

Metabolic Engineering of *Escherichia coli* for Production of Enantiomerically Pure (*R*)-(-)-Hydroxycarboxylic Acids

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A heterologous metabolism of polyhydroxyalkanoate (PHA) biosynthesis and degradation was established in *Escherichia coli* by introducing the *Ralstonia eutropha* PHA biosynthesis operon along with the *R. eutropha* intracellular PHA depolymerase gene. By with this metabolically engineered *E. coli*, enantiomerically pure (*R*)-3-hydroxybutyric acid (R3HB) could be efficiently produced from glucose. By employing a two-plasmid system, developed as the PHA biosynthesis operon on a medium-copy-number plasmid and the PHA depolymerase gene on a high-copy-number plasmid, R3HB could be produced with a yield of 49.5% (85.6% of the maximum theoretical yield) from glucose. By integration of the PHA biosynthesis genes into the chromosome of *E. coli* and by introducing a plasmid containing the PHA depolymerase gene, R3HB could be produced without plasmid instability in the absence of antibiotics. This strategy can be used for the production of various enantiomerically pure (*R*)-hydroxycarboxylic acids from renewable resources.

Polyhydroxyalkanoates (PHAs) are a group of completely biodegradable polyesters that are synthesized and accumulated by various bacteria (1, 5, 9, 27). More than 140 kinds of carboxylic acids hydroxylated at the 3-, 4-, 5-, or 6-position, all in the (*R*)-configuration if they possess a chiral center on the position of hydroxyl group, can be incorporated into PHAs by employing different bacteria under various culture conditions (5, 9, 28). Therefore, it was reasoned that various enantiomerically pure (*R*)-(-)-hydroxycarboxylic acids (RHAs) might be conveniently prepared by depolymerizing biosynthesized PHAs. These RHAs contain two functional groups that are convenient to modify for the synthesis of various chiral compounds, especially fine chemicals such as antibiotics, vitamins, perfumes, and pheromones (3, 9, 21, 23). For example, (*R*)-(-)-3-hydroxybutyric acid (R3HB) is an important precursor of 4-acetoxazetidinone, which in turn is used to make carbapenem antibiotics, which have close to a billion-dollar market. Poly-(*R*)-(-)-3-hydroxybutyrate (PHB) is the most ubiquitous member of the PHAs. Methods for producing R3HB by chemical digestion of PHB have been reported (13, 23, 24). In these methods, however, organic solvents were used in large amounts, and the production efficiency was rather low due to complicated processes.

The metabolism for the synthesis and degradation of PHB plays an important role in many bacteria for the reservation and reutilization of excess carbon and energy sources and reducing power (1). The metabolic pathway for the synthesis and degradation of PHB in *Ralstonia eutropha* was already proposed (1, 5, 11, 18, 22, 25). PHB is synthesized from acetyl-

coenzyme A (CoA) by three sequential enzymatic reactions catalyzed by β -ketothiolase, acetoacetyl-CoA reductase, and PHA synthase (18, 22, 25). When the conditions are met, PHB is depolymerized to R3HB by intracellular PHA depolymerase and oligomer hydrolase (1, 5, 11). Then the R3HB dehydrogenase converts R3HB to acetoacetate, which is further metabolized in the cell. By employing this cyclic nature of PHB synthesis and degradation, we recently demonstrated that R3HB could be efficiently produced in naturally PHA-producing bacteria by providing appropriate environmental conditions (lowering the pH, for example) that support high activity of intracellular PHA depolymerase and no activity of R3HB dehydrogenase (11).

It has previously been demonstrated that recombinant *E. coli* strains harboring the *Ralstonia eutropha* PHA biosynthesis genes (18, 22, 25) were able to accumulate a large amount of PHB, up to 90% of dry cell weight (4, 10, 25). Recently, Saegusa et al. (19) cloned the intracellular PHA depolymerase gene from *R. eutropha*. It was therefore reasoned that R3HB and other RHAs may be efficiently produced by establishing a heterologous PHA biosynthesis and degradation pathway in *Escherichia coli*, as depicted in Fig. 1.

