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Biosynthesis of Poly(3-hydroxybutyrate-*co*-3-hydroxyalkanoates) by Metabolically Engineered *Escherichia coli* Strains

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Abstract

Biosynthesis of polyhydroxyalkanoates (PHAs) consisting of 3-hydroxyalkanoates (3HAs) of 4 to 10 carbon atoms was examined in metabolically engineered *Escherichia coli* strains. When the *fadA* and/or *fadB* mutant *E. coli* strains harboring the plasmid containing the *Pseudomonas* sp. 61-3 *phaC2* gene and the *Ralstonia eutropha phaAB* genes were cultured in Luria-Bertani (LB) medium supplemented with 2 g/L of sodium decanoate, all the recombinant *E. coli* strains synthesized PHAs consisting of C4, C6, C8, and C10 monomer units. The monomer composition of PHA was dependent on the *E. coli* strain used. When the *fadA* mutant *E. coli* was employed, PHA containing up to 63 mol% of 3-hydroyhexanoate was produced. In *fadB* and *fadAB* mutant *E. coli* strains, 3-hydroxybutyrate (3HB) was efficiently incorporated into PHA up to 86 mol%. Cultivation of recombinant *fadA* and/or *fadB* mutant *E. coli* strains in LB medium containing 10 g/L of sodium gluconate and 2 g/L of sodium decanoate resulted in the production of PHA copolymer containing a very high fraction of 3HB up to 95 mol%. Since the material properties of PHA copolymer consisting of a large fraction of 3HB and a small fraction of medium-chain-length 3HA are similar to those of low-density polyethylene, recombinant *E. coli* strains constructed in this study should be useful for the production of PHAs suitable for various commercial applications.

Index Entries: Polyhydroxyalkanoates; *Escherichia coli*; β-oxidation; sodium decanoate; copolymer.

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Introduction

Polyhydroxyalkanoates (PHAs) are accumulated in numerous Gram-positive and Gram-negative bacteria as a carbon/energy storage material under the nutrient-limiting condition in the presence of excess carbon source *(1–5)*. PHAs have been drawing much attention as a family of completely biodegradable plastics and elastomers *(4,6)*. More than 150 kinds of (*R*)-hydroxyalkanoic acid monomers have been found to be assimilated into bacterial PHAs *(7)*. The monomer composition of PHAs highly depends on the metabolic capability of host microorganism and on the substrate specificity of PHA synthase *(8)*.

Bacterial PHAs have been generally classified into two groups depending on the number of carbon atoms in the monomer units: short-chain-length (SCL) PHAs consisting of monomers having C3–C5 atoms, and medium-chain-length (MCL) PHAs consisting of monomers having C6–C14 atoms *(3,5)*. Recently, PHAs containing both SCL and MCL monomer units have also been found in several bacteria *(9–15)*. Poly(3-hydroxybutyrate) (P[3HB]) is the most extensively studied member of SCL-PHAs, and it is rather stiff and crystalline, thus making it difficult to process and be commercially utilized. On the other hand, MCL-PHAs possess low crystallinity and high elasticity, which makes them good candidates for biodegradable rubber and elastomer. The copolymer of randomly incorporated 3-hydroxybutyrate (3HB) and 3-hydroxyhexanoate (3HHx), P(3HB-*co*-3HHx), was recently found to be accumulated in several aeromonads cultured on oils and fatty acids *(13,14)*. The material properties of this copolymer were dependent on the 3HHx mole fraction; the copolymer containing 20 mol% of 3HHx shows properties similar to low-density polyethylene (LDPE) *(16)*. Therefore, the incorporation of MCL monomer unit into the P(3HB) polymer backbone improves the ductility and processibility suitable for commercial applications *(16)*. Furthermore, copolymers consisting of 3HB and a small amount of MCL monomers (e.g., 95 mol% 3HB and 5 mol% MCL monomers) were shown to be more flexible material than P(3HB-co-3HHx), having material properties similar to those of LDPE *(17)*.

