

## Poly[(*R*)-3-Hydroxybutyrate] Formation in *Escherichia coli* from Glucose through an Enoyl-CoA Hydratase-Mediated Pathway

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**In this study, a new metabolic pathway for the synthesis of poly[(*R*)-3-hydroxybutyrate] [P(3HB)] was constructed in a recombinant *Escherichia coli* strain that utilized forward and reverse reactions catalyzed by two substrate-specific enoyl-CoA hydratases, *R*-hydratase (PhaJ) and *S*-hydratase (FadB), to epimerize (*S*)-3HB-CoA to (*R*)-3HB-CoA via a crotonyl-CoA intermediate. The *R*-hydratase gene (*phaJ<sub>Ac</sub>*) from *Aeromonas caviae* was coexpressed with the PHA synthase gene (*phaC<sub>Re</sub>*) and 3-ketothiolase gene (*phaA<sub>Re</sub>*) from *Ralstonia eutropha* in *fadR* mutant *E. coli* strains (CAG18497 and LS5218), which had constitutive levels of the  $\beta$ -oxidation multienzyme FadB<sub>Ec</sub>. When grown on glucose as the sole carbon source, the cells accumulated P(3HB) up to an amount 6.5 wt% of the dry cell weight, whereas the control cells without *phaJ<sub>Ac</sub>* or *fadR* mutation accumulated significantly smaller amounts of P(3HB). These results suggest that PhaJ<sub>Ac</sub> and FadB<sub>Ec</sub> played an important role in supplying monomers for P(3HB) synthesis in the pathway. Furthermore, by using this pathway, a P(3HB)-concentration-dependent fluorescent staining screening technique was developed to rapidly identify cells that possess active *R*-hydratase.**

**[Key words:** (*R*)-specific enoyl-CoA hydratase, poly[(*R*)-3-hydroxybutyrate], *Escherichia coli*, metabolic engineering, fatty acid  $\beta$ -oxidation]

Polyhydroxyalkanoates (PHAs) are biopolyesters synthesized by a wide variety of bacteria as an intracellular carbon and energy storage material (1). PHAs can be used as biodegradable thermoplastics for a wide range of agricultural, marine and medical applications and have received increased attention because they are produced from renewable resources such as sugars and vegetable oils.

Of all the PHAs, poly[(*R*)-3-hydroxybutyrate] [P(3HB)] is the most extensively characterized polymer. P(3HB) was discovered in 1926 in *Bacillus megaterium* (2). Subsequently, biochemical investigations on the P(3HB) biosynthesis pathway have focused on several natural producers such as *Ralstonia eutropha* (3) and *Zoogloea ramigera* (4). These bacteria possess the P(3HB) biosynthesis pathway (three-step pathway), which consists of three enzymatic reactions, and use acetyl coenzyme A (acetyl-CoA) as the starting material (Fig. 1A). The first reaction consists of the condensation of two acetyl-CoA molecules into acetoacetyl-CoA by 3-ketothiolase (encoded by *phaA*). The second reaction is the reduction of acetoacetyl-CoA to (*R*)-3-hydroxybutyryl-CoA [(*R*)-3HB-CoA] by an NADPH-dependent acetoacetyl-CoA reductase (encoded by *phaB*). Lastly, (*R*)-3HB-CoA molecules are polymerized into P(3HB) by PHA

synthase (encoded by *phaC*).

Several bacteria are proposed to use an alternative pathway for P(3HB) formation. In 1969, Moskowitz and Merrick reported the feasibility of P(3HB) formation through an enoyl-CoA hydratase-mediated pathway (hydratase pathway) in the photosynthetic bacterium *Rhodospirillum rubrum* (5). The hydratase pathway includes two substrate-specific hydratases: one is specific for the *R* enantiomer [(*R*)-specific enoyl-CoA hydratase, *R*-hydratase] and the other is specific for the *S* enantiomer [(*S*)-specific enoyl-CoA hydratase, *S*-hydratase]. The following metabolic route for supplying (*R*)-3HB-CoA monomers in *R. rubrum* has been proposed (Fig. 1B): two acetyl-CoA molecules undergo a condensation reaction to form acetoacetyl-CoA by 3-ketothiolase. Acetoacetyl-CoA is then reduced to (*S*)-3HB-CoA by an NADH-dependent acetoacetyl-CoA reductase. (*S*)-3HB-CoA then undergoes an epimerization to form (*R*)-3HB-CoA via a crotonyl-CoA intermediate by forward and reverse reactions catalyzed by the two different hydratases. The *R*-hydratase gene (*phaJ<sub>Rt</sub>*) and PHA synthase gene (*phaC<sub>Rt</sub>*) have been cloned from *R. rubrum* and their products have been characterized (6, 7). However, other genes associated with this pathway have not yet been identified. The only other bacterium proposed to use this pathway for P(3HB) production is the methanol-utilizing bacterium *Methylobacterium rhodesianum* (8).