In this article, we report the development of metabolically engineered *E. coli* strains harboring a heterologous PHA synthesis and degradation pathway and a novel strategy for the production of enantiomerically pure RHAs by these strains.

MATERIALS AND METHODS

Bacterial strains, plasmids, PCR primers, and growth condition. The bacterial strains, plasmids, and PCR primers used in this study are listed in Table 1. *R. eutropha* H16 was grown at 30°C in nutrient broth (NB; Difco laboratories, Detroit, Mich.). Recombinant *E. coli* XL1 Blue transformed with various plasmids were grown at 37°C in Luria-Bertani medium (LB; 10 g of Bacto-tryptone, 5 g of Bacto-yeast extract, and 10 g of sodium chloride per liter) supplemented with 20 g of glucose per liter or in a chemically defined medium (R medium) (4)

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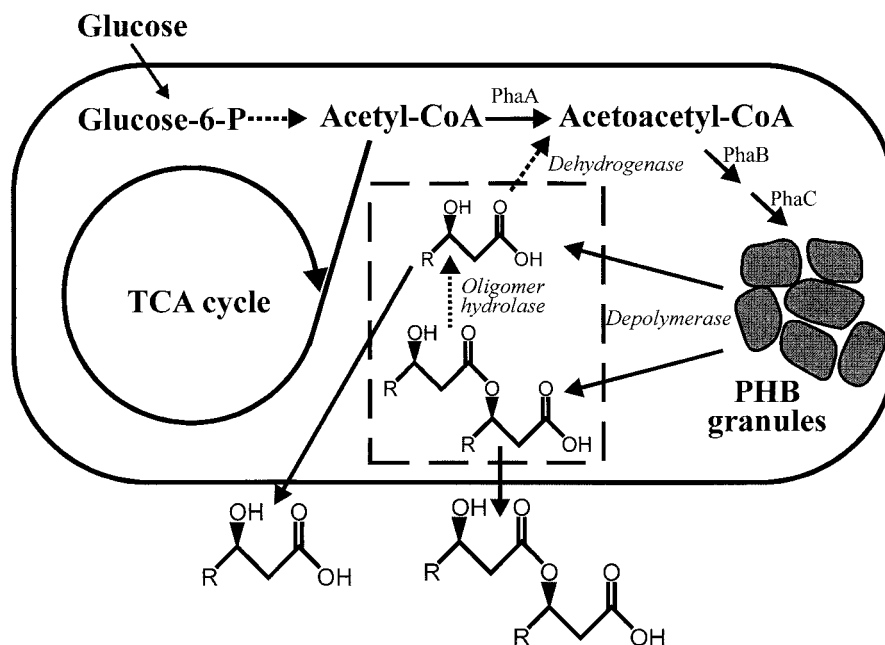


FIG. 1. Poly-(*R*)-(-)-3-hydroxybutyrate (PHB) metabolism and the metabolic engineering strategy for the production of (*R*)-(-)-3-hydroxybutyric acid in *E. coli*. Abbreviations: PhaA, β -ketothiolase; PhaB, acetoacetyl-CoA reductase; PhaC, PHA synthase.

supplemented with 20 g of glucose per liter and 20 mg of thiamine per liter. When specified, ampicillin (100 μ g/ml) and/or chloramphenicol (50 μ g/ml) was added for the maintenance of plasmids.

Batch cultures were carried out in a 2.5-liter jar fermentor (KoBiotech Co., Incheon, Korea) containing 1.2 liters of R medium supplemented with 20 g of glucose per liter and 20 mg of thiamine per liter at 37°C and 500 rpm without pH

control. For the production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymers, cells were cultured in R medium containing 10 g of glucose per liter and 1 g of propionic acid per liter (26). Plasmid stability was measured as described previously (12) by comparing the number of colonies appearing on antibiotic- and non-antibiotic-containing plates after serially subculturing the recombinant *E. coli* cells in LB medium.

