

Properties of Engineered Poly-3-Hydroxyalkanoates Produced in Recombinant *Escherichia coli* Strains

QUN REN, NICOLAS SIERRO, MICHELE KELLERHALS, BIRGIT KESSLER,
AND BERNARD WITTHOLT*

Institute of Biotechnology, Swiss Federal Institute of Technology, CH-8093 Zürich, Switzerland

Received 14 October 1999/Accepted 20 January 2000

To prepare medium-chain-length poly-3-hydroxyalkanoates (PHAs) with altered physical properties, we generated recombinant *Escherichia coli* strains that synthesized PHAs with altered monomer compositions. Experiments with different substrates (fatty acids with different chain lengths) or different *E. coli* hosts failed to produce PHAs with altered physical properties. Therefore, we engineered a new potential PHA synthetic pathway, in which ketoacyl-coenzyme A (CoA) intermediates derived from the β -oxidation cycle are accumulated and led to the PHA polymerase precursor R-3-hydroxyalkanoates in *E. coli* hosts. By introducing the poly-3-hydroxybutyrate acetoacetyl-CoA reductase (PhbB) from *Ralstonia eutropha* and blocking the ketoacyl-CoA degradation step of the β -oxidation, the ketoacyl-CoA intermediate was accumulated and reduced to the PHA precursor. Introduction of the *phbB* gene not only caused significant changes in the monomer composition but also caused changes of the physical properties of the PHA, such as increase of polymer size and loss of the melting point. The present study demonstrates that pathway engineering can be a useful approach for producing PHAs with engineered physical properties.

Poly-3-hydroxyalkanoates (PHAs), which are produced as intracellular storage material by many bacterial species, have attracted considerable attention due to their potential applications as biodegradable plastics and as sources of valuable chiral synthons (13). Based on detailed surveys, Steinbüchel and coworkers have classified PHAs into two groups (38), which are synthesized via different pathways (Fig. 1A). One group, short-chain-length PHAs (scl-PHAs), contains 3-hydroxyalkanoate monomers with chain lengths from C₄ to C₅. Another group, medium-chain-length PHAs (mcl-PHAs), are characterized by 3-hydroxyalkanoate monomers with chain lengths from C₆ to C₁₄.

It is known that PHAs are synthesized by polymerization of coenzyme A (CoA)-linked R-3-hydroxy fatty acids through one of several PHA polymerases (PhaC) (see a recent review [21]). The synthesis of such CoA substrates can occur by a variety of pathways, the simplest of which uses β -ketothiolase (PhbA) and acetoacetyl-CoA reductase (PhbB) to synthesize precursors for scl-PHA polymerases such as poly-3-hydroxybutyrate (PHB) synthase (PhbC) in *Ralstonia eutropha* (25, 26) (Fig. 1A). mcl-PHA CoA-linked precursors can be generated through fatty acid β -oxidation that produces acyl-CoA intermediates such as enoyl-CoA, 3-ketoacyl-CoA, and/or S-3-hydroxyacyl-CoA when fatty acids are used as sole carbon sources (14, 16, 39, 40) (Fig. 1A).

Effort has been devoted in recent years to increasing PHA yields and productivity. Achieving production costs that are in the same range as those of chemically synthesized plastics may be feasible, given the recent creation of PHA-producing transgenic plants (22, 27, 40, 41). Equally important in the production of PHAs is the control of the fundamental properties of the polymer. The production of PHA with specific monomer units has demonstrated that the monomer unit composition significantly affects the final properties of the polymer product

(41). To influence rationally these properties in the production of organisms, it is necessary to control and direct the production of PHA precursors. Various approaches have been used to modify PHA monomer compositions. One example is cofeeding of different substrates, such as for the production of poly-3-hydroxybutyrate-co-3-hydroxyvalerate (5), and blending polymer poly(3-hydroxy-5-phenylvalerates) and poly-3-hydroxyalkanoates (19). So far, modification of monomer compositions of mcl-PHAs has not been feasible since mcl-PHA precursors are derived from fatty acid metabolism and their concentrations are difficult to control.

Biosynthesis of these polymers in host organisms that do not naturally produce PHA allows modulation of biosynthetic enzymes and therefore allows one to achieve substrate levels that are difficult to achieve in a native host strain for the study of PHA production (18). In addition, introduction of specific genes into an organism having a suitably modified PHA synthetic pathway may allow extension and regulation of the range of compounds that can be produced. *Escherichia coli* is one of these useful hosts.

Previously, it has been reported that *E. coli* strains defective in the β -oxidation pathway are able to accumulate mcl-PHA when equipped with a PHA polymerase (29, 31). There are two PHA polymerases that are encoded by *phaC1* and *phaC2* in *Pseudomonas oleovorans* and *P. aeruginosa* (16, 39). The presence of one of these two polymerases is sufficient for PHA production in *E. coli* fatty acid degradation mutants (29, 31). Recently, it was found that inhibition of the β -oxidation thiolase step resulted in an increase in the availability of substrate for PHA synthesis (30). Therefore, engineering of *E. coli* to extend the range and distribution of precursors which can be channeled into mcl-PHA may lead to interesting PHA variants for application and development studies and is likely to extend the available chiral synthon pool as well.

In this study, we selected two *E. coli* mutants, JMU193 (*fadR fadB*) and JMU194 (*fadR fadA*), to investigate PHA accumulation. Our results revealed that ketoacyl-CoA intermediates of the β -oxidation cycle are potential precursors for PHA synthesis. By modifying relevant enzyme and substrate levels, we

* Corresponding author. Mailing address: Institute of Biotechnology, ETH Hönggerberg, CH-8093, Zürich, Switzerland. Phone: 41-1-6333286. Fax: 41-1-6331051. E-mail: bw@biotech.biol.ethz.ch.

