant strains harboring pTV118NpctC1(STQK)ldhDABdPRe. In particular, E. coli XL1-Blue/pTV118NpctC1(STQK)ldhDABdPRe accumulated 15.7 wt% P(28.5% LA-co-3HB), resulting in a higher polymer content and LA fraction than those of other recombinant strains harboring pTV118NpctC1(STQK)ABdPRe. This indicates that polymers with relatively high LA fractions would be synthesized by introducing the ldhD gene, even into E. coli strains. Further, E. coli DH5α harboring both pTV118NpctC1(STQK)AB and pRKmAX-ldhD accumulated 45.3 wt% LA-based polymer (LA fraction: 19.8 mol%) (Table 2), resulting in a much higher polymer content than that of E. coli DH5α harboring pTV118NpctC1(STQK)ldhDABdPRe (7.6 wt%, LA fraction: 14.3 mol%) (Fig. 3). pTV118NpctC1(STQK) ldhDABdPRe was constructed by eliminating PRe, the promoter of the phaCAB operon in R. eutropha, between the pct and phaC1(STQK) genes, from pTV118NpctC1(STQK)AB and inserting the ldhD gene. Thus, the difference in the plasmids was presumed to affect the expression level of these genes, including the phaC1(STQK) gene.

In conclusion, we succeeded in enhancing LA fractions of P(LA-co-3HB)s synthesized by various E. coli strains through the introduction of the ldhD gene from Lb. acetotolerans HT. This is the first report on LA-based polymer synthesis by exogenous δ-LDH gene introduction in E. coli. The introduction of an exogenous δ-LDH gene (ldhD) into E. coli would be preferable for effective LA-based polymer production and cell growth under microaerobic conditions. This method enables us to enhance the LA fraction of the LA-based polymer without using a host such as a pflA-knockout strain.

Acknowledgments

This work was partly supported by the Sasakawa Scientific Research Grant from The Japan Science Society. We would like to thank Editage (www.editage.jp) for English language editing.

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