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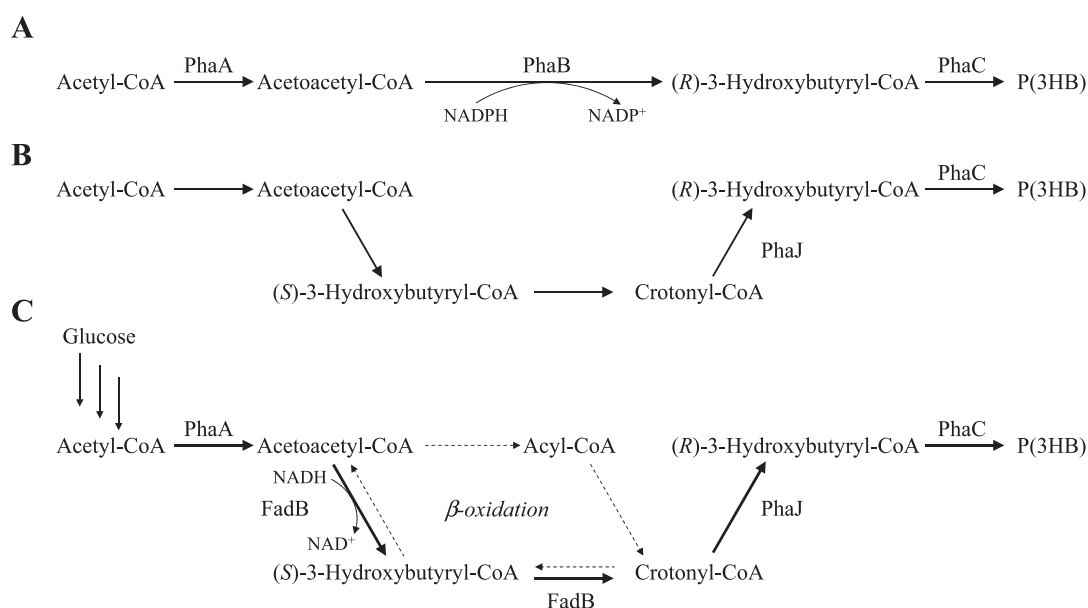


FIG. 1. Metabolic pathways for P(3HB) biosynthesis. (A) Standard three-step P(3HB) production pathway in *R. eutropha*. (B) Hydratase-mediated P(3HB) synthesis pathway (hydratase pathway) proposed in *R. rubrum*. (C) Hydratase-mediated P(3HB) synthesis pathway (hydratase pathway) designed in recombinant *E. coli*. The dotted arrows indicate the forward direction of fatty acid  $\beta$ -oxidation. Abbreviations: PhaA, 3-ketothiolase; PhaB, acetoacetyl-CoA reductase; PhaC, PHA synthase; PhaJ, *R*-hydratase; FadB,  $\beta$ -oxidation multienzyme that exhibits *S*-hydratase and 3-hydroxyacyl-CoA dehydrogenase activities.

Although the hydratase pathway was previously proposed, P(3HB) formation via this pathway has not been demonstrated. In this study, we present the first evidence of P(3HB) formation via a hydratase pathway in recombinant *Escherichia coli* by using strains with mutations in fatty acid  $\beta$ -oxidation and heterologous P(3HB) biosynthesis enzymes.

## MATERIALS AND METHODS

**Bacterial strains and plasmids** The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* DH5 $\alpha$  was used for all genetic manipulations. In addition to strain DH5 $\alpha$ , two *fadR*-deficient strains of *E. coli*, namely, LS5218 (9) and CAG18497 (10), were used for P(3HB) accumulation. The product of *fadR* (FadR) is a transcription factor that negatively regulates fatty acid  $\beta$ -oxidation (11). The strains LS5218 and CAG18497 were developed using chemical mutagenesis and the site-specific insertion of Tn10 into *fadR*, respectively. Thus, they are expected to have different expression levels of the genes involved in fatty

acid  $\beta$ -oxidation.

**Plasmid construction** The plasmid pGEM''*phaCA*<sub>Re</sub>*J*<sub>Ac</sub> was constructed from the plasmid pGEM''-*phaCAB*<sub>Re</sub> (12) harboring the *R. eutropha phaCAB*<sub>Re</sub> genes as well as the *R. eutropha* promoter and terminator regions (Fig. 2). To remove the NADPH-dependent acetoacetyl-CoA reductase gene (*phaB*<sub>Re</sub>) from pGEM''-*phaCAB*<sub>Re</sub>, the entire region outside *phaB*<sub>Re</sub> was amplified by PCR using the plasmid as a template (13). The primers used were 5'-ACCGAATTCCCTGGTTCAACCAGTCGGCAGCCGGC-3', where the underlined sequence indicates an *EcoRI* site, and 5'-GATTAAGCTTGTATATCGTCGCCGGTCCGCGCCAA-3', where the underlined sequence indicates a *HindIII* site. The PCR product (7.2 kb) was allowed to self-ligate to yield the plasmid pGEM''*phaCA*<sub>Re</sub>. The plasmid pUCJ (14), which harbors the *Aeromonas caviae R*-hydratase gene (*phaJ*<sub>Ac</sub>) was digested with *EcoRI* and *HindIII* and the 0.5-kb DNA fragment carrying *phaJ*<sub>Ac</sub> was purified. Finally, the 0.5-kb DNA fragment was ligated to *EcoRI*-*HindIII*-digested pGEM''*phaCA*<sub>Re</sub> to yield the plasmid pGEM''*phaCA*<sub>Re</sub>*J*<sub>Ac</sub>.

