JOURNAL OF BACTERIOLOGY, Feb. 1998, p. 667–673 Vol. 180, No. 3
0021-9193/98/$04.00+0

Expression and Characterization of (*R*)-Specific Enoyl Coenzyme A Hydratase Involved in Polyhydroxyalkanoate Biosynthesis by *Aeromonas caviae*

TOSHIAKI FUKUI,[1] NAOFUMI SHIOMI,[2] AND YOSHIHARU DOI[1]*

Polymer Chemistry Laboratory, The Institute of Physical and Chemical Research (RIKEN), Hirosawa 2-1, Wako-shi, Saitama 351-01,[1] and Department of Human Sciences, Kobe College, Okadayama 4-1, Nishinomiya-shi, Hyogo 662,[2] Japan

Received 13 August 1997/Accepted 25 November 1997

Complementation analysis of a polyhydroxyalkanoate (PHA)-negative mutant of *Aeromonas caviae* proved that ORF3 in the *pha* locus (a 402-bp gene located downstream of the PHA synthase gene) participates in PHA biosynthesis on alkanoic acids, and the ORF3 gene is here referred to as $phaJ_{Ac}$. *Escherichia coli* BL21(DE3) carrying $phaJ_{Ac}$ under the control of the T7 promoter overexpressed enoyl coenzyme A (enoyl-CoA) hydratase, which was purified by one-step anion-exchange chromatography. The N-terminal amino acid sequence of the purified hydratase corresponded to the amino acid sequence deduced from the nucleotide sequence of $phaJ_{Ac}$ except for the initial Met residue. The enoyl-CoA hydratase encoded by $phaJ_{Ac}$ exhibited (*R*)-specific hydration activity toward *trans*-2-enoyl-CoA with four to six carbon atoms. These results have demonstrated that (*R*)-specific hydration of 2-enoyl-CoA catalyzed by the translated product of $phaJ_{Ac}$ is a channeling pathway for supplying (*R*)-3-hydroxyacyl-CoA monomer units from fatty acid β-oxidation to poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) biosynthesis in *A. caviae*.

Polyhydroxyalkanoates (PHA) occur naturally in a wide variety of bacteria as a material for storage of carbon and energy from renewable carbon resources under conditions of restricted growth. They have recently attracted industrial attention because of their potential properties as biodegradable thermoplastics. A great deal of research has been undertaken with the aim of understanding the mechanism of PHA biosynthesis (1, 7), and numerous advances have been made from recent molecular analysis of PHA biosynthesis genes (29, 30).

In *Alcaligenes eutrophus*, two molecules of acetyl coenzymeA (acetyl-CoA) derived from various carbon sources are converted to (*R*)-3-hydroxybutyryl-CoA [(*R*)-(3HB)-CoA] via dimerization catalyzed by β-ketothiolase and subsequent (*R*)-specific reduction catalyzed by NADPH-acetoacetyl-CoA reductase. The resultant (*R*)-3HB-CoA molecules are then polymerized into poly(3-hydroxybutyrate) [P(3HB)] by the function of PHA synthase. The genes encoding the three enzymes are organized in a single operon as *phbCAB*, consisting of the genes for PHA synthase, thiolase, and reductase, respectively (21, 25, 28). This operon has been introduced into several prokaryotes and eukaryotes, resulting in P(3HB) accumulation (18, 22, 29, 34). Pseudomonads belonging to rRNA homology group I accumulated PHA consisting of medium-chain-length (C_5 to C_{14}) 3-hydroxyalkanoate (3HA) units from simple carbon sources, such as sugars (13, 15, 32) or *n*-alkanoic acids, *n*-alkanols, or *n*-alkanes (4, 12, 14, 16). In the production of PHA from alkanoic acids with six to nine carbon atoms by *Pseudomonas oleovorans*, the major monomer unit in PHA has the same chain length as the alkanoic acids fed as a carbon source, but monomer units with more or fewer carbon atoms than the acid fed are also generally present in the PHA formed. It has been proposed that acyl-CoA intermediates of the β-oxidation pathway are channeled to PHA biosynthesis (6a, 9); however, few details have been elucidated.

Aeromonas caviae isolated from soil has been reported to produce a random copolyester of 3HB and (*R*)-3-hydroxyhexanoate [(*R*)-3HHx], P(3HB-*co*-3HHx), from alkanoic acids with even carbon numbers or from plant oils (8, 26). On the basis of the fact that this bacterium does not accumulate any PHA from sugars, C_4 and C_6 (*R*)-3HA units were proposed to be supplied from the β-oxidation intermediates, similar to the PHA biosynthesis pathway in pseudomonads on alkanoic acids (8). Recently, we have cloned and analyzed the PHA biosynthesis genes of *A. caviae* and have suggested that ORF3 located downstream of the PHA synthase gene ($phaC_{Ac}$) encodes (*R*)-specific enoyl-CoA hydratase (11). In this paper, we report direct evidence that ORF3 is essential for PHA biosynthesis from alkanoic acids in *A. caviae*. Furthermore, ORF3, referred to as $phaJ_{Ac}$, is overexpressed in *Escherichia coli*, and characteristics of the translated product are investigated.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *A. caviae* strains were cultivated at 30°C in a nutrient-rich medium containing 10 g of meat extract, 10 g of polypeptone, and 2 g of yeast extract in 1 liter of distilled water, and *E. coli* strains were grown at 37°C on Luria-Bertani (LB) medium (23). Kanamycin (50 mg/liter) or ampicillin (50 mg/liter) was added to the medium when necessary.

