

# Expression of 3-Ketoacyl-Acyl Carrier Protein Reductase (*fabG*) Genes Enhances Production of Polyhydroxyalkanoate Copolymer from Glucose in Recombinant *Escherichia coli* JM109

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Received 21 December 2004/Accepted 21 February 2005

**Polyhydroxyalkanoates (PHAs) are biologically produced polyesters that have potential application as biodegradable plastics. Especially important are the short-chain-length-medium-chain-length (SCL-MCL) PHA copolymers, which have properties ranging from thermoplastic to elastomeric, depending on the ratio of SCL to MCL monomers incorporated into the copolymer. Because of the potential wide range of applications for SCL-MCL PHA copolymers, it is important to develop and characterize metabolic pathways for SCL-MCL PHA production. In previous studies, coexpression of PHA synthase genes and the 3-ketoacyl-acyl carrier protein reductase gene (*fabG*) in recombinant *Escherichia coli* has been shown to enhance PHA production from related carbon sources such as fatty acids. In this study, a new *fabG* gene from *Pseudomonas* sp. 61-3 was cloned and its gene product characterized. Results indicate that the *Pseudomonas* sp. 61-3 and *E. coli* FabG proteins have different substrate specificities in vitro. The current study also presents the first evidence that coexpression of *fabG* genes from either *E. coli* or *Pseudomonas* sp. 61-3 with *fabH*(F87T) and PHA synthase genes can enhance the production of SCL-MCL PHA copolymers from nonrelated carbon sources. Differences in the substrate specificities of the FabG proteins were reflected in the monomer composition of the polymers produced by recombinant *E. coli*. SCL-MCL PHA copolymer isolated from a recombinant *E. coli* strain had improved physical properties compared to the SCL homopolymer poly-3-hydroxybutyrate. This study defines a pathway to produce SCL-MCL PHA copolymer from the fatty acid biosynthesis that may impact on PHA production in recombinant organisms.**

Polyhydroxyalkanoates (PHAs) are biodegradable polyesters that are produced by some bacteria grown under nutrient limitation in the presence of excess carbon and have attracted research interest because they can be used as biodegradable plastics that can be produced from renewable resources (11, 23). PHAs can be divided into three main types based on the number of carbons in the monomer units incorporated into the polymer chain. Short-chain-length (SCL) PHA consists of monomers from C3 to C5 in length, medium-chain-length (MCL) PHA consists of monomers from C6 to C14 in length, and SCL-MCL PHA copolymer consists of both SCL and MCL monomer units. Polymers composed of SCL monomers such as poly-3-hydroxybutyrate [P(3HB)] have thermoplastic properties but are generally brittle and must be processed in a special manner to improve their properties (2, 7), while PHA polymers composed of MCL subunits have elastomeric properties that may be improved with the addition of nanocomposite materials (5, 6). SCL-MCL PHA copolymers have qualities between the SCL and MCL PHA polymers, depending on the ratio of SCL and MCL monomers, and because of superior physical and

thermal properties have a wide array of uses (15). It has been shown that an SCL-MCL PHA copolymer with a high mol% SCL monomer composition and a low mol% MCL monomer composition has properties similar to polyethylene (1). Because of the many possible applications of SCL-MCL PHA, it is important to examine and define the enzymes and metabolic pathways for SCL-MCL PHA production.

In our previous study, we demonstrated that coexpression of mutant 3-ketoacyl-acyl carrier protein (ACP) synthase III genes (*fabH*) with PHA synthase genes (*phaC*) leads to the production of SCL-MCL PHA in recombinant *Escherichia coli* grown in the presence of excess glucose (16). That study proposes that substrates for PHA production are derived from the fatty acid biosynthesis pathway (Fig. 1A) and also proposes that the 3-ketoacyl-ACP reductase (FabG) would be necessary to provide monomers for PHA production from the fatty acid biosynthesis pathway in recombinant *E. coli* (16).

FabG is a member of the ketoacyl reductase family of proteins and is an essential enzyme for type II fatty acid biosynthesis in *E. coli* that catalyzes an NADPH-dependent reduction of 3-ketoacyl-ACP to the (*R*)-3-hydroxyacyl isomer (10). Previous studies have also shown that coexpression of the *fabG* genes from *E. coli* and *Pseudomonas aeruginosa* with type II PHA synthase genes enables recombinant *E. coli* to accumulate similar MCL PHA copolymers when grown in the pres-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
<i>Pseudomonas</i> sp. 61-3	Wild type	JCM 10015
<i>E. coli</i>		
JM109	<i>recA1 endA1 gyrA96 thi-1 hsdR17</i> ( $r_K^- m_K^+$ ) <i>supE44 relA1 <math>\lambda^- lac</math></i> [F' <i>proAB lacI<sup>q</sup> Z<math>\Delta</math>M15</i> ]	Takara
BL21(DE3)	F <sup>-</sup> <i>ompT hsdS<sub>B</sub></i> ( $r_B^- m_B^-$ ) <i>gal dcm</i> (DE3)	Novagen
pBluescript II KS+	<i>lacPOZ</i> , T7 and T3 promoters, Amp <sup>r</sup>	Stratagene
pPFAG	pBluescript II KS+ derivative, <i>Pseudomonas</i> sp. 61-3 <i>fabG</i>	This study
pGEMC1AB	pGEM derivative, <i>Pseudomonas</i> sp. 61-3 <i>phaC1</i> , <i>phaAB</i> , Amp <sup>r</sup>	27
pGEMC1AB-SCQM	pGEM derivative, <i>phaC1</i> (SCQM), <i>phaAB</i> , Amp <sup>r</sup>	27
pGEMC1AB-STQK	pGEM derivative, <i>phaC1</i> (STQK), <i>phaAB</i> , Amp <sup>r</sup>	27
pBBR1-MCS2	Broad-host-range vector, <i>lacPOZ'</i> , Km <sup>r</sup>	8
pBBRC1	pBBR1-MCS2 derivative, <i>Pseudomonas</i> sp. 61-3 <i>phaC1</i>	This study
pBBRSCQM	pBBR1-MCS2 derivative, <i>phaC1</i> (SCQM)	This study
pBBRSTQK	pBBR1-MCS2 derivative, <i>phaC1</i> (STQK)	This study
pCR2.1-TOPO	TA cloning vector, Km <sup>r</sup> , Amp <sup>r</sup>	Invitrogen
pCECFG+	pCR2.1-TOPO derivative, <i>E. coli fabG</i>	This study
pCPSFG+	pCR2.1-TOPO derivative, <i>Pseudomonas</i> sp. 61-3 <i>fabG</i>	This study
pBBRSCQMGEC	pBBR1-MCS2 derivative, <i>phaC1</i> (SCQM), <i>E. coli fabG</i>	This study
pBBRSTQKGEC	pBBR1-MCS2 derivative, <i>phaC1</i> (STQK), <i>E. coli fabG</i>	This study
pBBRSCQMGPS	pBBR1-MCS2 derivative, <i>phaC1</i> (SCQM), <i>Pseudomonas</i> sp. 61-3 <i>fabG</i>	This study
pBBRSTQKGPS	pBBR1-MCS2 derivative, <i>phaC1</i> (STQK), <i>Pseudomonas</i> sp. 61-3 <i>fabG</i>	This study
pTrcFabH(F87T)	pTrc99A derivative, <i>fabH</i> (F87T), Amp <sup>r</sup>	16
pET-15b	Amp <sup>r</sup> , T7 <i>lac</i> promoter, His tag, N terminus	Novagen
pETGE	pET-15b derivative, <i>E. coli fabG</i>	This study
pETGP	pET-15b derivative, <i>Pseudomonas</i> sp. 61-3 <i>fabG</i>	This study

