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Two NADH-dependent (*S*)-3-hydroxyacyl-CoA dehydrogenases from polyhydroxyalkanoate-producing *Ralstonia eutropha*

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Ralstonia eutropha **H16 contains both NADH- and NADPH-dependent reduction activities to acetoacetyl-CoA, and the NADPH-dependent activity is mediated by PhaB paralogs with (*R*)-stereospecificity providing (*R*)-3-hydroxybutyryl (3HB)-CoA monomer for poly((*R*)-3-hydroxybutyrate) synthesis. In contrast, the gene encoding the NADH-dependent enzyme has not been identified to date. This study focused on the NADH-dependent dehydrogenase with (*S*)-stereospecificity in *R. eutropha*, as the (*S*)-specific reduction of acetoacetyl-CoA potentially competed with the polyester biosynthesis via (*R*)-3HB-CoA. The NADH-dependent reduction activity decreased to one-half when the gene for H16_A0282 (PaaH1), one of two homologs of clostridial NADH-3HB-CoA dehydrogenase, was deleted. The enzyme responsible for the remaining activity was partially purified and identified as H16_A0602 (Had) belonging to a different family from PaaH1. Gene disruption analysis elucidated that most of the NADH-dependent activity was mediated by PaaH1 and Had. The kinetic analysis using the recombinant enzymes indicated that PaaH1 and Had were both NADH-dependent 3-hydroxyacyl-CoA dehydrogenases with rather broad substrate specificity to 3-oxoacyl-CoAs of C_4 to C_8. The deletion of *had* in the *R. eutropha* strain previously engineered for biosynthesis of poly((*R*)-3-hydroxybutyrate-*co*-(*R*)-3-hydroxyhexanoate) led to decrease in the C_6 composition of the copolyester synthesized from soybean oil, suggesting the role of Had in (*S*)-specific reduction of 3-oxohexanoyl-CoA with reverse β-oxidation direction. Crotonase ((*S*)-specific enoyl-CoA hydratase) in *R. eutropha* H16 was also partially purified and identified as H16_A3307.**

[**Key words:** Polyhydroxyalkanoates; Poly(3-hydroxybutyrate); *Ralstonia eutropha*; NADH-3-hydroxyacyl-CoA dehydrogenase; NADPH-acetoacetyl-CoA reductase; Crotonase]

A number of bacteria synthesize polyhydroxyalkanoates (PHAs) as intracellular storage materials from renewable carbon sources under unbalanced growth conditions (1,2). As PHAs are biodegradable bio-based polymeric materials and several kinds of PHAs are thermoplastics with similar properties to common petroleum-based plastics, they have attracted industrial attention as eco-friendly plastics (3). Poly[(*R*)-3-hydroxybutyrate] [P(3HB)] is the most widely distributed PHAs in nature. In many P(3HB)-producing microbes, two molecules of acetyl-CoA are condensed by β-ketothiolase (PhaA), and the resulting acetoacetyl-CoA is reduced by NADPH-dependent acetoacetyl-CoA reductase (PhaB1) with (*R*)-stereospecificity to form (*R*)-3-hydroxybutyryl (3HB)-CoA. (*R*)-3HB-CoA is then polymerized by PHA synthase (PhaC) to the high molecular weight polyester. *Ralstonia eutropha* (*Cupriavidus necator*) strain H16 has been well studied as an efficient producer of P(3HB) from several carbon sources such as fructose, gluconate, fatty acids, and vegetable oils (4). The P(3HB) biosynthesis genes are clustered as *phaCAB1* operon on the chromosome 1 (5). There have been several studies regarding production of PHA copolymers showing more flexible properties than P(3HB) homopolymer by feeding precursor compounds to *R. eutropha*, and metabolic engineering of this bacterium (1,4,6–9).

It has been reported by Haywood et al. (10) in 1988 that the cell extract of *R. eutropha* H16 contained both NADPH- and NADH-dependent reduction activities to acetoacetyl-CoA, and the enzymes were individually purified and characterized. Later studies elucidated that most of the NADPH-dependent activity was mediated by PhaB1, as the activity was markedly reduced by disruption of *phaB1* (11,12). When the cells grown on fructose, PhaB1 and a weakly expressed paralog PhaB3 were involved in P(3HB) biosynthesis by providing the (*R*)-3HB-CoA monomer from acetyl-CoA (11). In contrast, the gene encoding the previously purified NADH-dependent enzyme has not been identified. The enzyme catalyzing the reversible oxidoreduction between acetoacetyl-CoA and (*S*)-3HB-CoA with NAD(H) dependency has been known as 3HB-CoA dehydrogenase. In solventogenic *Clostridium acetobutylicum*, 3HB-CoA dehydrogenase (Hbd) has a role in formation of (*S*)-3HB-CoA from acetoacetyl-CoA, and the resulting (*S*)-3HB-CoA is reduced to *n*-butanol or butyrate via butyryl-CoA (13,14). Disruption of *hbd* in *C. acetobutylicum* blocked the *n*-butanol/butyrate formation pathway, changing the strain to an ethanol producer (15). Recently, Machado et al. (16) have directed attention to a homolog of clostridial Hbd from *R. eutropha*, annotated as PaaH1, as a promising NADH-dependent 3-hydroxyacyl (3HA)-CoA dehydrogenase by referencing the results by Haywood et al. (10), and applied it in an artificial pathway for biosynthesis of linear higher

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alcohols by recombinant *Escherichia coli* (10). Kim et al. (17) have reported the crystal structure and NADH-dependent 3HB-CoA dehydrogenase activity of PaaH1. However, it should be noted that there has been no experimental evidence for that PaaH1 was corresponded to the NADH-dependent enzyme purified by Haywood et al. (10) to date.

