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Modification of β-oxidation pathway in *Ralstonia eutropha* for production of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) from soybean oil

Chayatip Insomphun,[1] Jun Mifune,[1] Izumi Orita,[1] Keiji Numata,[2] Satoshi Nakamura,[1] and Toshiaki Fukui[1,*]

Department of Bioengineering, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta, Midori-ku, Yokohama 226-8501, Japan[1] *and Enzyme Research Team, RIKEN Biomass Engineering Program, RIKEN, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan*[2]

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Ralstonia eutropha **H16 is a useful platform for metabolic engineering aiming at efficient production of polyhydroxyalkanaotes being attracted as practical bioplastics. This study focused on bifunctional (*S*)-specific 2-enoyl-CoA hydratase/(*S*)-3-hydroxyacyl-CoA dehydrogenase encoded by *fadB* to obtain information regarding β-oxidation in this bacterium and to achieve compositional regulation of poly((*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] synthesized from soybean oil. In addition to two FadB homologs (FadB1 and FadB′) encoded within the previously identified β-oxidation gene clusters on the chromosome 1, a gene of third homolog (FadB2) was found on chromosome 2 of *R. eutropha*. The *fadB* homologs were disrupted in *R. eutropha* strain NSDG expressing a mutant gene of PHA synthase from *Aeromonas caviae*. The gene disruptions affected neither growth nor PHA production on fructose. On soybean oil, *fadB′* deletion led to reduction of PHA quantity attributed to decrease of 3HB unit, while *fadB1* deletion slightly increased 3HHx composition without serious negative impact on both cell growth and PHA biosynthesis. Double deletion of *fadB1* and *fadB′* significantly impaired the cell growth and PHA biosynthesis, indicating the major roles of *fadB1* and *fadB′* in β-oxidation. When *fadB1* was deleted in several engineered strains of *R. eutropha* possessing additional (*R*)-enoyl-CoA hydratase gene(s), the net amounts of 3HHx unit in the PHA fractions showed 6–21% increase probably due to slightly enhanced supply of medium-chain-length 2-enoyl-CoAs through the partially impaired β-oxidation. These results demonstrated that modification of β-oxidation by *fadB1* deletion was effective for increasing 3HHx composition in the copolyesters produced from soybean oil.**

[**Key words:** Polyhydroxyalkanoate; Bioplastic; *Ralstonia eutropha*; β-Oxidation; Vegetable oil]

Polyhydroxyalkanoates (PHAs) are carbon and energy storage materials synthesized by a wide variety of bacteria. PHAs have attracted attention as eco-friendly thermoplastics because they can be produced from renewable biomass resources and are completely degraded to CO_2 and H_2O by environmental microbes unlike many petroleum-based plastics (1–3).

Many bacteria and some archaea synthesized poly[(*R*)-3-hydroxybutyrate] [P(3HB)] generally via a three step reaction; condensation of two acetyl-CoA molecules and subsequent (*R*)-specific reduction to provide (*R*)-3-hydroxybutyryl-CoA (3HB-CoA) by β-ketothiolase (PhaA) and NADPH-dependent acetoacetyl-CoA reductase (PhaB), respectively, and polymerization of (*R*)-3HB-CoA by PHA synthase (PhaC). Several kinds of bacteria can biosynthesize copolyesters composed of (*R*)-3-hydroxyalkanoates (3HAs) with different chain length. *Pseudomonas* spp. possess type II PHA synthase with broad substrate specificity, thus they accumulate PHAs composed of medium-chain-length (C_6–C_{12}) 3HA units from various carbon sources (4–8). *Aeromonas caviae* can synthesize poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] from vegetable oils and fatty acids of even-carbon numbers (9), because PHA synthase in this bacterium ($PhaC_{Ac}$) showed polymerization activity to (*R*)-3HA-CoAs from C_4 to C_7 (10–12).

When vegetable oil or fatty acid was fed as a carbon source, the acyl moieties incorporated into the cells are degraded to acetyl-CoA molecules through β-oxidation mediated by acyl-CoA synthetase, acyl-CoA dehydrogenase, (*S*)-specific enoyl-CoA hydratase, (*S*)-3HA-CoA dehydrogenase, and 3-oxoacyl-CoA thiolase (13). It has been clarified that, in *Escherichia coli* and *Pseudomonas* spp., a trifunctional enzyme complex FadBA is functional in β-oxidation (14–17). This complex is composed of two subunits in an $\alpha_2\beta_2$ conformation, in which the large subunit α encoded by *fadB* exhibits (*S*)-specific enoyl-CoA hydratase and (*S*)-3HA-CoA dehydrogenase activity and the small subunit β encoded by *fadA* is 3-oxoacyl-CoA thiolase (14,16). The linkage between fatty acid β-oxidation and PHA biosynthesis is mediated by (*R*)-specific enoyl-CoA hydratase (PhaJ) providing (*R*)-3HA-CoA monomers from 2-enoyl-CoA intermediates in β-oxidation (12,18,19). It has been reported that 3-oxoacyl-acyl carrier protein reductases (FabG) from pseudomonads and *E. coli* showed an ability to supply (*R*)-3HA-CoAs from the corresponding 3-oxoacyl-CoAs for biosynthesis of medium-chain-length PHAs in recombinant *E. coli* (20–22), however, the contribution of FabG to PHA biosynthesis by natural PHA producers has not been demonstrated. Previous studies

* Corresponding author. Tel./fax: +81 45 924 5766.
E-mail address: tfukui@bio.titech.ac.jp (T. Fukui).