were digested with BamHI and SacII, and a DNA fragment harboring the *phaC1* gene or one of its mutant derivatives was ligated into the same sites of the pBBR1-MCS2 plasmid to create pBBR1-*phaC1*, pBBR1SCQM, or pBBRSTQK. The *E. coli fabG* gene was amplified from *E. coli* JM109 genomic DNA by PCR with the following primers: for the N terminus, 5'-GCT CTA GAG AGG AAA ATC ATG AAT TTT GAA GG-3', and for the C terminus, 5'-TCA GAC CAT GTA CAT CCC GCC GTT CAC-3'. The subsequent PCR product was cloned into the TA-cloning vector pCR2.1-TOPO (Invitrogen, Carlsbad, CA). The insertion, overall correctness, and orientation of the gene were determined by DNA sequencing and restriction digestion. The *Pseudomonas* sp. 61-3 *fabG* gene was amplified from *Pseudomonas* sp. 61-3 genomic DNA by PCR with the following primers: for the N terminus, 5'-CCT CTA ACC CTC AAT ACC CCA G-3', and for the C terminus, 5'-TTG AAG GGA TCC GTC ACA T-3'. As with the *E. coli fabG* gene PCR product, the *Pseudomonas* sp. 61-3 *fabG* PCR product was cloned into pCR2.1-TOPO to produce pCECFG+ and pCPSFG+, respectively, and the insertion and orientation of the gene were determined by DNA sequencing and restriction digestion. The *fabG* genes were isolated from the pCECFG+ and pCPSFG+ plasmids with HincII, and the DNA fragments were subcloned into the SmaI site of the pBBRC1, pBBRSCQM, and pBBRSTQK plasmids. The orientation of the insert was determined by sequence analysis.

The following DNA primers were used to amplify the *fabG* genes from *E. coli* and *Pseudomonas* sp. 61-3 in order to construct the expression vectors pETGE and pETGP for the production of His-tagged FabG<sub>Ec</sub> and FabG<sub>Ps</sub>: 5'-GAA ACA TAT GAA TTT TGA AG GAA AAA TCG CAC TGG-3' for the N terminus of *fabG*<sub>Ec</sub>, where bold lettering indicates the introduction of an NdeI restriction site; 5'-GCG GAT CCC GGT CAG ACC ATG TAC ATC CCG CCG TTC ACA TG-3' for the C terminus of *fabG*<sub>Ec</sub>, where bold lettering indicates the introduction of a BamHI site into the primer; 5'-GAA GCT CAT ATG AGT CTG CAA GGT AAA GTT GCA C-3' for the N terminus of *fabG*<sub>Ps</sub>; and 5'-GAA GGG ATC CGT CAC ATT TAA CTC ATG TAC ATC CCG C-3' for the C terminus of *fabG*<sub>Ps</sub>. The PCR products were purified and digested with NdeI and BamHI and inserted into the same sites of the pET15b plasmid (Novagen, Madison, WI) to make either the *E. coli fabG* expression plasmid pETGE or the *Pseudomonas* sp. 61-3 *fabG* expression plasmid pETGP (Table 1).

**Overexpression of *fabG* genes and purification of His-tagged recombinant FabG proteins.** *E. coli* BL21(DE3) (Novagen, Madison, WI) was transformed with either the pETGE plasmid harboring the *E. coli fabG* gene or pETGP harboring the *Pseudomonas* sp. 61-3 *fabG* gene. Individual colonies were selected based on their resistance to ampicillin on LB ampicillin plates grown overnight at 37°C and used to inoculate starter cultures of 1.75 ml of LB medium that were grown overnight in a rotary shaker (200 rpm). Each starter culture was used to

inoculate 100 ml of LB medium in 500-ml Sakaguchi flasks supplemented with 100  $\mu$ g ml<sup>-1</sup> ampicillin. The cultures were grown for 3 h at 37°C, and the expression of the *fabG* genes was induced by the addition of 0.1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG), after which the cultures were incubated for an additional 2 h with constant shaking at 37°C before harvesting by centrifugation.

The harvested cells were washed with cold 50 mM phosphate buffer and resuspended in 4 ml of the same buffer. The cells were broken on ice by sonication with a model UD-200 ultrasonic disruptor (TOMY, Tokyo, Japan) by pulsing four times for 5 s each and centrifuged to purify the cell lysates. The cell lysates were subjected to purification via Ni column chromatography with the His-Bind purification kit (Novagen). The purified, His-tagged FabG proteins were eluted according to the manufacturer's protocol, and the purity of the final product was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Single bands with molecular masses approximately equivalent to predicted molecular masses of the FabG proteins on the gels after staining with Rapid CBB stain (Kanto Chemical, Tokyo, Japan) indicated that the proteins were purified to electrophoretic homogeneity (data not shown).

**Assay for substrate specificity of FabG proteins.** Purified His-tagged *E. coli* FabG and *Pseudomonas* sp. 61-3 FabG proteins were assayed for substrate specificity using a modified form of a previously developed method to evaluate the stereospecificity of enoyl-CoA hydratases (29). Reaction mixtures consisted of 0.25 mM *trans*-2-enoyl-CoA (C4, C6, C8, C10, or C12), 0.5 mM NADP<sup>+</sup>, and 1.0 U of hydratase (either PhaJ1 or PhaJ4) in 400  $\mu$ l of 50 mM Tris-HCl (pH 8.0). Reactions were initiated by the addition of 1 U of either His-tagged *E. coli* FabG or His-tagged *Pseudomonas* sp. 61-3 FabG. Reactions were carried out at room temperature for up to 2 min, and substrate specificity was monitored by the increase in absorbance at 340 nm, which is due to the formation of NADPH linked with the oxidation of 3-hydroxyacyl-CoA substrates produced by PhaJ proteins to 3-ketoacyl-CoA.