Given the competition of the NADH-dependent reduction of acetoacetyl-CoA to (*S*)-3HB-CoA with the PhaB1-driven P(3HB) biosynthesis in *R. eutropha*, understanding of the details of the NADH-dependent conversion pathway and the role in carbon/energy metabolisms in *R. eutropha* are important to apply this bacterium as a platform for production of value-added compounds, including PHAs, from biomass feedstocks. In this study, two dehydrogenases responsible for the NADH-dependent reduction of acetoacetyl-CoA in *R. eutropha* were identified. The catalytic properties of these two enzymes indicated that they were NADH-(*S*)-3HA-CoA dehydrogenase with rather broad substrate specificity. The study further investigated the effects of the gene deletion on PHA biosynthesis, as well as identified crotonase, converting (*S*)-3HB-CoA to crotonyl-CoA, in *R. eutropha*.

MATERIALS AND METHODS

Bacterial strains and plasmids 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 Lysogeny broth (LB) medium for general gene manipulation and transconjugation. Kanamycin (100 mg/L for *E. coli* and 200 mg/L for *R. eutropha* strains) or ampicillin (100 mg/L for *E. coli*) was added to the medium when necessary.

Construction of gene-deleted strains of *R. eutropha* DNA manipulations were carried out according to standard procedures, and PCR reactions were performed with KOD-Plus ver.2 DNA polymerase (Toyobo, Osaka, Japan). The sequences of oligonucleotide primers used for PCR amplification are shown in Supplementary Table S1. Plasmids for deletion of *h16_A0282*, *h16_A1102*, or *h16_A0602* from the chromosome of *R. eutropha* were constructed as below; the target gene region along with approximately 1-kbp of the flanking upstream and downstream regions was amplified by PCR with genomic DNA of *R. eutropha* H16 as a template and the appropriate primer sets. The amplified fragment was digested by BamHI, and then inserted into pK18mobsacB (18) at the corresponding site. This plasmid was used as a template for inverse PCR to remove the coding region, and the resulting fragment consisting of the upstream region, vector backbone, and downstream region was 5′-phosphorylated and self-ligated. *E. coli* S17-1 (19) harboring the deletion vector pK18ms-ΔA0282, pK18ms-ΔA1102, or pK18ms-ΔA0602 was used to transform *R. eutropha* H16 and TT013 (20) by transconjugation, and the single deletion strains formed by homologous recombination were selected as described previously (12). The double deletion strains were constructed from H16ΔA0282 or TT013ΔA0282 as the parent strain by using the deletion vectors pK18ms-ΔA1102 and pK18ms-ΔA0602.

PHA production by *R. eutropha* *R. eutropha* strains were cultivated at 30 °C in 100 mL of a nitrogen-limited mineral salts (MB) medium composed of 0.9 g of Na_2HPO_4 $12H_2O$, 0.15 g of KH_2PO_4, 0.05 g of NH_4Cl, 0.02 g of $MgSO_4$ $7H_2O$, and 0.1 mL of trace-element solution (21) in 100 mL of deionized water. A filter-sterilized solution of fructose was added to the medium at a final concentration of 0.5% (*w*/*v*) as a sole carbon source. Soybean oil was directly added to the medium at 1.0% (*v*/*v*). Kanamycin was added at the final concentration of 300 mg/L, when necessary. After the cultivation for 72 h with reciprocal shaking (115 strokes/min), the cells were harvested, washed once with cold deionized water, and then lyophilized. The cellular PHA content and composition were determined by gas chromatography (GC) after direct methanolysis of the dried cells in the presence of 15% sulfuric acid as described previously (12,21).

Enzyme assay Acetoacetyl-CoA and crotonyl-CoA were purchased from Sigma–Aldrich (St. Louis, MO, USA), and *trans*-2-enoyl-CoAs and 3-oxoacyl-CoAs of C_6 and C_8 were chemo-enzymatically synthesized from the corresponding *trans*-2-alkenoic acids (Tokyo Chemical Industry, Tokyo, Japan) and purified as described previously (22).

The activities of NAD(P)H-dependent reduction of 3-oxoacyl-CoAs were assayed in the mixture composed of 100 μM NAD(P)H, 40 μM substrate, and enzyme solution with appropriate dilution in 500 μL of 100 mM Tris–HCl buffer (pH 8.0). The consumption of NAD(P)H accompanied by decrease in absorbance at 340 nm was monitored at 30 °C ($\varepsilon_{340} = 6.22 \times 10^3\ M^{-1}\ cm^{-1}$ for NAD(P)H).

Overall 2-enoyl-CoA hydratase activity was assayed by monitoring hydration of crotonyl-CoA as decrease in absorbance at 263 nm derived from the enoyl-thioester bond (20). (*R*)-specific 2-enoyl-CoA hydratase (*R*-hydrratase) activity was determined by coupled assay using endogenous PHA synthase in the crude cell extract of *R. eutropha*, in which (*R*)-3HB-CoA formed from crotonyl-CoA by *R*-hydratase was polymerized to P(3HB) by PHA synthase. The release of CoA-SH accompanied by the polymerization was monitored by using 5,5′-dithio-bis(2-nitrobenzoic acid), as described previously (20).