A. General pathways for mcl-PHA and scl-PHA synthesis.

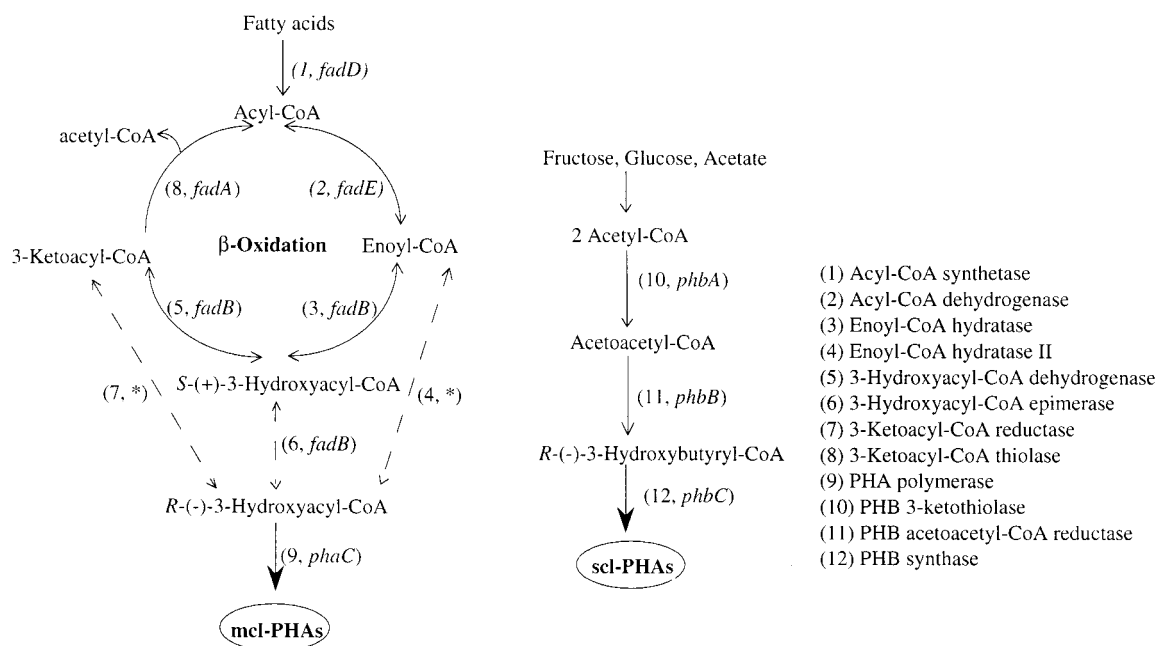


FIG. 1. General and modified pathways for the synthesis of mcl-PHA precursors. (A) Possible links between mcl-PHA synthesis and fatty acid degradation (left) and the general pathway for PHB synthesis in *R. eutropha* (right) are outlined. All enzymatic activities are numbered as indicated on the right. Genes encoding these enzymes that have not been identified are indicated with an asterisk. Dashed lines indicate pathways or reactions that are theoretically possible but might not take place due to limited concentrations of the β -oxidation intermediates. (B) Modified pathway for mcl-PHA synthesis in the *E. coli fadB* mutant. The *fadB* mutation blocks β -oxidation at the 3-hydroxyacyl-CoA dehydrogenase step (cross), which channels substrates to mcl-PHA. The introduced *phaC* gene enables mcl-PHA synthesis, if sufficient substrate is available. The thick dashed arrow indicates the PHB acetoacetyl-CoA reductase activity encoded by *phbB*, cloned into the host. Reduction of ketoacyl-CoA intermediates is theoretically possible but might not take place due to limited 3-ketoacyl-CoA concentrations or limited enzyme activity for the mcl acyl-CoA β -oxidation intermediates. (C) Construct analogous to that seen in panel B with the host *E. coli fadA* mutant. This modified pathway was designed to increase the supply of mcl-PHA precursors by introduction of *phbB* in addition to blocking β -oxidation at the 3-ketoacyl-CoA thiolase. The thick solid arrow indicates the postulated channeling of ketoacyl-CoA from β -oxidation to PHA. The cross indicates a block of 3-ketoacyl-CoA thiolase.

were able to redirect ketoacyl-CoA β -oxidation intermediates into mcl-PHA, and polymers with altered monomer composition and different physical properties were obtained.

MATERIALS AND METHODS

Strains and plasmids. The following *E. coli* strains were used in this study: JMU193 (*fadR::Tn10 fadB64*) (32) and JMU194 (*fadR::Tn10 fadA30*) (32). Plasmids pETQ2 (31) and pBTC2 carrying *phaC2* and pET901 carrying *phbB* were used to construct recombinants capable of PHA synthesis. pGec404 carrying *phaC2* (16), pBS20 carrying *phbCAB* (36), pBCKS (Promega), pUC19 (43), pVLT35 (3), and pCK01 (6) were used for the construction of pBTC2 and pET901. pETQ102, pET104, pBTC2', and pET901' were intermediate plasmids during the construction of pBTC2 and pET901.

Recombinant DNA techniques. Isolation and analysis of plasmid DNA were carried out according to the method of Sambrook et al. (33). Transformations of *E. coli* competent cells were done according to standard procedures (33). Plasmids pBTC2 and pET901 were constructed as illustrated in Fig. 2.

Media and culture conditions. *E. coli* recombinants were grown at 37°C in complex Luria-Bertani (LB) medium (33). To allow accumulation of intracellular PHA, 0.1NE2 minimal medium (15) was used, 0.2% (wt/vol) yeast extract was added to the media as the carbon source to support growth. Fatty acids were prepared as previously described (17) and supplied as indicated in the medium for PHA production and possible carbon sources. Cells were induced with 200 μ M isopropyl- β -D-thiogalactopyranoside (IPTG) at the early exponential phase. If necessary, the following antibiotics were added: kanamycin, 50 μ g/ml; ampicillin, 100 μ g/ml; streptomycin, 50 μ g/ml; and chloramphenicol, 30 μ g/ml. Cell growth was monitored by measuring optical density at 450 nm (42).

PHB acetoacetyl-CoA reductase assay. For enzymatic analysis, precultures were grown in LB medium for 8 h at 37°C and then transferred into 0.1NE2 minimal medium with 0.2% (wt/vol) yeast extract with a 1:100 dilution. Cells were induced with 200 μ M IPTG at early exponential phase and harvested at late exponential phase, washed once with 0.1 M Tris-HCl (pH 7.5), and resuspended in a 10% volume of the same buffer. Cells were then disrupted in 200- μ l aliquots by

sonication. Protein concentrations were determined by Bio-Rad protein assay. Acetoacetyl-CoA reductase activity was monitored at 340 nm by the oxidation of NADPH during reduction of acetoacetyl-CoA (35). Then, 25 μ l of crude extract and 25 μ l of 10 mM NADPH were mixed with 500 μ l of buffer (120 mM potassium phosphate [pH 5.5], 24 mM $MgCl_2$, 1 mM dithiothreitol), and 455 μ l of H_2O . The reaction was started by adding 5 μ l of 7 mM acetoacetyl-CoA. One unit is defined as 1 μ mol of NADPH depletion/min.

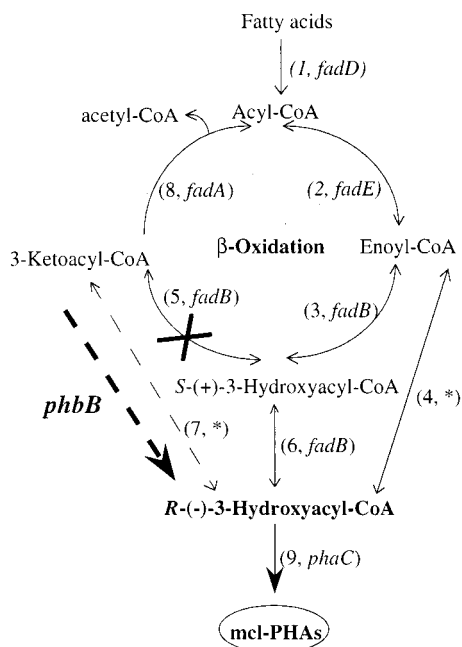
Incorporation of monomers into preexisting PHA. Cells were cultivated on 0.1NE2 minimal medium with 0.2% (wt/vol) yeast extract and 2 mM hexadecanoate as described above. A pulse of 0.5 mM pentadecanoate was added to the culture at the time indicated. PHA compositions were analyzed by gas chromatography as previously described (20), and C_7 and C_9 monomer incorporation rates in preexisting C-even number PHA were determined.

Extraction of PHA. Lyophilized cells (500 to 1,000 mg) were extracted with 150 ml of methylene chloride (CH_2Cl_2) for at least 8 h at 50°C. The cell extract was filtered, and the solvent was evaporated (Buechi Rotavapor) until <15 ml of extract remained. PHA was recrystallized by dropping the extract into 10 volumes of well-stirred, ice-cold methanol. Methanol solutions were then filtered through Zetapor filter membranes (CUNO, Inc., Meriden, Conn.). Polymer was recovered from the filters by dissolving it in chloroform and evaporating the solvent. The polymer was stored in the dark at room temperature for further analysis.

Determination of PHA. To determine the presence and composition of PHA, lyophilized cells or isolated PHA were treated with H_2SO_4 -methanol (85/15 [vol/vol]) in $CHCl_3$ (containing 0.1 mg of methyl benzoate/ml) as previously described (20). The methanolized PHA monomers were analyzed by using a CP-Sil 5 CB column (Chrompack) on a gas chromatograph (Fison) (20). Splitless injection, an attenuation of 1, and a range of 0 were used to reach maximum sensitivity.