**PHA production from glucose in recombinant *E. coli*.** Plasmids harboring either the *E. coli fabG* gene or the *Pseudomonas* sp. 61-3 *fabG* gene with the wild-type *phaC1* gene, *phaC1*(STQK) gene, or *phaC1*(SCQM) gene were co-transformed with pTrcFabH(F87T) into *E. coli* JM109. Transformants were isolated, and the presence of the pTrcFabH plasmid and a plasmid harboring *phaC1* and *fabG* was confirmed by restriction digestion and PCR. Single colonies of confirmed transformants were cultured overnight in 1.75 ml of LB medium and used to inoculate 500-ml culture flasks with either 100 ml LB medium or 100 ml of M9 medium supplemented with glucose. Cultures were incubated at 30°C and constantly shaken at 150 rpm for 5 h, at which time expression of the *fabH*(F87T) gene was induced by the addition of 1 mM IPTG. Cultures grown in

LB medium were incubated with constant shaking at 30°C for an additional 3 h, at which time glucose was added to a final concentration of 2 g ml<sup>-1</sup>. The cells were grown for a total of 96 h before harvesting by centrifugation. PHA contents were determined by gas chromatography (GC) analysis of lyophilized cells as previously described (16).

**GPC analysis of PHA polymers.** Cell materials for gel permeation chromatography (GPC) analysis were prepared and lyophilized as described for GC analysis except that a total of 2 liters of liquid culture per sample was harvested by centrifugation. The lyophilized cells were added to 100 ml of chloroform and were stirred in a covered beaker at room temperature for 48 h to extract the polymers. The chloroform-polymer solution was filtered first through filter paper to remove cell debris and then through a 0.45- $\mu$ m polytetrafluoroethylene membrane to remove any residual solid materials. The chloroform was evaporated using a rotary vacuum evaporator (Eyela, Tokyo, Japan), and the isolated polymer was washed with 20 ml of methanol. The polymer was allowed to dry at room temperature and was redissolved in 20 ml of chloroform. The polymer was precipitated by the addition of 10 times the volume of methanol and collected by filtering the solution through a 0.45- $\mu$ m polytetrafluoroethylene membrane. The membrane and polymers were allowed to dry at room temperature, and the polymers were dissolved by the addition of chloroform and collected in a beaker. The chloroform was allowed to evaporate at room temperature in a fume hood for 48 h, and the polymer cast film was weighed and used for GPC analysis. Molecular mass data of polyesters were obtained by GPC analysis using the Shimadzu 10A system with a RID-10A refractive-index detector with serial columns of ShodexK802 and K806 M as described previously (9).

**Determination of PHA polymer composition by NMR.** Twenty mg of polymer isolated from *E. coli* JM109 harboring pBBRSTOKGEC and pTrcFabH(F87T) was dissolved in 1 ml of CDCl<sub>3</sub> and subjected to both <sup>1</sup>H and <sup>13</sup>C nuclear magnetic resonance (NMR) analysis. <sup>1</sup>H NMR spectra were recorded using a JEOL  $\alpha$ -400 spectrometer with a 5.0- $\mu$ s pulse width (45° pulse angle), 3-s pulse repetition, 7,500-Hz spectra width, and 16K data points. For <sup>13</sup>C NMR analysis, data were collected using a JEOL ECP-500 spectrometer with a 7.0- $\mu$ s pulse width (45° pulse angle), 5-s pulse repetition, 25,000-Hz spectra width, and 64K data points. Tetramethylsilane (Me<sub>4</sub>Si) was used as an internal chemical shift standard.

**Determination of thermal properties of SCL-MCL PHA polymers produced by recombinant *E. coli*.** The thermal data were recorded on a PerkinElmer Pyris 1 differential scanning calorimeter equipped with a liquid nitrogen cooling accessory. Data was collected under a nitrogen flow of 20 ml min<sup>-1</sup>. Melt-quenched polyester samples (ca. 3 mg) encapsulated in aluminum pans were heated from -30°C to 200°C at a rate of 20°C min<sup>-1</sup>, and the heat flow curves were recorded. The observed melting temperatures were determined from the positions of the endothermic peaks.

## RESULTS

**Cloning and identification of a novel 3-ketoacyl-ACP reductase (*fabG*) gene from *Pseudomonas* sp. 61-3.** *Pseudomonas* sp. 61-3 is a nonpathogenic bacterium isolated from soil that can produce a blend of P(3HB) homopolymer and random SCL-MCL PHA copolymers from both sugars and alkanolic acids (14). Previous studies have shown that 3-ketoacyl reductases can be used for monomer supply in PHA biosynthesis (21, 24), so we set out to investigate the potential role of reductase enzymes from *Pseudomonas* sp. 61-3. Southern blot analysis was performed using genomic DNA fragments from digestions with several restriction enzymes, and a 1.2-kbp EcoRI fragment that cross-hybridized with a heterologous *E. coli fabG* (*EcfabG*) PCR probe was cloned into pBluescript SK+ at the same restriction sites. The insert was sequenced and found to contain the entire open reading frame corresponding to the *Pseudomonas* sp. 61-3 *fabG* gene (DDBJ, AB193436). The predicted amino acid sequence of the *Pseudomonas* sp. 61-3 FabG was found to be 83% identical and 91% similar to the *Pseudomonas aeruginosa* FabG and 66% identical and 79% similar to the *E. coli* FabG protein via ClustalW alignment. The *Pseudomonas* sp. 61-3 *fabG* (*PsfabG*) gene product was

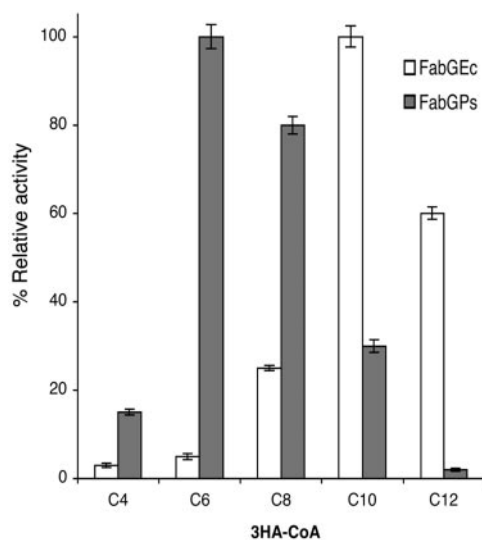


FIG. 2. FabG substrate specificity. Data represent results for three independent experiments  $\pm$  standard deviations. Results are presented as relative reactivities toward a specific substrate. FabGec, *E. coli* FabG protein-relative activities for substrates; FabGPs, *Pseudomonas* sp. 61-3 FabG protein-relative activities for substrates. C4, 3-hydroxybutyryl-CoA; C6, 3-hydroxyhexanoyl-CoA; C8, 3-hydroxyoctanoyl-CoA; C10, 3-hydroxydecanoyl-CoA; C12, 3-hydroxydodecanoyl-CoA.

further characterized and used to assess its potential role in the production of SCL-MCL PHA from glucose.

