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ORIGINAL PAPER

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Characterization and cloning of an (*R*)-specific *trans*-2,3-enoylacyl-CoA hydratase from *Rhodospirillum rubrum* and use of this enzyme for PHA production in *Escherichia coli*

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Abstract An (*R*)-*trans*-2,3-enoylacyl-CoA hydratase was purified to near-homogeneity from *Rhodospirillum rubrum*. Protein sequencing of enriched protein fractions allowed the construction of a degenerate oligonucleotide. The gene encoding the (*R*)-specific hydratase activity was cloned following three rounds of colony hybridization using the oligonucleotide, and overexpression of the gene in *E. coli* led to the purification of the enzyme to homogeneity. The purified enzyme used crotonyl-CoA, *trans*-2,3-pentenoyl-CoA, and *trans*-2,3-hexenoyl-CoA with approximately equal specificity as substrates in the hydration reaction. However, no activity was observed using *trans*-2,3-octenoyl-CoA as a substrate, but this compound did partially inhibit crotonyl-CoA hydration. Based on the nucleotide sequence, the protein has a monomeric molecular weight of 15.4 kDa and is a homotetramer in its native form as determined by gel filtration chromatography and native PAGE. The hydratase was expressed together with the PHA synthase from *Thiocapsa pfennigii* in *E. coli* strain DH5α. Growth of these strains on oleic acid resulted in the production of the terpolyester poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxyhexanoate).

Introduction

Polyhydroxyalkanoates (PHA) are produced by many different microorganisms as a means of carbon and energy storage. PHAs represent a renewable, biodegradable source of plastics and can be used as a substitute for petroleum-derived plastics, if produced economically. The most commonly described PHA, polyhydroxybutyrate (PHB), is synthesized from (*R*)-β-hydroxybutyryl-CoA by the enzyme PHA synthase. This substrate can be produced by several routes. The classical pathway, first described in *Ralstonia eutropha*, utilizes acetyl-CoA condensation by β-ketothiolase followed by reduction of acetoacetyl-CoA by an (*R*)-specific acetoacetyl-CoA reductase (Steinbüchel and Schlegel 1991).

Another source of acyl-CoA intermediates for PHA biosynthesis can be derived from fatty acid β-oxidation (Brandl et al. 1988; Eggink et al. 1992, 1995; Kranz et al. 1997; Lageveen et al. 1988; Page and Manchak 1995; de Waard et al. 1993). In fact, several different microorganisms are capable of producing PHAs from oils and alkanoic acids (Anderson and Dawes 1990; Brandl et al. 1988, 1989; Eggink et al. 1995; Lageveen et al. 1988). This can result in the production of medium-chain-length PHAs (MCL-PHAs). MCL-PHAs possess different physical properties than shorter-chain PHAs and therefore can be used for other applications. Some of these organisms possess an (*R*)-specific *trans*-2,3-enoylacyl-CoA hydratase which allows them to utilize intermediates from β-oxidation for PHA production (Fukui and Doi 1997; Fukui et al. 1998; Moskowitz and Merrick 1969). This enzyme catalyzes the hydration of *trans*-2,3-enoylacyl-CoAs to their corresponding (*R*)-β-hydroxyacyl-CoAs, which can then be utilized by PHA synthase for polymer production. Such an enzyme has been described in the photosynthetic, PHA producing, purple bacterium *Rhodospirillum rubrum*, though it has not definitively been demonstrated that this enzyme is essential for PHA biosynthesis (Moskowitz and Merrick 1969).

A similar hydratase activity has recently been described in the microorganism *Aeromonas caviae*. Work by Fukui et al. (Fukui and Doi 1997; Fukui et al. 1998) describes the cloning and characterization of an (*R*)-*trans*-2,3-enoylacyl-CoA hydratase ($phaJ_{Ac}$) from this organism. These investigators were able to demonstrate that $phaJ_{Ac}$ could be used to complement a mutant of *A. caviae* that was unable to

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produce poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) [P(3HB-*co*-3HHx)] when grown in the presence of the dodecanoic acid (Fukui et al. 1998). These results provide direct evidence that define the link between β-oxidation and PHA production in this organism (Fukui et al. 1998).

β-Oxidation results in the transient production of *trans*-2,3-enoylacyl-CoA intermediates which might potentially be utilized for polymer production in recombinant organisms such as microbes and plants. Unfortunately, from a metabolic engineering point of view, the (*S*)-isoforms of β-hydroxyacyl-CoAs transiently produced from β-oxidation cannot be utilized as substrates for PHA production since the stereospecificity of the PHA synthase is for (*R*)-β-hydroxyacyl-CoAs. Therefore, utilization of a gene encoding an (*R*)-*trans*-2,3-enoylacyl-CoA hydratase could allow PHA production via β-oxidation in genetically modified organisms that contain this enzyme plus a PHA synthase.

In this report we discuss a reverse genetics approach to cloning the (*R*)-*trans*-2,3-enoylacyl-CoA hydratase from *R. rubrum*. We also describe the biochemical and kinetic properties of the purified protein. Lastly, we demonstrate in recombinant *E. coli,* an engineered pathway which utilizes the two enzymatic activities of the hydratase together with a PHA synthase to produce poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate-*co*-3-hydroxyhexanoate) [poly(3HB-*co*-3HV-*co*-3HHx)] from oleic acid.

Materials and methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1.

Growth and culture conditions

For purposes such as DNA isolation, as well as growth and propagation, *E. coli* strains were grown on Luria Bertani (LB) medium (Sambrook et al. 1989) with the appropriate antibiotic. Carbenicillin was used in these studies for selection at a concentration of 100 μg/ml. *R. rubrum* was obtained from the American Tissue Culture Collection (ATCC No. 25903). It was cultured in R8AH medium. The following components were added per liter of medium: 2.5 g malic acid, 1.0 g yeast extract, 1.25 g $(NH)_2SO_4$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.07 g $CaCl_2 \cdot 2H_2O$, 0.01 g ferric citrate, 0.02 g EDTA, 0.6 g KH_2PO_4, 0.9 g K_2HPO_4, 1 ml trace elements (consisting of the following in 100 ml: 0.3 g ferric citrate, 2 mg $MnSO_4 \cdot H_2O$, 1 mg H_3BO_3, 1 mg $CuSO_4 \cdot 5H_2O$, 2 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1 mg $ZnSO_4$, 0.05 g EDTA, 0.02 $CaCl_2 \cdot 2H_2O$), 7.5 ml vitamin solution (consisting of the following in 100 ml: 0.2 g nicotinic acid, 0.2 g nicotinamide, 0.4 g thiamine HCl, 8 mg biotin). NaOH was used to adjust the pH of the medium to 6.9. Cultures were incubated at 30 °C approximately 5 inches (12.5 cm) from a 90 W incandescent light bulb under anaerobic conditions. Anaerobic conditions were achieved by completely filling the culture bottles with medium, capping them, and wrapping them with parafilm. Each day, the cultures were shaken by hand to disperse the culture. Precultures were grown in 50-ml bottles for approximately 3–4 days and 2 ml of this culture was then used to inoculate a 1-l culture.

