FabG Mediates Polyhydroxyalkanoate Production from Both Related and Nonrelated Carbon Sources in Recombinant *Escherichia coli* LS5218

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Polyhydroxyalkanoates (PHAs) composed of a mixture of short-chain-length-medium-chain-length (SCL-MCL) hydroxyacyl monomers are biologically produced polyesters that have properties ranging from thermoplastic to elastomeric, dependent on the molar ratio of SCL to MCL monomers incorporated into the copolymer. Because of the potential wide range of properties and applications for SCL-MCL PHA copolymers, it is important to develop and characterize novel metabolic pathways for SCL-MCL PHA production. The current study shows that coexpression of *fabG* genes from either *E. coli* or *Pseudomonas* sp. 61-3 with *fabH*(F87T) and PHA synthase genes enhances the production of SCL-MCL PHA copolymer from both related and nonrelated carbon sources in *Escherichia coli* LS5218, indicating the flexibility of FabG as a monomer-supplying enzyme for biological PHA production.

Introduction

Polyhydroxyalkanoates (PHAs) are biodegradable polyesters that are produced by some bacteria grown under nutrient limitation in the presence of excess carbon that have attracted research interest because they can be used as biodegradable plastics (*1*, *2*). PHAs can be divided into three main types based on the lengths of the pendant groups of the monomer units incorporated into the polymer chain. Short-chain-length (SCL) PHA consists of monomers C3−C5 in length. Medium-chain-length (MCL) PHA consists of monomers C6−C14 in length, and SCL-MCL PHA copolymer consists of both SCL and MCL monomer units. Polymers composed of SCL subunits have thermoplastic properties, whereas polymers composed of MCL subunits have elastomeric properties. SCL-MCL PHA copolymers have qualities between those of the SCL and MCL PHA polymers depending on the ratio of SCL and MCL monomers and therefore may have a wide array of uses. Previous studies have shown that SCL-MCL PHA copolymer consisting of mainly 3-hydroxybutyrate (3HB) and a small molar percent of MCL monomer had properties similar to those of polypropylene (*3*). The SCL-MCL PHA copolymers have a wide array of desirable physical properties that could translate into commercially viable products (*4*). Although production of both SCL and MCL copolymers has been achieved in various recombinant bacteria (*1*), economically efficient production of SCL-MCL PHA copolymers from renewable biomass has remained difficult. The development of methods to produce PHAs from both nonrelated and related renewable carbon sources will be important for economical production of PHAs. Nonrelated carbon sources are dissimilar to the final PHA product and include substrates such as sugars. On the other hand, related carbon sources are similar in chemical structure to the final PHA product and include substrates such as fatty acids. Development and understanding of the metabolic pathways that process these carbon sources will be important for the design of recombinant organisms to have flexibility in their ability to produce PHAs.

Escherichia coli presents a model organism in which to study designed metabolic pathways to produce PHAs (*5*). Although *E. coli* is normally incapable of producing SCL-MCL PHA from nonrelated carbon sources, a previous study demonstrated that coexpression of mutant 3-ketoacyl acyl carrier protein synthase III genes (*fabH*) with PHA synthase genes (*phaC*) led to the production of SCL-MCL PHA in recombinant *E. coli* JM109 grown in the presence of excess glucose by intercepting intermediates from the fatty acid biosynthesis pathway (Figure 1A) via transacylation of the 3-ketoacyl-ACP monomers to the 3-ketoacyl-CoA substrates (*6*). Additional coexpression of 3-ketoacyl acyl carrier protein (ACP) reductases (FabG) enhanced the conversion of these 3-ketoacyl-CoA monomers to the (*R*)-3-hydroxyacyl-CoA monomers necessary for PHA production (*7*).