**Substrate specificity of FabG proteins.** Although the natural reaction of the FabG protein is the reduction of 3-ketoacyl-ACP to form (*R*)-3-hydroxyacyl-ACP in fatty acid biosynthesis (Fig. 1A), it was shown previously that FabG is capable of recognizing both (*R*)-3-ketoacyl-CoA and (*R*)-3-hydroxyacyl-CoA as substrates (Fig. 1B) (25). In this study, we used an assay previously developed to characterize stereoselectivity in *P. aeruginosa* enoyl-CoA hydratase (PhaJ) proteins (29) to assay the substrate specificity of the FabG proteins. PhaJ proteins can convert enoyl-CoAs into (*R*)-3-hydroxyacyl-CoAs (29). For example, PhaJ1 can convert crotonyl-CoA to (*R*)-3-hydroxybutyryl-CoA and PhaJ4 can convert hexenyl-CoA to (*R*)-3-hydroxyhexanoyl-CoA. In turn, these 3-hydroxyacyl-CoA forms can be converted to (*R*)-3-ketoacyl-CoA with the subsequent reduction of NADP<sup>+</sup> to NADPH via FabG. In order to assess the substrate specificities of the FabG proteins, recombinant FabG proteins from *Pseudomonas* sp. 61-3 and *E. coli* were produced and purified and their substrate specificities were determined for C4 to C12 substrates. The results indicated that the *E. coli* FabG protein has high substrate specificity toward C8 to C12 substrates, with the highest substrate specificity toward C10 compounds (Fig. 2). In contrast to the *E. coli* FabG protein, the *Pseudomonas* sp. 61-3 FabG protein displayed higher substrate specificity toward C6 to C10 compounds, with the highest specificity for the C6 substrate (Fig. 2). In addition to their preferred substrate specificities, both proteins were able to react with a broad (C4 to C12) range of substrates, with the *Pseudomonas* sp. 61-3 FabG protein having a stronger preference for C4 substrates than the *E. coli* FabG protein (Fig. 2).

TABLE 2. Effects of coexpression of *fabG*, *fabH*(F87T), and *phaC1* on PHA production in recombinant *E. coli* JM109 grown on LB medium and glucose<sup>a</sup>

Relevant gene(s)	CDW <sup>b</sup> (g liter <sup>-1</sup> )	PHA content (wt% of CDW) <sup>c</sup>	PHA composition (mol%) <sup>c</sup>			
			3HB (C4)	3HHx (C6)	3HO (C8)	3HD (C10)
61-3 <i>phaC1</i>	2.2 ± 0.7	ND <sup>d</sup>				
61-3 <i>phaC1</i> , <i>EcfabG</i>	1.5 ± 0.4	ND				
61-3 <i>phaC1</i> , <i>EcfabH</i> (F87T)	2.1 ± 0.1	0.11 ± 0.02	75.6 ± 4	24.4 ± 1		
61-3 <i>phaC1</i> , <i>EcfabG</i> , <i>EcfabH</i> (F87T)	0.66 ± 0.02	0.64 ± 0.14	66.9 ± 1.1	22.4 ± 1.2	7.4 ± 0.5	3.3 ± 0.3
61-3 <i>phaC1</i> , <i>PsfabG</i>	1.6 ± 0.1	ND				
61-3 <i>phaC1</i> , <i>PsfabG</i> , <i>EcfabH</i> (F87T)	1.8 ± 0.6	0.08 ± 0.05	96.3 ± 6.3	3.7 ± 6.3		

<sup>a</sup> Cells were cultivated in LB medium. IPTG and 2 g of glucose ml<sup>-1</sup> were added after 5 and 8 h of cultivation, respectively, and the cells were grown for 96 h total as described in Materials and Methods. The results are the averages and the standard deviations of at least three independent experiments.

<sup>b</sup> CDW, cellular dry weight.

<sup>c</sup> 3HHx, 3-hydroxyhexanoate, 3HO, 3-hydroxyoctanoate; 3HD, 3-hydroxydecanoate.

<sup>d</sup> ND, not detected.

<sup>e</sup> wt% of CDW, grams of PHA per gram of CDW.

**PHA production from glucose is enhanced by the coexpression of *fabG* genes with the *Pseudomonas* sp. 61-3 *phaC1* gene and *fabH*(F87T) in *E. coli* JM109.** In our previous study, the coexpression of mutant *fabH* genes, which contained single amino acid substitutions that arose from a point mutation in the codon encoding the Phe at position 87, with a PHA synthase enabled recombinant *E. coli* to accumulate SCL-MCL PHA when grown in the presence of excess glucose (16). Based on those results, the pathway depicted in Fig. 1A was proposed for the supply of monomers for PHA biosynthesis from glucose via fatty acid biosynthesis. In this model, both FabH and FabG are necessary for SCL and MCL monomer supply for SCL-MCL PHA production from fatty acid biosynthesis. However, the previous study used only native levels of FabG produced by the recombinant strain to reduce the 3-ketoacyl-CoA substrates to R-3-hydroxybutyryl-CoA substrates. It was predicted that the additional overexpression of *fabG* would further enhance the SCL-MCL monomer supply from fatty acid biosynthesis. In order to test this, *E. coli* JM109 was transformed with the pTrcFabH(F87T) plasmid and either pBBR1C1GEC harboring the *Pseudomonas* sp. 61-3 *phaC1* gene and the *EcfabG* gene or pBBRC1GPS harboring the *Pseudomonas* sp. 61-3 *phaC1* gene and the *PsfabG* gene. The *fabH*(F87T) gene was used because when it was coexpressed with the wild-type *Pseudomonas* sp. 61-3 *phaC1* gene, it supplied the broadest substrate range of MCL monomers and produced the highest percentage of cellular dry weight PHA and the highest cell yield compared to other strains used in the previous study (16). The cells were assayed for PHA production by GC analysis, and the results are shown in Table 2. Control strains harboring only the *phaC1* gene or the *phaC1* gene and the *fabG* gene from either *E. coli* or *Pseudomonas* sp. 61-3 were unable to accumulate any detectable PHA. A strain that harbored only the *phaC1* gene with the *fabH*(F87T) gene was able to accumulate 0.11% of the cellular dry weight of SCL-MCL PHA copolymer consisting of approximately 75 mol% C4 monomer and 25 mol% C6 monomer, similar to results previously reported (16). The additional expression of *EcfabG* led to a sixfold increase in accumulated PHA (0.64% of cellular dry weight). Furthermore, the expression of *EcfabG* broadened the amount and types of monomers incorporated into the SCL-MCL PHA copolymer (66.9 mol% C4, 22.4 mol% C6, 7.4 mol% C8, and 3.3 mol% C10). These results indicated that

coexpression of the *EcfabG* gene with the *phaC1* and *fabH*(F87T) genes in recombinant *E. coli* JM109 leads to an enhancement in the amount of polymer produced and the incorporation of a much broader variety of MCL monomers into the SCL-MCL PHA copolymer.