TABLE 1. Strains, plasmids, and PCR primers

Strain, plasmid, or primer	Relevant features	Source or reference ^a
Strains		
<i>E. coli</i> XL1-Blue	<i>supE44 hsdR17 recA1 endA1 gyrA96 thi relA1 lac F'</i> [<i>proAB</i> ⁺ <i>lacI</i> ^f <i>lacZ</i> Δ <i>M15</i> Tn10(<i>tet</i> ^r)]	Stratagene
<i>E. coli</i> B	Wild type; ATCC 11303	ATCC
<i>E. coli</i> B-PHA+	B (ATCC 11303) <i>pta::phaCAB</i> , Km ^r	This study
<i>R. eutropha</i> H16	Wild type; ATCC 17699	ATCC
Plasmids		
pACYC184	Cloning vector; Cm ^r Tc ^r	NEB
pUC19	Cloning vector; Ap ^r	NEB
pSYL105	Expression vector containing <i>R. eutropha phaC_{Re}</i> , <i>phaA_{Re}</i> ; and <i>phaB_{Re}</i> ; <i>hok/sok</i> locus ^b ; Ap ^r	10
p5184	pACYC184 carrying <i>Xba</i> I fragment of pSYL105 containing <i>phaC_{Re}</i> , <i>phaA_{Re}</i> , and <i>phaB_{Re}</i> as an operon and <i>hok/sok</i> locus	This study
pSYL105Red	pSYL105 containing <i>phaZi_{Re}</i>	This study
pTrcEBG	pTrc99A containing <i>red</i> operon of bacteriophage λ	8
pUC19Red	pUC19 containing <i>phaZi_{Re}</i>	This study
pUCRed_stb	pUC19Red containing <i>hok/sok</i> locus	This study
pUC19pta	pUC19 containing segments of <i>E. coli pta</i> gene	This study
pUC19pta-PHA	pUC19pta containing <i>R. eutropha</i> PHA biosynthesis operon	This study
Primers^c		
phaZ-XB/F	5'-GCTCTAGAGGATCCTTGTGTTTTCCGCAGCAACAGAT-3'	Genotech
phaZ-BH/R	5'-GCGGATCCAAGCTTACCTGGTGGCCGAGGC-3'	Genotech
pta-P1	5'-GCGAATTCTTTAAAGACGCGCGCATTTCCTAAACT-3'	Genotech
pta-P2	5'-GCGGTACCGAGCTCCGGGTTGATCGCACAGTCA-3'	Genotech
pta-P3	5'-GGCGAGCTCGCGCATGCCGACCGCTGAACAGCTG-3'	Genotech
pta-P4	5'-GCAAGCTTTTTAAAGCGCAGTTAAGCAAGATAATC-3'	Genotech

^a Stratagene, Stratagene Cloning Systems, La Jolla, Calif.; ATCC, American Type Culture Collection, Rockville, Md.; NEB, New England Biolabs, Beverly, Mass.

^b *parB* (*hok/sok*) locus of plasmid R1.

^c Primers were synthesized at Genotech, Daejeon, Korea.

Genetic manipulation. Chromosomal DNA of *R. eutropha* was prepared by Marmur's method (15). PCR was carried out with an automatic thermal cycler (Takara Shuzo Co., Kyoto, Japan). Isolation and purification of plasmid DNA, restriction digestion, ligation, gel electrophoresis, and transformation were carried out as described by Sambrook et al. (20).

Integration of the *R. eutropha* PHA biosynthesis genes into the chromosome of *E. coli* B was carried out as previously reported (8, 29). The *E. coli* phosphotransacetylase (*pta*) gene was selected for the site of integration of the *R. eutropha* PHA biosynthesis genes. Plasmid pTrcEBG (8) containing the *red* operon of bacteriophage λ was transformed into *E. coli* B. Transformants were prepared as electroporation-competent cells after induction with 1 mM isopropylthiogalactopyranoside (IPTG) for the expression of the *red* operon. The *pta* gene was amplified as two fragments from the chromosomal DNA of *E. coli* B by PCR with two pairs of primer sets, *pta*-P1 plus *pta*-P2 and *pta*-P3 plus *pta*-P4 (Table 1). Plasmid pUC19pta was prepared by inserting these two PCR products digested with *Eco*RI-*Kpn*I and *Sph*I-*Hind*III, respectively, into pUC19.