FabG is a member of the ketoacyl reductase (KAR) family of proteins and is an essential enzyme for type II fatty acid biosynthesis in bacteria. The natural reaction for FabG is the catalysis of the NADPH-dependent reduction of 3-ketoacyl-ACP to (*R*)-3-hydroxyacyl-ACP (*8*). In terms of PHA production, previous studies have demonstrated that coexpression of the *fabG* genes from *E. coli* and *Pseudomonas aeruginosa* with PHA synthase genes enabled recombinant *E. coli* to accumulate

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10.1021/bp070303y CCC: $40.75 © 2008 American Chemical Society and American Institute of Chemical Engineers
Published on Web 01/24/2008

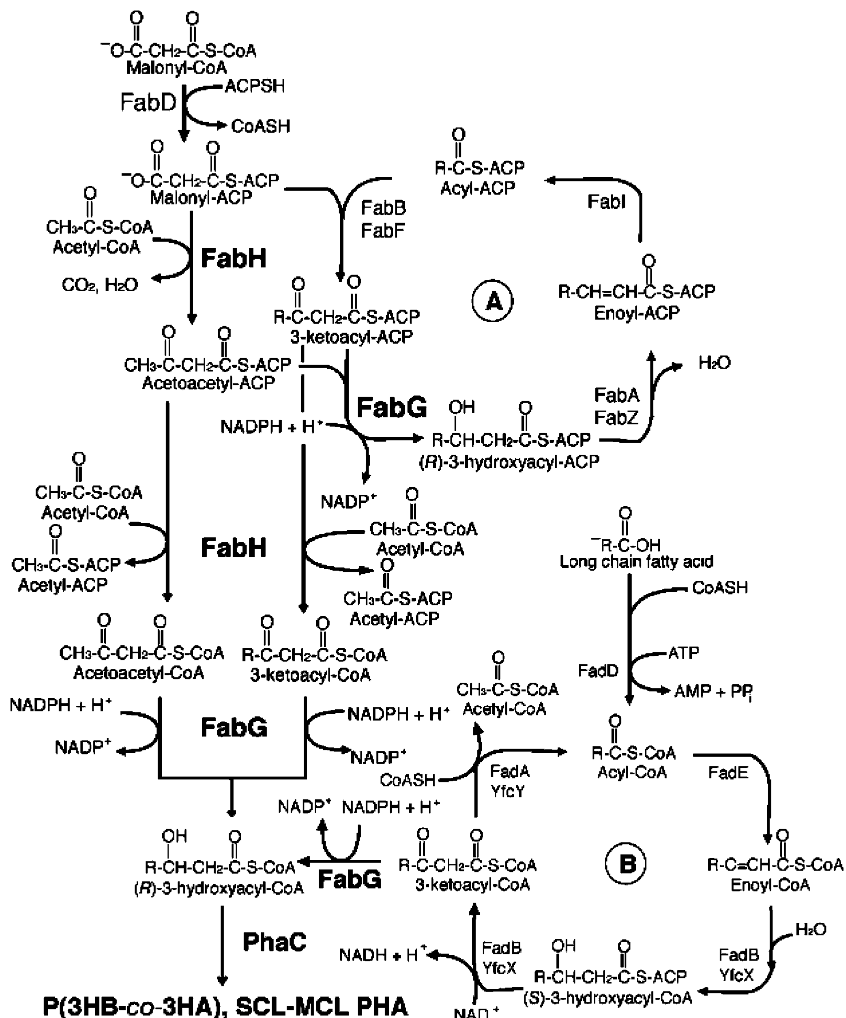

Figure 1. Proposed metabolic pathways for PHA production mediated by FabG in recombinant *E. coli* LS5218. (1) Fatty acid biosynthesis pathway. FabG acts as a reductase in both the fatty acid biosynthesis pathway and in PHA production. (2) β-oxidation pathway. FabG intercepts 3-ketoacyl-CoA intermediates from the β-oxidation pathway to produce (*R*)-3-hydroxyacyl-CoA monomers for PHA synthesis.

MCL PHA copolymer when grown in the presence of excess fatty acids such as decanoate or dodecanoate (*9*−*11*). In cells grown in the presence of fatty acids, FabG acts to intercept 3-ketoacyl-CoA intermediates from the β-oxidation pathway to produce (*R*)-3-hydroxyacyl-CoA substrates, which are subsequently incorporated into a PHA polymer by PHA synthase (Figure 1B). We previously cloned and characterized the *fabG* gene from *Pseudomonas* sp. 61-3 and found that this enzyme was capable of enhancing monomer supply for PHA production when coexpressed with engineered *fabH* genes and in vitro evolved PHA synthase genes (*7*). In the current study, the ability of both *Pseudomonas* sp. 61-3 and *E. coli* FabG proteins to enhance monomer supply for PHA biosynthesis from both the β-oxidation and the fatty acid biosynthesis pathways was investigated using the *fadR*-deficient *E. coli* strain LS5218. The current study presents the first evidence that coexpression of *fabG* genes with *fabH*(F87T) and PHA synthase genes leads to enhanced SCL-MCL PHA production from both related and nonrelated carbon substrates in recombinant *E. coli* LS5218.