Chemical mutagenesis and isolation of a PHA-negative mutant of *A. caviae*. *A. caviae* FA440 was treated with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (50 μg/ml) and then inoculated on MM agar medium composed of 3 g of K_2HPO_4, 7 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$, 0.1 g of yeast extract, 5 g of glucose, and 15 g of agar in 1 liter of distilled water (pH 7.0). Colonies grown at 30°C for 5 days were replicated on MPA agar medium composed of 7 g of K_2HPO_4, 3 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of yeast extract, 1.5 g of palmitic acid, 10 ml of Triton X-100, and 15 g of agar in 1 liter of distilled water (pH 7.0). The small amount of yeast extract (0.1 g/liter) was added to enhance cell growth. PHA-negative mutants, which were not stained with Sudan Black B (24) on MPA agar medium, were isolated.

DNA manipulation. Basic recombinant DNA techniques, such as preparation and purification of plasmid DNA, restriction endonuclease digestion, agarose gel electrophoresis, and transformation of *E. coli*, were carried out as described by

* Corresponding author. Mailing address: Polymer Chemistry Laboratory, The Institute of Physical and Chemical Research (RIKEN), Hirosawa 2-1, Wako-shi, Saitama 351-01, Japan. Phone: 81-48-467-9402. Fax: 81-48-462-4667. E-mail: ydoi@postman.riken.go.jp.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference(s)
A. caviae		
FA440	Wild type	FERM P-3432; 8, 26
AC004	PHA-negative mutant of FA440	This study
E. coli		
DH5α	*deoR endA1 gyrA96 hsdR17*(r_K^- m_K^+) *recA1 relA1 supE44 thi-1* Δ(*lacZYA-argFV169*) ϕ80 Δ*lac*ZΔM15 F^- r^-	Clontech
S17-1	*recA* and *tra* genes of plasmid RP4 integrated into chromosome; auxotrophic for proline and thiamine	27
BL21(DE3)	F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)	Novagen
pJRD215	Cosmid; *Km*^{r} *Sm*^{r} RSF1010 replicon; Mob^+	5
pJRDEE32	3.2-kbp insert (*Eco*RI fragment containing *phaC*$_{Ac}$, ORF1, and ORF3 [*phaJ*$_{Ac}$]) in pJRD215	11
pJRDEE32d1	2.8-kbp insert (*Eco*RI fragment containing *phaC*$_{Ac}$ and ORF3 [*phaJ*$_{Ac}$]) in pJRD215	11
pJRDEE32d3	2.8-kbp insert (*Eco*RI fragment containing *phaC*$_{Ac}$ and ORF1) in pJRD215	11
pJRDEE32d13	2.4-kbp insert (*Eco*RI fragment containing *phaC*$_{Ac}$) in pJRD215	11
pJRDG13	1.4-kbp insert (*Eco*RI fragment containing ORF1 and ORF3 [*phaJ*$_{Ac}$]) in pJRD215	This study
pEE32	3.2-kbp insert (*Eco*RI fragment containing *phaC*$_{Ac}$, ORF1, and ORF3 [*phaJ*$_{Ac}$]) in pUC18	11
pET-3a	*Ap*^{r}; contains a T7 promoter with start codon and designed ribosome binding site	Novagen
pETNB3	0.4-kbp insert (PCR-amplified *Nde*I-*Bam*HI fragment of ORF3 [*phaJ*$_{Ac}$]) in pET-3a	This study

Sambrook et al. (23). Transconjugation of *A. caviae* with *E. coli* S17-1 harboring broad-host-range plasmids was performed as described by Friedrich et al. (10).

Construction of pJRDG13. Two *Bgl*II sites across *phaC*$_{Ac}$ were created on pEE32 (11) by site-directed mutagenesis with the unique-site elimination procedure (6) using mutagenic primers M2 (5′-GACGCTACGGGCTAGATCTC GCCTCGGGTGTG-3′) and M5 (5′-GCGGCTCAACCCAGATCTTGCCTGC CCAACAG-3′), which corresponded to the sequences from nucleotides 2647 to 2678 and 4430 to 4461, respectively (11) (the created *Bgl*II sites are underlined). The resultant plasmid was digested with *Bgl*II and self-ligated to delete the coding region of *phaC*$_{Ac}$. The *Eco*RI restriction fragment excised from the *phaC*$_{Ac}$-deleted plasmid was inserted into pJRD215 (5) to form pJRDG13, harboring ORF1 and *phaJ*$_{Ac}$ (ORF3) of *A. caviae.*

Production and analysis of PHA. *A. caviae* strains were first cultivated in 100 ml of nutrient-rich medium on a reciprocal shaker (130 strokes/min) at 30°C for 12 h. Then harvested and washed cells were transferred into 100 ml of nitrogen-free mineral salt medium (pH 7.0), which was composed of 0.9 g of $Na_2HPO_4 \cdot 12H_2O$, 0.15 g of KH_2PO_4, 0.02 g of $MgSO_4 \cdot 7H_2O$, and 0.1 ml of trace element solution (15), and incubated at 30°C for 48 h. Sodium dodecanoate (1%) was added as a carbon substrate for PHA biosynthesis. For maintenance of broad-host-range plasmids in *A. caviae*, kanamycin was added to the medium at a concentration of 50 mg/liter. Cellular PHA contents were determined by gas chromatography after methanolysis of dried cells in the presence of 15% sulfuric acid, as described previously (15).

Construction of expression plasmid pETNB3. To construct a plasmid for the overexpression of *phaJ*$_{Ac}$, an *Nde*I site was introduced into the proposed translational start codon of *phaJ*$_{Ac}$ by PCR. The 427-bp fragment was amplified with primers P3N (5′-GCCATATGAGCGCACAATCCCTGGAAGTAG-3′) and P3C (5′-CTGGGATCCGCCGGTGCTTAAGGCAGCTTG-3′), corresponding to the sequences from nucleotides 4470 to 4499 and 4867 to 4896 (complementary sequence), respectively (11) (underlined sequences show an *Nde*I site in P3N and a *Bam*HI site in P3C). The PCR product was purified, digested with *Nde*I and *Bam*HI, and subcloned into pET-3a. The resultant plasmid was termed pETNB3.