pGEM''*phaC*<sub>Re</sub>*J*<sub>Ac</sub> was constructed from pGEM''*phaCA*<sub>Re</sub>*J*<sub>Ac</sub> by deleting the 3-ketothiolase gene (*phaA*<sub>Re</sub>). To this end, pGEM''*phaCA*<sub>Re</sub>*J*<sub>Ac</sub> was digested with *AatI*, and the resulting 6.7-kb

TABLE 1. Bacterial strains and plasmids used in this study

| Strain and plasmid                                       | Relevant characteristics  | Source or reference |
|--|---|---------------------|
| Strain   |   |                     |
| <i>Escherichia coli</i> DH5 $\alpha$                     | <i>deoR endA1 gyrA96 hsdR17</i> ( $r_K^- m_K^+$ ) <i>relA1 supeE thi-1</i>  | Takara              |
| LS5218   | $\Delta$ ( <i>lacZYA-argFV169</i> ) $\phi$ 80 $\Delta$ <i>lacZ</i> $\Delta$ M15F- $\lambda$ - <i>fadR601, atoC2</i> (Con)               | 9                   |
| CAG18497   | <i>fadR13::Tn10</i>   | 10                  |
| Plasmid  |   |                     |
| pGEM-T   | Ap <sup>r</sup> <i>lacPOZ</i> T7 and SP6 promoter   | Promega             |
| pGEM''- <i>phaCAB</i> <sub>Re</sub>                      | pGEM-T derivative; <i>pha</i> <sub>Re</sub> promoter, <i>phaC</i> <sub>Re</sub> , <i>phaA</i> <sub>Re</sub> , <i>phaB</i> <sub>Re</sub> | 12                  |
| pGEM'' <i>phaCA</i> <sub>Re</sub>                        | pGEM-T derivative; <i>pha</i> <sub>Re</sub> promoter, <i>phaC</i> <sub>Re</sub> , <i>phaA</i> <sub>Re</sub>                             | This study          |
| pGEM'' <i>phaC</i> <sub>Re</sub> <i>J</i> <sub>Ac</sub>  | pGEM-T derivative; <i>pha</i> <sub>Re</sub> promoter, <i>phaC</i> <sub>Re</sub> , <i>phaJ</i> <sub>Ac</sub>                             | This study          |
| pGEM'' <i>phaCA</i> <sub>Re</sub> <i>J</i> <sub>Ac</sub> | pGEM-T derivative; <i>pha</i> <sub>Re</sub> promoter, <i>phaC</i> <sub>Re</sub> , <i>phaA</i> <sub>Re</sub> , <i>phaJ</i> <sub>Ac</sub> | This study          |

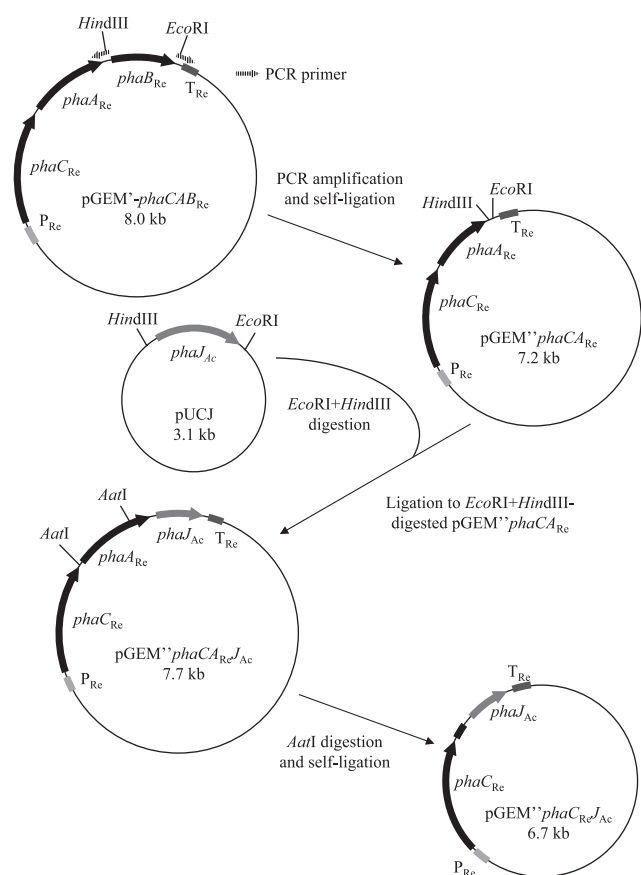


FIG. 2. Strategy of constructing pGEM''phaCA<sub>Re</sub>, pGEM''phaCA<sub>Re</sub>J<sub>Ac</sub>, and pGEM''phaC<sub>Re</sub>J<sub>Ac</sub>.

DNA fragment was allowed to self-ligate to yield pGEM''phaC<sub>Re</sub>J<sub>Ac</sub>.