Molecular weight determination. The molecular weight measurements were done by gel permeation chromatography. Samples were dissolved in tetrahydrofuran and run through a PL-Gel C 5- μ m column. Signals were detected using capillary viscosimetry (Viscotek 502), light scattering (small angle laser light scattering detector, KMX-6), and light refraction (differential high-temperature

B. Modified pathway for mcl-PHA synthesis in *E. coli* *fadRfadB* JMU193(*phaC*, *phbB*)



C. Modified pathway for mcl-PHA synthesis in *E. coli* *fadRfadA* JMU194(*phaC*, *phbB*)

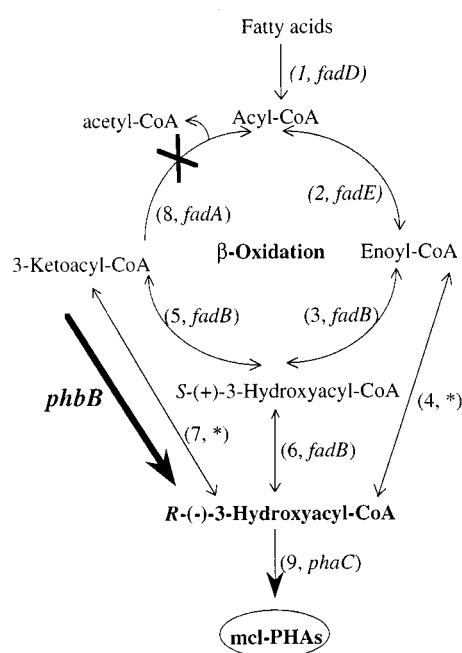


FIG. 1—Continued.

refractometer [Knauer] measurements. The column was calibrated with polystyrene standards.

DSC measurement. Differential scanning calorimetry (DSC) spectra (melting temperatures T_m , enthalpy of fusion ΔH_m , and glass transition temperatures T_g) were recorded in a temperature range of -60 to 80°C on a Netzsch DSC 200 Perkin-Elmer model DSC-4 equipped with a cooling accessory under a nitrogen flow of 30 ml/min. PHA samples of 3 mg were encapsulated in aluminum pans and heated from -60 to 80°C at a rate of $10^\circ\text{C}/\text{min}$. The T_g was taken as the midpoint of the heating capacity change.

RESULTS

In this study *E. coli fadR fadA* and *fadR fadB* mutants (JMU194 and JMU193, respectively) were used through the whole study. The *fadR* gene encodes a protein that exerts negative control over genes necessary for fatty acid oxidation (2, 4, 17, 23). A mutation in *fadR* derepresses transcription of these genes, as a result of which the *fad* genes are constitutively expressed, rendering *E. coli* capable of growth on mcl fatty acids (2, 4). The *fadA* gene encodes 3-ketoacyl-CoA thiolase (Fig. 1), and the *fadB* gene encodes four enzyme activities (Fig. 1): enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, Δ^3 -*cis*- Δ^2 -*trans*-enoyl-CoA isomerase (not included in Fig. 1), and 3-hydroxyacyl-CoA epimerase (2, 4, 17, 23). Mutations in *fadA* or *fadB* block fatty acid oxidation and result in accumulation of specific intermediates.

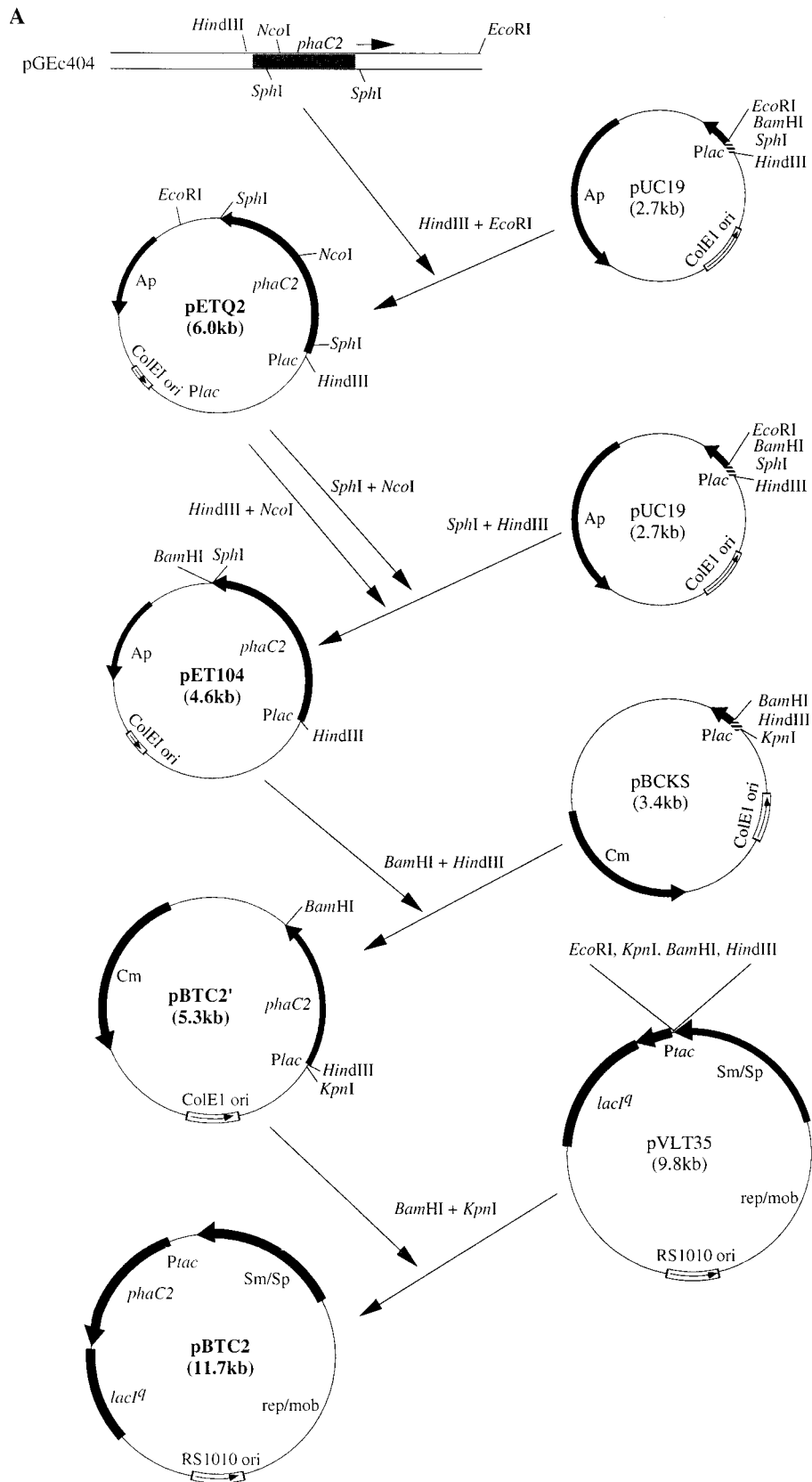
Utilization of different substrates for PHA formation in a *fadR fadB* negative recombinant *E. coli*. The *E. coli* fatty acid catabolism mutant JMU193, which lacks 3-hydroxyacyl-CoA dehydrogenase (24, 37) (Fig. 1B), was equipped with the PHA polymerase 2 from *P. oleovorans* GPo1 (16) and analyzed for PHA formation from a number of different fatty acids ranging from C_6 to C_{18} . Table 1 shows that the highest PHA contents (ca. 4% of cell dry weight [cdw]) were obtained during growth

in the presence of longer-chain-length alkanooates of C_{16} to C_{18} . However, the composition of PHAs formed from the tested alkanooates did not vary significantly with changes of alkanooate chain length. The polymer produced from odd-chain-length fatty acids consisted mainly of 3-hydroxyheptanoate (C_7) and 3-hydroxynonanoate (C_9), while the dominant even-chain-length monomers are 3-hydroxyhexanoate (C_6), 3-hydroxyoctanoate (C_8), and 3-hydroxydecanoate (C_{10}).

From these results it can be concluded that different-chain-length alkanooates supplied to a given *E. coli* recombinant lead to essentially identical C_6 to C_{10} acyl-CoA levels, resulting in a rather constant PHA monomer composition.

PHA formation by *E. coli* recombinants defective in different steps of the β -oxidation. Since the metabolic status, including the concentrations of metabolites and the rate of metabolite formation, may differ from one strain to another, it is plausible that the intracellular content and the composition of mcl-PHAs formed from such intermediates will vary from strain to strain. Thus, *E. coli* JMU194 (*fadR fadA*), a mutant which lacks 3-ketoacyl-CoA thiolase activity (24, 37) (Fig. 1C), was compared with JMU193 for PHA formation. To control the expression of *phaC2*, plasmid pBTC2 (Fig. 2A) containing *phaC2* of *P. oleovorans* GPo1 expressed through the *Ptac* promoter was introduced in JMU193 and JMU194, and the recombinants were cultivated on medium containing hexadecanoate.