To prepare the *R. eutropha* PHA biosynthesis operon, pSYL105 (10) was digested with *Eco*RI, made blunt ended, and digested with *Bam*HI. Plasmid pUC19pta-PHA was constructed by inserting the PHA biosynthesis operon into the *Sma*I and *Bam*HI sites of pUC19pta. Finally, the DNA cassette containing the PHA biosynthesis operon and the homologous regions of the *pta* gene was prepared by digesting pUC19pta-PHA with *Dra*I and mixed with electroporation-competent cells. After electroporation, the desired transformants were selected by identifying PHB-accumulating colonies on LB plates containing 20 g of glucose per liter at 37°C. From the selected transformants, pTrcEBG was removed by cultivating cells in the absence of ampicillin.

Analytical procedures. Cell concentration (grams of dry cell weight per liter of culture broth) and PHB concentration were determined as previously described (4). The PHB content was defined as the percentage of the ratio of PHB concentration to the cell concentration. Residual cell concentration was calculated by subtracting PHB concentration from the cell concentration. The RHA concentration was measured by high-performance liquid chromatography (HPLC) as previously described (11). When PHB was degraded by an intracellular PHA depolymerase, mainly dimers of R3HB were produced and excreted into the medium along with R3HB monomers.

Dimers of R3HB were hydrolyzed to R3HB monomers by mild heat treatment under alkaline condition as follows. To a 1.5-ml microcentrifuge tube containing 500 μ l of cell-free supernatant, 500 μ l of 10 N NaOH was added. The tightly sealed tube was incubated at 95°C for 2 h. After cooling to room temperature, the reaction mixture was diluted with an appropriate amount (2- to 50-fold) of distilled water. The resulting solution was filtered and analyzed by HPLC. The monomer yield was defined as the percentage of the ratio of the amount of R3HB formed after heat treatment to the total amount of glucose consumed. The theoretical yield of R3HB from glucose is 57.8%.

RESULTS AND DISCUSSION

Metabolic engineering of *E. coli*. To establish a heterologous metabolism of PHA biosynthesis and degradation in *E. coli*, plasmids that contain the genes encoding the PHA biosynthetic enzymes and an intracellular PHA depolymerase were constructed as follows. The *R. eutropha* intracellular PHA depolymerase gene (*phaZi_{Re}*), including its native promoter region, was amplified from *R. eutropha* chromosomal DNA by PCR with primers *phaZ*-XB/F and *phaZ*-BH/R. Plasmid pUC19Red was constructed by inserting the *Bam*HI-digested PCR product into the *Bam*HI site of plasmid pUC19 (Fig. 2). The 1.4-kbp *Hind*III-digested fragment of pUC19Red containing the *phaZi_{Re}* gene was cloned into the *Hind*III site of pSYL105 (10) harboring the *R. eutropha* PHA biosynthesis genes to construct pSYL105Red (Fig. 2). These plasmids thus contain the genes coding for four enzymes responsible for PHA biosynthesis and degradation under the control of their native promoters and ribosome-binding sites: β -ketothiolase (*phaA_{Re}*), NADPH-dependent acetoacetyl-CoA reductase (*phaB_{Re}*), and PHA synthase (*phaC_{Re}*) as an operon and an intracellular PHA depolymerase (*phaZi_{Re}*).