**P(3HB) synthesis** The cells were cultivated in 500-ml flasks with 100 ml of M9 medium (15) containing glucose (20 g/l) and ampicillin (100 mg/l) on a reciprocal shaker (130 strokes per min) at 30°C for 72 h. After cultivation, the collected cells were washed with water and then lyophilized.

**Polymer characterization** P(3HB) content in the lyophilized cells was determined by analytical high-performance liquid chromatography (HPLC) after cellular P(3HB) was converted to crotonic acid by treatment with hot concentrated sulfuric acid (H<sub>2</sub>SO<sub>4</sub>) (16).

The polymers that had accumulated in the cells were extracted with chloroform for 72 h at room temperature and purified by precipitation with methanol. Molecular weight and molecular weight distribution were determined by gel permeation chromatography (GPC) at 40°C, using a Shimadzu 10A GPC system and a 10A refractive index detector with Shodex K-806M and K-802 columns. Chloroform was used as the eluent at a flow rate of 0.8 ml/min, and sample concentrations of 1.0 mg/ml were applied. Polystyrene standards with low polydispersity were used to make a calibration curve.

**Cell staining** Lyophilized cell staining by Nile blue A was performed according to the method by Ostle *et al.* (17). Cells fixed on a glass plate were stained with a Nile blue A solution at 55°C, and then washed with 8% acetate aqueous solution. The sample was exposed to UV light and observed by fluorescent microscopy.

For viable-cell staining, Nile red was used as a dye (18). Nile red dissolved in dimethylsulfoxide (DMSO) was added to M9 medium agar plates containing glucose (20 g/l), yeast extract (1 g/l) and ampicillin (100 mg/l) to give a final concentration of 0.5 mg of

dye per liter of medium. The cells were exposed to light with a wavelength of 420–500 nm using a Dark Reader DR-45M2 (Clare Chemical Research, Denver, CO, USA) after 72 h of cultivation to visualize strains able to produce P(3HB).

**Enzyme activity assay** The recombinant *E. coli* strains were inoculated into 100 ml of M9 medium, containing glucose (20 g/l) and ampicillin (100 mg/l), and cultivated for 24 h at 30°C. Cells were harvested and resuspended in 2.5 ml of ice-cold 20 mM potassium phosphate buffer (pH 7.2, containing 1 mM EDTA). Subsequently, cells were disrupted by sonication (20 kHz, 60 W, 2.5 min), after which the broken cells were centrifuged and the supernatant was used for an enzyme assay.

SR-Hydrotase activity was determined by the hydration of crotonyl-CoA (Sigma, St. Louis, MO, USA) (19). A 5 µl volume of diluted crude extract was added to 895 µl of 200 mM potassium phosphate buffer (pH 8.0) containing 30 µM crotonyl-CoA in a quartz cuvette with a 1.0-cm light path, and the decrease in absorbance at 263 nm was measured at 30°C. The  $\epsilon_{263}$  of the enoyl-thioester bond was  $6.7 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ . One unit of enzyme activity was defined as the amount of enzyme required to catalyze the decrease of 1 µmol of substrate in 1 min. Protein concentration was determined by the Bradford assay (Bio-Rad, Richmond, CA, USA) with bovine serum albumin as the standard.

## RESULTS AND DISCUSSION

### Design of hydratase pathway for synthesis of P(3HB)

**in *E. coli*** To show the feasibility of P(3HB) formation by the hydratase pathway, we designed an artificial metabolic pathway in *E. coli* using inherent and heterologous enzymes as shown in Fig. 1C. As a first step in this pathway, two acetyl-CoA molecules from glycolysis are condensed into acetoacetyl-CoA by the 3-ketothiolase of *R. eutropha* (PhaA<sub>Re</sub>). Following this, acetoacetyl-CoA is converted into crotonyl-CoA in two subsequent reactions catalyzed by the NADH-dependent (*S*)-specific 3-hydroxyacyl-CoA dehydrogenase and *S*-hydratase that reside in the inherent multi-enzyme FadB<sub>Ec</sub> of fatty acid  $\beta$ -oxidation. Crotonyl-CoA is then converted to (*R*)-3HB-CoA by the *R*-hydratase of *A. caviae* (PhaJ<sub>Ac</sub>). Lastly, (*R*)-3HB-CoA is polymerized into P(3HB) by the PHA synthase of *R. eutropha* (PhaC<sub>Re</sub>). To express the genes of the three heterologous enzymes (PhaA<sub>Re</sub>, PhaJ<sub>Ac</sub> and PhaC<sub>Re</sub>) necessary for this pathway, the expression plasmid pGEM''phaCA<sub>Re</sub>J<sub>Ac</sub> was constructed (Fig. 2).

In this study, we used the *R*-hydratase PhaJ<sub>Ac</sub>, because it is the most well-characterized enzyme among the bacterial *R*-hydratases and has narrow substrate specificity toward enoyl-CoA substrates with acyl chain lengths of C4 to C6 (19). This enzyme provides C4 and C6 monomers from fatty acid  $\beta$ -oxidation for the synthesis of a P(3HB-co-3-hydroxyhexanoate) copolymer in *A. caviae* (20). As for *R. rubrum*, the *R*-hydratase PhaJ<sub>R</sub> has been proposed to function in the hydratase pathway for P(3HB) synthesis. Although PhaJ<sub>Ac</sub> and PhaJ<sub>R</sub> work in different pathways, their amino acid identity is as high as 45% (6).