The additional expression of the *PsfabG* with the *phaC1* and *fabH*(F87T) genes led to a slight decrease in the total amount of PHA accumulated by the cells and also changed both the amount and the type of monomers incorporated into the SCL-MCL PHA copolymer produced (Table 2). This result showed that the substrate specificity of the *Pseudomonas* sp. 61-3 FabG protein is also a determinant for the type of monomer supplied for the production of PHA from glucose in recombinant *E. coli* JM109.

**PHA production from glucose is further enhanced by the coexpression of *fabG* genes with the genetically engineered PHA synthase genes and *fabH*(F87T) in *E. coli* JM109.** Previously, our lab developed several highly active mutant type II PHA synthases via in vitro evolutionary engineering and saturation point mutagenesis (27). Two mutants obtained from that study were used to further characterize the involvement of the fatty acid biosynthesis genes in SCL-MCL PHA copolymer production from glucose in *E. coli*. The first mutant PHA synthase [PhaC1(STQK)] contains two point mutations. One of the point mutations changes amino acid 325 of the *Pseudomonas* sp. 61-3 PhaC1 enzyme from Ser to Thr, and a secondary point mutation changes the Gln at position 481 to Lys. The mutations at S325T/Q481K led to an 8.7-fold increase in activity toward 3-hydroxybutyrate-CoA and a 2.8-fold increase in activity toward 3-hydroxydecanoate-CoA in an in vitro assay (26). The second mutant PHA synthase [PhaC1(SCQM)] also had two point mutations, one changing the Ser at 325 to Cys and the second changing Gln at 481 to Met. These two mutants were found to be highly active for the production of P(3HB) homopolymer compared to the wild-type enzyme (27). Furthermore, these mutants also possessed the capacity to produce SCL-MCL PHA copolymer when grown in the presence of dodecanoate (26).

It was predicted that coexpression of highly active mutant forms of PHA synthases with the *fabH*(F87T) and *fabG* genes would further enhance SCL-MCL PHA production in *E. coli* JM109 from glucose. To test this, plasmids harboring the genetically engineered *phaC1* genes alone (pBBRSTQK and

TABLE 3. Effects of coexpression of *fabG*, *fabH*(F87T), and genetically modified *phaC1* genes on PHA production in recombinant *E. coli* JM109 grown on LB medium and glucose<sup>a</sup>

Relevant gene(s)	CDW <sup>b</sup> (g liter <sup>-1</sup> )	PHA content (% of CDW)	PHA composition (mol%) <sup>c</sup>				
			3HB (C4)	3HHx (C6)	3HO (C8)	3HD (C10)	3HDD (C12)
<i>phaC1</i> (STQK)	1.7 ± 0.02	ND <sup>d</sup>					
<i>phaC1</i> (STQK), <i>EcfabG</i>	1.8 ± 0.2	0.10 ± 0.008	100				
<i>phaC1</i> (STQK), <i>EcfabH</i> (F87T)	2.1 ± 0.02	0.96 ± 0.07	90.5 ± 2	6.0 ± 0.3	2.0 ± 0.9	1.3 ± 0.9	1.2 ± 0.3
<i>phaC1</i> (STQK), <i>EcfabG</i> , <i>EcfabH</i> (F87T)	0.91 ± 0.07	4.5 ± 0.8	94.1 ± 0.1	4.6 ± 0.1	0.9 ± 0.01	0.4 ± 0.02	
<i>phaC1</i> (STQK), <i>PsfabG</i>	1.2 ± 0.8	0.06 ± 0.004	100				
<i>phaC1</i> (STQK), <i>PsfabG</i> , <i>EcfabH</i> (F87T)	2.2 ± 0.06	0.67 ± 0.1	96.3 ± 3.3	3.7 ± 3.3			
<i>phaC1</i> (SCQM)	1.7 ± 0.05	ND					
<i>phaC1</i> (SCQM), <i>EcfabG</i>	1.7 ± 0.09	0.02 ± 0.02	100				
<i>phaC1</i> (SCQM), <i>EcfabH</i> (F87T)	2.1 ± 0.04	0.62 ± 0.22	88.6 ± 0.6	8.3 ± 0.2	1.9 ± 0.9	0.7 ± 0.3	0.5 ± 0.3
<i>phaC1</i> (SCQM), <i>EcfabG</i> , <i>EcfabH</i> (F87T)	1.0 ± 0.4	2.3 ± 0.5	91.3 ± 0.1	6.7 ± 0.1	1.4 ± 0.04	0.6 ± 0.02	0
<i>phaC1</i> (SCQM), <i>PsfabG</i>	1.9 ± 0.5	0.2 ± 0.5	100				
<i>phaC1</i> (SCQM), <i>PsfabG</i> , <i>EcfabH</i> (F87T)	1.4 ± 0.8	1.4 ± 0.5	95.5 ± 5.2	4.5 ± 2.0			

<sup>a</sup> Cells were cultivated in LB medium. IPTG and 2 g of glucose ml<sup>-1</sup> were added after 5 and 8 h of cultivation, respectively, and the cells were grown for 96 h total as described in Materials and Methods. The results are the averages and the standard deviations of at least three independent experiments.

<sup>b</sup> CDW, cellular dry weight.

<sup>c</sup> 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate; 3HD, 3-hydroxydecanoate; 3HDD, 3-hydroxydodecanoate.

<sup>d</sup> ND, not detected.

pBBRSCQM), plasmids harboring the genetically engineered *phaC1* genes with the *EcfabG* genes (pBBRSTQKGEC and pBBRSCQMGECC), and plasmids harboring the genetically engineered *phaC1* genes with the *PsfabG* genes (pBBRSTQKGPS and pBBRSCQMGPS) were transformed with or without the pTrcFabH(F87T) plasmid into *E. coli* JM109. The relevant genotypes and phenotypes of the strains are described in Table 3. The ability of the transformed *E. coli* JM109 strains to accumulate SCL-MCL PHA copolymer from glucose was assessed by GC as described in Materials and Methods, and the results are shown in Table 3. Control strains harboring plasmids expressing either *phaC1*(STQK) or *phaC1*(SCQM) alone were unable to accumulate detectable levels of PHA in *E. coli* JM109. Strains harboring either *EcfabG* or *PsfabG* and *phaC1*(STQK) or *phaC1*(SCQM) were able to accumulate a small amount of P(3HB) homopolymer. A strain coexpressing *E. coli fabH*(F87T) with *phaC1*(STQK) was able to produce 0.96% of the cellular dry weight of SCL-MCL PHA copolymer consisting of 90.7 mol% C4, 6.0 mol% C6, 2.0 mol% C8, 1.3 mol% C10, and 1.2 mol% C12 (Table 3). This result indicates that the *PhaC1*(STQK) enzyme was able to broaden the number and type of substrates that could be incorporated into the SCL-MCL PHA copolymer and increase the yield compared to SCL-MCL PHA copolymer produced by the wild-type PHA synthase. The additional coexpression of the *EcfabG* gene with the *E. coli fabH*(F87T) and *phaC1*(STQK) genes resulted in a marked increase in PHA content (up to 4.5% of cellular dry weight) in addition to a change in the mol% composition of the SCL-MCL copolymer produced in this strain, with a shift toward the shorter-chain-length monomers compared to the strain that harbored only the *fabH*(F87T) and *phaC1*(STQK) genes (Table 3). These results contrast with those obtained by coexpressing the *PsfabG* gene with both the *E. coli fabH*(F87T) and the *phaC1*(STQK) genes, an event which produced an