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indicated that inhibition of β-oxidation by acrylic acid and disruption of *fadB* greatly affected composition of PHA copolymers from fatty acids (15,23–25). For example, disruption of *fadB* or *fadBA* in *Pseudomonas putida* KT2442 led to decrease of C_8 unit and increase of longer C_{10} or C_{12} unit in medium-chain-length PHA copolymers produced from decanoic or dodecanoic acid (23,24). The well-studied organism for PHA biosynthesis is the gram-negative bacterium *Ralstonia eutropha*. This bacterium could accumulate P(3HB) up to 80% of its cell dry weight under nutrient limitation conditions (3). Moreover, to overcome the brittle property of P(3HB) homopolymer, many efforts have been made for biosynthesis of copolyesters consisting of 3HB with a second monomer unit by using this bacterium (1–3).

We have focused on engineering of *R. eutropha* for efficient production of P(3HB-*co*-3HHx) from renewable carbon sources without additional supplement of precursor compounds, because this copolyester is one of the most practical PHAs with higher flexibility and lower melting point than P(3HB) (9). It has been demonstrated that excellent ability to synthesize P(3HB-*co*-3HHx) from soybean oil was conferred to *R. eutropha* by replacement of *phaC1* and *phaA* in *pha* operon on chromosome 1 by $phaC_{NSDG}$ (encoding the N149S/D171G mutant of $PhaC_{Ac}$ that can incorporate (*R*)-3HHx unit into PHA more efficiently than the parent enzyme (26)) and $phaJ_{Ac}$ from *A. caviae* (27). Further investigation elucidated that endogenous PhaJ4a partially acted on providing (*R*)-3HHx-CoA via β-oxidation along with PhaB1 in *R. eutropha* (18) (Fig. 1). Budde et al. also engineered *R. eutropha* for production of P(3HB-*co*-3HHx) from palm oil using *phaC* and *phaJ* genes derived from *Rhodococcus aetherivorans* and *Pseudomonas aeruginosa*, respectively (28).

Nevertheless, there has been only little information regarding β-oxidation pathway in *R. eutropha*. Brigham et al. recently identified two gene clusters involved in β-oxidation in this bacterium by DNA microarray analysis (29), although functions of each gene within the clusters have not been investigated. In addition, as physical and mechanical properties of copolymers were highly depended on the composition, compositional regulation of PHAs synthesized via biological processes is a quite important subject to achieve the practical applications.

This study investigated the effects of disruption of *fadB* homologs in *R. eutropha* on P(3HB-*co*-3HHx) biosynthesis through β-oxidation, in order to obtain information regarding β-oxidation in this bacterium as well as to apply the modification of β-oxidation for compositional regulation of P(3HB-*co*-3HHx) produced from soybean oil.

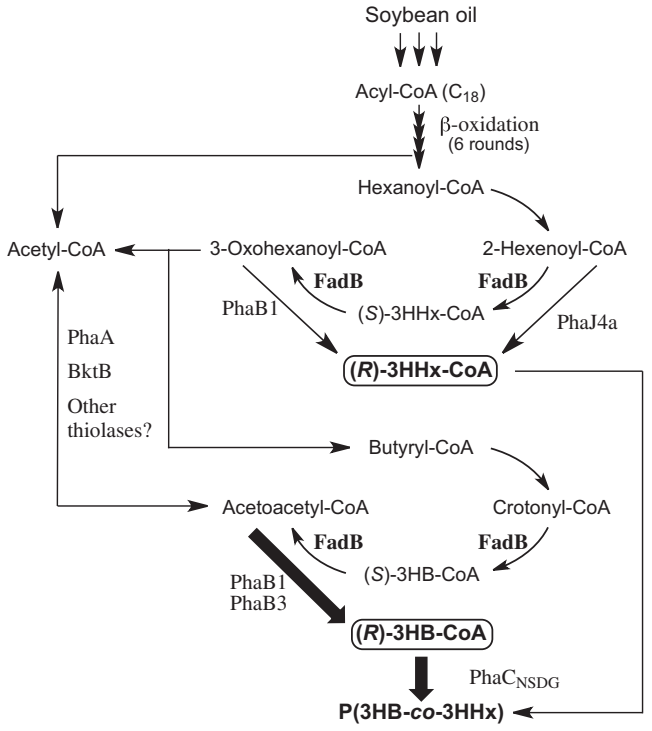

FIG. 1. Proposed P(3HB-*co*-3HHx) biosynthesis pathway from soybean oil in *R. eutropha* strain NSDG.