Note that a different cofactor is required during P(3HB) biosynthesis between the well-characterized three-step pathway from *R. eutropha* and the hydratase pathway. In the three-step pathway, NADPH is required as a cofactor to reduce acetoacetyl-CoA by PhaB, whereas, in the hydratase pathway, NADH is required as a cofactor to reduce acetoacetyl-CoA by the multi-enzyme FadB.

TABLE 2. P(3HB) accumulation and specific activities in recombinant *E. coli*<sup>a</sup>

| Plasmid (relevant markers)   | Strain       | Gene expression <sup>b</sup> |             |             |             | Dry cell weight (g/l) | P(3HB) content (wt%) | SR-Hydratase activity (U/mg) |
|--|--------------|------------------------------|-------------|-------------|-------------|-----------------------|----------------------|------------------------------|
|  |              | <i>phaA</i>                  | <i>fadB</i> | <i>phaJ</i> | <i>phaC</i> |                       |                      |                              |
| pGEM'' <i>phaCA</i> <sub>Re</sub> <i>J</i> <sub>Ac</sub> ( <i>phaC</i> <sub>Re</sub> , <i>phaA</i> <sub>Re</sub> , <i>phaJ</i> <sub>Ac</sub> ) | DH5 $\alpha$ | ++                           | –           | ++          | ++          | 1.1 $\pm$ 0.04        | 0.20 $\pm$ 0.02      | 46 $\pm$ 9                   |
|  | CAG18497     | ++                           | +           | ++          | ++          | 0.95 $\pm$ 0.01       | 6.5 $\pm$ 1.2        | 141 $\pm$ 14                 |
|  | LS5218       | ++                           | +           | ++          | ++          | 1.2 $\pm$ 0.2         | 1.6 $\pm$ 0.2        | 52 $\pm$ 23                  |
| pGEM'' <i>phaCA</i> <sub>Re</sub> ( <i>phaC</i> <sub>Re</sub> , <i>phaA</i> <sub>Re</sub> )  | DH5 $\alpha$ | ++                           | –           | –           | ++          | 0.85 $\pm$ 0.04       | 0.73 $\pm$ 0.06      | 0.012 $\pm$ 0.01             |
|  | CAG18497     | ++                           | +           | –           | ++          | 0.94 $\pm$ 0.10       | 0.49 $\pm$ 0.20      | 0.10 $\pm$ 0.02              |
|  | LS5218       | ++                           | +           | –           | ++          | 1.2 $\pm$ 0.2         | 0.20 $\pm$ 0.09      | 0.22 $\pm$ 0.04              |
| pGEM'' <i>phaC</i> <sub>Re</sub> <i>J</i> <sub>Ac</sub> ( <i>phaC</i> <sub>Re</sub> , <i>phaJ</i> <sub>Ac</sub> )                              | DH5 $\alpha$ | –                            | –           | ++          | ++          | 0.75 $\pm$ 0.08       | 0.15 $\pm$ 0.01      | 81 $\pm$ 25                  |
|  | CAG18497     | –                            | +           | ++          | ++          | 0.76 $\pm$ 0.05       | 1.5 $\pm$ 0.2        | 168 $\pm$ 40                 |
|  | LS5218       | –                            | +           | ++          | ++          | 0.70 $\pm$ 0.04       | 1.2 $\pm$ 0.3        | 128 $\pm$ 23                 |

<sup>a</sup> The results are the averages and the standard deviations of at least three independent experiments.

<sup>b</sup> Gene expression levels are indicated as follows: ++, plasmid-based expression; +, chromosomal gene expression; –, no expression or repression in presence of glucose.

**P(3HB) synthesis through hydratase pathway in *E. coli* strain DH5 $\alpha$**  *E. coli* strain DH5 $\alpha$  was used as a host for hydratase-mediated P(3HB) production. pGEM''*phaCA*<sub>Re</sub>*J*<sub>Ac</sub> carrying *phaC*<sub>Re</sub>, *phaA*<sub>Re</sub> and *phaJ*<sub>Ac</sub> was introduced into *E. coli* strain DH5 $\alpha$  and the transformed cells were grown on glucose for 72 h at 30°C. As a control, recombinant DH5 $\alpha$  cells expressing only *phaC*<sub>Re</sub> and *phaA*<sub>Re</sub> (pGEM''*phaCA*<sub>Re</sub>) or *phaC*<sub>Re</sub> and *phaJ*<sub>Ac</sub> (pGEM''*phaC*<sub>Re</sub>*J*<sub>Ac</sub>) were grown in parallel. All cells were subjected to HPLC analysis to measure the P(3HB) content of the cells. The results are shown in Table 2. Cells expressing *phaJ*<sub>Ac</sub> and *phaA*<sub>Re</sub> (pGEM''*phaCA*<sub>Re</sub>*J*<sub>Ac</sub>) accumulated a very small amount of P(3HB) (0.2 wt% of dry cell weight). However, *phaJ*<sub>Ac</sub> expression showed no enhancement of P(3HB) accumulation, suggesting that the designed hydratase pathway was incapable of P(3HB) production in strain DH5 $\alpha$ .