SCL-MCL copolymer with a dramatic shift toward C4 and C6 monomer incorporation compared to strains expressing the *E. coli fabG* gene. This indicates that the monomer-supplying enzymes are important factors in determining the composition of SCL-MCL PHA copolymers. Similar results were obtained when coexpression of the *phaC1*(SCQM) gene was performed with the fatty acid biosynthesis genes (Table 3). Overall, these results indicate that the coexpression of *fabG* genes with the *fabH*(F87T) and PHA synthase genes results in the enhanced production of SCL-MCL PHA copolymer in *E. coli* JM109 grown in the presence of excess glucose.

**Effect of medium composition on PHA production in *E. coli* JM109.** Because LB is an undefined medium, it was difficult to discern whether or not all of the PHA produced by the recombinant *E. coli* strains was a result of the glucose addition to the medium or from the incorporation of an undefined carbon source. Therefore, a defined medium (M9) with a known amount of glucose was used to examine whether recombinant *E. coli* strains harboring plasmids expressing the *fabG*, *fabH*(F87T), and either the *phaC1*(STQK) or *phaC1*(SCQM) genes were capable of producing SCL-MCL PHA copolymer in *E. coli* JM109 directly when glucose was the sole carbon source. The results are shown in Table 4. The wild-type *phaC1* expression plasmid was not used in this experiment because the amount of PHA accumulation in strains harboring plasmids expressing this gene is lower than it is in those strains expressing the highly active genetically engineered PHA synthase genes (Tables 2 and 3).

The cellular dry weights were much lower for all of the strains grown in M9 than those for cells grown in LB medium (Tables 3 and 4). Recombinant JM109 expressing only *phaC1*(STQK) or *phaC1*(SCQM) failed to accumulate detectable PHA. However, recombinant JM109 strains expressing *phaC1*(STQK) and *EcfabH*(F87T) or *phaC1*(SCQM) and

TABLE 4. PHA accumulation in recombinant JM109 *E. coli* cells grown in M9 medium with glucose as the sole carbon source<sup>a</sup>

Relevant gene(s)	CDW <sup>b</sup> (g liter <sup>-1</sup> )	PHA content (% of CDW)	PHA composition (mol%) <sup>c</sup>	
			3HB (C4)	3HHx (C6)
<i>phaC1</i> (STQK)	0.50 ± 0.03	ND <sup>d</sup>		
<i>phaC1</i> (STQK), <i>EcfabH</i> (F87T)	0.54 ± 0.01	2.0 ± 0.3	98.5 ± 1	1.5 ± 0.2
<i>phaC1</i> (STQK), <i>EcfabG</i>	0.54 ± 0.02	0.08 ± 0.02	100	
<i>phaC1</i> (STQK), <i>PsfabG</i>	0.48 ± 0.01	0.06 ± 0.04	100	
<i>phaC1</i> (STQK), <i>EcfabG</i> , <i>EcfabH</i> (F87T)	0.42 ± 0.01	2.8 ± 0.4	97.5 ± 0.5	2.5 ± 0.5
<i>phaC1</i> (STQK), <i>PsfabG</i> , <i>EcfabH</i> (F87T)	0.42 ± 0.06	2.1 ± 0.4	96.9 ± 0.8	3.1 ± 0.8
<i>phaC1</i> (SCQM)	0.48 ± 0.02	ND		
<i>phaC1</i> (SCQM), <i>EcfabH</i> (F87T)	0.41 ± 0.03	3.0 ± 0.2	97.4 ± 0.2	2.6 ± 0.1
<i>phaC1</i> (SCQM), <i>EcfabG</i>	0.56 ± 0.01	0.01 ± 0.01	100	
<i>phaC1</i> (SCQM), <i>PsfabG</i>	0.46 ± 0.02	0.14 ± 0.05	100	
<i>phaC1</i> (SCQM), <i>EcfabG</i> , <i>EcfabH</i> (F87T)	0.49 ± 0.02	1.5 ± 0.2	96.7 ± 0.1	3.3 ± 0.1
<i>phaC1</i> (SCQM), <i>PsfabG</i> , <i>EcfabH</i> (F87T)	0.48 ± 0.01	0.5 ± 0.2	100	

<sup>a</sup> Cells were cultivated in M9 medium with 2 g of glucose ml<sup>-1</sup>. IPTG was added after 8 h of cultivation, and the cells were grown for 96 h total as described in Materials and Methods. The results are the averages and the standard deviations of at least three independent experiments.

<sup>b</sup> CDW, cellular dry weight.

<sup>c</sup> 3HHx, 3-hydroxyhexanoate. 3HO (3-hydroxyoctanoate) was not detectable in the cells.

<sup>d</sup> ND, not detected.

*EcfabH*(F87T) were able to accumulate SCL-MCL PHA copolymers composed of 98.5 mol% C4, 1.5 mol% C6, 97.4 mol% C4, and 2.6 mol% C6, respectively. The recombinant JM109 strains expressing *phaC1*(STQK) and either *EcfabG* or *PsfabG* were able to accumulate a small amount of P(3HB). Recombinant JM109 strains expressing *phaC1*(STQK), *EcfabH*(F87T) and *EcfabG* or *phaC1*(STQK), *EcfabH*(F87T), and *PsfabG* accumulated SCL-MCL PHA copolymers composed of 97.5 mol% C4, 2.5 mol% C6, 96.9 mol% C4, and 3.1 mol% C6, respectively, while recombinant JM109 strains expressing *phaC1*(SCQM), *EcfabH*(F87T) and *EcfabG* or *phaC1*(SCQM), *EcfabH*(F87T), and *PsfabG* accumulated SCL-MCL PHA copolymers composed of C4 and C6 monomers (Table 4). These results indicate that the overexpression of fatty acid biosynthesis enzymes leads to the production of SCL and MCL monomers for SCL-MCL PHA copolymer production from glucose in *E. coli*.