Enoylacyl-CoA hydratase assays

A spectrophotometric assay similar to the one reported by Moskowitz and Merrick (1969), based on the hydration of enoylacyl-CoA, was routinely used to measure hydratase activity. The assay was carried out in 1 ml total volume and monitored at 260 nm (ϵ = 6.7/mM × cm). Components were added in the following order to the stated final concentrations: 100 mM *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS), pH 8.5, 32 μM crotonyl-CoA and enzyme to initiate the reaction. To determine kinetic constants for *trans*-2,3-enoylacyl-CoA substrates (see Table 6), the concentration of substrate was varied from 5 to 80 μM. All rates were the average of duplicate measurements and the data fitted to the normal Michaelis-Menten equation using the commercial software GraFit (Leatherbarrow 1990). Coupled assays based on NAD(P)H oxidation to $NAD(P)^+$ using (*R*)-β-hydroxybutyryl-CoA reductase

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
R. rubrum		
ATCC 25903	Derivative of *R. rubrum* strain S1	ATCC center
E. coli		
DH5α	*deoR endA1 gyrA96 hsdR17*($r_K^- m_K^+$) *recA1 relA1 supE44 thi-1* Δ(*lacZYA-argFV169*) ϕ80 Δ*lac*ZΔM15F$^-$r$^-$	(Sambrook et al. 1989)
Plasmids		
pSP72	Cloning and *in vitro* transcription vector	Promega
pSE380	A *trc* expression vector with super-polylinker	Invitrogen
pSK28	Genomic fragment from *T. pfennigii* containing the PHA synthase subunits $phaC_{Tp}$ and $phaE_{Tp}$ cloned into pBluescript SK	A. Steinbüchel and M. Liebergesell, generous gift
pMON25653	An *E. coli* expression vector containing *phbB* fused behind the G10 leader sequence (Olins et al. 1988) and the *tac* promoter (De Boer et al. 1983)	This study
pMON25893	$phaE_{Tp}$ subcloned as a *Bgl*II/*Eco*RI fragment into pSP72	This study
pMON25894	$phaC_{Tp}$ subcloned as a *Bgl*II/*Eco*RI fragment into pSP72	This study
pMON25895	$phaE_{Tp}$ subcloned as a *Sph*I/*Bam*HI fragment into pSE380	This study
pMON25896	$phaC_{Tp}$ subcloned as a *Bgl*II/*Bam*HI fragment into pMON25895	This study
pMON37186	Derivative of pMON25896 containing $phaJ_{Rr}$ subcloned as an *Eco*RI/*Not* I fragment from pMON37187	This study
pMON37187	A 1.35 kb *Hin*dIII fragment from *R. rubrum* containing $phaJ_{Rr}$ and ORF2 inserted into pSP72	This study
pMON37189	*Not*I/*Xho*I deletion of pMON37187 to remove ORF2	This study

(PhbB) from *R. eutropha* (described below), or (*S*)-β-hydroxyacyl-CoA dehydrogenase from yeast (Sigma) were used to determine the stereospecificity of the 3-hydroxyacyl-CoA product being produced from the hydratase reaction, and were monitored at 340 nm. One unit is defined as 1 μmol product produced per minute.

The reductase enzyme, PhbB, from *R. eutropha* was purified in the following manner. Plasmid pMON25653 was used to transform *E. coli* strain DH5α. Four milliliters of an overnight culture was used to inoculate a 200-ml culture. The absorbance of the culture was monitored and upon reaching an $A_{600} = 0.5$–0.6, the culture was induced with 1 mM isopropyl thiogalactoside (IPTG) for an additional 2 h before being harvested. The cell pellet was resuspended in extraction buffer (10 mM Tris, pH = 7.8, 1 mM dithiothreitol (DTT), 5% glycerol) and sonicated using a microtip probe at maximum setting for a total of 6 min (three bursts at 2 min each, resting on ice for 2 min between bursts). The sample was desalted using a PD-10 column (Pharmacia), equilibrated with Tris buffer (10 mM Tris, 1 mM DTT, pH 7.8), filtered through a 0.45-μm filter and loaded onto a MonoQ HR5/5 column (Pharmacia) also equilibrated with the Tris buffer. The enzyme was eluted with a 0–0.1 M KCl gradient (10 ml) at a flow rate of 1 ml/min followed by a 0.1–0.25 M KCl gradient (40 ml) at a flow rate of 1 ml/min. Active fractions were concentrated and frozen for storage at −80 °C.

Substrate synthesis

Preparation of *trans*-2,3-pentenoyl-CoA, *trans*-2,3-hexenoyl-CoA, and *trans*-2,3-octenoyl-CoA was accomplished using a procedure similar to that described by Schulz (1974). The synthesis involves a mixed condensation reaction starting with commercially available pentenoic, hexenoic, and octenoic acids (Aldrich). To 0.1 mmol *trans*-2,3-pentenoic, *trans*-2,3-hexenoic, or *trans*-2,3-octenoic acid was added 4 ml degassed tetrahydrofuran (THF) and 0.15 mmol triethylamine. In a separate vessel, 1.5 ml of degassed THF was added to 0.1 mmol ethyl chloroformate. Both solutions were kept sealed under nitrogen at 4 °C. The ethyl chloroformate solution was added dropwise to the enoic acid solution over a 10-min period with constant stirring at 4 °C. A small volume of degassed THF was used to rinse out the ethyl chloroformate flask and was then added to the reaction mixture. The mixture was allowed to react further for an additional 10–20 min to complete the formation of the mixed anhydride, and then filtered with glass wool. The glass wool was rinsed with additional degassed THF, and the rinse added to the filtrate. The clarified solution was concentrated by rotary evaporation to 4–5 ml, and kept cool at 4 °C.

A total of 0.1 mmol CoA was dissolved in 1.5 ml degassed deionized water and 3 ml degassed THF. The pH of this solution was adjusted to 8 with 1 M $NaHCO_3$. The flask containing the CoA solution was then sealed around the pH electrode, and degassed with a stream of nitrogen. The mixed anhydride was added dropwise over a 30-min period to the CoA solution at room temperature with constant stirring, and the pH maintained between 8 and 9 using 1 M $NaHCO_3$ and 0.1% formic acid. A small volume of degassed THF was used to rinse the mixed anhydride flask, and this was added to the reaction mixture. The solution was allowed to react for an additional 1.5-h with the pH maintained just above 8. The reaction mixture was reduced to pH 5 using 0.1% formic acid and concentrated to 20–25 ml by rotary evaporation. The pH was readjusted to 5 with 1 M $NaHCO_3$, and the volume reduced to 1–2 ml using a SpeedVac (Savant).