The hydratase pathway relies on substrate-specific hydration and dehydration reactions catalyzed by PhaJ<sub>Ac</sub> (R-hydratase) and FadB<sub>Ec</sub> (S-hydratase) to provide monomers for P(3HB) biosynthesis. The SR-hydratase in *E. coli* DH5 $\alpha$  showing activity toward crotonyl-CoA was assayed using the crude extracts from the cells expressing *phaJ*<sub>Ac</sub> and a control strain lacking *phaJ*<sub>Ac</sub>. The recombinant cells expressing *phaJ*<sub>Ac</sub>, which were expected to have both PhaJ<sub>Ac</sub> and FadB<sub>Ec</sub> activities, exhibited high activity (46–81 U/mg), whereas the cells lacking *phaJ*<sub>Ac</sub>, which were expected to only have FadB<sub>Ec</sub> activity, exhibited very low activity (0.012 U/mg). On the basis of this result, the expression of *phaJ*<sub>Ac</sub> was found to be the main contributor to the high hydratase activity because the hydratase activity was high in the recombinant strains expressing *phaJ*<sub>Ac</sub>. These results also indicate that there was little contribution to hydratase activity from the native level of FadB<sub>Ec</sub> (S-hydratase) produced in *E. coli* DH5 $\alpha$  under the growth conditions of this experiment. The enzymes involved in fatty acid  $\beta$ -oxidation are induced by the presence of fatty acids in the growth medium. Thus, the very low level of FadB<sub>Ec</sub> expression led to the low P(3HB) production in DH5 $\alpha$  when grown on glucose as a carbon source. In addition, FadB<sub>Ec</sub> has lower activity toward C4 substrates than longer substrates such as C8 and C10 (21). Thus, the reaction catalyzed by FadB<sub>Ec</sub> could be the rate-limiting step in the designed hydratase pathway. By elevating the active concentration of FadB<sub>Ec</sub> in the cells and increasing metabolic flux through FadB<sub>Ec</sub>, P(3HB) for-

mation through the hydratase pathway could be increased (see below).

**P(3HB) synthesis through hydratase pathway in *fadR* mutant *E. coli* strains** The product of *fadR* (FadR) is a transcription factor that negatively regulates fatty acid  $\beta$ -oxidation in *E. coli* (11). Strains that lack *fadR* constitutively express the enzymes involved in fatty acid  $\beta$ -oxidation, despite the presence or absence of fatty acids in the growth media. Two *fadR* mutant *E. coli* strains (namely, LS5218 and CAG18497) were used to examine the effects of constitutively active fatty acid  $\beta$ -oxidation on P(3HB) production via the hydratase pathway. The hydratase activities of these two strains (0.10–0.22 U/mg) were higher than that of DH5 $\alpha$  (0.012 U/mg) even when the cells lacking *phaJ*<sub>Ac</sub> were grown on glucose (Table 2).

The results of P(3HB) production via the hydratase pathway in the *fadR* mutants (LS5218 and CAG18497) are summarized in Table 2. Both of the *fadR* mutants were able to accumulate P(3HB) up to an amount 6.5 wt% of the dry cell weight, which is significantly higher than that accumulated by DH5 $\alpha$  (0.2 wt%). These results indicate that the designed hydratase pathway is functional in the *fadR* mutants and that the reactions catalyzed by FadB<sub>Ec</sub> are the rate-limiting steps in the hydratase pathway. It was also observed that the *fadR* mutants without the expression of *phaJ*<sub>Ac</sub> accumulated small amounts of P(3HB) (0.20–0.49 wt%), indicating that PhaJ<sub>Ac</sub> plays an important role in supplying monomers for P(3HB) formation in the hydratase pathway.

Of the two *fadR* mutants harboring *phaC*<sub>Re</sub>, *phaA*<sub>Re</sub>, and *phaJ*<sub>Ac</sub>, the CAG18497 strain exhibited a higher degree of P(3HB) accumulation (6.5 wt%) than the LS5218 strain (1.6 wt%). The low degree of P(3HB) accumulation may have resulted from a mutation other than the *fadR* mutation in the

TABLE 3. Molecular weights of P(3HB) synthesized by recombinant *E. coli*<sup>a</sup>

| Strain   | Plasmid  | Molecular weight <sup>b</sup> |                         |           |
|----------|--|-------------------------------|-------------------------|-----------|
|          |  | $M_n$ ( $\times 10^6$ )       | $M_w$ ( $\times 10^6$ ) | $M_w/M_n$ |
| LS5218   | pGEM'' <i>phaCA</i> <sub>Re</sub> <i>J</i> <sub>Ac</sub> | 1.1                           | 1.8                     | 1.7       |
| CAG18497 | pGEM'' <i>phaCA</i> <sub>Re</sub> <i>J</i> <sub>Ac</sub> | 1.2                           | 2.1                     | 1.7       |

<sup>a</sup> Cells were cultivated at 30°C for 72 h in M9 medium containing glucose (20 g/l) as the sole carbon source.