Materials and Methods

Bacterial Strains, Plasmids, and Cultivation Conditions. The bacterial strains and plasmids used in this study are listed in Table 1. All transformations and DNA manipulations were carried out using *E. coli* JM109 (Promega, USA). For production of PHA, *E. coli* LS5218 was used as a host strain. For PHA production from nonrelated carbon sources, the strains were grown at 30 °C in either Luria−Bertani (LB) medium supplemented with glucose to a final concentration of 2 mg mL^{-1} or M9 medium supplemented with 0.001% thiamine and glucose to a final concentration of 2 mg mL^{-1}. The composition of M9 media has been described previously (*7*). For PHA production from related carbon sources, the strains were grown at 30 °C in M9 medium supplemented with 0.001% (v/v) thiamine and 0.25% (w/v) dodecanoate. For plasmid selection in recombinant *E. coli* strains, 100 μg of ampicillin and/or 50 μg of kanamycin were used as indicated.

Isolation, Analysis, and Manipulation of DNA. DNA sequences of plasmid constructs were confirmed by dye termination cycle sequencing using a Beckman Coulter CEQ 2000 sequencer (Beckman Coulter, USA). All other genetic techniques were performed as previously described (*12*).

PHA Production from Related and Nonrelated Carbon Sources 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 LS5218 *E. coli* cells. Transformants were isolated, and the presence of the pTrcFabH plasmid and a plasmid harboring *phaC1* and *fabG* was confirmed by restriction digest and PCR. Single colonies of confirmed transformants were cultured overnight in 1.75 mL of LB and used to inoculate 500-mL culture flasks with either

Table 1. Bacterial Strains and Plasmids Used in This Study

strain or plasmids	relevant characteristics	ref
E. coli LS5218	*fadR601, atoC*(Con)	15
pBBRC1	pBBR1MCS-2 derivative, *Pseudomonas* sp. 61-3 *phaC1*	7
pBBRSCQM	pBBR1MCS-2 derivative, *phaC1* (SCQM)	7
pBBRSTQK	pBBR1MCS-2 derivative, *phaC1* (STQK)	7
pBBRC1GEC	pBBR1MCS-2 derivative, *phaC1*, *E. coli fabG*	7
pBBRSCQMGEC	pBBR1MCS-2 derivative, *phaC1* (SCQM), *E. coli fabG*	7
pBBRSTQKGEC	pBBR1MCS-2 derivative, *phaC1* (STQK), *E. coli fabG*	7
pBBRC1GPS	pBBR1MCS-2 derivative, *phaC1*, *Pseudomonas* sp. 61-3 *fabG*	7
pBBRSCQMGPS	pBBR1MCS-2 derivative, *phaC1* (SCQM), *Pseudomonas* sp. 61-3 *fabG*	7
pBBRSTQKGPS	pBBR1MCS-2 derivative, *phaC1* (STQK), *Pseudomonas* sp. 61-3 *fabG*	7
pTrcFabH(F87T)	pTrc99A derivative; *fabH*(F87T), Amp^r	6

100 mL of LB or 100 mL of M9 medium supplemented with either glucose or dodecanoate as carbon sources. 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 media were incubated with constant shaking at 30 °C for an additional 3 h, at which time glucose was added to a final concentration of 0.2 g mL^{-1}. 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 (*6*).