Recently, the PHA biosynthesis genes were cloned from *Pseudomonas* sp. 61-3, which is able to produce a random PHA copolymer consisting of 3HAs having C4–C12 carbon atoms *(18)*. The heterologous expression of the *Pseudomonas* sp. 61-3 PHA synthase gene ($phaC1_{Ps}$ or $PhaC2_{Ps}$) in recombinant *Ralstonia eutropha* PHB^{-4} and *Pseudomonas putida* GPp104 resulted in the accumulation of copolymer consisting of C4–C12 monomers from fatty acids, but the 3HB monomer fraction in coplymers was lower than 50 mol% *(18)*. The additional introduction of the *R. eutropha* $phaAB_{Re}$ genes along with the $phaC1_{Ps}$ gene allowed production of copolymer having a high 3HB fraction up to 85 mol% *(19)*. In addition, a copolymer having a very high 3HB fraction of 94 mol% could be produced from glucose by expressing the $phaC1_{Ps}$ and $phaAB_{Re}$ genes in *Pseudomonas* sp. 61-3 deficient in P(3HB) synthase gene ($phbC_{ps}$) *(17)*.

This kind of copolymer consisting of C4–C12 monomers could also be produced in recombinant *Escherichia coli* by the introduction of the $phaC1_{Ps}$ gene and one of the *P. aeruginosa* (*R*)-specific enoyl-CoA hydratase genes ($phaJ1_{Pa}$ or $phaJ2_{Pa}$). However, the mole fraction of 3HB in the copolymer was lower than 10 mol%, resulting in a material property quite similar to that of MCL-PHA *(20)*. In principle, the intermediates of fatty acid metabolism can be converted to 3-hydroxyacyl-CoAs (3HA-CoAs) of different carbon numbers depending on the supplied fatty acids *(4)*. Recently, it has been reported that the *fad* mutant *E. coli* strains could supply various MCL monomers, the number of carbon atoms of which were the same and reduced by two or four compared with those of supplied fatty acids *(21–26)*. This means that *E. coli* impaired in the β-oxidation pathway can supply acetyl-CoAs, the major precursors for 3HB, along with MCL monomers from fatty acids. Therefore, in the present study, we investigated the biosynthesis of copolymers composed of 3HB and a small amount of MCL monomers in *fadA* and/or *fadB* mutant *E. coli* strains by the coexpression of the *Pseudomonas* sp. 61-3 $phaC2_{Ps}$ gene and the *R. eutropha* $phaAB_{Re}$ genes.

Materials and Methods

Bacterial Strains and Plasmids

The strains used are provided in Table 1. All cloning was carried out using *E. coli* XL1-Blue as a host strain. The *E. coli* strains defective in fatty acid β-oxidation pathway were previously described (21,26) and used for the production of PHA: WA101 (W3110 *fadA::Km*), WB101 (W3110 *fadB::Km*), and WAB101 (W3110 *fadBA::Km*).

Plasmid Construction

The plasmids used are also provided in Table 1. All DNA manipulations including restriction digestion, ligation, and agarose gel electrophoresis were carried out by standard procedures *(27)*. The sequences of the primers used in polymerase chain reaction (PCR) are given in Table 2. PCR was performed by a PCR Thermal Cycler MP (Takara Shuzo, Shiga, Japan) using an Expand™ High Fidelity PCR System (Roche, Mannheim, Germany). DNA sequencing was carried out using a Bigdye terminator cycle sequencing kit (Perkin-Elmer, Boston, MA) and *Taq* polymerase using ABI Prism™ 377 DNA sequencer (Perkin-Elmer). The procedures for constructing the plasmids are illustrated in Fig. 1. pBlueReAB was constructed by the ligation of *Pst*I-digested fragment containing the *R. eutropha* $phaAB_{Re}$ genes from pSYL107 *(28)* with *Pst*I-digested pBluescript SK(–). The *gntT104* promoter, which can transcribe the genes constitutively owing to the substitutional mutation in the internal operator of *gntT* promoter, was used for the constitutive expression of genes *(21,26,29)*. The PCR product of the *gntT104* promoter containing the *E. coli* ribosomal binding site was amplified using *E. coli* W3110 chromosomal DNA as a template. The *Pseudomonas* sp. 61-3 $phaC2_{Ps}$ gene was amplified using pBSEB50 *(18)* as a template.