**Physical characterization of SCL-MCL PHA copolymer isolated from recombinant *E. coli*.** Polymer was isolated from a JM109 strain harboring the pTrcFabH(F87T) and pBBRSTQKGEC plasmids as described in Materials and Methods. This polymer was chosen as a representative polymer produced by fatty acid biosynthetic enzymes and a mutant PHA synthase and was characterized by NMR spectroscopy, GPC, and differential scanning calorimetry. The results are shown in Table 5.

In order to determine the structure of the isolated polymer

and to show that the polymer was a true copolymer rather than a blend of polymers, NMR was used. The mol% fractions of the secondary (C6) and tertiary (C8) monomer units were determined from the intensity ratio of the main-chain methylene proton resonance to methyl proton resonance in the <sup>1</sup>H NMR spectra (data not shown). Supporting information for tertiary (C8) monomer units was obtained by <sup>13</sup>C NMR analysis (data not shown), which was used to prove that the isolated SCL-MCL PHA was a random copolymer rather than a blend of polymers. The degree of randomness of the copolymer was determined by comparing the obtained spectra with the experimentally obtained spectra and simulated spectra in which random distribution was assumed (3). The R factor, a number defining the statistical regression, was 0.998 (near 1.000) as determined by the least-squares method, and the <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra of polymer isolated from *E. coli* JM109 harboring the pTrcFabH(F87T) and pBBRSTQKGEC plasmids revealed that the polymer was composed of 95.1 mol% 3HB, 3.6 mol% 3HHx, and 1.3 mol% 3HO. Although the 3HD fraction was detectable by GC analysis, it was not detected by NMR spectroscopy. However, overall, these mol% values were similar to those obtained by GC analysis (Tables 3 and 5).

The weight-average molecular weight ( $M_w$ ) of the polymer was determined by GPC and revealed that the  $M_w$  of the SCL-MCL PHA copolymer was slightly lower than that of P(3HB) homopolymer but that the relative distributions of the polydispersity indices ( $M_w/M_n$ ) were similar for the SCL-MCL

TABLE 5. Physical characteristics of SCL-MCL PHA copolymer produced using *fabH*(F87T) and *EcfabG* as monomer suppliers<sup>a</sup>

Relevant gene or source	PHA composition (mol%) <sup>b</sup>			Mol wt <sup>c</sup>		Thermal properties <sup>d</sup>		
	3HB (C4)	3HHx (C6)	3HO (C8)	$M_w$ (× 10 <sup>5</sup> )	$M_w/M_n$	$T_c$ (°C)	$T_m$ (°C)	$\Delta H_m$ (J/g)
<i>R. eutropha</i> P(3HB)	100			4.4	2.7	51	171	52
<i>phaC1</i> (STQK), <i>fabH</i> (F87T), <i>EcfabG</i>	95.1	3.6	1.3	2.0	1.9	70	135, 152	32

<sup>a</sup> Cells were cultivated in LB medium. IPTG and 10 g liter<sup>-1</sup> glucose were added after 5 h and 8 h of cultivation, respectively.

<sup>b</sup> PHA composition was determined by NMR analysis. 3HHx, 3-hydroxyhexanoate, 3HO, 3-hydroxyoctanoate.

<sup>c</sup>  $M_w$ , weight-average molecular weight;  $M_n$ , number-average molecular weight;  $M_w/M_n$ , polydispersity index.

<sup>d</sup>  $T_c$ , crystallization temperature;  $T_m$ , melting temperature;  $\Delta H_m$ , enthalpy of fusion.

PHA copolymer and P(3HB) (Table 5). This result indicates that the relative distributions of the polymers isolated from each strain were similar despite the difference in the overall molecular weights.

Thermal properties of the SCL-MCL PHA copolymer isolated from recombinant *E. coli* harboring the pTrcFabH(F87T) and pBBRSTQKGEC plasmids were determined by differential scanning calorimetry/DSC analysis and compared with the thermal properties of the P(3HB) homopolymer (Table 5). For the SCL-MCL PHA copolymer, two melting temperature peaks were observed as opposed to the single melting temperature peak observed for the P(3HB) homopolymer. The lower temperature melting peaks observed for the SCL-MCL PHA copolymers are from their respective original crystals, while the higher temperature melting peaks arose from the recrystallization of the copolymer during the heating process. The thermal properties for each polymer are summarized in Table 5. As determined by NMR analysis, the SCL-MCL PHA copolymer isolated from the recombinant *E. coli* strain harboring the pTrcFabH(F87T) and pBBRSTQKGEC plasmids consisted of polymers composed of 95.1 mol% 3HB, 3.6 mol% 3HHx, and 1.3 mol% 3HO monomer units as determined by NMR analysis. The addition of 4.9 mol% 3HHx and 3HO MCL-monomer units to the PHA copolymer lowered the melting temperature to 150°C from 170°C compared to the P(3HB) homopolymer sample (Table 5). In addition, the enthalpy of fusion was lowered to 32 J/g compared to 52 J/g for the P(3HB) homopolymer. These results indicate that the polymer is an SCL-MCL PHA copolymer and that the addition of MCL monomers within the copolyester dramatically alters the thermal properties of the polymer. Based on previous studies, these changes in thermal properties correspond to improved physical properties (1).

## DISCUSSION

Two important factors must be taken into consideration for the use of PHAs as bulk commodity plastics: the properties of the polymer and the cost of production. Polymer properties are greatly influenced by their monomer composition. P(3HB) homopolymer made of SCL monomer units is a stiff and brittle thermoplastic. On the other hand, SCL-MCL PHA copolymers with a high mol% of SCL monomers along with a low mol% of MCL monomers have properties similar to polypropylene and polyethylene and many potential uses as bulk commodity plastics (1, 15). Therefore, it is of special interest to develop pathways and enzymes for the production of SCL-MCL PHA copolymers with various MCL monomer compositions.

There are many factors that influence the cost of production of SCL-MCL PHA copolymers, but one of the key components is the cost of the carbon feedstock. One way to reduce the cost is by using inexpensive substrates such as sugars or molasses (11, 23). Another way is to bypass these carbon sources by using photosynthetic organisms and to use CO<sub>2</sub> as a carbon source for the production of SCL-MCL PHA copolymers. However, the lack of defined and usable pathways has limited the application of these methods for the economical production of PHAs. The current study represents a preliminary step to addressing these issues by defining a pathway for SCL-MCL

PHA copolymer production from nonrelated carbon sources via the fatty acid biosynthesis pathway.