Enzyme assay. Enoyl-CoA hydratase activity was assayed by the hydration of crotonyl-CoA (19, 31). A 10-μl volume of enzyme solution was added to 290 μl of 50 mM Tris-HCl (pH 8.0) containing 0.25 mM crotonyl-CoA (Sigma) in quartz cuvettes with a 0.1-cm light path, and the decrease in absorbance at 263 nm was measured at 30°C. The ε_{263} of the enoyl-thioester bond is taken to be 6.7×10^3 M^{-1} cm^{-1} (31). Other C_5, C_6, and C_8 *trans*-2-enoyl-CoA substrates used for the hydratase assay were synthesized from a lithium salt of CoA and the corresponding *trans*-2-alkenoic acids (Tokyo Kasei) based on a mixed-anhydride method and were purified with a Sep-Pak C_{18} column (Waters), as described by Valentin and Steinbüchel (33). Protein concentrations were determined by the method of Bradford (2) by using Bio-Rad assay solution and bovine serum albumin as the standard.

Expression of *phaJ*$_{Ac}$ in *E. coli*. The expression plasmid pETNB3 was transformed into *E. coli* BL21(DE3). A 1-ml overnight culture of the cells was inoculated into 100 ml of LB medium containing 100 mg of ampicillin per liter and cultivated at 30°C. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM when the absorbance at 600 nm reached 0.6, and cultivation was continued for an additional 2 h at 30°C. The cells grown in four 100-ml cultures were harvested, sonicated in buffer A (20 mM HEPES, pH 7.2), and subjected to centrifugation (20,000 × *g*, 30 min, 4°C). The obtained soluble protein fraction was loaded directly onto a HiLoad Q-Sepharose HP 16/10 column (Pharmacia) equilibrated with buffer A and was eluted with a linear gradient (250 ml) of 0 to 1.0 M NaCl (2.5 ml/min). The enoyl-CoA hydratase assay was done for each fraction (5 ml) with crotonyl-CoA used as a substrate, and the active fractions were combined, concentrated by ultrafiltration with a Centriplus 10 (Amicon), and desalted with a Sephadex G-25 column (Pharmacia). The purified enzyme was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (17). The N-terminal amino acid sequence of the enzyme was determined with a protein sequencer (model 610A; Perkin-Elmer) as instructed by the manufacturer.

Determination of molecular mass. The molecular mass of the native enzyme was evaluated by gel filtration chromatography with a Superdex 75 HR 10/30 column (Pharmacia) in buffer A containing 0.15 M NaCl. The molecular mass of the subunit was determined by SDS-PAGE and matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOFMS).

RESULTS

Complementation analysis of *A. caviae* PHA-negative mutants. Chemical mutagenesis was carried out to generate PHA-negative mutants of *A. caviae* FA440, and five mutants incapable of accumulating PHA on an MPA agar plate containing palmitic acid were isolated after 10^4 colonies were screened. One such mutant, *A. caviae* AC004, was used for further analysis. The wild-type strain of *A. caviae* produced P(3HB-*co*-3HHx), up to 29 wt% of the dry cell weight from dodecanoate as a carbon substrate by two-step fermentation, whereas mutant AC004 could not synthesize any PHA under the same condition. As shown in Fig. 1b, the PHA^- phenotype of AC004 was able to revert to PHA^+ by introduction of pJRDEE32, harboring a 3.2-kbp fragment of *A. caviae* genomic DNA (ORF1, *phaC*$_{Ac}$, and ORF3) (11), indicating that AC004 contains a mutation within the 3.2-kbp region in its chromosome. Various deletion clones of pJRDEE32 were then introduced into AC004, and the ability of the recombinant strains to synthesize PHA was investigated to identify the mutation. The transconjugants of AC004 harboring pJRDEE32d3 or pJRDEE32d13 did not accumulate any PHA or accumulated only a trace amount. In contrast, both pJRDEE32d1 and pJRDG13, harboring ORF3, could complement the PHA-negative mutation of AC004. These results have revealed that AC004 does not lack the PHA synthase encoded by *phaC*$_{Ac}$ but

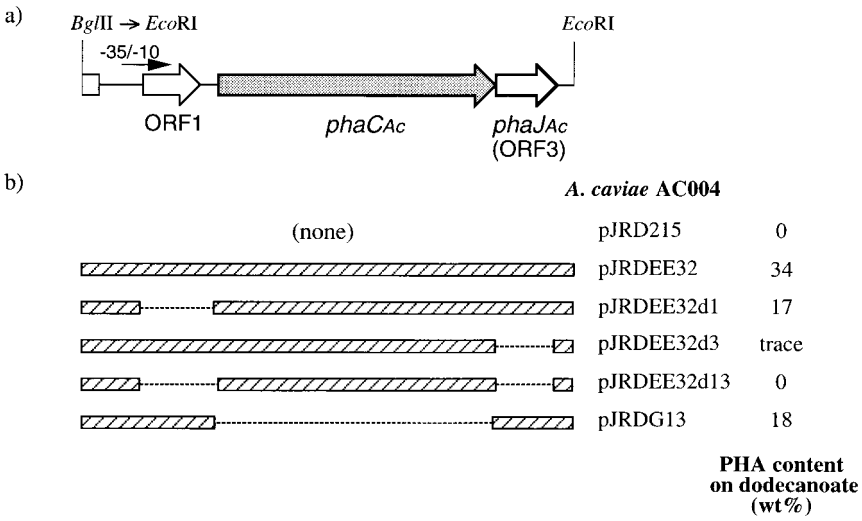

FIG. 1. (a) Schematic drawing of a 3.2-kbp *Eco*RI fragment containing ORF1, $phaC_{Ac}$, and $phaJ_{Ac}$ (ORF3) with a putative promoter region from *A. caviae* FA440. (b) The ability of PJRDEE32 and its deleted clones to complement a PHA-negative mutant of *A. caviae* (AC004). PHA production was carried out on 1% dodecanoate by two-step fermentation, as described in the text.