Purification of product was accomplished using a semi-prep C8 reverse-phase column (Beckman ultrasphere, 10 mm × 25 cm) using a linear gradient of 5–45% acetonitrile (95–55% 50 mM ammonium acetate, pH 6.0) over 25 min. The flow rate was 4.0 ml/min with peak detection at 260 nm. *Trans*-2,3-pentenoyl-CoA, *trans*-2,3-hexenoyl-CoA, and *trans*-2,3-octenoyl-CoA typically eluted around 12.5, 15.5, and 18.5 min, respectively. Pooled fractions were concentrated to approximately 10 mM based on absorbance at 260 nm ($\epsilon = 22/\text{mM} \times \text{cm}$ for all three enoyl-CoAs) using a SpeedVac. The solutions were kept in this form for subsequent hydratase assays and stored at −80 °C. Stability is for greater than 6 months under these conditions. Yields for the whole procedure based on CoA are 30–60%.

Protein purification of an (*R*)-*trans*–2,3-enoylacyl-CoA hydratase from *R. rubrum*

All the steps in the protein purification were carried out at 4 °C unless otherwise stated. Active fractions were routinely concentrated using a Centriprep 10 filter (Amicon) and desalted using a PD-10 column (Pharmacia). For the steps described below, a total of 8 l *R. rubrum* culture were harvested, which yielded 18.3 g wet weight of cellular material.

Step 1: Preparation of a crude lysate

The cells were sonicated in extraction buffer (4 ml per gram of wet cells, 50 mM Tris, 2 mM DTT, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0 with HCl) using a blunt-tip probe at maximum setting for a total of 6 min (three bursts at 2 min each, resting on ice for 2 min between bursts). DNA was precipitated by adding 0.22 vol. of a 10% streptomycin sulfate solution (10 g streptomycin sulfate in a total of 100 ml extraction buffer) and incubating on ice for 30 min. The sample was centrifuged at 10,000 *g* for 15 min.

Step 2: Ammonium sulfate precipitation and 70 °C heat step

Ammonium sulfate was added to the extract in a stepwise manner. The first ammonium sulfate cut was to 35% saturation. Centrifugation was carried out at 10,000 *g* for 15 min. The resulting supernatant was then raised to 55% saturation. Again, centrifugation was carried out at 10,000 *g* for 15 min. The supernatant was then raised to 75% saturation before being centrifuged at 10,000 *g* for 15 min. The resulting pellets from the 55% and 75% ammonium sulfate cuts were combined by resuspending them in potassium phosphate buffer (10 mM KH_2PO_4, 2 mM DTT, 1 mM EDTA, pH 7.0 with KOH). The sample was placed in dialysis tubing with a 10,000-molecular-weight cutoff and dialyzed for 2 h in 1 l of potassium phosphate buffer, followed by overnight dialysis in 1 l of the same buffer. The sample was incubated at 70 °C for 5 min and the precipitated protein removed by centrifugation at 30,000 *g* for 30 min.

Step 3: MonoQ HR10/10 separation

The supernatant from step 2 was filtered through a 0.45-μm filter, equilibrated with Tris buffer (10 mM Tris, 1 mM DTT, pH 7.8) using a PD-10 desalting column (Pharmacia) and loaded onto a MonoQ HR10/10 column (Pharmacia) also equilibrated with Tris buffer. The enzyme was eluted stepwise with a 0–0.1 M KCl gradient (15 ml) at a flow rate of 3 ml/min followed by a 0.1–0.35 M KCl gradient (60 ml) at a flow rate of 3 ml/min. The active hydratase fractions were pooled and concentrated.

Step 4: Phenyl superose HR5/5 separation

Solid ammonium sulfate was added to the pooled, concentrated, hydratase fractions to a final concentration of 1 M. The solution was then filtered through a 0.45-μm filter and loaded onto a phenyl superose HR5/5 column (Pharmacia) equilibrated with sodium phosphate buffer (20 mM $Na_2HPO_4 \cdot 7H_2O$, 1 mM DTT, pH 7.0). The enzyme was eluted with a 1–0.5 M $(NH_4)_2SO_4$ gradient (10 ml) at a flow rate of 0.5 ml/min followed by a 0.5–0.25 M $(NH_4)_2SO_4$ gradient (30 ml) at a flow rate of 0.5 ml/min. The hydratase fractions were pooled and concentrated.

Step 5: MonoQ HR5/5 separation

The hydratase from the previous step was desalted with Tris buffer (10 mM Tris, 1 mM DTT, pH 7.8) using a PD-10 desalting

column, filtered through a 0.45-μm filter and loaded onto a MonoQ HR5/5 column (Pharmacia) pre-equilibrated with Tris buffer. The enzyme was eluted stepwise with a 0–0.1 M KCl gradient (10 ml) at a flow rate of 1 ml/min followed by a 0.1–0.25 M KCl gradient (40 ml) at a flow rate of 1 ml/min. Fractions were concentrated and then frozen at −80 °C for later analysis.

Protein expression and purification from *E. coli*

E. coli strain DH5α was transformed with the high-copy-number plasmid pMON37189. Generation of a crude lysate, ammonium sulfate precipitation, and heat treatment were carried out as previously described for the purification of the protein from *R. rubrum*. Following the heat treatment of the sample, purification proceeded in the following manner:

Step 1: Phenyl superose separation

Solid ammonium sulfate was added to a final concentration of 1 M to the solution containing the hydratase. The solution was filtered through a 0.45-μm filter and loaded onto a preparative phenyl superose column (25 cm × 2 cm) equilibrated with sodium phosphate buffer (20 mM $Na_2HPO_4{\cdot}7H_2O$, 1 mM DTT, 1 M $(NH_4)_2SO_4$, pH 7.0). The enzyme was eluted stepwise with a 1–0.5 M $(NH_4)_2SO_4$ gradient (30 ml) at a flow rate of 3.0 ml/min followed by a 0.5–0 M $(NH_4)_2SO_4$ gradient (60 ml) at a flow rate of 3 ml/min. The hydratase fractions were pooled and concentrated.

Step 2: MonoQ separation

The hydratase was desalted using a PD-10 desalting column in Tris buffer (10 mM Tris, 1 mM DTT, pH 7.8) and filtered through a 0.45-μm filter. It was then loaded onto a MonoQ HR10/10 column (Pharmacia) equilibrated with Tris buffer. The enzyme was eluted with a 0–0.05 M KCl gradient (6 ml) at a flow rate of 3 ml/min followed by a 0.05–0.15 M KCl gradient (60 ml) at a flow rate of 3 ml/min. A total of 527 mg protein was applied to the column, which had a hypothetical binding capacity of approximately 520 mg. After the sample was applied to the column it was noted that the majority of the activity was found in the void volume, indicating the possibility that the column was overloaded. The net result was that the majority of the activity had not been retained by the column. The void volume together with the active fractions which were later eluted from the column were pooled, equilibrated in Tris buffer, and passed over the column a second time. The protein in this second pass was retained by the column and it was eluted in the stepwise manner described above. The active hydratase fractions were concentrated and stored at −80 °C for later analysis.