Table 1
Bacterial Strains and Plasmids Used

Strains or plasmids	Relevant characteristics	References or source
E. coli strains		
XL1-Blue	*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*, *suppE44*, *relA1*, *l*$^{-}$, *lac*$^{-}$, *F'[proAB lacl*^{q} *lacZDM15, Tn10 (tet)r]*	Stratagene [a]
W3110	*F*$^{-}$ *mcrA mcrB IN(rrnD*$^{-}$*rrnE)1*l^{-}	KCTC [b]
WA101	W3110 (*fadA::Km*)	*21,26*
WB101	W3110 (*fadB::Km*)	*26*
WAB101	W3110 (*fadBA::Km*)	*26*
Plasmids		
pBluescript SK(–)	Ap^{r}; *lacZ*; cloning vehicle	Stratagene
pBSEB50	pBluescript II KS(+) derivative; *Pseudomonas* sp. 61-3 PHA biosynthesis genes ($phaC1_{Ps}$, $phaZ_{Ps}$, $phaC2_{Ps}$)	*18*
pSYL107	pBluescript KS(–) derivative; *R. eutropha* PHA biosynthesis genes ($phaCAB_{Re}$), *parB*, *E. coli ftsZ* gene	*28*
pJC4	pGEM-7Zf(+) derivative; *Alcaligenes latus* PHA biosynthesis genes, *parB* (*hok/sok*) locus of plasmid R1	*30*
pBlueReAB	pBlueScript SK (–) derivative; $phaAB_{Re}$	This study
pUCstb	pUC19 derivative; *parB* (*hok/sok*) locus of plasmid R1	This study
p104613C2ReABstb	pBlueScript SK (–) derivative; *gntT104* promoter, $phaC2_{Ps}$, $phaAB_{Re}$, *parB*	This study

[a] Stratagene Cloning System, La Jolla, CA.
[b] Korean Collection for Type Cultures, Daejeon, Republic of Korea.

Table 2
Primers Used in PCR Experiments[a]

Primer	Primer sequence	Gene	Template
1	5-CGC**GGATCC**AATAAGGAGATA**TCTAGA**TGAGAGAGAAACCAACGCCG	*phaC2*$_{Ps}$	pBSEB50
2	5-C**GGATCC**CCGGGTACCGAGCTCGAATTCTCAGCGCACGCGCACGTAGGTA	*phaC2*$_{Ps}$	pBSEB50
3	5-G**ACTAGT**TGAAAGGTGTGCGCGATCTCAC	*gntT104* promoter	*E. coli* W3110 chromosome
4	5-GC**GGATCC**CATTTGTTATGGGCGACGTCAATTT	*gntT104* promoter	*E. coli* W3110 chromosome

[a] Restriction enzyme sites are shown in bold. The ribosome binding site is underlined.

Gene fragment containing *gntT104* promoter and *Pseudomonas* sp. 61-3 $phaC2_{Ps}$ gene was cloned into pBlueReAB to make p104613C2ReAB, as shown in Fig. 1. Finally, to increase plasmid stability, the *parB* (*hok/sok*) locus of plasmid R1 from pJC4 *(30)* was introduced into p104613C2ReAB to make p104613C2ReABstb (Fig. 1).

Culture Conditions

E. coli XL1-Blue was grown at 37°C in Luria-Bertani (LB) medium (containing 10 g/L of tryptone, 5 g/L of yeast extract, and 5 g/L of NaCl). Recombinant *E. coli* strains for the PHA production were cultivated at 30°C for 72 h in LB medium containing two different carbon sources: (1) 2 g/L of sodium decanoate (Sigma, St. Louis, MO); and (2) 10 g/L of sodium gluconate (Junsei, Tokyo, Japan) plus 2 g/L of sodium decanoate. All the flask cultures were carried out in triplicate in a rotary shaker at 250 rpm. For the cultivation of recombinant *E. coli* strains, ampicillin (50 mg/L) was added to the medium.