contains a mutation within the ORF3 region. This is clear evidence that ORF3 ($phaJ_{Ac}$) is essential for PHA biosynthesis on alkanoic acids by *A. caviae.*

Expression of $phaJ_{Ac}$ in *E. coli*. When $phaJ_{Ac}$ (ORF3) was expressed in *E. coli* DH5α together with $phaC_{Ac}$ under the control of the native promoter, a higher enoyl-CoA hydratase activity was detected in the supernatant of the recombinant strain than in the control strain (11), suggesting that $phaJ_{Ac}$ is a gene encoding enoyl-CoA hydratase. For further investigation of the translated product of $phaJ_{Ac}$, we constructed an expression plasmid, pETNB3, in which $phaJ_{Ac}$ is oriented in the T7 promoter and designed ribosome binding site of pET-3a (Fig. 2). *E. coli* BL21(DE3) was then transformed with pETNB3 and cultured until mid-log phase at 30°C. After the addition of 0.4 mM IPTG and further cultivation for 2 h, a very high enoyl-CoA hydratase activity (1.7×10^3 U/mg) was detected in the soluble protein fraction without formation of an inclusion body. This activity was more than 10^3-fold higher than that in strain DH5α carrying $phaC_{Ac}$ and $phaJ_{Ac}$ under the control of the native promoter region. A protein of 15.5 kDa, which is in reasonable agreement with the approximate mass of the predicted $phaJ_{Ac}$ product (14.1 kDa), was observed in the soluble fraction from BL21(DE3)/pETNB3 as determined by SDS-PAGE analysis (Fig. 3, lane 2).

The soluble protein fraction prepared from the cells grown in four 100-ml cultures was loaded onto a Q-Sepharose column, and the enoyl-CoA hydratase activity was eluted from a linear gradient of NaCl (Fig. 4). SDS-PAGE analysis revealed that the combined active fraction was electrophoretically homogeneous (Fig. 3, lane 3). The hydratase activity could be recovered with a high yield (65% of total activity), and the purified enzyme showed a threefold-higher specific activity than the crude one, as given in Table 2. When this protein was subjected to Edman degradation, an N-terminal amino acid sequence (SAQSLEVGQKARLSKRFGAA) which corresponded to the amino acid sequence deduced from the nucleotide sequence of $phaJ_{Ac}$ (11) except for the initial Met residue was obtained.

Characteristics of the $phaJ_{Ac}$ product. The molecular mass of the purified enoyl-CoA hydratase was determined as 13,963 Da by MALDI-TOFMS analysis, and the obtained value was in good agreement with a value (13,954 Da) calculated from the

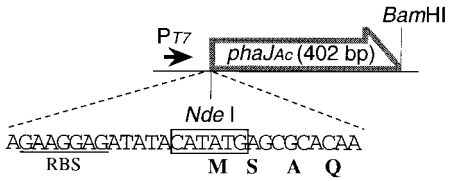

FIG. 2. Construction of plasmid pETNB3 for overexpression of $phaJ_{Ac}$ in *E. coli* BL21(DE3). A designed ribosome binding site (RBS) from pET-3a is indicated (underlined). P_{T7}, T7 promoter in pET-3a.

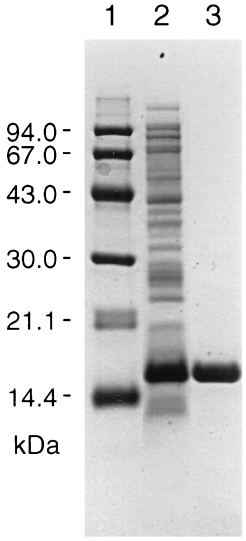

FIG. 3. SDS-PAGE analysis of enoyl-CoA hydratase from *E. coli* BL21(DE3)/pETNB3. Lanes: 1, molecular mass standard proteins, with the masses indicated on the left (from top to bottom, phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor); 2, crude extract proteins of *E. coli* BL21(DE3)/pETNB3; 3, purified enoyl-CoA hydratase after chromatography on Q-Sepharose.

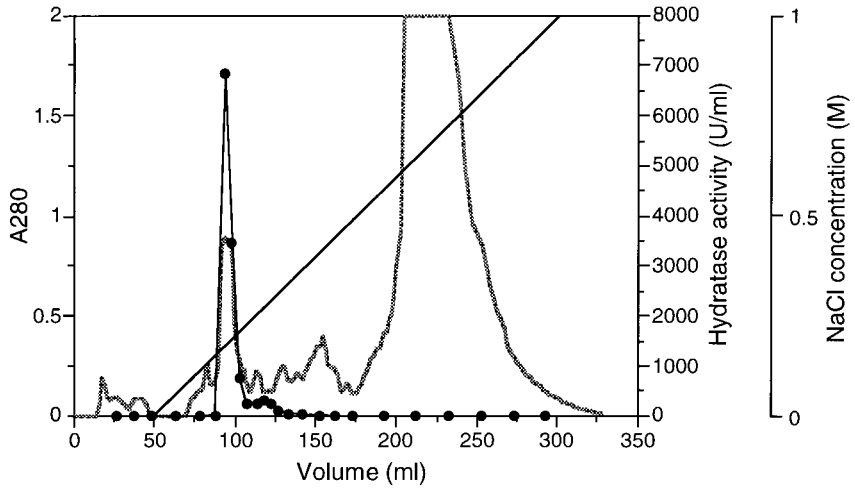

FIG. 4. Elution profile of enoyl-CoA hydratase from *E. coli* BL21(DE3)/pETNB3. The soluble protein fraction from the cells grown in four 100-ml cultures was applied to a Q-Sepharose column. ▨, absorbance at 280 nm; ——, concentration of NaCl; ●, enoyl-CoA hydratase activity toward crotonyl-CoA.