Determination of molecular mass

The molecular mass of the native enzyme was determined by native PAGE and gel filtration chromatography using a Superose 12 HR10/30 column (Pharmacia) using 0.05 M phosphate and 0.15 M NaCl as eluting buffer.

Protein electrophoresis and detection

SDS-PAGE and native PAGE was performed using precast gradient gels (10–20%), or homogeneous gels (12.5%), respectively, from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). Proteins were visualized by Coomassie staining using the ISS Pro-Blue protein detection kit from Integrated Separation Systems (Natick, Mass.). Alternatively, precast (12.5%) gels from Amersham Pharmacia Biotech (Piscataway, NJ) were also used and protein detection was performed by silver staining using the Amersham Pharmacia PhastGel system. Samples for SDS-PAGE were mixed 1:5 with gel loading buffer (0.06 M Tris, pH 6.8, 2% SDS, 10% glycerol, 0.025% bromophenol blue, and 2% β-mercaptoethanol) and incubated at 100 °C for 5 min before being loaded onto a gel.

Protein sequencing

Protein sequencing was carried out on proteolytic products following tryptic digestion of isolated proteins. N-terminal protein sequencing was carried out by automated Edman degradation.

DNA hybridization and sequencing

Southern blot hybridization and colony hybridization were performed with Hybond-N membranes (Amersham, Arlington Heights, Ill.) by standard techniques using the degenerate oligonucleotide Int-11 (Table 2) (Sambrook et al. 1989). The nucleotide sequence of cloned fragments were determined by the dideoxy chain-termination method on an Applied Biosystems (Foster City, Calif.) ABI Prism 377 DNA Sequenator according to the manufacturer's instructions. Nucleotide sequences and deduced amino acid sequences were compared to sequence information in GenBank release 110.0 (Benson et al. 1998) using the BLAST algorithm (Altschul et al. 1990) via a worldwide web server.

Nucleotide sequence accession number

The nucleotide sequence for the 1.4 kb *Hin*dIII fragment from *R. rubrum* reported here has been deposited in the GenBank database under GenBank accession number AF156879 (Benson et al. 1998).

Polymer production in recombinant *E. coli*

The plasmid vector pMON37186 was used to transform *E. coli* strain DH5α to produce PHA from alkanoic acids. Plasmid pMON37186 was derived in the following manner. The PCR

Table 2 Oligonucleotides used in this study. Underlined regions denote restriction enzyme sites or Shine-Delgarno (*S.D.*) ribosomal binding sites which were introduced into the oligonucleotides. Nucleotides enclosed in *parentheses* are equally represented in that particular nucleic acid position of the oligonucleotide in order to introduce degeneracy

Primer name	Oligonucleotide sequence[a]
5′-PhaE	5′-GA AGATCT GCATGC TCATGA ACGATACGGCCAACAAGACCAGC *Bgl*II *Sph*I *Bsp*HI
3′-PhaE	5′-G GAATTC GGATCC CGGCCGACATCACTGGCCGGTGGT *Eco*RI *Bam*HI
5′-PhaC	5′-GA AGATCT AGGAG GCATGC TCATGA CCCCATTCCCGATCGACATCCGG *Bgl*II S.D. *Sph*I *Bsp*HI
3′-PhaC	5′-G GAATTC GGATCC GTGGGTCGACCCGGCTCAGCCGCG *Eco*RI *Bam*HI
Int-11	5′-GGCGC(C/G)ATCTA(T/C)GT(C/G)AA(C/T)CAG

[a] Oligonucleotides were ordered from Life Technologies (Grand Island, New York)

primers 5′-PhaC together with 3′-PhaC (Table 2) and 5′-PhaE together with 3′-PhaE (Table 2) were used to amplify the genes $phaC_{Tp}$ and $phaE_{Tp}$, respectively, from pSK28. PCR was performed using Vent polymerase (New England Biolabs Inc., Beverly, Mass.) for 25 cycles using a melting temperature of 96 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min using a Perkin Elmer Cetus, DNA thermocycler. The PCR products were digested with *Bgl*II/*Eco*RI and subcloned into pSP72 to produce pMON25893 and pMON25894. Plasmid pMON25895 was made by subcloning the $phaE_{Tp}$ gene as an *Sph*I/*Bam*HI fragment from pMON25893. The plasmid pMON25896 contains $phaC_{Tp}$ subcloned as a *Bgl*II/*Bam*HI fragment from pMON25894. Plasmid vector pMON25896 contains both subunits of PHA synthase from *T. pfennigii* cloned behind the IPTG inducible *trc* promoter creating a two-gene operon. An artificial Shine-Delgarno sequence (nucleotide sequence AGGAGG) was incorporated into primer 5′-PhaC (Table 2) and allows translational initiation of the PhaC subunit. The hydratase was subcloned from pMON37187 into pMON25896 as an *Eco*RI/*Not*I fragment to produce pMON37186.

Cultures for polymer production were grown in the following manner. *E. coli* strain DH5α harboring plasmid pMON37186 or the negative control plasmids pMON25896 or pMON37189 were grown overnight in LB medium. Ten milliliters of the overnight culture was transferred to 1 l M9 medium supplemented with 0.1% of the appropriate carbon source. The culture was incubated at 37 °C for 8 h. IPTG was then added to the culture to a final volume of 1 mM. The culture was then incubated at 37 °C for an additional 16 h before being harvested for polymer analysis.

Polymer analysis

Polymer analysis and quantitation were carried out using gas chromatography (GC) following methanolysis of the PHA, based on the method of Braunegg et al. (1978) as modified by Brandl et al. (1988). Additional modifications are outlined below. Peaks identified as being methyl ester derivatives of PHAs were confirmed by mass spectroscopy. Methanolysis was carried out directly on lyophilized bacterial samples. Samples for analysis were collected by centrifugation and washed with phosphate buffer saline (0.01 M KH_2PO_4, 0.1 M Na_2HPO_4, 1.37 M NaCl, 0.027 M KCl, pH of solution 7.4). The samples were then recentrifuged and the supernatant discarded. The pellet was washed with ethanol, recentrifuged, and the supernatant discarded. The resulting pellet was frozen and then lyophilized. Methanolysis was typically carried out on approximately 20 mg lyophilized bacterial sample. It was dissolved in 1 ml chloroform containing methyl benzoate as an internal standard. To this, 1 ml 15% H_2SO_4 in methanol (v/v) was added and the sample was incubated at 100 °C for 2.5 hours. The sample was then cooled to room temperature and 0.5 ml water was added. This was vortexed for 20 s and then centrifuged for 30 s at 1000 *g* in a clinical centrifuge to separate the phases. The chloroform phase was removed to a new vial and approximately 50 mg sodium sulfate was added. The sample was briefly vortexed and the supernatant transferred to a GC vial for analysis.