Gel Permeation Chromatography (GPC) Analysis of PHA Polymers. Cell materials for GPC analysis were prepared and lyophilized as described for GC analysis except that a total of 2 L 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 polymer. The chloroform−polymer solution was filtered first through filter paper to remove cell debris and then through a 0.45 μm PTFE membrane to remove any residual solid materials. The chloroform was evaporated using a rotary vacuum evaporator, 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. This was repeated three times with the final wash step using hexane and then resuspended in chloroform once more. The polymer was again filtered through a 0.45 μm PTFE membrane, and the chloroform was evaporated at room temperature over 3 days in a fume hood, resulting in a gel-like solid. Molecular mass data of polyesters were obtained by GPC analysis using a Shimadzu 10A system with RID-10A refractive-index detector with serial columns of ShodexK806M and K802 as described previously (*13*).

Determination of PHA Polymer Composition by Nuclear Magnetic Resonance (NMR). Purified polymer (20 mg) isolated from *E. coli* LS5218 harboring pBBRSTQKGEC and pTrcFabH(F87T) was dissolved in 1 mL of $CDCl_3$ and subjected to both ^{1}H and ^{13}C NMR analysis. ^{1}H and ^{13}C NMR spectra were recorded using a Bruker BioSpin AVANCE 600 NMR spectrometer operating at 150 MHz as previously described (*14*).

Determination of Thermal Properties of SCL-MCL PHA Polymers Produced by Recombinant *E. coli*. The thermal data were recorded on a Perkin-Elmer Pyris 1 differential scanning calorimeter (DSC) equipped with a liquid nitrogen cooling accessory. Data was collected under a nitrogen flow of 20 mL min^{-1}. Melt-quenched polyester samples (ca. 6.2 mg) encapsulated in aluminum pans were heated from −50 to 200 °C at a rate of 20 °C min^{-1}, and the heat flow curves were recorded. The glass transition temperature (T_g) and melting temperatures (T_m) were determined from the position of the endothermic peaks.

Results

PHA Accumulation in Recombinant *E. coli* LS5218 from Glucose, a Nonrelated Carbon Source. *E. coli* LS5218 carries two significant mutations that have been shown to enhance PHA production (*15*). The first is the *fadR601* mutation. The *fadR* gene product encodes a transcriptional regulator protein that represses the transcription of genes necessary for long-chain fatty acid oxidation and the glyoxylate pathway and induces transcription of genes necessary for unsaturated fatty acid biosynthesis. Interruption of expression of *fadR* derepresses transcription of the genes involved in the oxidation of fatty acids while depressing the expression of enzymes involved in fatty acid biosynthesis. The second mutation of significance in *E. coli* LS5218 is the *atoC*(Con) mutation. The *atoC* gene encodes a protein that is a positive activator of genes necessary for the medium-chain fatty acid utilization (ATO) system in *E. coli*. The *atoC*(Con) mutation causes constitutive expression of the *atoC* gene; thus activation of the genes involved in medium-chain length fatty acid uptake and utilization in *E. coli*.

It was shown previously that overexpression of the *E. coli fabG* genes facilitated the production of monomers for PHA production via the β-oxidation pathway in *E. coli* LS5218 from related fatty acids (*9*). For the synthesis of the SCL-MCL PHA copolymer we utilized two highly active genetically engineered forms of the PHA synthase. Previously, our lab developed several highly active mutant PHA synthases via in vitro evolutionary engineering and saturation point mutagenesis (*16*). Two highly active mutant enzymes obtained from that study were used in this study to enhance 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 changed amino acid 325 of the *Pseudomonas* sp. 61-3 PhaC1 enzyme from Ser to Thr and a secondary point mutation changed the Gln at position 481 to Lys. The mutations at ST/QK 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 (*17*). The second mutant PHA synthase, PhaC1(SCQM), also has two point mutations; one changed the Ser at position 325 to Cys and the second mutation changed the Gln at position 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 (*16*). Furthermore, these mutants also possessed the capacity to produce SCL-MCL PHA copolymer. In the current study, *E. coli* LS5218 transformed with *E. coli fabH*(F87T), one of the three PHA synthase genes [wild-type *Pseudomonas* sp. 61-3 *phaC1, phaC1*(STQK), or *phaC1*(SCQM)] and either *EcfabG* or *PsfabG* were used to examine the effects of the *fadR* and *ato*(Con) mutations on PHA production from glucose.