Enzyme purification from *R. eutropha* NADH-dependent dehydrogenase catalyzing reduction of acetoacetyl-CoA was purified from *R. eutropha* H16ΔA0282 cultivated in MB medium containing 0.5% (*w*/*v*) fructose for 26 h. The cells in 3.2 L culture broth were harvested and disrupted by a high pressure cell disruptor (one-shot model) (Constant Systems, Northants, UK) at 20,000 psi in 10 mL of

TABLE 1. Strains and plasmids used in this study.

Strain or plasmid	Relevant markers	Source or reference
Escherichia coli		
S17-1	F⁻, *thi*, *pro*, *hsdR*, [RP4-2 Tc::Mu Km::Tn7 (Tp Sm)]	19
DH5α	F⁻, *deoR*, *endA*1, *gyrA*96, *hsdR*17($r_K^- m_K^+$), *recA*1, *relA*1, *supE*44, *thi-1*, Δ(*lacZYA-argF*), U169, ϕ80*lacZ*ΔM15	Clontech
BL21(DE3)	*E. coli* B, F⁻, *dcm*, *ompT*, hsdS($r_B^- m_B^-$), gal, λ(DE3)	Novagen
Ralstonia eutropha		
H16	Wild type	DSM428
H16ΔA0282	H16 derivative; Δ*paaH1*	This study
H16ΔA1102	H16 derivative; Δ*paaH2*	This study
H16ΔA0602	H16 derivative; Δ*had*	This study
H16ΔΔA0282_A1102	H16Δ0282 derivative, Δ*paaH2*	This study
H16ΔΔA0282_A0602	H16Δ0282 derivative, Δ*had*	This study
TT013	Δ*phaC*::$phaC_{NSDG}$-$phaJ_{Ac}$-*phaJ4a*, Δ*phaA*	20
TT013ΔA0282	TT013 derivative; Δ*paaH1*	This study
TT013ΔA1102	TT013 derivative; Δ*paaH2*	This study
TT013ΔA0602	TT013 derivative; Δ*had*	This study
TT013ΔΔA0282_A1102	TT013Δ0282 derivative, Δ*paaH2*	This study
TT013ΔΔA0282_A0602	TT013Δ0282 derivative, Δ*had*	This study
Plasmid		
pK18mobsacB	pMB1 ori, RP4 *mob*, Km^r, modified *sacB*, *lacZ*α	18
pUC118	Amp^r, cloning vector	Takara Bio
pET15b	Amp^r, P_{T7}, His-Tag	Novagen
pK18ms-ΔA0282	pK18mobsacB derivative; *paaH1 del*	This study
pK18ms-ΔA1102	pK18mobsacB derivative; *paaH2 del*	This study
pK18ms-ΔA0602	pK18mobsacB derivative; *had del*	This study
pET-A0282	pET15b derivative; *paaH1*	This study
pET-A0602	pET15b derivative; *had*	This study

The postfix *del* indicates inserts used for targeted gene deletion. *Ac*, *Aeromonas caviae*; $phaC_{NSDG}$, a gene encoding N149S/D171G mutant of PHA synthase from *A. caviae* (31).

50 mM Tris−HCl buffer (pH 7.5). The soluble fraction was obtained by centrifugation (8000 ×g) at 4 °C for 30 min followed by ultracentrifugation (150,000 ×g) at 4 °C for 1 h. The protein having 3HB-CoA dehydrogenase activity in the soluble fraction was purified by series of chromatography using an anion-exchange column HiLoad 16/10 Q Sepharose HP (GE Healthcare Life Sciences, Pittsburgh, PA, USA) and 50 mM Tris−HCl buffer (pH 7.5) with linear gradient of NaCl from 0 to 300 mM, a hydrophobic column HiTrap Phenyl FF 5 mL (GE Healthcare Life Sciences) and 50 mM Tris−HCl buffer (pH 7.5) with linear gradient of $(NH_4)_2SO_4$ from 30 to 0%, and a gel-filtration column Superdex 200 10/300 GL (GE Healthcare Life Sciences) and 50 mM Tris−HCl buffer (pH 7.5) containing 150 mM NaCl. The proteins in the active fractions after the gel-filtration was precipitated by 10% trichloroacetic acid, washed with cold acetone, and then separated by SDS-PAGE performed by a standard procedure. The major protein band of 30 kDa was cut out from the gel, and then subjected to in-gel digestion with trypsin followed by MALDI-TOF-MS analysis using Ultraflex TOF/TOF (Bruker, Billerica, MA, USA) for identification of the protein.

Crotonase was purified from *R. eutropha* H16 grown in 3.0 L of MB medium containing 0.5% fructose for 36 h. The preparation of cell extract and enzyme purification by chromatography were carried out by the same procedures as described above, except for linear gradient of NaCl from 0 to 1.0 M in the anion-exchange chromatography. The active fractions obtained by the gel-filtration chromatography was further subjected to the second anion-exchange chromatography using Resource Q (1 mL) column (GE Healthcare Life Sciences) and 50 mM Tris−HCl buffer (pH 7.5) with linear gradient of NaCl from 0 to 0.5 M. The final active fraction was subjected to SDS-PAGE, and the proteins were transferred to PVDF membrane by electroblotting. The *N*-terminal amino acid sequence of the major 26 kDa protein was determined by a protein sequencer PPSQ-21 (Shimadzu, Kyoto, Japan).