Analytical Procedures

Cell growth was monitored by measuring the absorbance at 600 nm (OD_{600} (DU Series 600 Spectrophotometer; Beckman, Fullerton, CA). PHA concentration and monomer composition were determined by gas chromatography (Donam, Seoul, Korea) equipped with a fused silica capillary column (Supelco SPB™-5, 30 m × 0.32 mm id, 0.25 μm film; Bellefonte, PA) using benzoic acid as an internal standard *(31)*. Cell concentration, defined as dry cell weight per liter of culture broth, was determined as previously described *(14)*. The residual cell concentration was defined as the cell concentration minus PHA concentration. The PHA content (wt%) was defined as the percentage of the ratio of PHA concentration to cell concentration.

Results

PHA Production in Flask Cultures

Recombinant *E. coli* strains WA101, WB101, and WAB101 harboring p104613C2ReABstb were cultured in LB medium containing 2 g/L of sodium decanoate at 30°C. These recombinant *E. coli* strains were also cultured in LB medium containing 2 g/L of sodium decanoate plus 10 g/L of sodium gluconate. The results of flask cultures are summarized in Table 3. When sodium decanoate was used as a sole carbon source, all the recombinant *E. coli* strains accumulated poly(3-hydroxybutyrate-*co*-3-hydroxyalkanoates) (P[3HB-*co*-3HAs]) consisting of various monomers including 3HB, 3HHx, 3-hydroxyoctanoate (3HO), and 3-hydroxydecanoate (3HD). The mole fraction of 3HB and 3HHx in copolymer varied considerably depending on the host strain and the kind of mutation. When the *fadB* mutant *E. coli* strain was employed, PHA containing a high 3HB fraction up to 86 mol% was obtained. When the *fadA* mutant *E. coli* strain was used,