<sup>b</sup>  $M_n$ , Number-average molecular weight;  $M_w$ , weight-average molecular weight;  $M_w/M_n$ , polydispersity.

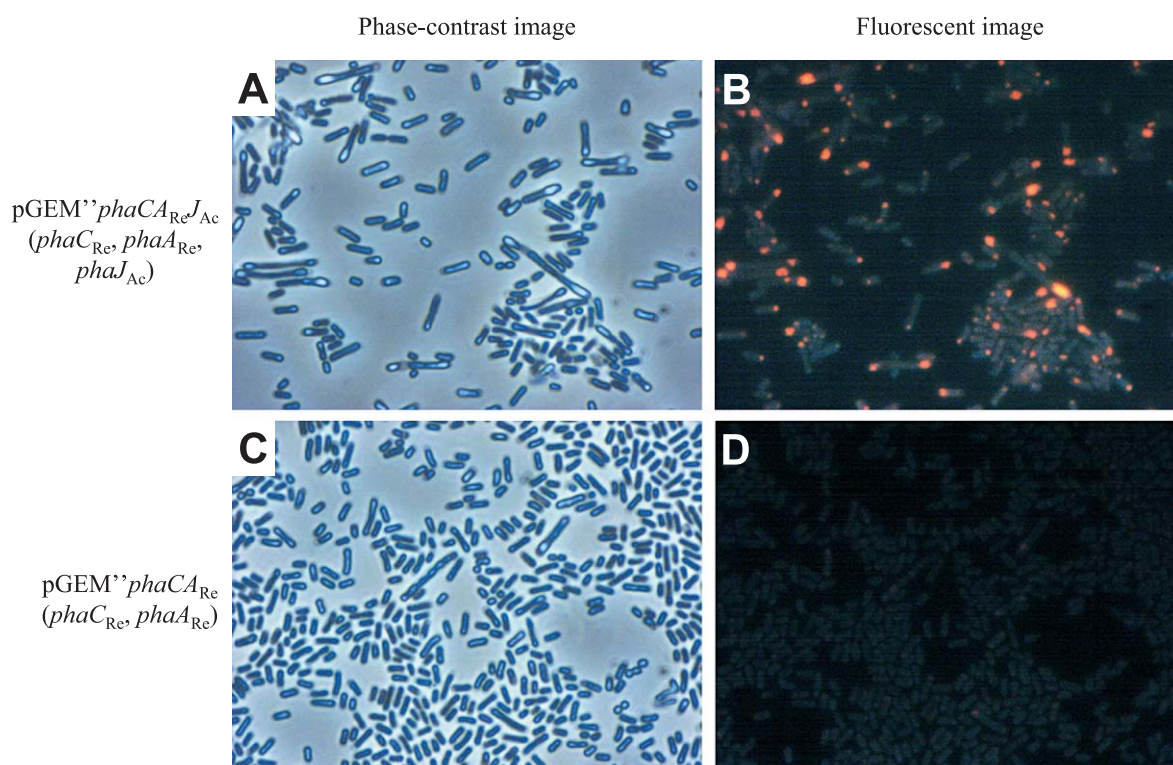


FIG. 3. Microscopic observation of recombinant *E. coli* CAG18497 cells stained by Nile blue A. The cells harboring pGEM''*phaCA*<sub>Re</sub><sup>J<sub>Ac</sub></sup> were observed by (A) phase-contrast and (B) fluorescent microscopies. The cells harboring pGEM''*phaCA*<sub>Re</sub> were observed by (C) phase-contrast and (D) fluorescent microscopies. P(3HB) granules fluoresced bright orange under UV light.

LS5218 strain. According to its genotype, the LS5218 strain has an *atoC* mutation that allows for the constitutive expression of a short-chain fatty acid degradation (*ato*) operon (22, 23). Because the enzymes encoded by the *ato* operon utilize intermediates of the P(3HB) hydratase-mediated pathway as substrates, the competitive reaction may reduce the flux of metabolites toward P(3HB) in the LS5218 strain. Thus, the *atoC* mutation may be unfavorable for P(3HB) synthesis by the hydratase-mediated pathway.

On the other hand, the *fadR* mutants deficient in *phaA*<sub>Re</sub> expression accumulated P(3HB) in the range of 1.2–1.5 wt%. This result suggests that PhaA<sub>Re</sub> can be complemented by other inherent 3-ketothiolases. Hence, the supply of (*R*)-3HB-CoA through the hydratase pathway in the *fadR* mutants is dependent on the concentrations of active PhaJ<sub>Ac</sub> and FadB<sub>Ec</sub>.