deduced amino acid sequence in which the initial Met was ignored. The N-terminal Met was deleted probably due to posttranslational modification in *E. coli* cells. The native molecular mass of the hydratase was estimated as 31,000 Da by gel filtration chromatography, indicating the formation of a homodimer.

The stereospecificity of the enoyl-CoA hydratase encoded by $phaJ_{Ac}$ was evaluated by two procedures. One procedure is hydration of crotonyl-CoA by the purified hydratase coupled with oxidation of the resulting 3HB-CoA catalyzed by (*S*)-specific 3-hydroxyacyl-CoA dehydrogenase from porcine heart (Sigma). The formation of NADH linked with the dehydrogenation of (*S*)-3HB-CoA was followed by monitoring the absorbance at 340 nm (19). Almost no difference in the increase of the absorbance was observed between the reactions in which the $phaJ_{Ac}$-derived hydratase was present and absent, while rapid formation of NADH was detected when crotonase [(*S*)-specific enoyl-CoA hydratase] from bovine liver (Sigma) was added instead of the hydratase from $phaJ_{Ac}$ (Fig. 5a). For the other procedure, a crude extract of *A. eutrophus* H16 (25) was added to the hydratase assay mixture as a crude PHA synthase solution together with 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) (33). The release of CoA-SH linked with the polymerization of (*R*)-3HB-CoA to P(3HB) by the function of the PHA synthase could be measured as an increase in the absorbance at 410 nm with time in the presence of DTNB. As shown in Fig. 5b, release of CoA-SH depending on the $phaJ_{Ac}$-derived hydratase and crotonyl-CoA was observed, in contrast to only the small change in absorbance caused by the addition of crotonase. From these results, it was evident that the enoyl-CoA hydratase translated from $phaJ_{Ac}$ has (*R*) specificity.

The apparent equilibrium constant K_{eq} ([3HB-CoA]/[crotonyl-CoA]) for the reaction catalyzed by the $phaJ_{Ac}$-derived hydratase was determined to be 2.2. The hydratase exhibited normal Michaelis-Menten kinetics, and Table 3 gives the K_m and V_{max} values for the hydratase-catalyzed reactions toward *trans*-2-enoyl-CoA with four, five, six, and eight carbon atoms. The K_m values were within 29 to 50 μM and were nearly constant, independent of the chain length of the substrates. In contrast, the V_{max} values decreased with increased carbon chain length of 2-enoyl-CoA. The V_{max} for crotonyl-CoA was 6.2×10^3 U/mg, and the hydratase showed a still-high activity for 2-hexenoyl-CoA ($V_{max} = 1.8 \times 10^3$ U/mg). However, the value for 2-octenoyl-CoA was much lower than those for the shorter substrates. The (*R*)-specific enoyl-CoA hydratase encoded by $phaJ_{Ac}$ showed a high activity toward C_4 to C_6 2-enoyl-CoA, which is consistent with the composition of PHA produced by *A. caviae* from alkanoic acids with even numbers of carbon atoms or from plant oils (8).

DISCUSSION

As the biosynthetic route to (*R*)-3HA-CoA from β-oxidation intermediates for PHA biosynthesis, three candidates have been proposed: (*R*)-specific hydration of 2-enoyl-CoA, (*R*)-specific reduction of 3-ketoacyl-CoA, or epimerization of (*S*)-3HA-CoA (6a, 9, 29). The results described here clearly indicate that (*R*)-specific enoyl-CoA hydratase encoded by $phaJ_{Ac}$ (ORF3) is involved in *A. caviae* PHA biosynthesis on alkanoic acids. The 2-enoyl-CoA intermediates with four to six carbon atoms derived from the β-oxidation of longer alkanoic acids can be converted to corresponding (*R*)-3HA-CoA by the hydratase. The resultant C_4 to C_6 (*R*)-3HA-CoA molecules are acceptable as substrates for PHA synthase of *A. caviae* en-

TABLE 2. Purification of enoyl-CoA hydratase from *E. coli* BL21(DE3)/pETNB3

Purification step	Vol (ml)	Protein		Total activity[a] (U)	Sp act[a] (U/mg)	Yield (%)	Purification (fold)
		mg	mg/ml				
None (crude enzyme)	10	101	10.1	1.7×10^5	1.7×10^3		
Q-Sepharose[b]	5	21.6	4.3	1.1×10^5	5.1×10^3	65	3

[a] Enoyl-CoA hydratase activity toward crotonyl-CoA (0.25 mM).
[b] Enzyme was concentrated and desalted after elution from a Q-Sepharose column.