MATERIALS AND METHODS

Bacterial strains and culture condition The bacterial strains and plasmids used in this study are listed in Table 1. *R. eutropha* strains were cultivated at 30°C in a nutrient-rich (NR) medium containing 10 g of meat extract, 10 g of polypeptone, and 2 g of yeast extract in 1 L of tap water. *E. coli* strains were grown at 37°C on a Luria–Bertani (LB) medium. Kanamycin (100 μg/ml for *E. coli* and 250 μg/ml for *R. eutropha* strains) or ampicillin (100 μg/ml for *E. coli*) was added to the medium when necessary.

Construction of recombinant strains The sequences of oligonucleotide primers used in this study are listed in Supplementary Table S1. For deletion of *fadB* genes from the chromosomes of *R. eutropha* strains, plasmids pK18msΔ*fadB1*, pK18msΔ*fadB2* and pK18msΔ*fadB′* were constructed as below. A coding region of *fadB1* (H16_A1526, 2082 bp) along with the upstream and downstream flanking regions (approximately 1 kbp each) was amplified by PCR with genomic DNA of *R. eutropha* H16 and fadB1up-F/fadB1down-R as template and a primer pair, respectively. The amplified fragment was digested by HindIII and XbaI, and then inserted into pK18mobsacB at the corresponding sites. Inverse PCR using fadB1up-R/fadB1down-F primers was done to remove the *fadB1* coding region, and the resulting fragment, in which the *fadB1*-flanking regions were attached to the each end of plasmid backbone, was 5′-phosphorylated and self-ligated to obtain pK18msΔ*fadB1*.

pK18msΔ*fadB2* and pK18msΔ*fadB′* were constructed in the same way. The primers used for the first PCR are fadB2up-F/fadB2down-R for *fadB2* (H16_B0724, 2079 bp) and fadB′up-Fw/fadB′down-R for *fadB′* (H16_A0461, 2424 bp), respectively. The PCR products were inserted into pK18mobsacB at BamHI/EcoRI and HindIII/XbaI sites, and inverse PCR was performed using fadB2up-R/fadB2down-F and fadB′up-R/fadB′down-F primer pairs for construction of pK18msΔ*fadB2* and pK18msΔ*fadB′*, respectively.

All the pK18mobsacB-based plasmids were transferred from *E. coli* S17-1 by conjugation to *R. eutropha* strains NSDG, MF02, MF03, MF04, TT011, TT012 or TT013. Isolation of strains generated by pop in-pop out recombination was performed as described previously (27). Double and triple disruptions of the fadB genes were

TABLE 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant marker	Source or reference
Escherichia coli		
S17-1	*thi pro hsdR recA* chromosomal RP4; Tra^+; Tmp^r Str/Spc^r	30
Ralstonia eutropha		
H16	Wild type	DSM428
NSDG	H16 derivative; $\Delta phaC_{Re}$::$phaC_{NSDG}$	27
NSDGΔfadB1	NSDG derivative; Δ*fadB1*	This study
NSDGΔfadB2	NSDG derivative; Δ*fadB2*	This study
NSDGΔfadB′	NSDG derivative; Δ*fadB′*	This study
NSDGΔΔfadB1B2	NSDG derivative; Δ*fadB1* Δ*fadB2*	This study
NSDGΔΔfadB1B′	NSDG derivative; Δ*fadB1* Δ*fadB′*	This study
NSDGΔΔΔfadB1B2B′	NSDG derivative; Δ*fadB1* Δ*fadB2* Δ*fadB′*	This study
MF02	$\Delta phaC_{Re}$::$phaC_{NSDG}$-$phaJ_{Ac}$	27
MF03	$\Delta phaC_{Re}$::$phaC_{NSDG}$-$phaJ_{Ac}$, Δ*phaA*	27
MF04	$\Delta phaC_{Re}$::$phaC_{NSDG}$-$phaJ_{Ac}$, Δ*phaA*::*bktB*	27
TT011	$\Delta phaC_{Re}$::$phaC_{NSDG}$-$phaJ4a_{Re}$, Δ*phaA*	18
TT012	$\Delta phaC_{Re}$::$phaC_{NSDG}$-$phaJ4b_{Re}$, Δ*phaA*	18
TT013	$\Delta phaC_{Re}$::$phaC_{NSDG}$-$phaJ_{Ac}$-$phaJ4a_{Re}$, Δ*phaA*	18
MF02ΔfadB1	MF02 derivative; Δ*fadB1*	This study
MF03ΔfadB1	MF03 derivative; Δ*fadB1*	This study
MF04ΔfadB1	MF04 derivative; Δ*fadB1*	This study
TT011ΔfadB1	TT011 derivative; Δ*fadB1*	This study
TT012ΔfadB1	TT012 derivative; Δ*fadB1*	This study
TT013ΔfadB1	TT013 derivative; Δ*fadB1*	This study
Plasmids		
pK18mobsacB	pMB1 *ori*, *mob*, Kan^r, *sacB*	31
pK18Δ*fadB1*	pK18mobsacB carrying *fadB1 del*	This study
pK18Δ*fadB2*	pK18mobsacB carrying *fadB2 del*	This study
pK18Δ*fadB′*	pK18mobsacB carrying *fadB′ del*	This study