Both recombinants exhibited similar growth behaviors on the medium used, with an exponential growth rate of 0.14 h^{-1} . However, *E. coli* JMU194(pBTC2) showed a sevenfold-higher monomer incorporation rate (60 nmol/mg of cdw/min) after 36 h of cultivation than did *E. coli* JMU193(pBTC2) (Table 2). There was also a significant difference in the PHA formed by the two recombinants: JMU194(pBTC2) accumulated 15% PHA, while JMU193(pBTC2) produced only 5% PHA after



B

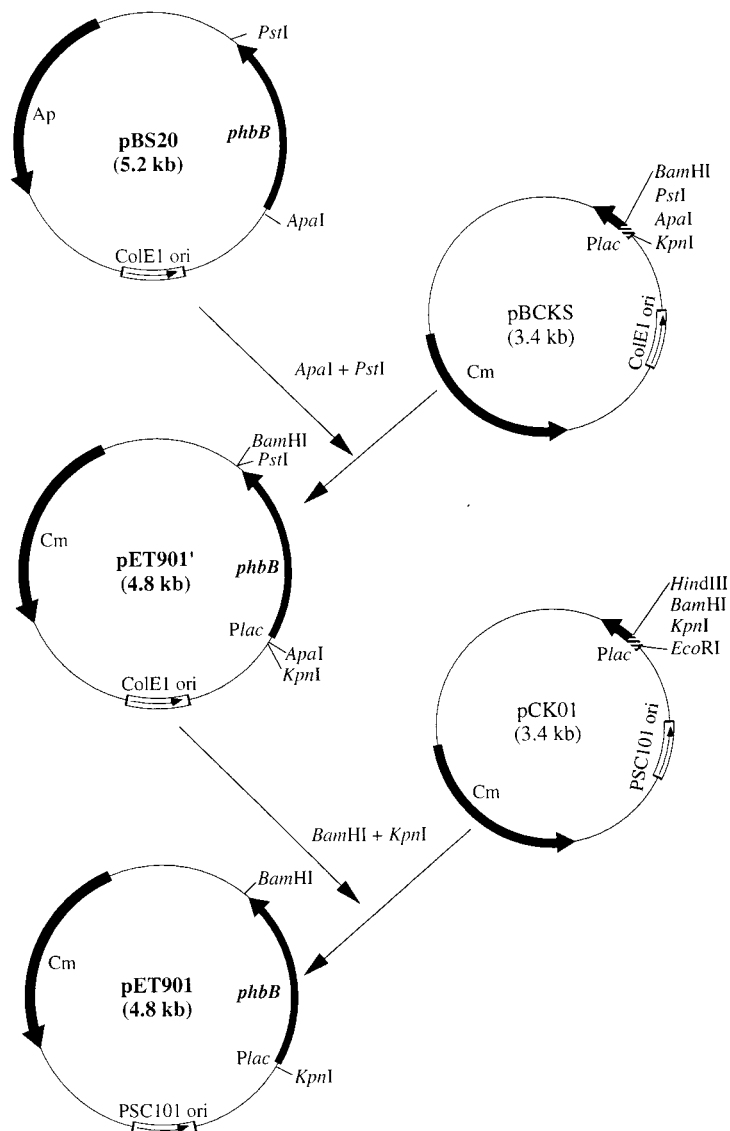


FIG. 2. Schematic representation of the strategy to construct plasmids pBTC2 and pET901. Only the relevant cloned gene(s) and restriction site(s) are shown. (A) pBTC2 contains the *phaC2* gene of *P. oleovorans* expressed through the *Ptac* promoter. (B) pET901 contains the *phbB* gene of *R. eutropha* expressed through the *Plac* promoter.

36 h of cultivation. Furthermore, the PHA formed by *E. coli* JMU194(pBTC2) contained significantly more C_6 and C_{10} monomers than did the PHA produced by JMU193(pBTC2) (Table 2).

Redirecting of ketoacyl-CoA intermediates from β -oxidation into the mcl-PHA synthetic pathway in *E. coli* JMU193 and JMU194 recombinants. As illustrated in Fig. 1C, it might be possible to redirect the carbon flux from the β -oxidation pathway to mcl-PHA synthesis by blocking step 8 to accumulate 3-ketoacyl-CoA intermediates and amplifying step 7 to permit the reduction of these intermediates to *R*-(-)-3-hydroxyacyl-CoA. It is known that the ketoacyl-CoA reductase encoded by *phbB* of *R. eutropha* (step 11 in Fig. 1A) has a substrate range of C_4 to C_6 acyl-CoAs (12), permitting it to convert ketoheyl-

CoA to *R*-(-)-3-hydroxyhexyl-CoA, which might be channeled into mcl-PHA by PHA polymerase.

Therefore, plasmid pET901, which contains *phbB* of *R. eutropha*, was constructed (Fig. 2B) and was subsequently introduced into *E. coli* recombinants JMU193(pBTC2) and JMU194(pBTC2). Ketoacyl-CoA reductase activities of 0.71 and 0.64 U/mg of total protein were found for JMU193 and JMU194 recombinants, respectively, which could result in an alteration in the intermediate concentrations of the β -oxidation cycle in these recombinants.

The effect of addition of pET901 on the characteristics of the PHA formed by JMU193 recombinants was slight. When hexadecanoate was supplied, with IPTG-induced *phaC2* and *phbB* expression, JMU193(pBTC2, pET901) produced 6%

TABLE 1. Formation of PHAs by *E. coli* JMU193(pETQ2) grown in the presence of *n*-alkanoates

Alkanoates ^a	PHA ^b (% of cdw)	Monomer composition (mol%) ^c				
		C ₆	C ₇	C ₈	C ₉	C ₁₀
Octanoate	0.3 ± 0.1	0 ^d	0	100	0	0
Nonanoate	0.2 ± 0.1	0	73	0	27	0
Decanoate	0.6 ± 0.1	0	0	83	0	17
Undecanoate	2.8 ± 0.5	0	72	0	28	0
Dodecanoate	2.8 ± 0.5	7	0	89	0	4
Tridecanoate	3.0 ± 0.8	0	71	0	29	0
Tetradecanoate	2.3 ± 0.9	7	0	86	0	7
Pentadecanoate	2.3 ± 0.7	0	77	0	23	0
Hexadecanoate	4.1 ± 1.8	6	0	88	0	6
Heptadecanoate	4.3 ± 1.2	0	75	0	25	0
Octadecanoate	3.5 ± 0.7	7	0	88	0	5

^a The *E. coli* recombinant was grown on 0.1NE2 minimal medium with 0.2% yeast extract and different alkanoates as indicated in this table.

^b The amount of PHA is given as a percentage (average ± the standard deviation) of the cdw.

^c The monomer composition is given as a molar percentage. C₆, 3-hydroxyhexanoate; C₇, 3-hydroxyheptanoate; C₈, 3-hydroxyoctanoate; C₉, 3-hydroxynonanoate; C₁₀, 3-hydroxydecanoate.

^d 0, <0.1%.

PHA, similar to the 5% PHA seen for JMU193(pBTC2, pCK01), and the expression of *phbB* in JMU193(pBTC2, pET901) hardly changed the PHA composition compared to JMU193(pBTC2, pCK01) (Table 2).

The results for JMU194(pBTC2, pET901), which synthesized PHA to 20%, were more interesting. Not only did this recombinant produce more PHA than the closely related strain JMU194(pBTC2, pCK01) (15% PHA), but it also produced a hexanoate-rich PHA. The higher PHA content can be attributed to different precursor concentrations since the monomer incorporation rates of the polymerase in both JMU194 recombinants were similar (Table 2). The increased hexanoate content, with a C₆:C₈:C₁₀ ratio that changed from 20:57:23 to 42:38:20 in JMU194(pBTC2, pET901) clearly depended on the expression of *phbB* (Table 2). To verify that this change was not caused by the accumulation of free C₆ monomers in the PHA samples, we also tested *E. coli* recombinants carrying only *PhbB* (pET901) and no PHA polymerase (with just

pVLT35). Table 2 shows that no PHA and no C₆ monomers were detected in either the JMU194 or JMU193 recombinants.