Preparation of His_6-tagged recombinant proteins The plasmids pET15b-A0282 and pET15b-A0602 for overexpression of *paaH1* and *had* in *E. coli*, respectively, were constructed by inserting an NdeI-BamHI restricted fragment of the coding region prepared by PCR into pET-15b at the corresponding sites. *E. coli* BL21(DE3) was transformed with each the expression plasmid, and the transformants were cultivated in LB medium at 37 °C on a reciprocal shaker (115 strokes/min). The gene expression was induced by addition of 0.5 mM IPTG when OD_{600} reached to 0.5, and the cultivation was continued for further 3 h at 37 °C. The cells were harvested, washed, and resuspended within 20 mM sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl and 30 mM imidazole, and then disrupted by the high pressure cell disruptor. The cell extract was subjected to Ni-affinity chromatography using HisTrap FF crude 1 mL (GE Healthcare Life Sciences), and the recombinant protein having a C-terminal His_6-tag was eluted by 20 mM sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl and 200 mM imidazole, and then used for further analyses after appropriate dilution.

RESULTS

NADH- and NADPH-dependent reduction activities toward acetoacetyl-CoA in *R. eutropha* The soluble extract of *R. eutropha* wild strain H16 grown on fructose showed both NADH- and NADPH-dependent activities of 0.33−0.51 U/mg and 0.36−0.41 U/mg, respectively, as reported previously (10−12). The stereospecificity of each activity was determined by monitoring increase of free CoA-SH in the presence of insoluble fraction of *R. eutropha* cells. As endogenous PHA synthase on P(3HB) granules in the insoluble fraction catalyzed polymerization of (*R*)-3HB-CoA, the formation of (*R*)-3HB-CoA from acetoacetyl-CoA could be detected by release of free CoA-SH during the subsequent polymerization. The addition of NADPH and acetoacetyl-CoA into the reaction mixture resulted in significant release of CoA-SH with comparable rate to NADPH oxidation in the standard 3HB-CoA dehydrogenase assay, in contrast, almost no release of CoA-SH was observed with NADH. These results were consistent with (*R*)-stereospecificity of the NADPH-dependent activity by PhaB1 whereas (*S*)-stereospecificity of the NADH-dependent reduction activity.

Participation of two Hbd_{Ca} homologs in NADH-3HB-CoA dehydrogenase activity in *R. eutropha* *R. eutropha* H16 genome harbors two genes of which products were homologous to Hbd from *C. acetobutylicum* (Hbd_{Ca}, 282 amino acids), which are H16_A0282 and H16_A1102 (54% and 38% identities to Hbd_{Ca}) assigned as PaaH1 and PaaH2, respectively (5). PaaH1 (284 amino acids), that has been already demonstrated to show NADH-dependent 3HB-CoA dehydrogenase activity (17), is composed of 3HCDH_N (NAD-binding) and 3HCDH (C-terminal) domains with the same organization as Hbd_{Ca}, while PaaH2 (507 amino acids) contains one 3HCDH_N domain and tandem of two 3HCDH domains (Fig. 1).

The reduction activity toward acetoacetyl-CoA in the soluble extracts of deletion strains H16ΔA0282 (Δ*paaH1*), H16ΔA1102 (Δ*paaH2*), and H16ΔΔA0282_A1102 (Δ*paaH1* Δ*paaH2*) were determined by using NADH or NADPH as a cofactor (Table 2). The NADH-dependent activity diminished to approximately 40% in H16Δ0282 and H16ΔΔA0282_A1102, whereas it was as high as in H16ΔA1102

TABLE 2. Relative NAD(P)H-dependent reduction activity toward acetoacetyl-CoA in *R. eutropha* H16 and the gene deletion strains.

Strain	Relevant genotype	Relative activity (%)			
		0.5% fructose		1% soybean oil	
		NADH	NADPH	NADH	NADPH
H16		100	100	100	100
H16ΔA0282	Δ*paaH1*	37.4	92.5	41.2	94.4
H16ΔA1102	Δ*paaH2*	119	111	92.5	132
H16ΔΔA0282_A1102	Δ*paaH1* Δ*paaH2*	39.8	114	47.4	92.8
H16ΔA0602	Δ*had*	73.9	95.3	66.7	92.0
H16ΔΔA0282_A0602	Δ*paaH1* Δ*had*	14.3	80.7	14.0	76.1

The cell free extracts were prepared from the cells cultivated in an MB medium containing 0.5% (*w*/*v*) fructose or 1% (*v*/*v*) soybean oil at 30 °C for 26 h (PHA production phase).

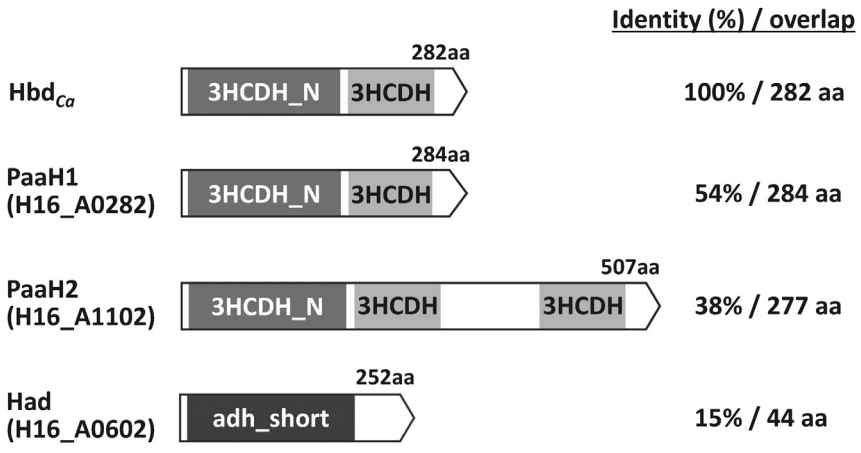

FIG. 1. Domain structures of Hbd_{Ca} and PaaH1, PaaH2, and Had from *R. eutropha*, and homology of PaaH1, PaaH2, and Had to Hbd_{Ca}. *Ca*, *C. acetobutylicum*.