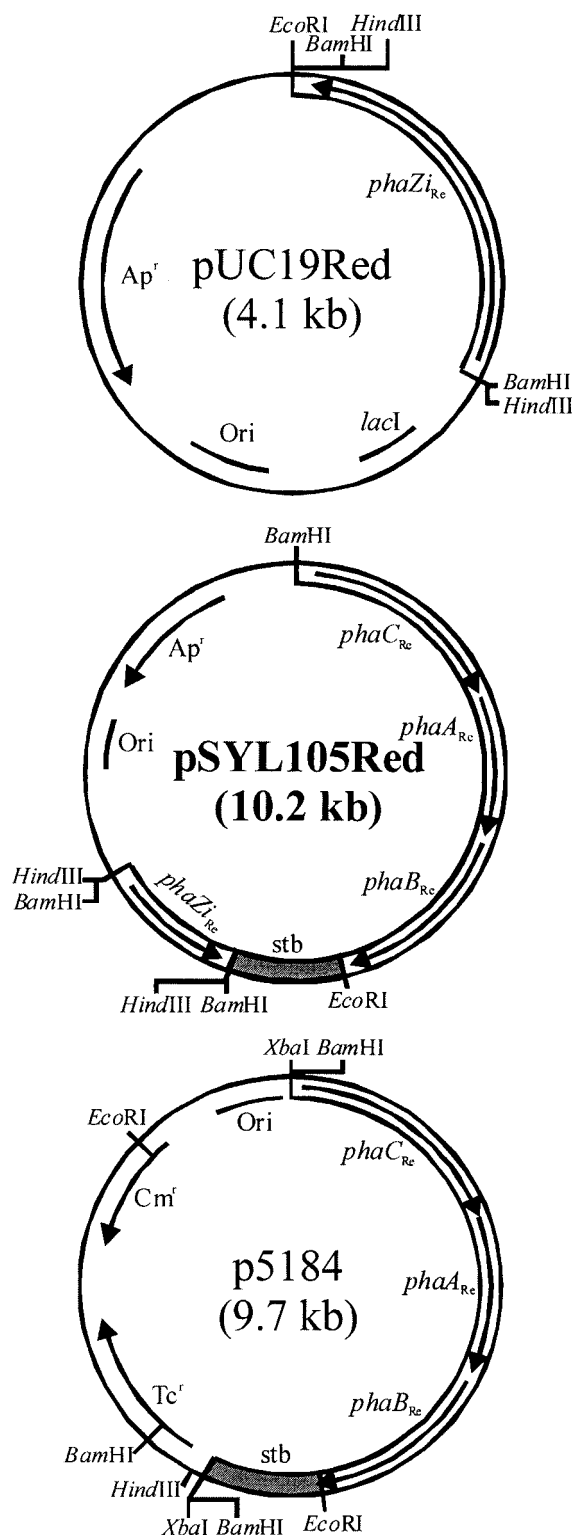


FIG. 2. Plasmids used in this study. Ap^r, ampicillin resistance gene; Cm^r, chloramphenicol resistance gene; *lacI*, lactose operon repressor; *ori*, origin of replication; *phaA*, β -ketothiolase; *phaB*, acetoacetyl-CoA reductase; *phaC*, PHA synthase; *phaZi*, intracellular PHA depolymerase; *stb*, the *hok/sok* locus of plasmid R1; Tc^r, tetracycline resistance gene.