The results are shown in Table 2. No PHA production was detected in any of the strains expressing just a PHA synthase gene [wild-type *phaC1, phaC1*(STQK), *phaC1*(SCQM)]. Co-

Table 2. Effects of Coexpression of *fabG, fabH*(F87T), and PHA Synthase Genes on PHA Production in Recombinant LS5218 *E. coli* Cells Grown in LB with Glucose[a]

relevant genes	CDW (g L^{-1})	PHA content (wt % of CDW)[b]	PHA composition (mol %)[c] 3HB (C4)	3HHx (C6)	3HO (C8)	3HD (C10)	3HDD (C12)
phaC1 (wild-type)	2.3 ± 0.1	ND[d]					
phaC1 (wild-type), *EcfabG*	2.3 ± 0.05	0.18 ± 0.07	100				
phaC1 (wild-type), *PsfabG*	2.6 ± 0.02	ND					
phaC1 (wild-type), *EcfabG, EcfabH* (F87T)	3.5 ± 0.2	0.10 ± 0.01	86.7 ± 2.0	13.3 ± 2.0			
phaC1 (wild-type), *PsfabG, EcfabH* (F87T)	3.1 ± 0.2	0.02 ± 0.01	100				
phaC1(STQK)	2.3 ± 0.1	ND					
phaC1(STQK), *EcfabH*(F87T)	2.8 ± 0.1	0.31 ± 0.03	100				
phaC1(STQK), *EcfabG*	3.3 ± 0.3	12.3 ± 1.0	99.5 ± 0.01	0.5 ± 0.01			
phaC1(STQK), *EcfabG, EcfabH*(F87T)	3.9 ± 0.3	3.6 ± 1.8	97.8 ± 0.8	2.2 ± 0.8			
phaC1(STQK), *PsfabG*	3.2 ± 0.2	10.4 ± 1.4	99.4 ± 0.07	0.6 ± 0.07			
phaC1(STQK), *PsfabG, EcfabH*(F87T)	3.3 ± 0.01	1.1 ± 0.1	94.2 ± 0.01	3.5 ± 0.2	0.8 ± 0.02	0.9 ± 0.1	0.6 ± 0.04
phaC1(SCQM)	2.3 ± 0.1	ND					
phaC1(SCQM), *EcfabH*(F87T)	3.1 ± 0.07	0.17 ± 0.02	100				
phaC1(SCQM), *EcfabG*	2.9 ± 0.1	2.0 ± 0.1	100				
phaC1(SCQM), *EcfabG, EcfabH*(F87T)	3.5 ± 0.1	2.0 ± 0.1	99.6 ± 0.7	0.4 ± 0.7			
phaC1(SCQM), *PsfabG*	2.8 ± 0.03	2.7 ± 0.09	99.0 ± 0.04	1.0 ± 0.04			
phaC1(SCQM), *PsfabG, EcfabH*(F87T)	3.5 ± 0.2	0.24 ± 0.1	95.7 ± 4.1	3.8 ± 3.5	0.8 ± 0.9		

[a] Cells were cultivated in LB medium. IPTG and 2 g of glucose mL^{-1} 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. [b] CDW, cell dry weight. [c] 3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctonoate; 3HD, 3-hydroxydecanoate; 3HDD, 3-hydroxydodecanoate. [d] ND, not detected.

expression of the wild-type *phaC1* and *EcfabG* genes led to a low level of accumulation (0.18% of cell dry weight) of poly-3-hydroxybutyrate [P(3HB)] homopolymer. The additional expression of *EcfabH*(F87T) with *phaC1* and *EcfabG* resulted in PHA accumulation to 0.1% of the cell dry weight. However, in this strain, the composition of the PHA shifted to form a SCL-MCL copolymer composed of 87 mol % C4 and 13 mol % C6 (Table 2). Coexpression of the *phaC1* and *EcfabH*(F87T) genes with the *PsfabG* gene led to low levels of P(3HB) production. Expression of the wild-type *phaC1* and *PsfabG* genes failed to accumulate detectable levels of PHA (Table 2).