(119%) when compared to the activity in the parent strain H16. No drastic change was observed for NADPH-dependent activity in all of the mutant strains examined. Namely, PaaH1 contributed more than half of the NADH-dependent reduction activity in the fructose-grown cells of *R. eutropha*, and the remaining activity was mediated by enzyme(s) other than PaaH2. The similar results were observed when the cells were cultivated on soybean oil (Table 2).

Identification of the second dehydrogenase The enzyme responsible for the remaining NADH-dependent activity in the fructose-grown cells of H16ΔA0282 was partially purified by series of chromatography, as shown by SDS-PAGE in Supplementary Fig. S1A. MALDI-TOF mass analysis of the major protein band of 30 kDa identified H16_A0602, and the minor 60 kDa band was also identified as the same protein. The 60-kDa protein was predicted to be a dimer form. H16_A0602 is belonging to short chain dehydrogenase family composed of single adh_short domain, being different from PaaH1 and PaaH2 but rather similar to NADPH-dependent acetoacetyl-CoA reductases PhaB1, PhaB2, and PhaB3 from *R. eutropha*, and 3-oxoacyl-ACP reductase FabG from various sources.

A single deletion strain H16ΔA0602 and a double deletion strain H16ΔΔA0282_A0602 lacking the gene for the newly identified dehydrogenase H16_A0602 were further constructed. The NADH-dependent activity in the cell extracts decreased to 67−74% in H16ΔA0602, and markedly reduced to 14% in H16ΔΔA0282_A0602 in comparison with that in H16, when the cells were grown on fructose or soybean oil. Apparently, H16_A0282 (PaaH1) and H16_A0602 (designated Had) were two major enzymes responsible for the NADH-dependent dehydrogenase activity toward acetoacetyl-CoA in *R. eutropha*.

Catalytic properties of PaaH1 and Had The recombinant proteins having C-terminal His_6-tag of PaaH1 and Had were produced in *E. coli* BL21(DE3) and purified by Ni-affinity chromatography. The cofactor and substrate specificities and kinetic parameters for reduction of 3-oxoacyl-CoAs (C_4, C_6, and C_8) were determined by using the recombinant enzymes. Both PaaH1 and Had were actually NADH-dependent, as the activity with NADPH were as low as 2.2−2.6% and 1.2%, respectively, of the respective activity with NADH. The reactions with NADH followed Michaelis−Menten kinetics for all the substrates examined. The determined kinetic parameters (Table 3) indicated that these enzymes were 3HA-CoA dehydrogenases with rather broad substrate specificity, as the catalytic efficiencies of PaaH1 and Had to the C_6 and C_8 substrates were about half and one-third of that to the C_4 substrate, respectively. Both showed high affinity towards the short and medium chain length substrates as indicated by low K_m values ranging from 6.5 μM to 17.5 μM. PaaH1 had in particular high affinity to the C_4 and C_6 substrates (9.4 μM and 8.3 μM, respectively), while Had showed the highest affinity to the C_8 substrate (6.5 μM) among the substrates examined.

Effects of deletion of the dehydrogenase gene(s) on PHA biosynthesis The roles of PaaH1, PaaH2, and Had in the growth and PHA biosynthesis in *R. eutropha* were investigated by cultivation of the single (Δ*paaH1*, Δ*paaH2*, and Δ*had*) and double (Δ*paaH1*Δ*paaH2* and Δ*paaH1*Δ*had*) deletion strains on fructose under a nitrogen-limited condition. However, no obvious difference was observed in the growth and PHA accumulation of these mutants from those of the parent strain H16. They all accumulated P(3HB) with 55−57 wt% of the dry cells (1.2 g-PHA/L) on fructose and 79−83 wt% (2.9−3.4 g-PHA/L) on soybean oil.

We next interested in the roles of the dehydrogenases in conversion of medium-chain-length CoA-thioesters through β-oxidation. *R. eutropha* strain TT013 was a previously constructed strain for production of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)], one kind of practical PHA with flexible properties, from vegetable oils (20). As reported previously, this strain produced the copolyester composed of 10 mol% 3HHx unit with 77 wt% of the dry cells from soybean oil (Table 4). It has been demonstrated that the C_6-monomer ((*R*)-3HHx-CoA) was provided by channeling of β-oxidation in this strain, that was (*R*)-specific hydration of 2-hexenoyl-CoA intermediate by plasmid-borne PhaJ4a derived from *R. eutropha* (medium chain-specific) and $PhaJ_{Ac}$ from *Aeromonas caviae* (short chain-specific) (20,23). The single and double deletions of the dehydrogenase gene(s) were introduced into TT013 as a parent strain. On soybean oil, TT013ΔA0282 (Δ*paaH1*), TT013ΔA1102 (Δ*paaH2*), and TT013ΔΔA0282_A1102 (Δ*paaH1*Δ*paaH2*) exhibited similar properties for growth and P(3HB-*co*-3HHx) biosynthesis to the parent strain TT013. The cell growth of and PHA accumulation (76−78 wt %) by the *had*-deleted strains TT013ΔA0602 and TT013ΔΔA0282_A0602 were also comparable to those by the parent strain, whereas it was of interest that the deletion of *had* resulted in reduction of 3HHx fraction to 5.2 mol%, suggesting some role of Had in β-oxidation by *R. eutropha*.