As shown in previous studies, FabG enzymes can convert 3-ketoacyl-CoA to (*R*)-3-hydroxyacyl-CoA for PHA production in recombinant bacteria (18, 21, 24). However, the enzymes used in these studies produced copolymers of similar MCL content when coexpressed with type II PHA synthases and the polymers produced had little or no SCL content. Polymers with low mol% SCL composition fall into the class of elastomers, which have limited applications. It is much more desirable to produce an SCL-MCL PHA copolymer with a relatively high mol% (ca. 90 to 95%) of SCL monomer with a low mol% (ca. 5 to 10%) of MCL monomers randomly distributed throughout the copolymer because the properties are most similar to those necessary to produce bulk commodity plastics (1, 15). In addition, production of MCL monomers from the related carbon sources is dependent on the flux through the  $\beta$ -oxidation pathway and requires the use of organisms with fully functional  $\beta$ -oxidation pathways.

The use of the fatty acid biosynthesis pathway for the supply of MCL monomers from nonrelated carbon sources represents an alternative to MCL monomer production from the  $\beta$ -oxidation pathway (Fig. 1B). FabG and FabH enzymes are important components in the type II fatty acid biosynthetic (FAS II) pathway that is inherent in bacteria and plants. In our previous study (16), it was suggested that the FabG enzyme was involved in PHA monomer supply from nonrelated carbon sources via the fatty acid biosynthesis pathway (Fig. 1A). The current study demonstrated for the first time the enhancement of SCL-MCL PHA copolymer production from glucose by coexpression of either the *E. coli fabG* gene or the *Pseudomonas* sp. 61-3 *fabG* gene with the *fabH*(F87T) and PHA synthase genes. Furthermore, an SCL-MCL PHA copolymer isolated from recombinant *E. coli* coexpressing the *E. coli fabG*, *fabH*(F87T), and *phaCI*(STQK) genes had improved properties compared to the P(3HB) homopolymer. The yields of PHA from nonrelated carbon sources attained in this study were rather low (between 0.5 and 5.0% cellular dry weight). This is likely due to the low transacylase activity associated with FabH (28). Currently, techniques such as in vitro evolution, which was used to develop the highly active PHA synthases used in this study (13, 26), and rational design, which was used to develop the FabH(F87T) protein used in this study (16), are being applied to the PHA monomer-supplying enzymes in our laboratory and may lead to improved production of SCL-MCL PHA copolymers. In addition, we have shown that coexpression of *fabH*(F87T) and genetically modified PHA synthase genes with other monomer-supplying enzyme genes can increase the yield of SCL-MCL PHA produced in recombinant *E. coli* (17), strategies that are currently under investigation in our lab.

Interestingly, coexpression of *fabG* with *fabH*(F87T) and PHA synthase genes led to an increase in the mol% of C4 monomer incorporated into the copolymer. This is in marked contrast to the in vitro substrate specificity data. This phenomenon likely occurs because, although the FabH(F87T) protein is capable of enhancing MCL monomer supply for PHA production in recombinant *E. coli*, the majority of monomers produced by this protein are still SCL (Tables 2 through 4), since the ratio of SCL to MCL monomers produced by the

mutant FabH protein is still skewed toward SCL monomer production. This study also showed that the medium used (LB or M9) could dramatically influence the composition and yields of the polymers produced (Tables 2 through 4). Based on these results, changing the medium would allow for the production of polymers with specific monomer compositions, dependent upon the desired properties.

Although *E. coli* may not represent the best organism to produce SCL-MCL PHA, it is an excellent organism to look at potential metabolic pathways for use in PHA production because it is easy to manipulate genetically, it grows rapidly, and it has a defined metabolic background. These qualities have allowed us to establish a defined system to produce SCL-MCL PHA copolymers from the fatty acid biosynthesis pathway in *E. coli*. The importance of establishing a route of monomer supply from nonrelated carbon sources must not be overlooked. Because of the ubiquity of the fatty acid biosynthesis pathway in all organisms, the defined use of both FabH and FabG enzymes as SCL-MCL PHA monomer-supplying enzymes provides a model that may be used in many different types of recombinant organisms. This is important because it may be possible to transfer these genes into chloroplasts, which harbor the proteins for fatty acid biosynthesis in photosynthetic organisms, thus facilitating the production of SCL-MCL PHA copolymers from carbon dioxide. In addition, it may be possible to overexpress these genes in native PHA-producing bacteria (*Aeromonas* sp., *Ralstonia* sp., and *Pseudomonas* sp.) to enhance PHA production from nonrelated carbon sources. These studies are currently under way in our laboratory and may lead to the more economical production of SCL-MCL PHA copolymers.

The current study has shown that *E. coli* FabG and *Pseudomonas* sp. 61-3 FabG have different substrates *in vitro* (Fig. 2) and that expression of these enzymes clearly influences the type of monomers incorporated *in vivo* into an SCL-MCL PHA copolymer in recombinant *E. coli* (Table 2 through 4). The FabG proteins share high sequence homology, so the current findings indicate that subtle differences in the amino acid sequences of FabG proteins can dramatically alter the substrate specificities of the enzymes. It is known that FabG enzymes from different organisms have different substrate specificities (12), and it may be possible to design new metabolic pathways capable of producing SCL-MCL PHA copolymers with specific compositions by incorporating FabG proteins with different substrate specificities. The crystal structures of the *E. coli* FabG (19, 20) and *Mycobacterium tuberculosis* FabG (4) enzymes are known and may allow for the use of a rational design strategy for changing substrate specificity in a manner similar to that used with FabH (16). Examination of the primary amino acid sequence of the *M. tuberculosis* FabG protein reveals a unique sequence at the C terminus from amino acids 243 through 247 (MGMGH). This is a conserved sequence unique to ketoacyl reductases from *Mycobacterium* sp. and may correlate with the specificity toward larger substrates by the FabG enzymes from *Mycobacterium* species (12). The use of such an enzyme or genetically modified FabG enzymes based on the crystal structures of the proteins may result in the production of the specific monomers necessary to control the composition and thus the properties of SCL-MCL PHA copolymers.

Our conclusion is that FabG plays an important role in enhancing the yield and determining the monomer composition of the SCL-MCL PHA copolymers produced from non-related carbon sources. Because previous studies have established that FabG enzymes are capable of supplying monomers for SCL-MCL PHA copolymers from related carbon sources (18, 21, 24), it may be possible to look at different biochemical pathways in the cell to supply monomers for SCL-MCL PHA copolymer production based on carbon source and growth conditions of the bacteria. This flexibility in carbon source usage could allow for the production of polymers with specific material properties by changing the carbon source and/or enzyme combinations for PHA synthesis and represents another step toward the improved production of useful biodegradable polyesters.

#### ACKNOWLEDGMENTS

We thank H. Abe and K. Matsumoto for helpful discussions regarding this work.

This work was supported by a grant from Ecomolecular Science Research (RIKEN). C. T. Nomura is supported by a JSPS Fellowship for Foreign Researchers (Japan Society for the Promotion of Science).

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