Heptadecanoate was also tested to determine whether the presence of *PhbB* led to PHAs with different monomer compositions. This was not the case: no significant difference was observed in the content and composition of PHAs produced by JMU194(pBTC2, pET901) and JMU194(pBTC2, pCK01). As expected, when the acyl source was heptadecanoate, JMU193(pBTC2, pCK01) and JMU193(pBTC2, pET901) again synthesized PHA with a similar monomer composition.

Physical properties of polymers produced by *E. coli* recombinants. PHAs produced by the *E. coli* recombinants used in this study were isolated by complete solvent extraction from lyophilized cells. The monomer compositions of the purified PHAs were essentially identical to the values estimated by gas chromatography analysis after methanolysis of whole cells.

PHAs synthesized by JMU193(pBTC2, pCK01) and JMU193(pBTC2, pET901) had similar compositions, and their molecular weights and polymerization degree (PD) were also similar (Table 2). The PHA isolated from JMU194(pBTC2, pCK01) had a molecular weight of about 70,000, whereas JMU194(pBTC2, pET901) formed a polymer with two molecular weight peaks at about 1,100,000 and about 97,000 (Fig. 3, Table 2). Furthermore, the PD for PHA with molecular weight at 97,000 was almost 2.5-fold higher than that for PHA synthesized by JMU194(pBTC2, pCK01) (Table 2).

The PHAs produced from JMU193(pBTC2, pCK01) and JMU193(pBTC2, pET901) showed a similar melting point (T_m), endotherm (ΔH_m), and glass transition temperature (T_g) (Table 2), whereas PHAs from two JMU194 recombinants exhibited a different DSC pattern: the PHAs isolated from JMU194(pBTC2, pCK01) showed T_m and ΔH_m , while the PHAs isolated from JMU194(pBTC2, pET901), which contained an active *PHB* acetoacyl-CoA reductase, did not show a melting endotherm (Fig. 4 and Table 2). Furthermore, this PHA exhibited a T_g value that was about 2.6°C higher than that for PHA from JMU194(pBTC2, pCK01) (Table 2).

DISCUSSION

The monomer composition of PHAs can affect the properties of the polymer (41). The development of *mcl*-PHA variants depends, therefore, on the range of monomers that can be

TABLE 2. PHAs produced by *E. coli* recombinants grown in the presence of hexadecanoate.

Strain ^a	Genotype	Rate of monomer incorporation into PHA ^b (nmol/mg of cdw/min)	PHA% ^c (wt/wt)	Monomer composition ^d (mol%)			M_w ^e (g/mol)	M_w/M_n ^e	PD ^f	T_g (°C)	T_m (°C)	ΔH_m (J/g)
				C ₆	C ₈	C ₁₀						
JMU193(pVLT35, pET901)	<i>phbB</i> ⁺	≤0.1	≤0.1									
JMU193(pBTC2, pCK01)	<i>phaC2</i> ⁺	9 ± 3	5 ± 0.5	11	76	13	59,800	2.14	198	-33.8	55.1	9.31
JMU193(pBTC2, pET901)	<i>phaC2</i> ⁺ <i>phbB</i> ⁺	12 ± 4	6 ± 0.4	10	76	14	61,200	2.10	205	-33.3	55.8	9.37
JMU194(pVLT35, pET901)	<i>phbB</i> ⁺	≤0.1	≤0.1									
JMU194(pBTC2, pCK01)	<i>phaC2</i> ⁺	60 ± 10	15 ± 4	20	57	23	71,300	3.03	165	-32.7	53.9	9.22
JMU194(pBTC2, pET901)	<i>phaC2</i> ⁺ <i>phbB</i> ⁺	50 ± 8	20 ± 5	42	38	20	97,000 and 1,100,000 ^g	1.80 ^g	405	-30.1	~ ^h	~ ^h

^a The recombinants were grown in 0.1NE2 minimal medium with 0.2% yeast extract and 2 mM hexadecanoate. Cells were induced with 200 μM IPTG at early exponential phase. Isolation of PHA and analysis of the PHA molecular weight and thermal properties were carried out as described in Materials and Methods. Average data of three independent experiments are given.

^b The monomer incorporation rate was assayed at 36 h by a pulse of 0.5 mM pentadecanoate for 1.5 h as described in Materials and Methods.

^c The amount of PHA is given as a weight percentage (average ± the standard deviation) of the cdw.

^d The monomer composition is given as a molar percentage. C₆, 3-hydroxyhexanoate; C₈, 3-hydroxyoctanoate; C₁₀, 3-hydroxydecanoate.

^e M_w , weight average molecular weight; M_n , number average molecular weight; M_w/M_n , polydispersity.

^f The PD was calculated based on M_n and the monomer composition.

^g This sample shows two peaks as indicated in Fig. 3. M_w/M_n refers to the low molecular weight peak.

^h ~, No melting point.

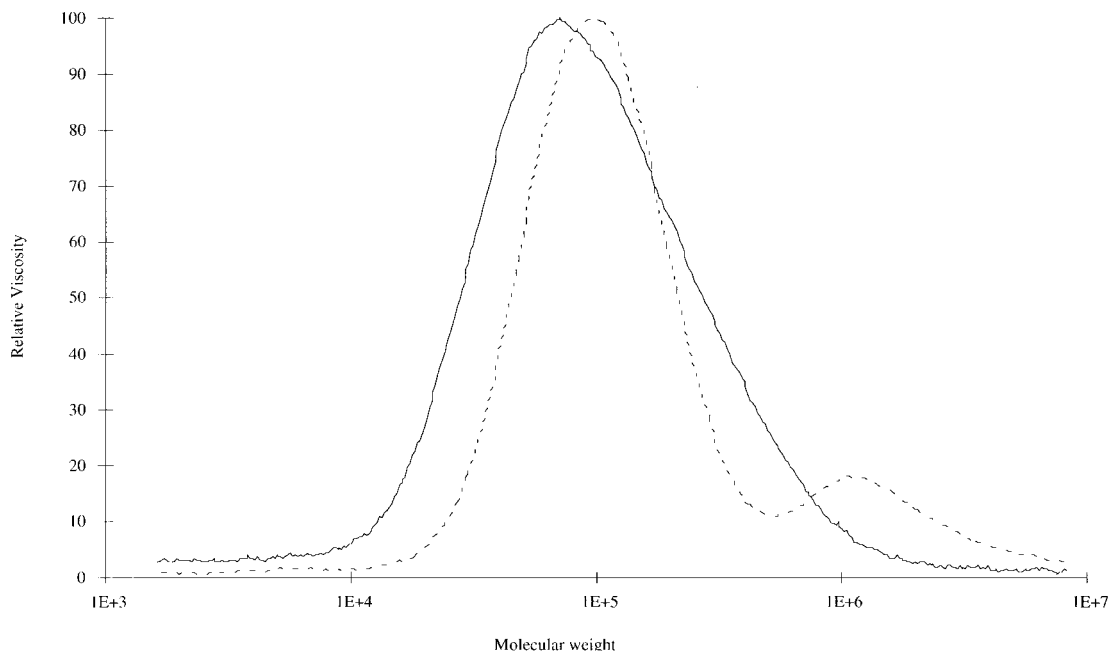


FIG. 3. Molecular weight of polymer isolated from JMU194 recombinants. *E. coli* JMU194 recombinants were grown in 0.1NE2 minimal medium with 0.2% yeast extract and 2 mM hexadecanoate. Cells were induced with 200 μ M IPTG in the early exponential phase and harvested in the stationary phase. PHA was isolated from cells and analyzed by gel permeation chromatography for the molecular weight as described in Materials and Methods. Solid line, PHA from JMU194(pBTC2, pCK01); dashed line, PHA from JMU194(pBTC2, pET901).

incorporated into PHA. Consequently, it is of interest to attempt to control rationally the monomer composition of new mcl-PHAs. This requires a better understanding of pathways involved in mcl-PHA synthesis and of the basic metabolic pathways that may provide PHA precursors. In this study, we set out to address two questions. One was whether ketoacyl-CoA of the β -oxidation can serve as precursor source for PHA synthesis. The second question was how the mcl-PHA monomer composition could be modified. Among the approaches we tested, pathway engineering by amplification of one potential PHA precursor supply route gave the most promising result. Other approaches, such as varying the substrates, did not lead to significant changes in PHA composition or physical properties (Table 2).