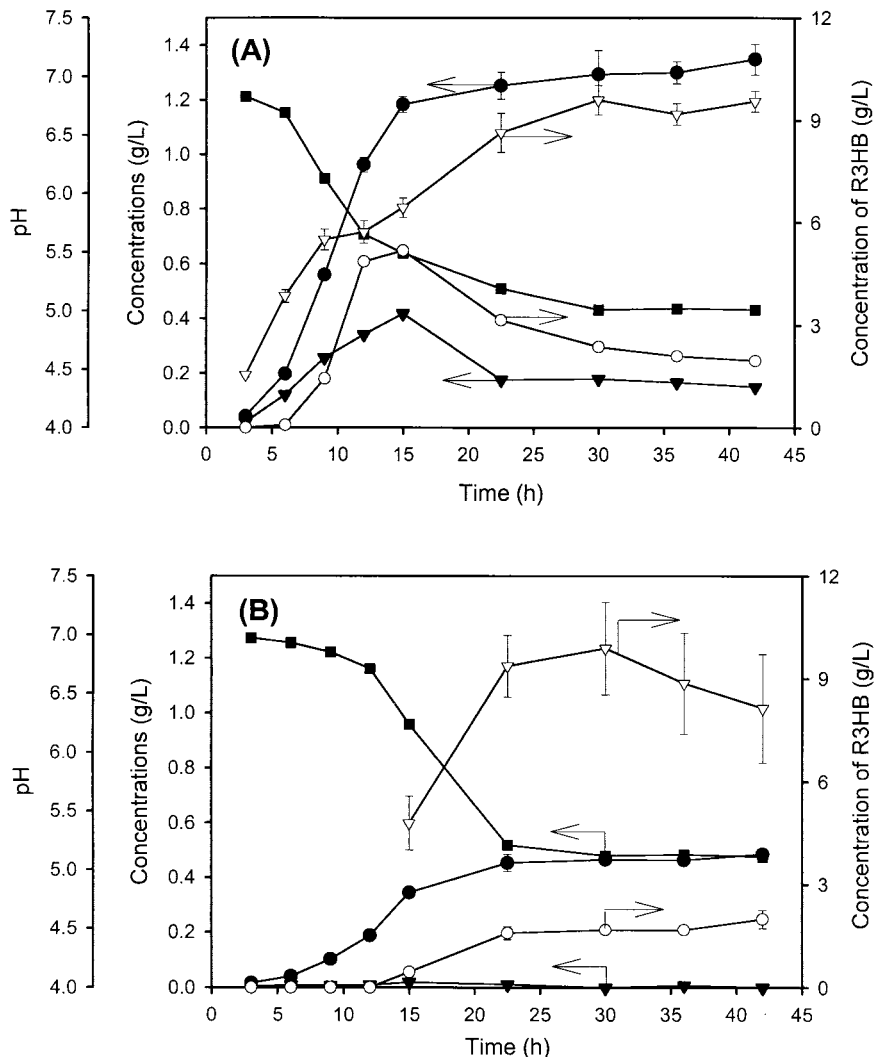


FIG. 3. Time profiles of cell concentration (●), PHB concentration (▼), pH (■), and R3HB concentration before (○) and after (▽) alkaline heat treatment during batch culture of recombinant *E. coli* harboring pSYL105Red (A) or p5184 and pUC19Red (B) in a chemically defined medium containing 20 g of glucose per liter.

Production of R3HB. To examine the possibility of R3HB production by this strategy, flask cultures of *E. coli* XL1-Blue harboring pSYL105Red were carried out. The recombinant *E. coli* strains produced R3HB monomers and dimers and excreted them into the medium. These results suggest that the heterologous PHA biosynthesis and degradation metabolism had been successfully established in *E. coli*. The final cell and PHB concentrations, the R3HB concentration after alkaline heat treatment, and the yield of R3HB determined after 51 h of cultivation were 1.3 g/liter, 0.45 g/liter, 8.3 g/liter, and 41.5%, respectively, when a complex medium was used, and 1.4 g/liter, 0.12 g/liter, 9.6 g/liter, and 48.0%, respectively, when a chemically defined medium was used. Throughout the culture, the PHB concentration was higher in complex medium than in chemically defined medium. However, more R3HB could be produced while less PHB remained in cells when a chemically defined medium was used, which is beneficial from the recovery point of view. Therefore, batch cultures were carried out in a chemically defined medium.

The results obtained by batch culture of *E. coli* XL1-Blue (pSYL105Red) are shown in Fig. 3A. Rapid production of R3HB started at the beginning of culture when a chemically defined medium was used. During the exponential growth phase (6 to 15 h), R3HB was produced mainly as a monomer up to 5.2 g/liter. The R3HB concentration after alkaline digestion was 6.4 g/liter at 15 h. During the stationary phase (after 15 h), the R3HB concentration decreased while the total R3HB concentration (after alkaline heat treatment) continued to increase. This phenomenon is likely to be due to the formation R3HB dimers from the monomers by esterification. Generally, the carboxylic acid group of R3HB can be readily esterified with alcohol groups to form esters (17). Since R3HB molecules possess both alcohol and carboxylic acid functional groups, the R3HB monomers produced can serve as reactants for reversible intermolecular esterification to form dimers. When R3HB was excreted mainly as monomers by the depolymerization of PHB during the exponential growth phase, the ratio of R3HB monomers to dimers was high enough for the