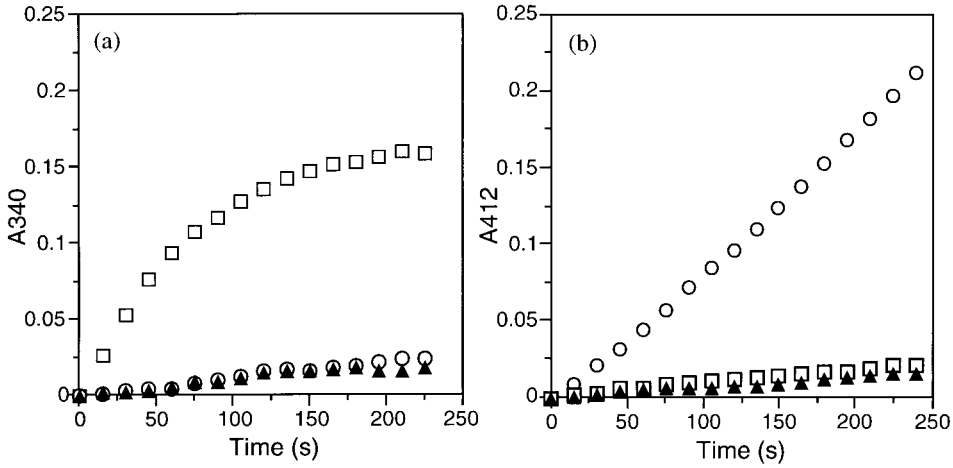

FIG. 5. Evaluation of stereospecificity of enoyl-CoA hydratase encoded by $phaJ_{Ac}$. (a) Hydration of crotonyl-CoA coupled with (*S*)-specific dehydrogenation of 3HB-CoA catalyzed by (*S*)-3HA-CoA dehydrogenase. The reaction mixture was composed of 0.25 mM crotonyl-CoA, 0.5 mM NAD^+, 6 mU of (*S*)-3HA-CoA dehydrogenase, and 1 U of hydratase in 400 μl of 50 mM Tris-HCl (pH 8.0). (b) Hydration of crotonyl-CoA coupled with (*R*)-specific polymerization of 3HB-CoA catalyzed by crude PHA synthase. The reaction mixture was composed of 0.25 mM crotonyl-CoA, 10 mM DTNB, 1.2 mU of PHA synthase (a crude extract of *A. eutrophus* H16 containing 10 μg of protein), and 1 U of hydratase in 400 μl of 125 mM potassium phosphate (pH 7.2). The crude extract of *A. eutrophus* H16 was prepared from cells grown on fructose for 30 h at 30°C, as described previously (25). Symbols: ○, addition of $phaJ_{Ac}$-derived enoyl-CoA hydratase; □, addition of crotonase [(*S*)-specific enoyl-CoA hydratase]; ▲, no addition of enoyl-CoA hydratase.

coded by $phaC_{Ac}$, and then they are polymerized to P(3HB-*co*-3HHx), as shown in Fig. 6. This is the first study to prove that the (*R*)-specific hydration of 2-enoyl-CoA is a channeling pathway for supplying (*R*)-3HA-CoA monomer units for PHA biosynthesis through the fatty acid β-oxidation pathway. The location of the gene encoding the (*R*)-hydratase within the *pha* locus is also a new finding.

Rhodospirillum rubrum is known to synthesize PHA consisting of short- and medium-chain-length 3HA (C_4 to C_6), like *A. caviae* (3), and the presence of two stereospecific [(*R*)- and

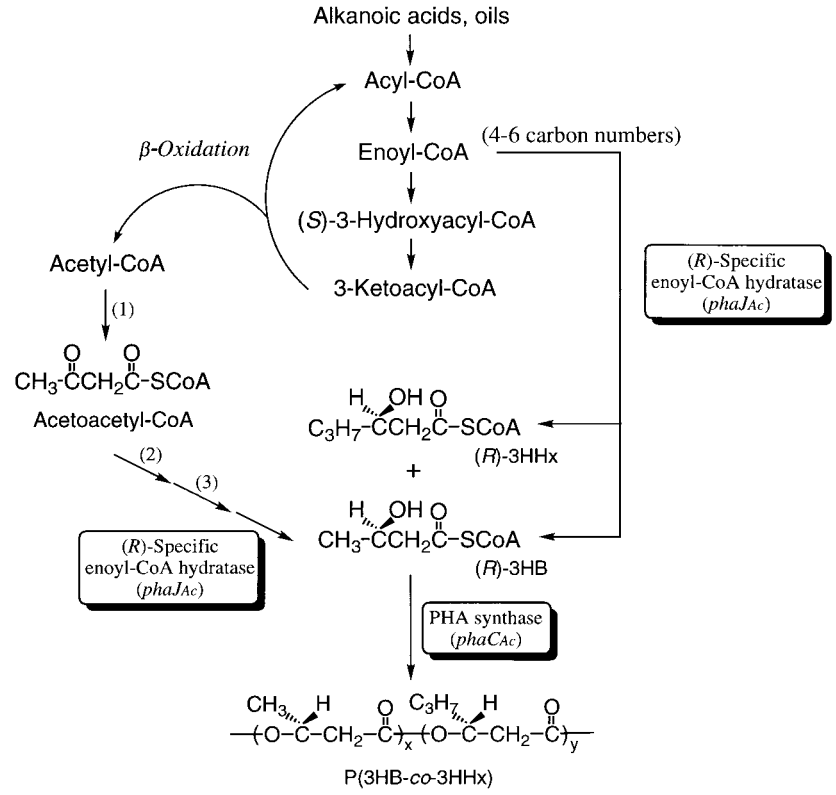

FIG. 6. Proposed pathway of P(3HB-*co*-3HHx) biosynthesis by *A. caviae* from alkanoic acids or oils. 1, β-ketothiolase; 2, NADH-acetoacetyl-CoA dehydrogenase; 3, crotonase [(*S*)-specific enoyl-CoA hydratase].