Although LS5218 strains coexpressing the *phaC1*(SCQM) gene with *EcfabH*(F87T) and *fabG* genes led to increases in the total amount of PHA accumulated compared to LS5218 strains expressing the wild-type *phaC1* gene with the fatty acid biosynthesis genes (Table 2), LS5218 strains coexpressing the *phaC1*(STQK) gene with the *fabG* genes led to dramatic increases (~68-fold) in the amount of PHA accumulated compared to LS5218 strains expressing the wild-type *phaC1* gene. There was also a dramatic shift in the PHA composition of all of the recombinant LS5218 strains toward the incorporation of the C4 monomer into SCL-MCL PHA copolymer when compared to the composition of PHA accumulated in the recombinant JM109 strains from previous studies (*6*, *7*, *18*).

Interestingly, the coexpression of *fabH*(F87T) with either the STQK or SCQM PHA synthase produced only P(3HB). Coexpression of *fabH*(F87T), either the *EcfabG* or *PsfabG* gene, and one of the mutant synthases resulted in the production of SCL-MCL PHA production (Table 2). However, the amount of total polymer accumulated in these strains was less than that accumulated in strains coexpressing either of the *fabG* genes with a mutant PHA synthase. These results indicate that both the type of monomer-supplying enzyme and the PHA synthase will affect polymer production in *E. coli* LS5218 and that the *fadR601* and *ato*(Con) mutations can affect the pathways for monomer incorporation into SCL-MCL PHA production, and thus, can significantly affect the composition of the polymers produced.

Effect of Media Composition on PHA Production in *E. coli* LS5218. Because LB is an undefined medium, it was difficult to discern whether all of the PHA produced by the recombinant *E. coli* strains was a result of the glucose addition to the media 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 *fabG, fabH*(F87T) and either the *phaC1*(STQK) or *phaC1*(SCQM) genes were capable of producing SCL-MCL PHA copolymer in both *E. coli* JM109 and LS5218 directly from glucose as the sole carbon source. The results are shown in Table 3. The wild-type *phaC1* expression plasmid was not used in this experiment because of the low amount of PHA accumulated in strains harboring plasmids expressing this gene compared with strains expressing the highly active genetically engineered PHA synthase genes (Tables 2 and 3).

The dry cell weights were much lower for all of the strains grown in M9 compared with cells grown in LB (Tables 2 and 3). For the recombinant LS5218 strains harboring plasmids expressing either *phaC1*(STQK) or *phaC1*(SCQM), a small amount of P(3HB) was accumulated. This is likely due to the constitutively active β-oxidation pathway supplying intermediates for PHA production in conjunction with native levels of *fabG* in *E. coli* (Figure 1). Recombinant *E. coli* LS5218 strains expressing the *phaC1*(STQK) or *phaC1*(SCQM) gene with *fabH*(F87T) were also able to accumulate low amounts of P(3HB) homopolymers. Overexpression of either *EcfabG* or *PsfabG* with either *phaC1*(STQK) or *phaC1*(SCQM) led to dramatic increases in P(3HB) production in the recombinant LS5218 strains compared to the recombinant JM109 strains overexpressing the same genes (*7*). Coexpression of *fabH*(F87T) with either *EcfabG* or *PsfabG* and *phaC1*(STQK) or *phaC1*-(SCQM) led to a slight decrease in overall PHA production compared to strains expressing *fabG* and PHA synthase genes but led to the production of SCL-MCL PHA copolymer consisting of C4, C6, and C8 monomers (Table 3). These results indicate that the genotype of the strain type can dramatically affect the type of polymer produced by the recombinant bacteria. Furthermore, these results demonstrate that the overproduction of fatty acid biosynthesis enzymes with engineered PHA synthases enhances the production of SCL and MCL monomers for SCL-MCL PHA copolymer production from glucose in various *E. coli* strains.

Table 3. PHA Accumulation in Recombinant *E. coli* LS5218 Cells Grown in M9 Media with Glucose as the Sole Carbon Source