The methyl esters of the hydrolyzed polymer were separated based on the method of Lageveen et al. (1988) with modifications. Methyl esters were separated on a DB5 column (J&W Scientific, length 30 m, internal diameter 0.25 mm, film thickness 0.25 μm) using a Varian 3400 GC instrument. The separation program used had the following parameters: 70 °C for 6 min, ramp to 130 °C at 30 °C per minute, next ramp to 300 °C at 50 °C per minute, and hold for 5 min.

Results

Partial purification of (*R*)-hydratase from *R. rubrum*

An (*R*)-*trans*-2,3-enoylacyl-CoA hydratase was purified to near-homogeneity from *R. rubrum* based on SDS-PAGE analysis with protein detection by silver staining. This was accomplished using a combination of ammonium sulfate cuts, a heat step, and hydrophobic and anion exchange chromatography. The results of the purification procedure are summarized in Table 3. SDS-PAGE analysis showed three protein bands of approximately 15, 30, and 68 kDa respectively (Fig. 1). It was observed that the 68-kDa protein could be eliminated by a longer incubation of the protein sample at 100 °C (approximately 7 min) in freshly prepared SDS gel loading buffer, prior to separation by SDS-PAGE. Hydrophobic exchange chromatography using a phenyl superose column resulted in a 20-fold increase in specific activity to over 2000 u/mg (Table 3). It was possible to further purify the enzyme by again passing it over a MonoQ column and using a shallower salt gradient. The final collected fractions had increased in specific activity from 12 u/mg in the crude extract to 4735 u/mg in the most purified fraction (Table 3). Enzymatic activity seemed to be associated with the 15-kDa band based on the relationship of observed activity of specific protein fractions to this protein band as observed on SDS-PAGE gels following silver staining.

N-Terminal and internal protein sequencing

N-terminal and internal protein sequence was obtained from all three isolated proteins. The protein sequence was used to design degenerate oligonucleotides in order to probe for the gene encoding the hydratase. The results of the protein sequencing from the 15- and 68-kDa proteins are presented in Table 4. The N-terminal protein sequences of the 15- and 68-kDa fragments were found to be the same following automated Edman degradation (Table 4). The 30-kDa protein was observed to have a different N-terminal protein sequence than the other two proteins and is an apparent impurity. An internal protein sequence was obtained from the 15-kDa protein following tryptic digestion (Table 4). None of

Table 3 Purification of $PhaJ_{Rr}$ from *R. rubrum*

Purification step	Protein (mg)	Volume (ml)	Total activity (μmol/min)	Specific activity (μmol/min × mg)	Percent recovery
1. Crude lysate	835	89	10169	12	100
2. 55–75% $(NH)_2SO_4$	102	13.5	3725	37	37
3. MonoQ HR10/10	9.9	21	1281	129	13
4. Phenyl superose HR5/5	0.6	2	1217	2028	12
5. MonoQ HR5/5	0.068	2	322	4735	3

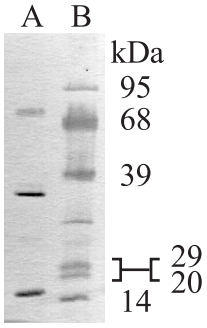

Fig. 1 SDS-PAGE analysis of the partially purified hydratase isolated from *R. rubrum*. Enriched hydratase from *R. rubrum* was loaded in lane A following the purification steps outlined in Table 3 and the Materials and methods section of the text. Molecular weight markers were loaded in lane B. Proteins were resolved on a 12.5% PhastGel (Amersham Pharmacia Biotech, Piscataway, NJ) and detected by silver staining

the protein sequences showed any significant similarity to any sequence reported in GenBank release 110.0.

Isolation of a gene encoding the (*R*)-hydratase activity from *R. rubrum*

The N-terminal and internal protein sequences were used to design degenerate DNA oligonucleotides. The oligonucleotide Int-11 (Table 2), based on the internal protein sequence from the 15-kDa protein, gave a specific signal when hybridized against *R. rubrum* genomic DNA digested with *Hin*dIII. A *Hin*dIII subgenomic library was constructed from 1.0- to 2.5-kb size selected DNA fragments, cloned into pSP72, and transformed into *E. coli* strain DH5α. The resulting colonies were screened by colony hybridization using the oligonucleotide Int-11. Two identical but independent clones of a 1.4-kb *Hin*dIII fragment were isolated following three rounds of colony hybridization.

The DNA sequence from both strands of the isolated *Hin*dIII fragment was determined by automated DNA sequencing and deposited in the GenBank database under GenBank accession number AF156879. Two possible open reading frames were determined from the DNA sequence (Fig. 2). ORF2 is a partial open reading

Table 4 N-Terminal and internal protein sequences of isolated proteins from *R. rubrum*. The amino acid sequence which is *underlined* was used to generate the degenerate oligonucleotide Int-11 (see Table 2)

Source	Protein sequence
N-terminal sequence from 15-kDa protein	SADDLILHYFEDIKEGQSAKL
N-terminal sequence from 68-kDa protein	SADDLILHYF
Internal sequence from 15-kDa protein	LPGPGAIYVNQSLK

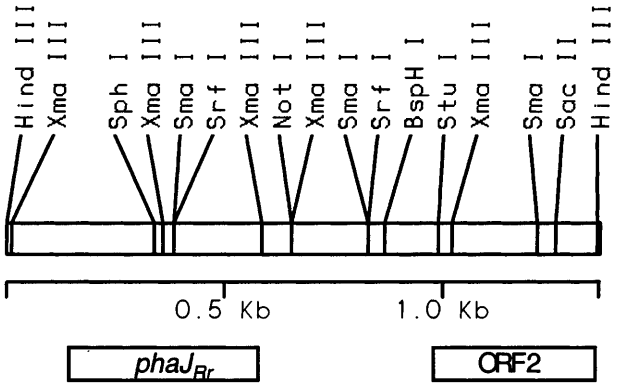

Fig. 2 Restriction map of the *R. rubrum* genomic fragment containing $phaJ_{Rr}$ and ORF2. A *Hin*dIII genomic fragment with homology to the oligonucleotide Int-11 from *R. rubrum* was isolated following three rounds of colony hybridization. The DNA sequence, as determined following automated DNA sequencing of both DNA strands, has been deposited in the GenBank database under GenBank accession number AF156879. ORF2 is a truncated open reading frame with similarity to *ribF* from *E. coli* (GenBank accession number 1786208). The gene, $phaJ_{Rr}$, encodes an (*R*)-*trans*-2,3-enoylacyl-CoA hydratase

frame encoding a 26-kDa truncated protein. ORF2 shows similarity (41% identity over 193 amino acids) to *ribF*, a putative regulatory protein from *E. coli* formerly known as *yaaC*. This open reading frame has been designated by accession number P08391 in the GenBank database. The predicted protein sequence of ORF2 shares no similarity with any of the amino acid sequences obtained from protein sequencing of the 15-, 30-, and 68-kDa partially purified proteins from *R. rubrum*.