**Molecular weight of P(3HB) synthesized through hydratase pathway** The molecular weights of P(3HB) polymers that were synthesized via the hydratase pathway were measured by GPC. Table 3 shows the number-average molecular weight ( $M_n$ ), weight-average molecular weight ( $M_w$ ) and polydispersity ( $M_w/M_n$ ) of P(3HB). The  $M_n$  of P(3HB) synthesized through the hydratase pathway were 1.1–1.2 × 10<sup>6</sup>, whereas the  $M_w$  were 1.8–2.1 × 10<sup>6</sup>. The polydispersities of these polymers were 1.7. These results demonstrate that a high-molecular-weight P(3HB) is produced by the hydratase pathway.

**Dye-enhanced visualization of P(3HB) in cells** Lyophilized CAG18497 cells were stained with Nile blue A and then observed by phase-contrast and fluorescence micros-

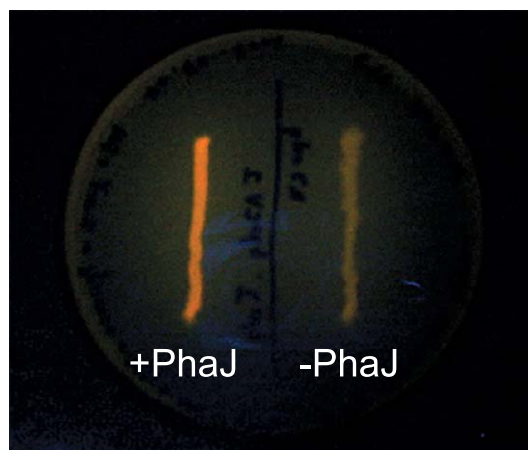


FIG. 4. Fluorescent Nile red staining of recombinant *E. coli* CAG18497 with and without expression of *R*-hydratase (PhaJ) on agar plate. The cells were grown on M9 medium (0.5 mg of Nile red/l) containing yeast extract (1 g/l) and glucose (20 g/l) and exposed to light of 420–500 nm after 72 h of cultivation. P(3HB)-accumulating cells fluoresced bright orange. +PhaJ; the cells harboring pGEM''*phaCA*<sub>Re</sub><sup>J<sub>Ac</sub></sup>, -PhaJ; the cells harboring pGEM''*phaCA*<sub>Re</sub>.

copies, as shown in Fig. 3. P(3HB) granules formed in the cells expressing *phaJ*<sub>Ac</sub> were visible by fluorescence microscopy and it was found that a small P(3HB) granule was formed at the leading edge of each cell, whereas P(3HB) granules were scarcely observed in the control cells without *phaJ*<sub>Ac</sub>.

Moreover, viable cells could be distinguished depending on the P(3HB) accumulation level determined by Nile red staining on agar plates using an excitation wavelength of 420–500 nm. As shown in Fig. 4, the cells with *phaJ<sub>Ac</sub>* were differentiated from the cells without *phaJ<sub>Ac</sub>*. Because one of the limiting factors for P(3HB) production is PhaJ activity in the hydratase pathway, this staining method provides a useful tool for the primary screening of cells carrying active PhaJ to distinguish them from those of a PhaJ-mutagenized library. In our previous study, we have created some substrate-specificity-altered PhaJ<sub>Ac</sub> enzymes by structure-based mutagenesis (14). By combining beneficial amino acid substitutions in PhaJ, it is hoped that further beneficial alterations can be achieved. However, thus far, there was no convenient technique to screen for active PhaJ enzymes. This method will enhance high-throughput screening of beneficial mutations in a PhaJ-mutagenized library by allowing us to screen for active PhaJ enzymes that produce P(3HB) via the hydratase pathway in *fadR* mutants *E. coli* strains.

On the other hand, previous visual inspection of Nile red stained cells has been performed under UV light, but the limits of detection were slightly lower because of the fluorescence in response to excitation by UV light (18). In addition, UV light may potentially damage cells and further mutate or damage DNA, thus hampering our ability to isolate active mutants. By utilizing visible wavelengths (420–500 nm) to identify cells that are capable of accumulating P(3HB), we avoid the complications associated with the use of UV light.

**Conclusion** In this study, we successfully showed that P(3HB) can be produced via hydratase-mediated P(3HB) synthesis from glucose in recombinant *E. coli*. The designed pathway consists of four enzymes, namely, PhaA<sub>Re</sub>, PhaC<sub>Re</sub>, PhaJ<sub>Ac</sub> (*R*-hydratase) and FadB<sub>Ec</sub> (*S*-hydratase). The coexpression of PhaJ<sub>Ac</sub>, PhaA<sub>Re</sub> and PhaC<sub>Re</sub> in *fadR* mutant *E. coli* strains, which had constitutive levels of FadB<sub>Ec</sub> activity, resulted in a degree of P(3HB) accumulation up to 6.5 wt% using glucose as the sole carbon source. Thus, the designed hydratase pathway was functional for P(3HB) synthesis in *fadR* mutant *E. coli* strains. Molecular weight analysis confirmed that P(3HB) synthesized through the hydratase pathway was of high molecular weight. Furthermore, by using the hydratase pathway, a novel screening technique was developed in which the cells with PhaJ activity were clearly distinguished from those without PhaJ activity by fluorescent staining dependent on P(3HB) accumulated through the hydratase pathway. Applying this approach to a PhaJ-mutagenized library, the high-throughput screening of active PhaJ can be conveniently performed *in vivo*.

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