Enoyl-CoA hydratase activities in *R. eutropha* and identification of crotonase Enoyl-CoA hydratase catalyzes reversible hydration/dehydration between 2-enoyl-CoAs and 3HA-CoAs. There are two kinds of the hydratases with opposite

TABLE 3. Kinetic parameters of recombinant PaaH1 and Had from *R. eutropha* for NADH-dependent reduction to 3-oxoacyl-CoAs of C_4 to C_8.

Enzyme	Substrate	K_m (μM)	V_{max} (U mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	k_{cat}/K_m ratio (C_n/C_4)
PaaH1 (H16_A0282)	Acetoacetyl-CoA (C_4)	9.4	168	84	8.9×10^6	1
	3-Oxohexanoyl-CoA (C_6)	8.3	86	43	5.2×10^6	0.58
	3-Oxooctanoyl-CoA (C_8)	12.5	106	53	4.2×10^6	0.47
Had (H16_A0602)	Acetoacetyl-CoA (C_4)	17.5	209	104	6.0×10^6	1
	3-Oxohexanoyl-CoA (C_6)	16.0	80	40	2.5×10^6	0.42
	3-Oxooctanoyl-CoA (C_8)	6.5	23	11	1.7×10^6	0.28

TABLE 4. P(3HB-*co*-3HHx) biosynthesis from soybean oil by *R. eutropha* TT013 and the gene deletion strains for NADH-dependent 3HA-CoA dehydrogenase(s).

Strain	Relevant genotype	Dry cell weight (g/L)	PHA content (wt%)	PHA (g/L)	3HHx composition (mol%)
TT013[a]		4.94 ± 0.16	77 ± 1.2	3.81 ± 0.07	10.8 ± 0.4
TT013ΔA0282	Δ*paaH1*	5.06 ± 0.27	78 ± 1.8	3.95 ± 0.24	11.7 ± 0.4
TT013ΔA1102	Δ*paaH2*	4.81 ± 0.23	81 ± 1.5	3.87 ± 0.18	9.8 ± 0.2
TT013ΔA0602	Δ*had*	4.46 ± 0.10	76 ± 4.1	3.41 ± 0.24	5.2 ± 0.2
TT013ΔΔA0282_A1102	Δ*paaH1* Δ*paaH2*	5.05 ± 0.14	81 ± 0.8	4.08 ± 0.09	10.7 ± 0.2
TT013ΔΔA0282_A0602	Δ*paaH1* Δ*had*	4.73 ± 0.03	78 ± 0.8	3.69 ± 0.05	5.6 ± 0.1

The cells were cultivated in an MB medium containing 1% (*v*/*v*) soybean oil at 30 °C for 72 h.

[a] Δ*phaC*::*phaC*$_{NSDG}$-*phaJ*$_{Ac}$-*phaJ4a*, Δ*phaA* (20).

stereospecificity; crotonase with (*S*)-specificity and *R*-hydratase encoded by *phaJ* with (*R*)-specificity. In the strain H16 grown on fructose, the overall enoyl-CoA hydratase activity (including both (*S*)- and (*R*)-specific activities) toward crotonyl-CoA was 2.1 U/mg-protein, while *R*-hydratase activity was determined to be 0.025 U/mg-protein in the fructose-grown cells. These results indicated that most of the overall enoyl-CoA hydratase activity was occupied by (*S*)-specific activity. Although the genome of *R. eutropha* H16 harbors a number of genes encoding *R*-hydratase homologous to PhaJ derived from *A. caviae* and *Pseudomonas aeruginosa* (5,20), the expression of the *phaJ* homolog genes appeared to be repressed on fructose.

The protein responsible for the overall hydratase activity was purified to 731-fold from the fructose-grown cells of *R. eutropha* H16 by series of chromatography (Supplementary Fig. S1B). We observed single activity peak throughout the all chromatography steps, and the major 26 kDa protein was identified as H16_A3307 by *N*-terminal amino acid sequencing. This was one of 47 proteins consisting of single ECH_1 (enoyl-CoA hydratase/isomerase) domain in *R. eutropha*, and here designated Crt2 because one homolog H16_B1189 has been annotated as Crt without experimental evidences. The partially purified enzyme showed 73% and 12% activity towards 2-hexenoyl-CoA and 2-ocetnoyl-CoA, respectively, of that to crotonyl-CoA.

DISCUSSION

NADH-dependent reduction activity to acetoacetyl-CoA in PHA-producing *R. eutropha* was mediated by two kinds of dehydrogenases, PaaH1 (H16_A0282) and Had (H16_A0602). The genome analysis of *R. eutropha* H16 has identified the presence of six proteins homologous to 3HB-CoA dehydrogenase derived from *Clostridium acetobutyricum* (Hbd_{Ca}) (24) including PaaH1. Three among the six homologs were dehydrogenase domains in FadB1, FadB2, and FadB′ which are bifunctional β-oxidation proteins ((*S*)-specific 2-enoyl-CoA hydratase/(*S*)-3HA-CoA dehydrogenase) (6,25). The remaining two homologs were PaaH2 and H16_B1652 estimated to be monofunctional dehydrogenase as well as PaaH1. The second identified enzyme Had was a member of short chain dehydrogenase family. There are 83 proteins classified into this family in *R. eutropha* proteome, including three paralogs of NADPH-dependent acetoacetyl-CoA reductase (PhaB1, PhaB2, and PhaB3) having (*R*)-stereospecificity (5,11). The phylogenetic tree of the proteins consisting of 3HCDH_N and 3HCDH domains, and those containing an adh_short domain is shown in Supplementary Fig. S2.