The β -oxidation intermediate ketoacyl-CoA is a potential precursor for PHA synthesis in *E. coli*. Introduction of the PHA polymerase into *E. coli* results in utilization of 3-hydroxyacyl-CoA precursors for the synthesis of PHA (29, 31, 34, 36). As illustrated in Fig. 1, when the β -oxidation is blocked at the last step (ketothiolase [FadA]), all upstream intermediates will accumulate (Fig. 1C). 3-Ketoacyl-CoA could be reduced to *R*-(-)-3-hydroxyacyl-CoA through a 3-ketoacyl-CoA reductase (step 7), *S*-(+)-3-hydroxyacyl-CoA may be epimerized (step 6, *fadB*), and enoyl-CoA may be hydrated to the corresponding *R*-(-)-3-hydroxyacyl-CoA (step 3, *fadB*). When the β -oxidation is blocked at step 5, catalyzed by 3-hydroxyacyl-CoA dehydrogenase (FadB), one of these possible mcl-PHA precursors, 3-ketoacyl-CoA, will not accumulate (Fig. 1B). In this study, we found that *E. coli* JMU194 (*fadR fadA*) *phaC*⁺ recombinant incorporated more C₆ and C₁₀ monomers into PHAs than the JMU193 (*fadR fadB*) *phaC*⁺ recombinant under the same growth conditions (Tables 1 and 2). The relative monomer content of PHA depends on the precursor concentration and the specificity of the PHA polymerase for each precursor. Since the specificity profile of the PHA polymerase

is presumably identical in both recombinants, differing PHA compositions must reflect the differences in relative intracellular precursor concentrations.

When the JMU193 (*fadR fadB*) *phaC*⁺ recombinant is provided with *phbB* (amplifying step 7 in Fig. 1B by using a PHB acetoacetyl-CoA reductase, step 11 in Fig. 1A), the polymer composition did not change very much compared with the strain carrying only *phaC* (Table 2). This is probably due to the fact that 3-ketoacyl-CoA cannot be accumulated in this strain due to the *fadB* mutation, as described above. This also explains why there was no increase in the PHA content in the JMU193 (*fadR fadB*) *phaC*⁺ recombinant after introduction of *phbB* (Table 2). Since *fadB*-negative *E. coli* strains carrying *phaC* are able to accumulate PHA even without this ketoacyl-CoA precursor route, there must be other precursor sources, such as enoyl-CoAs of β -oxidation as described previously (7–9), which are available for PHA synthesis in *E. coli*.

In contrast, introduction of *phbB* into the JMU194 (*fadR fadA*) *phaC*⁺ recombinant (Fig. 1C) resulted in increased PHA accumulation and a PHA which contained twofold-higher C₆ monomer than the PHA from the *fadR fadA* recombinant carrying only *phaC* (Table 2). This is compatible with the notion that in the JMU194 (*fadR fadA*) *phaC*⁺ recombinant there was accumulation of C₆ ketoacyl-CoA, which was then reduced by the PHB acetoacyl-CoA reductase and incorporated into mcl-PHA. These data strongly indicate that the 3-ketoacyl-CoA intermediate of the β -oxidation is a potential precursor source for PHA synthesis.

When grown on heptadecanoate, no C₅ monomers were found in the PHAs of *E. coli fadR fadA* strains carrying *phaC* and *phbB* (Table 2), although 3-hydroxypentanoyl-CoA (C₅) could in principle be formed via β -oxidation from the intermediate 3-ketopentanoyl-CoA by the PHB acetoacyl-CoA reductase. This is probably due to the low affinity of the PHA

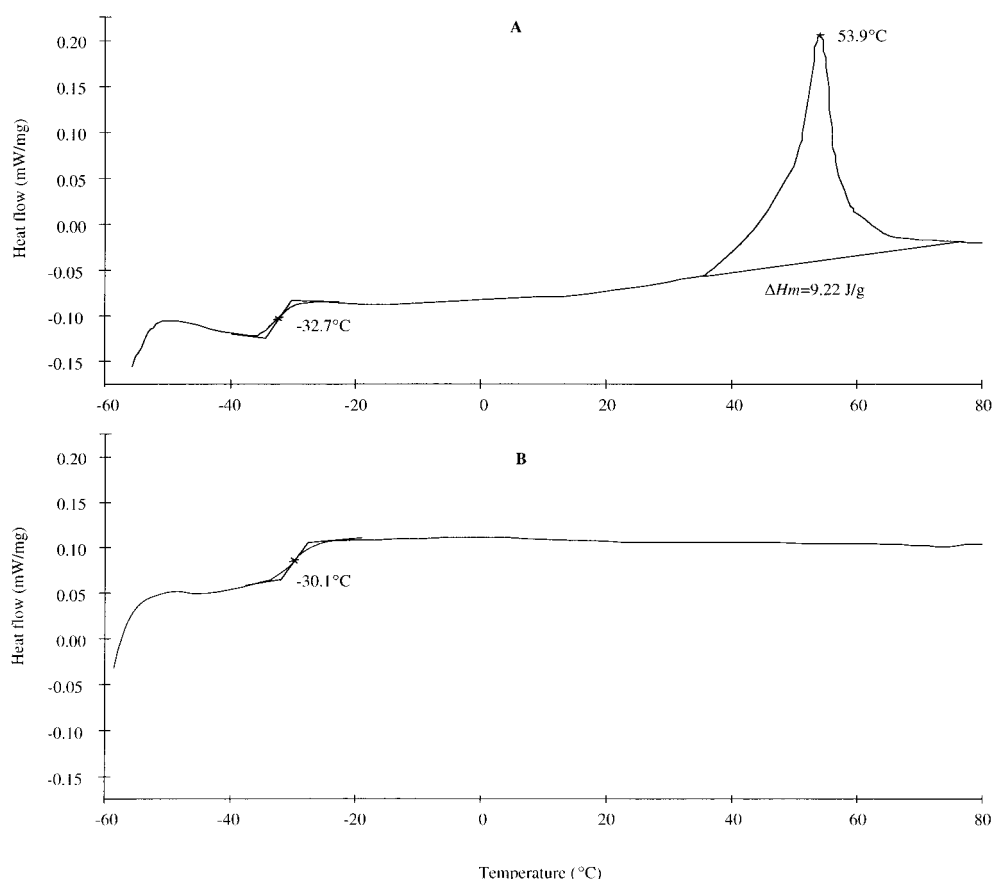


FIG. 4. DSC thermograms of the PHA samples from JMU194 recombinants. The PHA samples described in Fig. 3 were analyzed for thermal properties as described in Materials and Methods. (A) PHA from JMU194(pBTC2, pCK01). (B) PHA from JMU194(pBTC2, pET901).

polymerase for C_5 monomers (15), resulting in limited incorporation into PHA.

Physical properties of PHA can be changed by pathway engineering. We found significant differences in the physical properties of PHA isolated from *fadR fadA E. coli* JMU194 carrying *phaC* and from the same recombinant carrying additional *phbB* (Table 2).