relevant genes	CDW (g L^{-1})	PHA content (wt % of CDW)[b]	PHA composition (mol %)[c] 3HB (C4)	3HHx (C6)	3HO (C8)
phaC1(STQK)	0.61 ± 0.02	0.11 ± 0.003	100		
phaC1(STQK), *EcfabH*(F87T)	0.39 ± 0.02	1.5 ± 0.5	100		
phaC1(STQK), *EcfabG*	0.57 ± 0.2	5.1 ± 1.0	100		
phaC1(STQK), *PsfabG*	0.68 ± 0.03	4.8 ± 0.8	100		
phaC1(STQK), *EcfabG, EcfabH*(F87T)	0.43 ± 0.004	0.7 ± 0.08	96.1 ± 1.8	3.9 ± 0.6	
phaC1(STQK), *PsfabG, EcfabH*(F87T)	0.45 ± 0.05	0.3 ± 0.05	97.4 ± 2.2	2.6 ± 2.2	
phaC1(SCQM)	0.62 ± 0.02	0.30 ± 0.05	100		
phaC1(SCQM), *EcfabH*(F87T)	0.34 ± 0.03	0.13 ± 0.03	100		
phaC1(SCQM), *EcfabG*	0.65 ± 0.04	3.7 ± 1.6	99.5 ± 0.3	0.3 ± 0.5	0.2 ± 0.2
phaC1(SCQM), *PsfabG*	0.66 ± 0.02	3.5 ± 0.9	100		
phaC1(SCQM), *EcfabG, EcfabH*(F87T)	0.44 ± 0.01	0.29 ± 0.08	95.7 ± 4.2	3.3 ± 2.9	1.0 ± 0.6
phaC1(SCQM), *PsfabG, EcfabH*(F87T)	0.43 ± 0.02	0.14 ± 0.01	100		

[a] Cells were cultivated in M9 medium with 2 g of glucose mL^{-1}. 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. [b] CDW, cell dry weight. [c] 3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate, 3HO, 3-hydroxyoctonoate; 3HD, 3-hydroxydecanoate; 3HDD, 3-hydroxydodecanoate. [d] ND, not detected.

PHA Production from Related Carbon Sources. It was shown previously that overexpression of the *E. coli fabG* genes facilitated the production of monomers for PHA production via the β-oxidation pathway in *E. coli* LS5218 from fatty acids (*9*). To determine the effect of *fabG* expression on monomer supply solely from the β-oxidation pathway, recombinant *E. coli* LS5218 harboring plasmids expressing either *E. coli fabG* or *Pseudomonas* sp. 61-3 *fabG* with a PHA synthase gene were grown in the presence of dodecanoate as the sole carbon source. The PHA content and composition were assessed by GC analysis. The results are shown in Table 4. Strains harboring the highly active mutant PHA synthases accumulated more PHA than strains expressing the wild-type PHA synthase. The strain expressing the *phaC1*(STQK) gene with the *Pseudomonas* sp. 61-3 *fabG* gene produced a copolymer consisting of 25.5 mol % C4, 20.7 mol % C6, 28.6 mol % C8, 17.4 mol % C10, and 7.3 mol % C12 monomers as determined by GC analysis (Table 4). All combinations of *E. coli* LS5218 harboring engineered PHA synthase genes with *fabG* produced PHA copolymers with similar monomer compositions, whereas the strain expressing the wild-type *phaC1* produced a PHA copolymer with lower mol % C4 (Table 4). These results indicate that the type of PHA synthase can greatly influence the type of SCL-MCL PHA copolymer produced by the cells. However, comparison of these results with the results obtained from strains harboring either *phaC1*(STQK) or *phaC1*(SCQM) with *E. coli fabG* clearly show that the type of *fabG* expressed during SCL-MCL PHA copolymer composition greatly influenced the copolymer monomer composition since those strains expressing the *E. coli fabG* gene had consistently higher molar concentrations of C8, C10, and C12 monomers as compared to strains expressing the *Pseudomonas* sp. 61-3 *fabG* gene. The results also indicate that both the type of *fabG* and the PHA synthase gene expressed can affect the SCL-MCL PHA monomer composition.