NADH-dependent acetoacetyl-CoA reductase (3HB-CoA dehydrogenase) from *R. eutropha* H16 was purified and characterized 30 years ago (10), although the gene identification had been left for long time. Recent studies demonstrated that PaaH1, identified as a homolog of Hbd_{Ca} as above, showed 3HB-CoA dehydrogenase activity and could function in an engineered pathway for biosynthesis of higher alcohols (16,17). Nevertheless, it had been unclear whether PaaH1 corresponded to the enzyme previously purified from *R. eutropha*. We here demonstrated that PaaH1 was indeed responsible for the 3HB-CoA dehydrogenase activity, but not unique in *R. eutropha*; PaaH1 contributed 60% of the total activity and Had was functional as the second dehydrogenase mediating approximately 35% of the activity (Table 2). In the anion-exchange chromatogram reported by Haywood et al. (10), a minor activity peak was seen in fractions eluted at higher NaCl concentration. It was speculated that the major and minor activity peaks corresponded to PaaH1 and Had, respectively. The previous RNA-seq analysis of *R. eutropha* H16 detected similar expression levels of *paaH1* and *had* at the PHA-biosynthesis phase on fructose with RPKM (reads per kilobase per million mapped reads) values of 2552 and 2868, respectively (26). This was consistent with the similar activity levels of PaaH1 and Had observed in this study. Another Hbd_{Ca} homolog PaaH2 did not so contribute to the NADH-dependent dehydrogenase activity in *R. eutropha* (Table 2) despite the comparable gene expression (RPKM 1,947) to those of *paaH1* and *had* on fructose. PaaH2 might be a dehydrogenase converting 3HA-CoA longer than C_8 or other hydroxyl compounds. It has been reported that a recombinant form of FadB′ exhibited NAD^+-dependent oxidation activity to (*S*)-3HB-CoA (27). However, the results of enzyme assay for H16ΔΔA0282_A0602 (Table 2) suggested that contribution of the bifunctional β-oxidation proteins including FadB′ to the NADH-dependent dehydrogenase activity was not significant (Table 2). The limited contributions of FadB′ and FadB2 to the intracellular activity were consistent with the previous RNA-seq analysis of *R. eutropha* (26) showing the low expression levels of *fadB′* (RPKM $<$ 1000) and *fadB2* (RPKM $<$ 100) both on fructose and octanoate. Although the expression of *fadB1* was highly induced at the PHA biosynthesis phase on fructose (RPKM 5787), FadB1 was predicted to have no or only faint activity to the C_4 substrate unlike FadB′.

The crystal structure of PaaH1 was homologous to that of human L-3-hydroxyacyl-CoA dehydrogenase (17,28), while PhaB1 was similar to 3HA-acyl carrier protein (ACP) reductase from *E. coli* (29,30). In PhaB1, Gly35 and Arg40 were identified as residues interacted with 2′-phosphate group of $NADP^+$ (PDB: 3VZS, 4N5N) (29,30). Alignment of the amino acid sequences indicated that these residues were substituted by Leu37 and Gly42 in Had, respectively, while Asp33 bound to 2′-hydroxyl group of NAD^+ in PaaH1 (PDB: 4PZD) (17) was conserved in Had at the position of 36, which might cause the preference of Had to NAD(H). Although PaaH1 and Had shared only weak identity of 30% in the *N*-terminal regions forming the Rossmann fold structures (17,29), they interestingly showed similar catalytic properties to each other; both the enzymes exhibited NADH-dependent reduction activity to 3-oxoacyl-CoAs of C_4, C_6, and C_8 with high affinity (K_m values lower than 17.5 μM) (Table 3). The catalytic properties of PaaH1 agreed with the enhanced production of higher alcohols by using PaaH1 in engineered strains of *E. coli* (16), thus Had was also expected to be useful for production of compounds longer than C_4. Determination of crystal structure of Had and comparison of it with that of PaaH1 and PhaB1 will help us to understand structural factors responsible for the specificities to the substrate and cofactor.

We initially assumed that the disruption of the NADH-dependent dehydrogenase genes might enhance P(3HB) biosynthesis on fructose, because the (*S*)-specific reduction of acetoacetyl-CoA competes with (*R*)-specific reduction providing (*R*)-3HB-CoA monomer for P(3HB) biosynthesis. Contrary to the assumption, the double deletion of *paaH1* and *had* gave almost no effect on growth and P(3HB) production. Although there was a possibility for conversion of (*S*)-3HB-CoA to (*R*)-3HB-CoA by combination of (*S*)- and (*R*)-specific enoyl-CoA hydratases (crotonase and *R*-hydratase, respectively) as a bypass for generation of (*R*)-3HB unit (Supplementary Fig. S3), this was not feasible due to the much lower *R*-hydratase activity than crotonase activity (mediated by Crt2 shown in this study). Moreover, despite the lack of possible metabolic fate of crotonyl-CoA in the fructose-grown cells, the previous metabolomic analysis of *R. eutropha* indicated very low levels of intracellular concentration of crotonyl-CoA throughout the growth, PHA biosynthesis, and stationary phases on fructose (22). It was estimated that acetoacetyl-CoA was dominantly converted to P(3HB) via (*R*)-3HB-CoA owing to the high affinity to acetoacetyl-CoA (K_m values of 5.7 μM) and high expression level of PhaB1 (RPKM $>$ 22,000) (26).