E. coli JMU194 carrying *phaC* accumulated PHA with a molecular weight of about 70,000. After introduction and expression of the *phbB* gene, PHA with two molecular weight ranges was found (Fig. 3). This is probably not caused by formation of different groups of polymers (e.g., one group is mcl-PHA, another is poly-3-hydroxyhexanoate), since only one glass transition temperature was observed (Fig. 4) and, furthermore, only one functional polymerase was introduced into the recombinant. The most likely reason for the formation of the high-molecular-weight peak is changes in the polymer chain length. PHB chains typically contain 7,000 to 23,000 monomers (see review [1]), which is 5- to 10-fold more than that found in mcl-PHAs (24). Perhaps the increased C_6 content of JMU194 carrying *phaC* and *phbB* somehow alters the ratio of chain elongation to chain termination events, resulting in longer PHA chains, comparable to the PHB formed in *R. eutropha* and other PHB-producing organisms. Another possibility, however, is that the 1,100,000 peak of Fig. 3 is due not to longer polymer chains but to polymer aggregates due to the presence of stretches of C_6 monomers in the PHA chains. These stretches could perhaps facilitate strong noncovalent interactions among PHA chains and thus result in the forma-

tion of microgels as shown in Fig. 5. These microgels may not be able to dissolve in tetrahydrofuran completely, resulting in the high-molecular-weight peak. Further experiments have to be carried out to distinguish between these two possibilities.

The PHA isolated from JMU194 carrying *phaC* and *phbB* showed an approximately 2.5-fold-higher PD than that from JMU194 carrying only *phaC* (Table 2). This could be due to differences in the binding affinities of the pendant group of different PHA monomers to the PHA polymerase. Previously, it has been shown that the polymerase is located at the PHA granule interface between the hydrophilic cytoplasm and the relatively hydrophobic granules (10). Therefore, when the pendant group of a predominant monomer is shorter, the binding of the polymerase to the chain end may be stronger, leading to fewer chain transfers relative to chain propagation events. Thus, PHAs from JMU194(pBTC2, pET901) containing mostly propyl pendant groups showed higher PD than PHAs from JMU194(pBTC2, pCK01) containing mostly valeryl pendant groups. Similar results have been reported by Fuller and coworkers, who showed that the PD for PHA synthesized by *P. oleovorans* when grown on hexanoate and heptanoate was approximately twice as large as the PD for PHA which was produced when using octanoate and other longer-chain-length alkanates (11).

No melting point was observed for PHA isolated from JMU194(pBTC2, pET901), whereas PHA from JMU194(pBTC2, pCK01) showed a T_m value of about 54°C (Fig. 4). Loss of the melting point could be due to the fact that this polymer contained predominantly *n*-propyl side chains, which

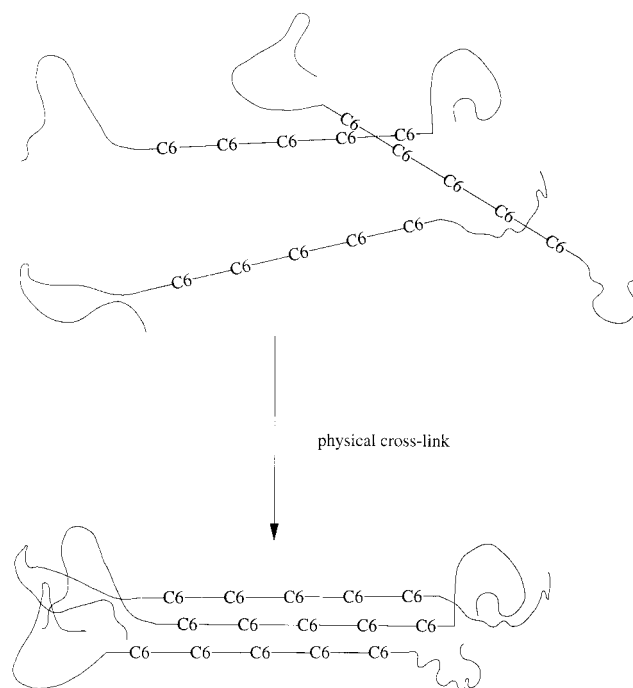


FIG. 5. Model for the structure of PHA isolated from JMU194(pBTC2, pET901). Straight lines indicate 3-hydroxyhexanoate (C_6) monomer-rich stretches; curved lines indicate stretches containing random C_6 , 3-hydroxyoctanoate (C_8), and 3-hydroxydecanoate (C_{10}) monomers.

may explain the inability of these samples to crystallize via close proximity of their main chains (possibly due to small quantities of longer side chain repeating units present in these samples), while having side chains which are too short to allow a layered packing order. Similar results were reported previously, showing that PHA produced by *P. oleovorans* from hexanoate and heptanoate did not have a clear melting point, whereas PHA produced from longer-chain alkanates did (1, 11, 28).

In this study, we showed that there is more than one precursor source available in *E. coli* for mcl-PHA synthesis. One is, as described previously, the well-established acyl-CoA route, involving β -oxidation of fatty acids from acyl-CoA to 3-hydroxyacyl-CoA via enoyl-CoA (7–9), following the β -oxidation cycle clockwise. The second, illustrated in the present study, involves an extension of the same cycle followed by formation of 3-hydroxyacyl-CoA via 3-ketoacyl-CoA. By engineering the second pathway in *E. coli*, PHAs with altered monomer compositions were produced. Physical characterization of the isolated polymers shows that the monomer composition plays an important role in controlling the physical properties of PHAs. This suggests that it will be possible to obtain PHAs with altered properties by modifying and regulating fatty acid metabolic genes and PHA synthetic genes in *E. coli*. The experience gained with precursor enrichments in *E. coli* will undoubtedly support the development of suitable mcl-PHA-producing transgenic plants.

ACKNOWLEDGMENTS

We thank M. Colussi for measuring molecular weight, D. Dennis for providing strains JMU193 and JMU194, and M. Held and K. Jung for helpful discussions.

This work was supported by grants from the Swiss Federal Office for Education and Science (BBW no. 96.0348) to Q.R.