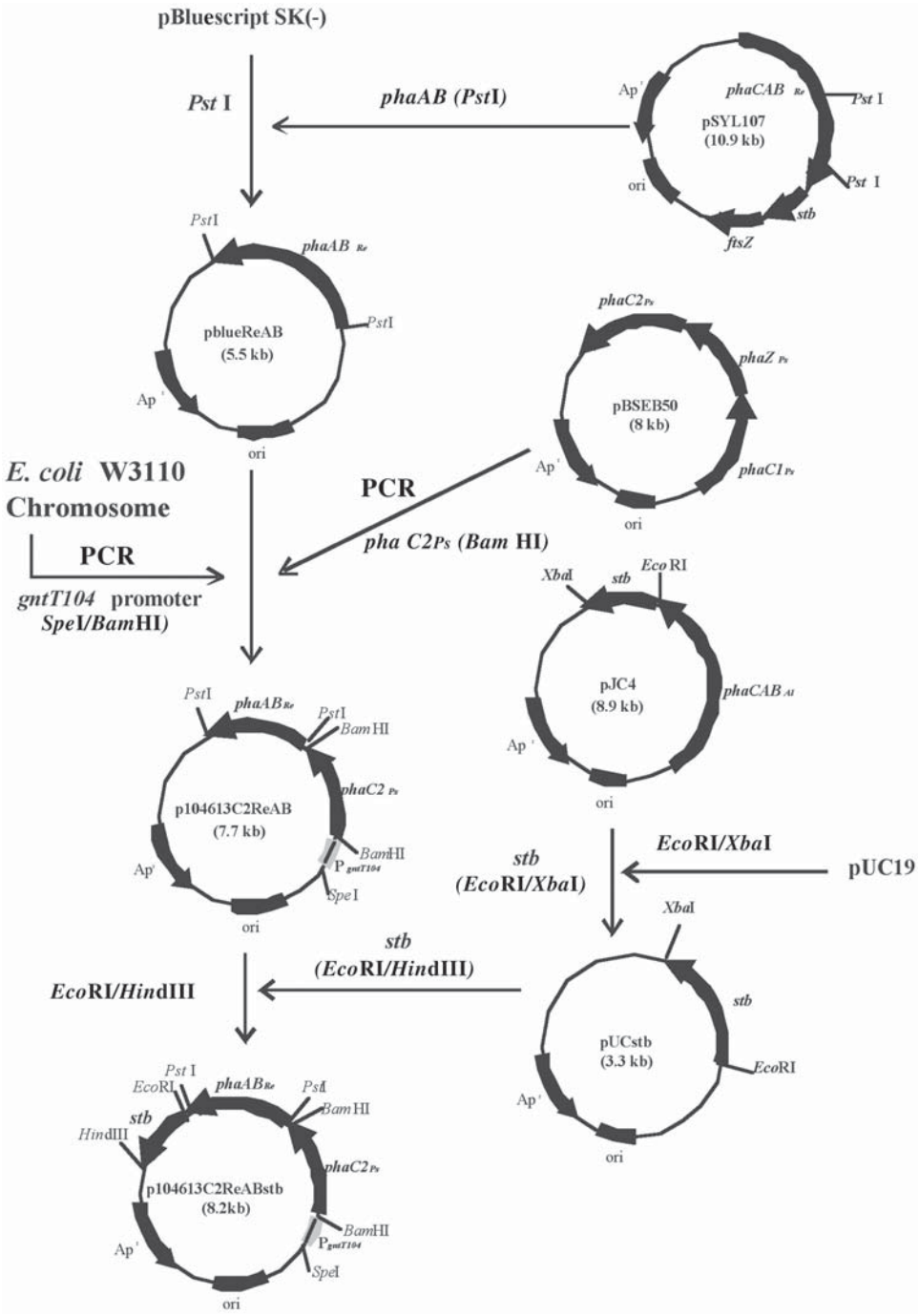

Fig. 1. Construction of plasmid p104613C2ReABstb, which is used to transform *fad* mutant *E. coli* strains for production of SCL-MCL PHA copolymers.

Table 3
Results of Flask Cultures of Recombinant *E. coli* Strains Harboring p104613C2ReABstb[a]

Strain	Carbon source[b]	Dry cell weight (g/L)	PHA concentration (g/L)	PHA content (wt%)	Composition (mol %)			
					3HB	3HHx	3HO	3HD
WA101	Decanoate	1.90 ± 0.02	0.60 ± 0.02	31.4 ± 2.0	34 ± 2	63 ± 2	3 ± 2	0
	Gluconate + decanoate	3.92 ± 0.08	1.90 ± 0.04	48.4 ± 2.0	89 ± 2	8 ± 2	2 ± 2	1 ± 2
WB101	Decanoate	1.20 ± 0.03	0.30 ± 0.02	25.5 ± 2.0	86 ± 2	10 ± 2	4 ± 2	0
	Gluconate + decanoate	3.48 ± 0.04	0.88 ± 0.02	25.3 ± 2.3	87 ± 2	11 ± 2	1 ± 2	1 ± 2
WAB101	Decanoate	1.25 ± 0.02	0.31 ± 0.03	25.0 ± 2.6	70 ± 2	26 ± 2	3 ± 2	1 ± 2
	Gluconate + decanoate	4.15 ± 0.05	1.34 ± 0.05	32.3 ± 1.6	95 ± 2	2 ± 2	2 ± 2	1 ± 2

[a] Cells were cultivated for 72 h in LB medium supplemented with different carbon sources. All experiments were carried out in triplicate.
[b] Sodium decanoate and sodium gluconate were added to concentrations of 2 g/L and 10 g/L, respectively.

PHA containing a high mole fraction of 3HHx up to 63 mol% was produced. Unexpectedly, only a very small amount of 3HD was incorporated into the copolymer, even though the *Pseudomonas* sp. 61-3 PHA synthase has a wide range of substrate specificity to monomers having C4–C12 atoms *(18)*.