TABLE 3. Substrate specificity of enoyl-CoA hydratase encoded by $phaJ_{Ac}$

Substrate	K_m (μM)	V_{max} (U/mg)
Crotonyl-CoA	29	6.2×10^3
2-Pentenoyl-CoA	36	2.8×10^3
2-Hexenoyl-CoA	34	1.8×10^3
2-Octenoyl-CoA	50	2.5

(*S*)-specific] enoyl-CoA hydratases in *R. rubrum* has been reported previously (19). As to their function in P(3HB) biosynthesis from acetate, it has been proposed that the (*R*)-3HB unit is supplied from two acetyl-CoA molecules via four-step reactions catalyzed by β-ketothiolase, NADH-acetoacetyl-CoA dehydrogenase, crotonase [(*S*)-specific enoyl-CoA hydratase], and (*R*)-specific enoyl-CoA hydratase. This pathway may function to supply the (*R*)-3HB unit from alkanoic acids in *A. caviae* together with the channeling route from the β-oxidation (Fig. 6), since a small fraction (3 to 5 mol%) of the 3HB unit was incorporated into copolyesters synthesized from alkanoic acids with odd numbers of carbon atoms (8). The activity toward 2-hexenoyl-CoA of the (*R*)-specific enoyl-CoA hydratase from *R. rubrum* was approximately one-third of the activity toward crotonyl-CoA (19), which is similar to the property of the enzyme encoded by $phaJ_{Ac}$ (Table 3). The (*R*)-specific hydratase of *R. rubrum* may also play an important role in PHA biosynthesis on alkanoic acids. However, further comparison of the properties of the (*R*)-specific hydratases of *A. caviae* and *R. rubrum* could not be made because of only a partial purification of the enzyme of *R. rubrum. Methylobacterium rhodesianum*, which is able to grow and to synthesize P(3HB) from methanol, has also been reported to possess two distinct crotonyl-CoA hydratases (20); however, the properties of the purified (*R*)-specific enzyme from *M. rhodesianum* (sigmoidal kinetics and a relatively high K_m) are quite different from those of the $phaJ_{Ac}$-derived hydratase. The participation of the (*R*)-hydratase in P(3HB) biosynthesis through the serine pathway in *M. rhodesianum* is still unclear.

PHA biosynthesis genes of *A. caviae* have been introduced into a PHA-negative mutant of *A. eutrophus*, and the ability of the transconjugants to synthesize PHA has been investigated (11). In some cases, the deletion of $phaJ_{Ac}$ resulted in an increase of the 3HHx fraction in P(3HB-*co*-3HHx) synthesized by the recombinant strains of *A. eutrophus*. This phenomenon was probably due to the higher hydration activity of the $phaJ_{Ac}$ product toward crotonyl-CoA than 2-hexenoyl-CoA. Expression of $phaJ_{Ac}$ in host cells may result in a higher concentration of (*R*)-3HB-CoA than of (*R*)-3HHx-CoA, which is reflected in the lower 3HHx unit content in the PHA produced. This result may suggest the possibility of changing the 3HHx fraction in bacterial polyesters by controlled expression of $phaJ_{Ac}$.

The $phaJ_{Ac}$ product has been suggested to have another function in the accumulation of PHA granules in cells, and ORF1 of *A. caviae* located upstream of $phaC_{Ac}$ may also take part in PHA biosynthesis and accumulation (11). Further study will be done to elucidate the characteristics and functions of the genes surrounding $phaC_{Ac}$.

ACKNOWLEDGMENT

This work was supported by CREST (Core Research for Evolutional Science and Technology) of Japan Science and Technology Corporation (JST).