ORF1 encodes a protein of 15,353 Da, based on its predicted protein sequence. This is similar in size to the 15 kDa fragment which was enriched in association with increased hydratase activity from *R. rubrum*. Additionally, the predicted N-terminal and internal protein sequences match the amino acid sequences obtained from protein sequencing the partially purified 15 kDa protein (Fig. 3).

The predicted amino acid sequence from ORF1 was used in a BLAST search (Altschul et al. 1990) to identify similar proteins in the public database, GenBank Release 110.0 (Benson et al. 1998). Two sequences showed significant similarity. A comparison of the amino acid sequences is shown in Fig. 3. The matching sequences were optimally aligned using the BESTFIT sequence alignment tool (Wisconsin Package version 9.1, Genetics Computer Group (GCG), Madison, Wis.). ORF1 has the greatest sequence similarity to a hypothetical protein from *Rhodobacter capsulatus* (accession number AF010496, 58% identity over 131 amino acids). This sequence was reported by Vlèek et. al. (1997) after sequencing a 189-kb contiguous sequence from *Rhodobacter capsulatus*. The predicted amino acid sequence also matched ORF3, $phaJ_{Ac}$, (accession number D88825, 45% identity over 131 amino acids), a known (*R*)-*trans*-2,3-enoylacyl-CoA hydratase identified from *A. caviae* which is involved in polyhydroxyalkanoate production (Fukui and Doi 1997).

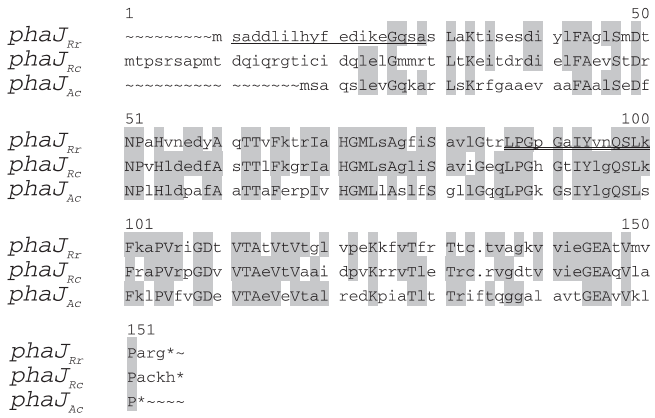

Fig. 3 Multiple alignment of hydratase and hydratase-like enzymes. PileUp (Wisconsin Package version 9.1, Genetics Computer Group (GCG), Madison, Wis.) was used to align the protein sequences of $phaJ_{Rr}$ from *R. rubrum* with other hydratase and hydratase-like proteins. The genes are: $phaJ_{Rr}$ from *R. rubrum* (this work, GenBank accession number AF156879), $phaJ_{Ac}$ from *A. caviae* (GenBank accession number D88825), and a probable hydratase labeled here as $phaJ_{Rc}$ from *R. capsulatus* (GenBank accession number AF010496). Letters which appear in *uppercase* are amino acids which are conserved in all three ORFs. Amino acids which have been *highlighted* are shared in at least two of the ORFs. *Underlined* amino acids represent the N-terminal sequence which was determined from the purified 15-kDa protein. *Double-underlined* amino acids were determined following tryptic digestion of the 15-kDa protein isolated from *R. rubrum*

E. coli strain DH5α was transformed with pMON37187 and tested for (*R*)-*trans*-2,3-enoylacyl-CoA hydratase activity relative to a negative control transformed with pSP72 alone. (*R*)-Specific hydratase activity was observed in crude extracts prepared from pMON37187 transformants compared to the plasmid control. To determine which ORF was responsible for encoding the hydratase activity, pMON37187 was digested with *Not*I and *Xho*I to remove the truncated ORF2 to produce pMON37189. *E. coli* strain DH5α transformed with pMON37189 still retained (*R*)-hydratase activity. On the basis of these observations the isolated ORF from *R. rubrum* was named $phaJ_{Rr}$, following the established nomenclature for this type of enzyme.

Overexpression, purification and native molecular weight determination of overexpressed $PhaJ_{Rr}$ protein from *E. coli*

The plasmid vector pMON37189 was used to overexpress the $PhaJ_{Rr}$ protein in *E. coli* strain DH5α. The (*R*)-*trans*-2,3-enoylacyl-CoA hydratase was purified to homogeneity based on SDS-PAGE with protein detection by Coomassie staining. A combination of ammonium sulfate cuts, a heat step, hydrophobic and anion exchange chromatography were used. Anion exchange chromatography using a MonoQ HR10/10 column resulted in an almost 50-fold increase in specific activity to over 3200 u/mg compared to the sample previously separated by hydrophobic exchange chromatography (Table 5). The final collected fractions had increased in specific activity from 34 u/mg in the crude lysate to 3236 u/mg in the most purified fraction (Table 5). An increase in specific activity was observed to be consistent with the enrichment of the 15-kDa protein, as was previously noted in the protein isolation of the hydratase from *R. rubrum*.

Native PAGE of the purified protein from *E. coli* found it to be approximately 65 kDa. Size exclusion chromatography was used to measure the native molecular weight of the protein more accurately, and it was observed to be 69.6 kDa relative to the molecular weight standards. These results would strongly suggest that the native protein is a homotetramer and, based on the predicted protein sequence, has a monomeric molecular weight of 15.4 kDa.

Enzymatic analysis of $PhaJ_{Rr}$

Overexpression of $phaJ_{Rr}$ in *E. coli* allowed the purification of the protein to homogeneity for enzymatic analysis. The enzyme was tested for stereospecificity using the coupled assay and found to give a rate less than 0.5% when the (*S*)-β-hydroxyacyl-CoA dehydrogenase was used, versus the *R*-specific acetoacetyl-CoA reductase. Thus, the hydratase is specific for formation of the (*R*)-stereoisomer.

The enzyme exhibited normal Michaelis-Menten kinetics. The K_m and V_{max} values for the hydratase with different *trans*-2,3-enoylacyl-CoA substrates are shown in Table 6. The K_m of the enzyme ranged from 9 μM to 21 μM, with the lowest K_m observed for 2-pentenoyl-CoA (Table 6). Crotonyl-CoA, which had the highest K_m value of 21 μM, was observed to have the greatest V_{max} value of 2846 μmol/mg × min, while the C5 and C6 substrates had similar V_{max} values of 798 and 986 μmol/mg × min, respectively (Table 6). These values result in V/K values between 89 and 133 for the various substrates (Table 6).