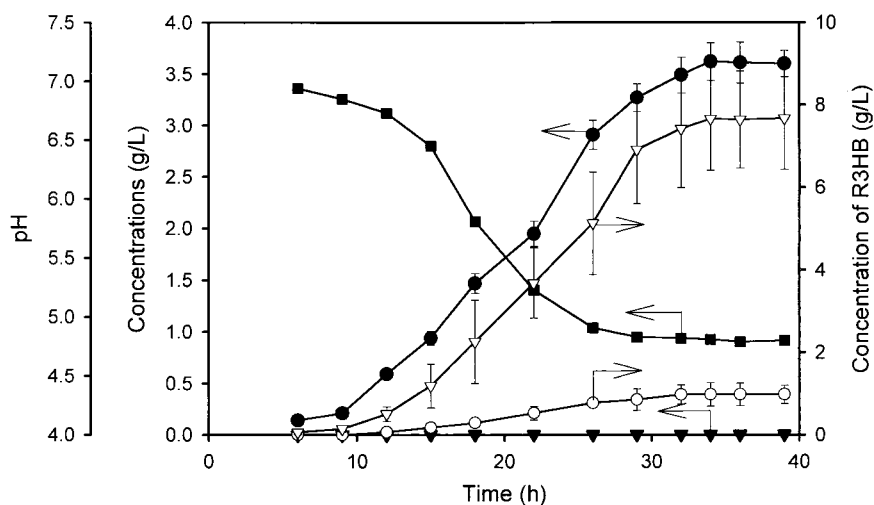


FIG. 4. Time profiles of cell concentration (●), PHB concentration (▼), pH (■), and R3HB concentration before (○) and after (▽) alkaline heat treatment during batch culture of recombinant *E. coli* B-PHA+ harboring pUCRed_stb in a chemically defined medium containing 20 g of glucose per liter.

monomers to undergo the esterification reaction. Nonetheless, our desired R3HB product could be successfully obtained by alkaline heat treatment.

Two-plasmid system. Even though R3HB could be produced by employing a single plasmid, there was some PHB remaining in the cell, which lowered the final monomer yield. It was reasoned that a higher intracellular PHA depolymerase activity would be required for the complete depolymerization of PHB. Therefore, a two-plasmid system in which the depolymerase gene is on a high-copy-number plasmid while the biosynthesis genes are on a medium-copy-number plasmid was developed to examine the possibility of increasing the monomer yield. For the expression of the PHA biosynthesis genes, plasmid p5184 (Fig. 2) was constructed by inserting the 5.5-kbp *Xba*I-digested fragment of pSYL105 containing the *R. eutropha* PHA biosynthesis operon and the *hok/sok* locus (6) to the *Xba*I site of medium-copy-number plasmid pACYC184. For the expression of the *phaZi_{Re}* gene, high-copy-number plasmid pUC19Red (Fig. 2) was used.

In the batch culture of recombinant *E. coli* harboring both p5184 and pUC19Red (Fig. 3B), cell growth was rather slow, and the final cell concentration was lower than that obtained with the single-plasmid system. This seemed to be due to the greater metabolic burden exerted by the presence of two plasmids compared with one plasmid. However, all PHB synthesized was immediately depolymerized to R3HB monomers and dimers without intracellular accumulation. Even though the copy number of the PHB biosynthesis genes was lower than that with the single-plasmid system, the maximum R3HB concentration after alkaline heat treatment obtained was 9.9 g/liter, which is slightly higher than that obtained with the single-plasmid system. The monomer yield was 49.5%, which is 85.6% of the maximum theoretical yield. It is notable that R3HB monomers were not dimerized, which is different from what was observed with the single-plasmid system. This seems to be because the R3HB concentration of less than 2 g/liter is not high enough to trigger the esterification reaction.