To increase further the 3HB fraction in the copolymer, sodium gluconate (10 g/L) was added to supply more acetyl-CoAs. By supplementing sodium gluconate, the 3HB fraction in the copolymer significantly increased. The effect of supplementing sodium gluconate was much more significant in the *fadAB* mutant *E. coli* strain; this resulted in a striking increase in the 3HB monomer fraction up to 95 mol% at the expense of the 3HHx monomer fraction. In addition, only a small amount of 3HD was incorporated into PHA by the addition of sodium gluconate in all the *fad* mutant *E. coli* strains.

Discussion

Recent studies suggest that the monomer composition of PHA is highly dependent on the metabolic capability of the host microorganism and substrate specificity of PHA synthase. In the present study, we developed recombinant *fad* mutant *E. coli* strains for the biosynthesis of PHA copolymer consisting of a high mole fraction of 3HB and a low mole fraction of MCL monomers. PHA has been routinely produced from the intermediates of the fatty acid metabolism by natural or recombinant bacteria using related or unrelated carbon sources such as glucose and fatty acid *(4)*. In *E. coli*, 3HB monomers have been efficiently generated by the expression of *phaAB* genes from acetyl-CoA *(26)*. It has been reported that recombinant *E. coli* expressing the MCL-PHA synthase gene could accumulate MCL-PHA from MCL fatty acids by the coexpression of the enoyl-CoA hydratase *(20)* or ketoacyl-ACP reductase gene *(21,25,32)*, or by inhibition of the β-oxidation pathway *(21–26)*. We have developed *E. coli* strains defective in the β-oxidation pathway for the production of SCL-MCL PHA copolymers. The *fad* mutant *E. coli* strains have been reported to be able to generate 3HA-CoAs, whose carbon numbers are reduced by two, four, six, or more, from the supplied fatty acids *(21–26)*. This means that acetyl-CoA, the starting metabolite for 3HB monomers, can also be generated by the *fad* mutant *E. coli* strain by supplying fatty acids. Recently, it has been reported that various enzymes homologous to Fad enzymes, such as YfcY and YfcX, are involved in the generation of 3HA-CoAs in the *fad* mutant *E. coli* strain *(24,33)*. Furthermore, *E. coli* MaoC has been revealed to have enoyl-CoA hydratase activity, which allows generation of (*R*)-3HA-CoAs in *fadB* mutant *E. coli* strain *(34)*.

The inherent substrate specificities of *Pseudomonas* sp. 61-3 PHA synthases ($PhaC1_{Ps}$ and $PhaC2_{Ps}$) are rather low toward 3HB monomer *(17,18)*, and the *fad* mutant *E. coli* strain used in our study cannot generate enough 3HB monomers from the β-oxidation pathway *(21,26)*. Therefore, we introduced the *R. eutropha* $phaAB_{Re}$ genes to generate enough more 3HB

monomers to increase the 3HB fraction in the copolymer. In addition, gluconate was provided as an auxiliary carbon source to supply more acetyl-CoAs, which are in turn converted into 3HB monomers in two reaction steps catalyzed by PhaA and PhaB. The metabolic pathways for PHA biosynthesis in several different *fad* mutant *E. coli* strains are shown in Fig. 2.

Previously, coexpression of the $phaAB_{Re}$ genes with the $phaC1_{Ps}$ gene in recombinant *R. eutropha* and pseudomonads resulted in the P(3HB-*co*-3HA) having a high mole fraction of 3HB, the material properties of which are tougher and flexible, thus making it suitable for a wide range of commercial applications *(17,19)*. As expected, when the $phaAB_{Re}$ and $phaC2_{Ps}$ genes were coexpressed in *fad* mutant *E. coli* strains and gluconate was added, copolymers consisting of a high mole fraction of 3HB were produced (Table 3), which have been suggested to have ductile material property similar to that of LDPE *(17)*. The mole fractions of monomer were dependent on the kind of *fad* mutation and host strain. The 3HHx fraction of the copolymer produced in the *fadA* mutant *E. coli* WA101 was much higher than that produced in the *fadB* and *fadAB* mutant *E. coli* strains. Previously, we reported that *E. coli* WA101 harboring only the $phaC2_{Ps}$ gene was able to produce MCL-PHA consisting of 3HHx, 3HO, and 3HD from sodium decanoate, in which 3HO and 3HD were the major components and 3HHx was a minor component *(21)*. The striking increase in the 3HHx mole fraction in the copolymer seems to be owing to the coexpression of the $phaB_{Re}$ gene because more 3HHx monomers can be generated by the reduction of 3-ketohexanoyl-CoA in *fadA* mutant *E. coli*, as previously reported by Ren et al. *(35)*. In the *fadB* and *fadAB* mutant *E. coli* strains, similar monomer compositions were observed with a slight increase in 3HHx fraction in *fadAB* mutant *E. coli*.