There was no detectable hydratase activity using *trans*-2,3-octenoyl-CoA as a substrate. To determine

Table 5 Purification of $PhaJ_{Rr}$ from *E. coli*

Purification step	Protein (mg)	Volume (ml)	Total activity (μmol/min)	Specific activity (μmol/min × mg)	Percent recovery
1. Crude lysate	1931	143	66591	34	100
2. 55–75% $(NH)_2SO_4$	879	34	63143	37	95
4. Phenyl superose	527	65	35779	68	54
5. MonoQ HR10/10	6.7	2.4	21731	3236	33

Table 6 In vitro kinetic analysis of $PhaJ_{Rr}$ (*ND* not detected)

Substrate	K_m (μM)	V_{max} (μmol/mg × min)	k_{cat} (1/s)	k_{cat}/K_m ($M^{-1}s^{-1}$)
Crotonyl-CoA	21.4 ± 1.7	2850 ± 90	728	3.4×10^7
trans-2,3-Pentenoyl-CoA	9.0 ± 1.2	798 ± 34	204	2.3×10^7
trans-2,3-Hexenoyl-CoA	9.1 ± 1.3	986 ± 37	252	2.8×10^7
trans-2,3-Octenoyl-CoA	ND	ND	ND	ND

whether the enzyme was able to bind this substrate, reactions were employed using crotonyl-CoA as a substrate at a final concentration of 31.6 μM and adding increasing amounts of *trans*-2,3-octenoyl-CoA, from 5.9 μM to 47.2 μM, to see if inhibition of the reaction would occur (Table 7). A 25% decrease in the relative reaction rate of the enzyme was observed when the concentration of *trans*-2,3-octenoyl-CoA was 47.2 μM (Table 7). Since the concentration of crotonyl-CoA was only slightly greater than its K_m value (31.6 μM vs. 21.4 μM), these results indicate modest inhibition, and suggest some competitive binding capacity to the enzyme.

Polymer production in recombinant *E. coli* strain DH5α

The plasmid vector pMON37186 was used to transform *E. coli* strain DH5α in the hopes of producing PHA from oleic acid. The plasmid pMON37186 contains the hydratase coupled with the two-subunit PHA synthase from *Thiocapsa pfennigii*. Growth of the culture in the presence of 0.1% oleic acid resulted in a total of 4.5% (cell dry weight) PHA production, based on direct methanolysis of the cellular material. The polymer was observed to be a terpolyester of poly(3HB-*co*-3HV-*co*-3HHx), consisting primarily of 3HB (96.3 mol% of the terpolyester), while 3HV and 3HHx were measured to be present at 2.2 and 1.5 mol%, respectively. Polymer was not observed in cultures transformed with the negative controls pMON25896 or pMON37189. The presence of 3HB, 3HV, and 3HHx was confirmed by GC-MS.

Discussion

In *R. rubrum*, a four-step reaction mechanism has been proposed for (*R*)-β-hydroxybutyrate production from acetate (Moskowitz and Merrick 1969). The pathway involves formation of acetoacetyl-CoA from two molecules of acetyl-CoA by a β-ketothiolase. Acetoacetyl-CoA is then reduced to (*S*)-β-hydroxybutyryl-CoA by an NADH-dependent acetoacetyl-CoA dehydrogenase. Crotonase, an (*S*)-specific enoyl-CoA hydratase, hydrates this compound to crotonyl-CoA, which is then converted to (*R*)-β-hydroxybutyryl-CoA by an (*R*)-specific enoyl-CoA hydratase (Moskowitz and Merrick 1969).

The (*R*)-specific hydratase activity could also account for the ability of *R. rubrum* to produce MCL-PHAs from alkanoic acids. β-Oxidation of fatty acids results in *trans*-2,3-enoylacyl-CoA intermediates which could be hydrated to (*R*)-β-hydoxyacyl-CoA for PHA production by the (*R*)-specific hydratase. It has been observed that *R. rubrum* accumulates MCL-PHAs when grown in the presence of alkanoic acids (Brandl et al. 1989). The various side chains of the polymer contained β-hydroxyhexanoate (3HHx) together with either β-hydroxybutyrate (3HB) or β-hydroxyvalerate (3HV), or both (Brandl et al. 1989). The hypothesis that PHA production is occurring via an (*R*)-specific hydratase, utilizing enoylacyl-CoA intermediates from β-oxidation, is complicated by the observation that even when *R. rubrum* is grown in the presence of decanoic acid, the polymer produced never consists of monomers greater than C6 (Brandl et al. 1989). An obvious explanation for this is that the substrate specificity of the biosynthetic enzymes limits what monomers can be incorporated into PHA. Indeed, our observation that the purified hydratase enzyme, $PhaJ_{Rr}$, is unable to utilize a C8 substrate, aids in the explanation of why monomers greater than C6 are not observed in MCL-PHAs synthesized from alkanoic acids in *R. rubrum* (Brandl et al. 1989). This supports the proposal that (*R*)-β-hydroxyacyl-CoA generated from the β-oxidation of alkanoic acids proceeds via an (*R*)-specific *trans*-2,3-enoylacyl-CoA hydratase, specifically, $PhaJ_{Rr}$.

Fukui and Doi (1997) have shown the close physical association of $phaJ_{Ac}$ with other genes involved in PHA production within the genome of *A. caviae*. The genomic fragment reported here from *R. rubrum*, containing $phaJ_{Rr}$, is too small to provide information as to whether or not this gene is associated with other PHA genes. However, both the hydratase and ORF2 which were cloned from *R. rubrum* share strong similarity to open reading frames from *R. capsulatus*. The open reading frames in *R. capsulatus* are organized similarly to what is observed in *R. rubrum*. Both ORF2 from *R. rubrum* and the similar sequence from *R. capsulatus* share sequence similarity to *ribF*, a riboflavin kinase/flavin mononu-

Table 7 Inhibition of $PhaJ_{Rr}$ by *trans*-2,3-octenoyl-CoA. The reaction rate of the enzyme was measured using crotonyl-CoA at a fixed concentration (31.6 μM) and varying concentrations of *trans*-2,3-octenoyl-CoA. The percentage relative rate of the enzyme in the presence of crotonyl-CoA alone is recorded. Values represent the average of duplicate measurements

trans-2,3-Octenoyl-CoA (mM)	% Relative rate of crotonyl-CoA
5.9	100
11.8	90
23.6	83
35.4	81
47.2	75

cleotide adenylyltransferase which is involved in the biosynthesis of riboflavin. The open reading frames from *R. capsulatus* were originally characterized as part of an effort to sequence a 189-kb continuous segment of the chromosome of *R. capsulatus* SB1003, and therefore the genes surrounding the open reading frames are known in this organism (Vlèek et al. 1997). Based on the characterization of the determined open reading frames in *R. capsulatus* SB1003 in this region, it is known that the closest PHA biosynthetic genes lie approximately 39.5 kb upstream of $phaJ_{Rc}$ and therefore, at least in *R. capsulatus* and possibly *R. rubrum*, *phaJ* is not closely associated with genes involved in PHA metabolism. If *R. capsulatus* does possess a functional (*R*)-specific *trans*-2,3-enoylacyl-CoA hydratase which utilizes intermediates of β-oxidation for PHA production, it would be interesting to study the relationship of this enzyme, as it contributes to PHA production, to the classical PHB pathway (i.e., the enzymes $PhbA_{Rr}$, $PhbB_{Rr}$, and $PhbC_{Rr}$) which has already been described in this organism and allows it to synthesize PHA from acetyl-CoA (Kranz et al. 1997).