The present results suggested some function of Had in β-oxidation by *R. eutropha*, because the deletion of *had* in the

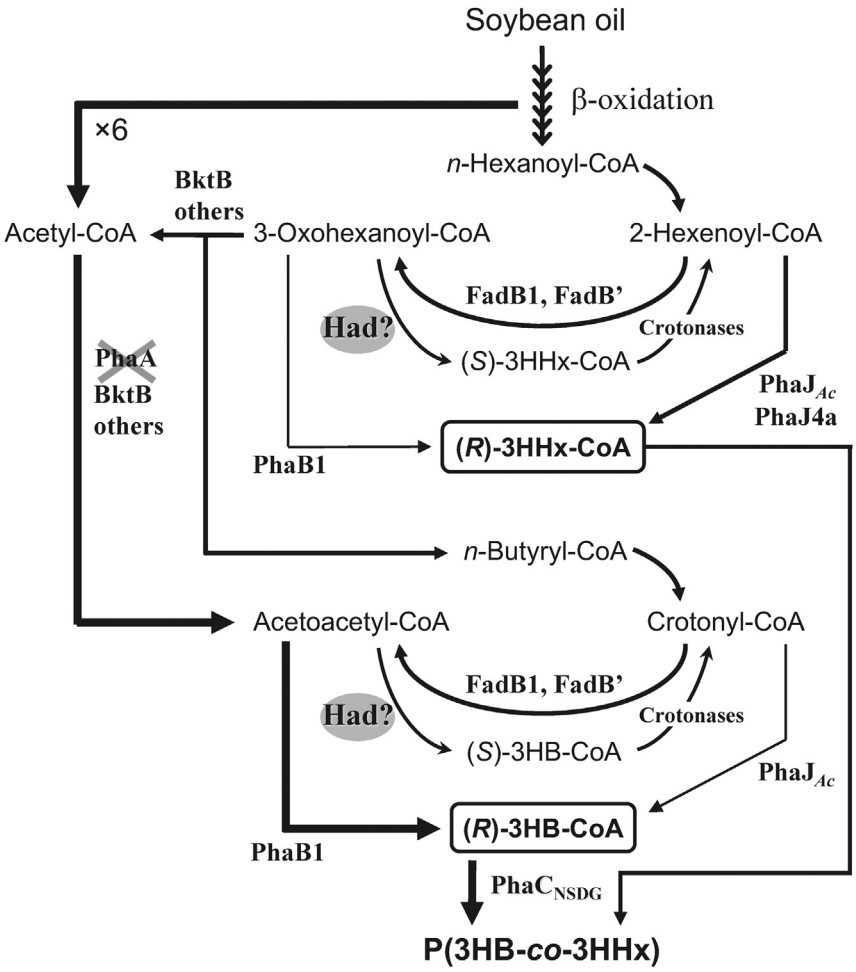

FIG. 2. P(3HB-*co*-3HHx) biosynthesis pathway from soybean oil through β-oxidation in *R. eutropha* strain TT013. PhaA and BktB, short-chain-specific and short-medium-chain-specific β-ketothiolases, respectively; PhaB1, NADPH-dependent acetoacetyl-CoA reductase; $PhaC_{NSDG}$, N149S/D171G mutant of PHA synthase from *A. caviae*; $PhaJ_{Ac}$, short-chain-specific *R*- hydratase from *A. caviae*; PhaJ4a, medium-chain-specific *R*- hydratase; Had, NADH-dependent (*S*)-3HA-CoA dehydrogenase. The width of the arrows shows the estimated relative flux, in which those mediated by PhaB1, BktB, $PhaJ_{Ac}$, and PhaJ4a are based on previous results (4,9,11,20).

engineered strain TT013 resulted in reduction of the C_6 composition from 10.8 mol% to 5.2—5.6 mol% in P(3HB-*co*-3HHx) synthesized from soybean oil (Table 4). Such compositional changes were not observed for the deletion of *paaH1*. This may suggest that the reaction catalyzed by Had for C_6-intermediates in β-oxidation was not oxidation of (*S*)-3HHx-CoA but the reverse direction reducing 3-oxohexanoyl-CoA to (*S*)-3HHx-CoA. The deletion of *had* might consequently weaken back-formation of 2-hexenoyl-CoA from 3-oxohexanoyl-CoA via (*S*)-3HHx-CoA, leading to reduced provision of (*R*)-3HHx-CoA monomer from 2-hexenoyl-CoA by *R*-hydratases. In β-oxidation by *R. eutropha*, bifunctional FadB1 and FadB′ played a role in formation of 3-oxoacyl-CoAs from 2-enoyl-CoAs (6), where the successive 2-step reactions by the bifunctional enzymes were estimated to proceed without release of (*S*)-3HA-CoA intermediates. Had may have a role in regulation of metabolic flux of β-oxidation by promoting the reverse β-oxidation reactions in combination with monofunctional crotonase(s) such as Crt2 (Fig. 2). However, when additional copies of *had* were introduced using a plasmid vector into the strain TT013, the change in 3HHx composition was not significant in the copolyester fraction synthesized from soybean oil (data not shown). The enzyme expressed from the chromosomal *had* might be enough for the functions under the condition examined. The details should be further investigated, and the knowledge for the functions of PaaH1 and Had would be useful for metabolic engineering of *R. eutropha* aiming production of not only PHAs but also other compounds from biomass feedstocks.

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