REFERENCES

- Ballistreri, A., G. Montaudo, G. Impallomeni, R. W. Lenz, Y. B. Kim, and R. C. Fuller. 1990. Sequence distribution of beta-hydroxyalkanoate units with higher alkyl groups in bacterial copolyesters. *Macromolecules* **23**:5059.
- Black, P. N., and C. C. DiRusso. 1994. Molecular and biochemical analyses of fatty acid transport, metabolism, and gene regulation in *Escherichia coli*. *Biochim. Biophys. Acta* **1210**:123–145.
- de Lorenzo, V., L. Eltis, B. Kessler, and K. N. Timmis. 1993. Analysis of *Pseudomonas* gene products using *lacI^q/P_{trp}-lac* plasmids and transposons that confer conditional phenotypes. *Gene* **123**:17–24.
- DiRusso, C. C., and W. D. Nunn. 1985. Cloning and characterization of a gene (*fadR*) involved in regulation of fatty acid metabolism in *Escherichia coli*. *J. Bacteriol.* **161**:583–588.
- Doi, Y., A. Tamaki, M. Kunioka, and K. Soga. 1987. Biosynthesis of terpolyesters of 3-hydroxybutyrate, 3-hydroxyvalerate, and 5-hydroxyvalerate in *Alcaligenes eutrophus* from 5-chloropentanoic and pentanoic acids. *Macromol. Chem. Rapid Commun.* **8**:631–635.
- Fernández, S., V. de Lorenzo, and J. Pérez-Martin. 1995. Activation of the transcriptional regulator XylR of *Pseudomonas putida* by release of repression between functional domains. *Mol. Microbiol.* **16**:205–213.
- 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.
- Fukui, T., N. Shiomi, and Y. Doi. 1998. Expression and characterization of (*R*)-specific enoyl coenzyme A hydratase involved in polyhydroxyalkanoate biosynthesis by *Aeromonas caviae*. *J. Bacteriol.* **180**:667–673.
- Fukui, T., S. Yokomizo, G. Kobayashi, and Y. Doi. 1999. Co-expression of polyhydroxyalkanoate synthase and (*R*)-enoyl-CoA hydratase genes of *Aeromonas caviae* establishes copolyester biosynthesis pathway in *Escherichia coli*. *FEMS Microbiol. Lett.* **170**:69–75.
- Gerngross, T. U., P. Reilly, J. Stubbe, A. J. Sinskey, and O. P. Peoples. 1993. Immunocytochemical analysis of poly-beta-hydroxybutyrate (PHB) synthase in *Alcaligenes eutrophus* H16: localization of the synthase enzyme at the surface of the PHB granules. *J. Bacteriol.* **175**:5289–5293.
- Gross, R. A., C. DeMello, R. W. Lenz, H. Brandl, and R. C. Fuller. 1989. Biosynthesis and characterization of poly(beta-hydroxyalkanoates) produced by *Pseudomonas oleovorans*. *Macromolecules* **22**:1106–1115.
- Haywood, G. W., A. J. Anderson, L. Chu, and E. A. Dawes. 1988. The role of NADH- and NADHP-linked acetoacetyl-CoA reductases in the poly-3-hydroxybutyrate synthesizing organism *Alcaligenes eutrophus*. *FEMS Microbiol. Lett.* **52**:259–264.
- Hrabak, O. 1992. Industrial production of poly- β -hydroxybutyrate. *FEMS Microbiol. Rev.* **103**:251–256.
- Huijberts, G. N. M., T. C. de Rijk, P. de Waard, and G. Eggink. 1995. ¹³C nuclear magnetic resonance studies of *Pseudomonas putida* fatty acid metabolic routes involved in poly(3-hydroxyalkanoate) synthesis. *J. Bacteriol.* **176**:1661–1666.
- Huisman, G. W., O. de Leeuw, G. Eggink, and B. Witholt. 1989. Synthesis of polyhydroxyalkanoates is a common feature of fluorescent pseudomonads. *Appl. Environ. Microbiol.* **55**:1949–1954.
- Huisman, G. W., E. Wonink, R. Meima, B. Kazemier, P. Terpstra, and B. Witholt. 1991. Metabolism of poly(3-hydroxyalkanoates) (PHAs) by *Pseudomonas oleovorans*. *J. Biol. Chem.* **266**:2191–2198.
- Jenkins, L. J., and W. D. Nunn. 1987. Genetic and molecular characterization of the genes involved in short-chain fatty acid degradation in *Escherichia coli*: the *ato* system. *J. Bacteriol.* **169**:42–52.
- Kidwell, J., H. E. Valentin, and D. Dennis. 1995. Regulated expression of the *Alcaligenes eutrophus* pha biosynthesis genes in *Escherichia coli*. *Appl. Environ. Microbiol.* **61**:1391–1398.
- Kim, Y. B., R. W. Lenz, and R. C. Fuller. 1991. Preparation and characterization of poly(beta-hydroxyalkanoates) obtained from *Pseudomonas oleovorans* grown with mixtures of 5-phenylvaleric acid and *n*-alkanoic acids. *Macromolecules* **24**:5256–5260.
- Lageveen, R. G., G. W. Huisman, H. Preusting, P. Ketelaar, G. Eggink, and B. Witholt. 1988. Formation of polyesters by *Pseudomonas oleovorans*: effect of substrates on formation and composition of poly-(*R*)-3-hydroxyalkanoates and poly-(*R*)-3-hydroxyalkanoates. *Appl. Environ. Microbiol.* **54**:2924–2932.
- Madison, L. L., and G. W. Huisman. 1999. Metabolic engineering of poly(3-hydroxyalkanoates): from DNA to plastic. *Microbiol. Mol. Biol. Rev.* **63**:21–53.
- Nawrath, C., Y. Poirier, and C. Somerville. 1994. Targeting of the polyhydroxybutyrate biosynthetic pathway to the plastids of *Arabidopsis thaliana* results in high levels of polymer accumulation. *Proc. Natl. Acad. Sci. USA* **91**:12760–12764.
- Nunn, W. D. 1986. Two-carbon compounds and fatty acids as carbon sources, p. 285–301. In F. C. Neidhardt et al. (ed.), *Escherichia coli* and *Salmonella typhimurium*. American Society for Microbiology, Washington, D.C.
- Overath, P., G. Pauli, and H. U. Schaerer. 1969. Fatty acid degradation in *Escherichia coli*: an inducible acyl-CoA synthetase, the mapping of *old*-mutations, and isolation of regulatory mutants. *Eur. J. Biochem.* **7**:559–574.
- Peoples, O. P., and A. J. Sinskey. 1989. Poly-beta-hydroxybutyrate biosynthesis in *Alcaligenes eutrophus* H16. Characterization of the genes encoding

- beta-ketothiolase and acetoacetyl-CoA reductase. *J. Biol. Chem.* **264**:15293–15297.
26. **Peoples, O. P., and A. J. Sinskey.** 1989. Poly-beta-hydroxybutyrate biosynthesis in *Alcaligenes eutrophus* H16. Identification and characterization of the PHB polymerase gene (*phbC*). *J. Biol. Chem.* **264**:15298–15303.
 27. **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.
 28. **Preusting, H., A. Nijenhuis, and B. Witholt.** 1990. Physical characteristics of poly(3-hydroxyalkanoates) and poly(3-hydroxyalkenoates) produced by *Pseudomonas oleovorans* grown on aliphatic hydrocarbons. *Macromolecules* **23**:4220–4224.
 29. **Qi, Q., B. H. A. Rehm, and A. Steinbüchel.** 1997. Synthesis of poly(3-hydroxyalkanoates) in *Escherichia coli* expressing the PHA synthase gene *phaC2* from *Pseudomonas aeruginosa*: comparison of PhaC1 and PhaC2. *FEMS Microbiol. Lett.* **157**:156–162.
 30. **Qi, Q. S., A. Steinbüchel, and B. H. A. Rehm.** 1998. Metabolic routing towards polyhydroxyalkanoic acid synthesis in recombinant *Escherichia coli* (*fadR*): inhibition of fatty acid beta-oxidation by acrylic acid. *FEMS Microbiol. Lett.* **167**:89–94.
 31. **Ren, Q.** 1997. Biosynthesis of medium chain length poly-3-hydroxyalkanoates: from *Pseudomonas* to *Escherichia coli*. Ph.D. thesis. Swiss Federal Institute of Technology, Zürich, Switzerland.
 32. **Rhie, H. G., and D. Dennis.** 1995. Role of *fadR* and *atoC*(Con) mutations in poly(3-hydroxybutyrate-co-3-hydroxyvalerate) synthesis in recombinant *pha⁺ Escherichia coli*. *Appl. Environ. Microbiol.* **61**:2487–2492.
 33. **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.
 34. **Schubert, P., A. Steinbüchel, and H. G. Schlegel.** 1988. Cloning of the *Alcaligenes eutrophus* genes for synthesis of poly-β-hydroxybutyrate (PHB) and synthesis of PHB in *E. coli*. *J. Bacteriol.* **170**:5837–5847.
 35. **Senior, P. J., and E. A. Dawes.** 1973. The regulation of poly-3-hydroxybutyrate metabolism in *Azotobacter beijerinckii*. *Biochem. J.* **134**:225–238.
 36. **Slater, S. C., W. H. Voige, and D. E. Dennis.** 1988. Cloning and expression in *E. coli* of the *Alcaligenes eutrophus* H16 poly-β-hydroxybutyrate biosynthetic pathway. *J. Bacteriol.* **170**:4431–4436.
 37. **Spratt, P.** 1984. Cloning, mapping, and expression of genes involved in the fatty acid-degradative multienzyme complex of *Escherichia coli*. *J. Bacteriol.* **158**:535–542.
 38. **Steinbüchel, A., and H. E. Valentin.** 1995. Diversity of bacterial polyhydroxyalkanoic acids. *FEMS Microbiol. Lett.* **128**:219–228.
 39. **Timm, A., and A. Steinbüchel.** 1992. Cloning and molecular analysis of the poly(3-hydroxyalkanoic acid) gene locus of *Pseudomonas aeruginosa* PAO1. *Eur. J. Biochem.* **209**:15–30.
 40. **van der Leij, F. R., and B. Witholt.** 1995. Strategies for the sustainable production of new biodegradable polyesters in plants: a review. *Can. J. Microbiol.* **41**:222–238.
 41. **Williams, S. F., and O. P. Peoples.** 1996. Biodegradable plastics from plants. *Chemtech* **26**:38–44.
 42. **Witholt, B.** 1972. Method for isolating mutants overproducing nicotinamide adenine dinucleotide and its precursors. *J. Bacteriol.* **109**:350–364.
 43. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Genes* **33**:103–119.