Conclusion

We developed several recombinant *E. coli* strains for the production of P(3HB-*co*-3HA) having various monomer compositions. By blocking the β-oxidation pathway and coexpressing the *phaAB* genes, various PHA monomers could be efficiently supplied. In addition, the monomer composition in the copolymer could be altered by supplying different carbon sources. The recombinant *E. coli* system that we developed should be useful for the production of copolymers consisting mainly of 3HB monomer and a small amount of MCL monomers, which can be used in a wide range of applications owing to their tougher and flexible material properties.

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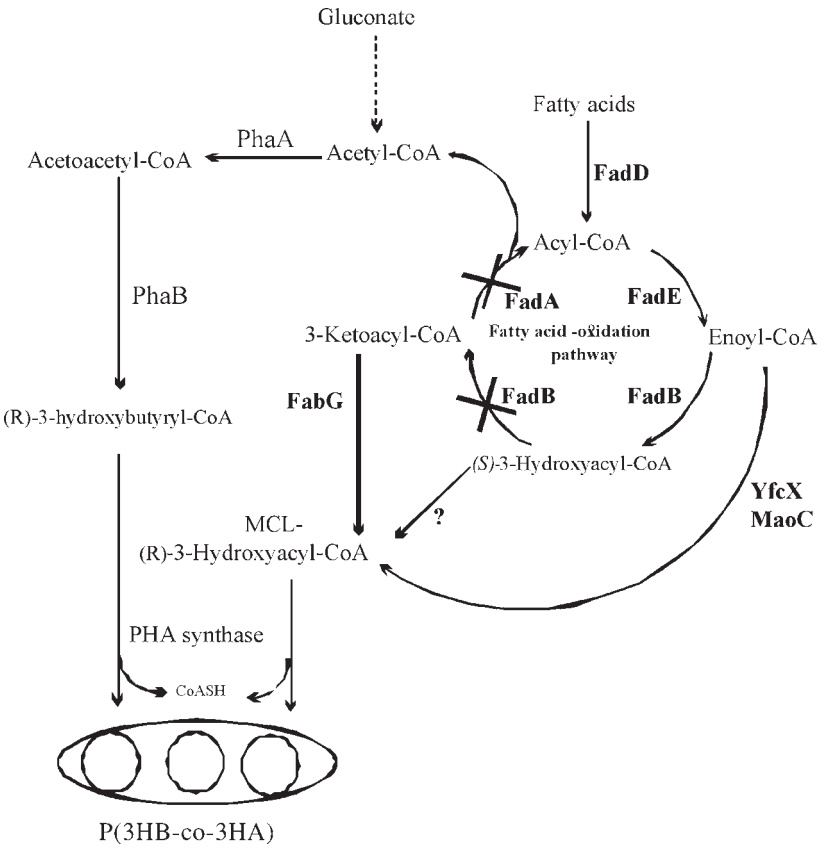

Fig. 2. Metabolic pathways for PHA biosyntheis in *fad* mutant *E. coli* strains used in this study. Enoyl-CoA hydratase, epimerase, and 3-ketoacyl-CoA or ACP reductase have been suggested to supply PHA precursors from inhibited b-oxidation pathway. The crosses indicate inactivation of corresponding enzymes. The question mark represents uncharacterized enzyme. Enzymes involved in the metabolic pathways shown have been described previously: FabG *(21,32)*, YfcX *(24,33)*, MaoC *(34)*, PhaA *(36)*, and PhaB *(36)*.

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