A *trans*-2,3-enoylacyl-CoA hydratase activity had previously been partially purified and characterized from *R. rubrum* (Moskowitz and Merrick 1969). These investigators observed that the enzyme had three times greater activity for crotonyl-CoA as a substrate than *trans*-2,3-hexenoyl-CoA. This observation is in approximate agreement with our results based on V_{max}. The substrate specificity of $PhaJ_{Ac}$ from *A. caviae* demonstrated that it had similar affinities for crotonyl-CoA, 2-pentenoyl-CoA, and 2-hexenoyl-CoA (Fukui et al. 1998), which is similar to the enzyme in this report. The K_m was measured at 29, 36 and 34 μM, respectively, for the *A. caviae* hydratase (Fukui et al. 1998). There was, however, slight variation in the V_{max} of the *A. caviae* enzyme for the various substrates. The greatest substrate turnover rate for $PhaJ_{Ac}$ was observed with crotonyl-CoA, and was approximately 3.4 times slower with *trans*-2,3-hexenoyl-CoA (Fukui et al. 1998). Measurable, but very little activity was observed with *trans*-2,3-octenoyl-CoA (Fukui et al. 1998). The kinetic properties we report here for $PhaJ_{Rr}$ from *R. rubrum* are in approximate agreement with what was previously observed in enriched enzyme preparations from *R. rubrum* reported by Moskowitz and Merrick (1969). The reasons for minor discrepancies might be contaminating activities present in the impure enzyme preparations. We observed that the purified enzyme had a higher affinity for binding *trans*-2,3-hexenoyl-CoA and *trans*-2,3-pentenoyl-CoA (9.0 and 9.1 μM, respectively) than crotonyl-CoA (21.4 μM). These values indicate that, at least *in vitro*, the hydratase enzyme from *R. rubrum* has greater affinity for C5 and C6 substrates than the enzyme from *A. caviae*. Additionally, the substrate number turnover (k_{cat}) of the enzyme from *R. rubrum* is slightly larger with all the substrates (C4–C6) than the (*R*)-specific hydratase from *A. caviae*. Unlike the hydratase reported from *A. caviae*, we never observed any activity when using the C8 substrate, *trans*-2,3-octenoyl-CoA, even in the presence of 100-fold excess enzyme. However, some binding of *trans*-2,3-octenoyl-CoA is apparent since it inhibits enzyme-catalyzed turnover of crotonyl-CoA.

The molecular weight of the hydratase protein from *R. rubrum* was observed to be approximately 15 kDa on the basis of SDS-PAGE of the purified protein. This is close to the predicted molecular weight of 15,353 Da based on the amino acid sequence. The native molecular weight of the *R. rubrum* hydratase was observed to be approximately 65 kDa based on native PAGE, and 69.6 kDa based on gel filtration chromatography. This strongly indicates that the protein is a homotetramer. The native enzyme from *A. caviae* was observed to be a homodimer of approximately 31 kDa (Fukui et al. 1998).

Observations surrounding the 68-kDa protein which were observed on SDS-PAGE gels during the purification of the hydratase from *R. rubrum* suggest that this protein is a stable homotetramer of the 15 kDa protein. This is confirmed by the observation that the 15-kDa and 68-kDa proteins share identical N-terminal amino acid sequences, and that the 68-kDa protein could routinely be observed if the sample was not sufficiently disrupted by the presence of fresh and adequate amounts of β-mercaptoethanol in the SDS-PAGE gel loading buffer. Additionally, the enzyme was observed to remain quite active following a 5-min incubation at 70 °C.

In this report, we demonstrated the use of $phaJ_{Rr}$ in conjunction with the PHA synthase from *T. pfennigii* for polymer production in recombinant *E. coli* strain DH5α. Growth of the bacteria in the presence of alkanoic acids resulted in 4.5% cell dry weight of poly(3HB-*co*-3HV-*co*-3HHx) production based on direct methanolysis of the cellular material. This is consistent with a recent report by Fukui et al. (1999) using the polyhydroxyalkanoate synthase and (*R*)-enoylacyl-CoA hydratase genes from *A. caviae* to produce polyesters in *E. coli*. This observation suggests that the hydratase is to some degree able to access the *trans*-2,3-enoylacyl-CoA intermediates generated from β-oxidation. The fact that β-hydroxyoctanoate was not observed in the polymer is consistent with the substrate limitation of $PhaJ_{Rr}$, since C8 incorporation into PHA has been observed with the PHA synthase from *T. pfennigii* (Liebergesell et al. 1993). Although the polymer yields from this system are quite low when compared to other recombinant systems which made use of *E. coli* β-oxidative mutants (Langenbach et al. 1997; Qi et al. 1997), they illustrate the utility of the enzyme for the production of PHAs from alkanoic acids in recombinant organisms. The reason for low polymer production could be low concentrations of free *trans*-2,3-enoylacyl-CoAs. Indeed, since β-oxidation in *E. coli* occurs through a multienzyme complex consisting of the FadA and FadB proteins, substrate channeling could limit the pool of free *trans*-2,3-enoylacyl-CoAs. This suggestion is supported by observations from other

groups that utilization of mutants in the β-oxidative pathway leads to increased polymer levels when combined with an MCL-PHA synthase (Fukui et. al. 1999; Langenbach et al. 1997; Qi et al. 1997).

MCL-PHA production in plants has been demonstrated by peroxisomal targeting of the PhaC1 synthase from *P. aeruginosa* in *Arabidopsis thaliana* (Mittendorf et al. 1998). PHA levels of 4 mg per g of dry weight were observed in the leaves of the transgenic *Arabidopsis*. Interestingly, the polymer produced consisted of 3-hydroxyalkanoic acids ranging from six to 16 carbons (Mittendorf et al. 1998). One possible limitation to the amount of polymer being produced via this pathway in plants may simply be the supply of fatty acids to the β-oxidation pathway (Mittendorf et al. 1998). Alternatively, flux towards PHA production could be limited by the proposed endogenous plant hydratase that converts *trans*-2,3-enoylacyl-CoA intermediates from β-oxidation into their corresponding (*R*)-3-β-hydroxyacyl-CoA molecules (Mittendorf et al. 1998). Introduction of *phaJ* from either *R. rubrum* or *A. caviae* could lead to increased PHA production from β-oxidation in plant tissues (Mittendorf et al. 1998; Van Der Leij and Witholt 1995).

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