As described above, all PHB was immediately depolymerized when the two-plasmid system was used, while some PHB remained in the cell when the single-plasmid system was used. This phenomenon can be explained as follows. We have previously reported that the crystallinity of PHB produced in recombinant *E. coli* is much higher than that of PHB produced in a natural PHB producer (7). PHB produced with a single-plasmid system seems to become partially crystalline because of its rapid accumulation before depolymerization. The intracellular PHA depolymerase cannot depolymerize crystalline PHB (19). Therefore, if PHB once accumulates in *E. coli* due to the higher PHA biosynthetic capacity compared with depolymerization capacity, some old PHB would become more crystalline and would not be a good substrate of the intracellular PHB depolymerase. When the two-plasmid system was used, the intracellular R3HB depolymerase activity was high enough to immediately degrade PHB produced by PHA biosynthesis enzymes present in lower copy numbers.

Integration of PHA biosynthesis genes into *E. coli* chromosome. One of the problems most frequently encountered during the industrial production of bioproducts by employing recombinant strains is plasmid instability. Addition of antibiotics to stabilize plasmids is not desirable, especially for the production of bulk products in a large-scale fermentor. The use of the *hok/sok* locus of plasmid R1 (6) is a good method to stabilize a plasmid by postsegregationally killing the plasmid-free cells, but it cannot be applied to the two-plasmid system. In the two-plasmid system that we used, only plasmid p5184 contains the *hok/sok* locus. In order to examine the stability of plasmids, *E. coli* XL1-Blue harboring p5184 and pUC19Red was serially subcultured for 30 generations without the addition of antibiotics. Plasmid p5184 containing the *hok/sok* locus was stably maintained for 30 generations. However, 85% of the cells lost pUC19Red after six generations (data not shown). Therefore, segregational instability of plasmid was found to be a problem in the absence of antibiotics.

To overcome this problem of plasmid instability, the PHA

biosynthesis operon was integrated into the chromosome of *E. coli* B by homologous recombination (29) to make *E. coli* B-PHA+. The phosphotransacetylase (*pta*) gene was selected for the site of integration because this can increase the flux towards the PHB biosynthesis pathway by eliminating the competing pathway consuming acetyl-CoA to form acetic acid. *E. coli* B-PHA+ was transformed with pUCRed_stb, which was constructed by cloning the *hok/sok* locus into pUC19Red. By batch fermentation of *E. coli* B-PHA+ harboring pUCRed_stb in the absence of antibiotics, R3HB was produced to a high level even though there was only a single copy of the PHA biosynthesis operon (Fig. 4). The plasmid was stably maintained until the end of fermentation. The final R3HB concentration and the yield obtained were 7.7 g/liter and 38.5%, respectively, after alkaline heat treatment.

To see if other RHAs could be produced by this strategy, production of R3HB and (*R*)-3-hydroxyvaleric acid (R3HV) by first making cells synthesize poly(3-hydroxybutyrate-co-3-hydroxyvalerate) was examined. By flask culture of *E. coli* B-PHA+(pUCRed_stb) in a chemically defined medium containing 10 g of glucose per liter and 1 g of propionic acid per liter, the final concentrations of R3HB and R3HV after alkaline heat treatment obtained in 48 h were 2.8 and 0.4 g/liter, respectively.

As demonstrated above, R3HB can be efficiently produced by employing metabolically engineered *E. coli* harboring the genes involved in the biosynthesis and degradation of PHA. By using a two-plasmid system, R3HB was most efficiently produced, with a yield of 49.5% from glucose. The problem of using antibiotics for the plasmid maintenance could be overcome by integrating the PHA biosynthesis operon into the *E. coli* chromosome and using the *hok/sok* locus of plasmid R1 in a plasmid harboring the PHA depolymerase gene. Various PHAs other than PHB can be produced in *E. coli* by engineering the metabolic pathways and by providing different substrates (2, 14, 16). More than 140 different monomer units can be incorporated into PHAs (28). Production of these RHAs will be possible by employing the strategy reported here and with the appropriate pairs of PHA biosynthesis and degradation genes having different substrate specificities. This was demonstrated by producing R3HB and R3HV by culturing a metabolically engineered *E. coli* in a medium containing glucose and propionic acid. This is a good example of "green chemistry" in that high-value fine chemicals are produced from renewable resources such as glucose and fatty acids.

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