REFERENCES

1. **Anderson, A. J., and E. A. Dawes.** 1990. Occurrence, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol. Rev. **54:**450–472.
2. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
3. **Brandl, H., E. J. Knee, R. C. Fuller, R. A. Gross, and R. W. Lenz.** 1989. Ability of the phototrophic bacterium *Rhodospirillum rubrum* to produce various poly(β-hydroxyalkanoates): potential source for biodegradable polyesters. Int. J. Biol. Macromol. **11:**49–55.
4. **Brandl, H., R. A. Gross, R. W. Lenz, and R. C. Fuller.** 1988. *Pseudomonas oleovorans* as a source of poly(3-hydroxyalkanoates) for potential application as biodegradable polyesters. Appl. Environ. Microbiol. **54:**1977–1982.
5. **Davison, J., M. Heusterspreute, N. Chevalier, V. Ha-Thi, and F. Brunel.** 1987. Vectors with restriction site banks. pJRD215, a wide-host-range cosmid vector with multiple cloning sites. Gene **51:**275–280.
6. **Deng, W. P., and J. A. Nickoloff.** 1992. Site-directed mutagenesis of virtually any plasmid by eliminating a unique site. Anal. Biochem. **200:**81–88.
6a. **de Waard, P., H. van der Wal, G. N. M. Huijberts, and G. Eggink.** 1993. Heteronuclear NMR analysis of unsaturated fatty acids in poly(3-hydroxyalkanoates). J. Biol. Chem. **268:**315–319.
7. **Doi, Y.** 1990. Microbial polyesters. VCH Publishers, New York, N.Y.
8. **Doi, Y., S. Kitamura, and H. Abe.** 1995. Microbial synthesis and characterization of poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate). Macromolecules **28:**4822–4828.
9. **Eggink, G., P. de Waard, and G. N. M. Huijberts.** 1992. The role of fatty acid biosynthesis and degradation in the supply of substrates for poly(3-hydroxyalkanoate) formation in *Pseudomonas putida*. FEMS Microbiol. Rev. **103:**159–164.
10. **Friedrich, B., C. Hogrefe, and H. G. Schlegel.** 1981. Naturally occurring genetic transfer of hydrogen-oxidizing ability between strains of *Alcaligenes eutrophus*. J. Bacteriol. **147:**198–205.
11. **Fukui, T., and Y. Doi.** 1997. Cloning and analysis of the poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) biosynthesis genes of *Aeromonas caviae*. J. Bacteriol. **179:**4821–4830.
12. **Gross, R. A., C. DeMello, R. W. Lenz, H. Brandl, and R. C. Fuller.** 1989. Biosynthesis and characterization of poly(β-hydroxyalkanoates) produced by *Pseudomonas oleovorans*. Macromolecules **22:**1106–1115.
13. **Huijberts, G. N. M., G. Eggink, P. de Waard, G. W. Huisman, and B. Witholt.** 1992. *Pseudomonas putida* KT2442 cultivated on glucose accumulates poly(3-hydroxyalkanoates) consisting of saturated and unsaturated monomers. Appl. Environ. Microbiol. **58:**536–544.
14. **Huisman, G. W., O. Leeuw, G. Eggink, and B. Witholt.** 1989. Synthesis of poly-3-hydroxyalkanoates is a common feature of fluorescent pseudomonads. Appl. Environ. Microbiol. **55:**1949–1954.
15. **Kato, M., H. J. Bao, C. K. Kang, T. Fukui, and Y. Doi.** 1996. Production of a novel copolyester of 3-hydroxybutyric acids and medium-chain-length 3-hydroxyalkanoic acid by *Pseudomonas* sp. 61-3 from sugars. Appl. Microbiol. Biotechnol. **45:**363–370.
16. **Kato, M., T. Fukui, and Y. Doi.** 1996. Biosynthesis of polyester blends by *Pseudomonas* sp. 61-3 from alkanoic acids. Bull. Chem. Soc. Jpn. **69:**515–520.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
18. **Leaf, T. A., M. S. Peterson, S. K. Stoup, D. Somers, and F. Srienc.** 1996. *Saccharomyces cerevisiae* expressing bacterial PHB synthase produces poly-3-hydroxybutyrate. Microbiology (Reading) **142:**1169–1180.
19. **Moskowitz, G. J., and J. M. Merrick.** 1969. Metabolism of poly-β-hydroxybutyrate. Enzymatic synthesis of D-(−)-β-hydroxybutyryl coenzyme A by an enoyl hydrase from *Rhodospirillum rubrum*. Biochemistry **8:**2748–2755.
20. **Mothes, G., and W. Babel.** 1995. *Methylobacterium rhodesianum* MB 126 possesses two stereospecific crotonyl-CoA hydratases. Can. J. Microbiol. **41:**68–72.
21. **Peoples, O. P., and A. J. Sinskey.** 1989. Poly-β-hydroxybutyrate biosynthesis in *Alcaligenes eutrophus* H16. Identification and characterization of the PHB polymerase gene (*phbC*). J. Biol. Chem. **264:**15298–15303.
22. **Poirier, Y., C. Nawrath, and C. Somerville.** 1995. Production of polyhydroxyalkanoates, a family of biodegradable plastics and elastomers, in bacteria and plants. Bio/Technology **13:**142–150.
23. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
24. **Schlegel, H. G., R. Lafferty, and I. Krauss.** 1970. The isolation of mutants not accumulating poly-β-hydroxybutyric acid. Arch. Mikrobiol. **71:**283–294.
25. **Schubert, P., A. Steinbüchel, and H. G. Schlegel.** 1988. Cloning of the *Alcaligenes eutrophus* poly-β-hydroxybutyrate synthetic pathway and synthesis of PHB in *Escherichia coli*. J. Bacteriol. **170:**5837–5847.
26. **Shimamura, E., K. Kasuya, G. Kobayashi, T. Shiotani, Y. Shima, and Y. Doi.** 1994. Physical properties and biodegradability of microbial poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate). Macromolecules **27:**878–880.
27. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for in vivo genetic engineering. Transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**784–791.

28. **Slater, S. C., W. H. Voige, and D. E. Dennis.** 1988. Cloning and expression in *Escherichia coli* of the *Alcaligenes eutrophus* H16 poly-β-hydroxybutyrate biosynthetic pathway. J. Bacteriol. **170:**4431–4436.
29. **Steinbüchel, A.** 1996. PHB and other polyhydroxyalkanoic acids, p. 403–464. *In* H. J. Rehm and G. Reed (ed.), Biotechnology. VCH Publishers, Weinheim, Germany.
30. **Steinbüchel, A., E. Hustede, M. Liebergesell, U. Pieper, A. Timm, and H. Valentin.** 1992. Molecular basis for biosynthesis and accumulation of polyhydroxyalkanoic acids in bacteria. FEMS Microbiol. Rev. **103:**217–230.
31. **Stern, J. R., A. Del Campillo, and I. Raw.** 1955. Enzymes of fatty acid metabolism. I. General introduction; crystalline crotonase. J. Biol. Chem. **218:**971–983.
32. **Timm, A., and A. Steinbüchel.** 1990. Formation of polyesters consisting of medium-chain-length 3-hydroxyalkanoic acids from gluconate by *Pseudomonas aeruginosa* and other fluorescent pseudomonads. Appl. Environ. Microbiol. **56:**3360–3367.
33. **Valentin, H. E., and A. Steinbüchel.** 1994. Application of enzymatically synthesized short-chain-length hydroxy fatty acid coenzyme A thioesters for assay of polyhydroxyalkanoic acid synthases. Appl. Microbiol. Biotechnol. **40:**699–709.
34. **Williams, M. D., J. A. Rahn, and D. H. Sherman.** 1996. Production of a polyhydroxyalkanoate biopolymer in insect cells with a modified eucaryotic fatty acid synthase. Appl. Environ. Microbiol. **62:**2540–2546.