Physical Characterization of SCL-MCL PHA Copolymer Isolated from a Related Carbon Source (Dodecanoate) in Recombinant *E. coli* LS5218. Polymer was isolated from an LS5218 strain harboring the pTrcFabH(F87T) and pBBRSTQKGEC plasmids as described in Materials and Methods. This polymer was chosen as a representative polymer produced by intercepting intermediates from the β-oxidation pathway with FabG for conversion to 3-hydroxyacyl intermediates followed by polymerization to PHA with a mutant PHA synthase. The isolated polymer was characterized by NMR, GPC, and DSC. 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 ^{1}H NMR spectra (Figure 2). Supporting information for tertiary (C8, C10, and C12) monomer units was obtained by ^{13}C NMR analysis. As shown in Figure 3, the ^{13}C NMR spectrum was used to show that the polymer was a random copolymer rather than a blend of polymers. Figure 3 also shows the chemical shift assignments for each of the carbonyl resonances. In a previous study by Shimamura et al. (*19*), the degree of randomness for P(3HB-*co*-3HHx) was determined on the basis of peak intensities for four carbonyl resonance lines (i.e., 3HB monomer next to 3HB, 3HB monomer next to a 3-hydroxyacyl (3HA) monomer, 3HA monomer next to a 3HB monomer, and 3HA monomer next to 3HA monomer). The randomness value could be estimated using this method since the 3HA resonance line adjacent to the 3HB resonance was clearly observed. We also compared the experimentally obtained spectra and simulated spectra in which random distribution was assumed (*20*). The results indicated that the polymer isolated from recombinant *E. coli* LS5218 grown on dodecanoate was a random copolymer.

The weight-average molecular weight (M_w) of the polymer was determined by GPC, which revealed that the M_w of the SCL-MCL PHA copolymer was slightly lower (1.2×10^5) than that of P(3HB) homopolymer (4.4×10^5), but that the polydispersity index (M_w/M_n) was closer to 1.0 for the SCL-MCL PHA copolymer as compared to the P(3HB) homopolymer (Table 3). This result indicates that the distribution of the polymers isolated from each strain were similar despite the difference in the overall molecular weights.

The thermal properties of the SCL-MCL PHA copolymer isolated from recombinant *E. coli* harboring the pTrcFabH-(F87T) and pBBRSTQKGEC plasmids were determined by DSC analysis and compared with the thermal properties of P(3HB) homopolymer. All data were determined from the endotherm profiles (Figure 3). For the SCL-MCL PHA copolymer, two melting temperature peaks were observed as opposed to the single melting temperature peak observed for P(3HB) homopolymer. No clear peak from crystallization was observed for the SCL-MCL PHA copolymer, although two peaks at 36 and 47 °C, respectively, were observed indicating molecular rearrangements of the polymer. The thermal properties for each polymer are also summarized in Table 5.

As determined by GC and NMR analysis, the SCL-MCL PHA copolymer isolated from the recombinant *E. coli* LS5218 strain harboring pTrcFabH(F87T) and pBBRSTQKGEC plas-

Table 4. PHA Accumulation from Dodecanoate in Recombinant LS5218 *E. coli* Cells Expressing *fabG* and PHA Synthase Genes in Recombinant LS5218 *E. coli* Cells[a]

relevant genes, media	CDW ($g\ L^{-1}$)	PHA content (wt % of CDW)[b]	PHA composition (mol %)[c] 3HB (C4)	3HHx (C6)	3HO (C8)	3HD (C10)	3HDD (C12)
phaC1(STQK), *PsfabG,* M9	0.8 ± 0.02	14.8 ± 0.5	25.5 ± 2.5	20.7 ± 0.9	28.6 ± 1.1	17.4 ± 2.0	7.3 ± 0.4
phaC1(SCQM), *PsfabG,* M9	0.6 ± 0.02	12.6 ± 1.8	25.3 ± 3.5	20.1 ± 0.9	28.5 ± 1.0	18.7 ± 1.1	7.2 ± 1.3
phaC1(wild-type), *PsfabG,* M9	0.5 ± 0.05	10.1 ± 0.1	11.4 ± 1.5	16.4 ± 0.2	47.5 ± 1.0	16.4 ± 0.3	8.4 ± 0.3
phaC1(STQK), *EcfabG,* M9	0.6 ± 0.06	14.7 ± 3.5	24.4 ± 0.3	21.3 ± 0.2	29.0 ± 0.3	17.3 ± 0.1	8.0 ± 0.2
phaC1(SCQM), *EcfabG,* M9	0.6 ± 0.05	10.3 ± 2.1	21.7 ± 0.5	19.7 ± 0.3	32.8 ± 0.1	18.2 ± 0.4	7.6 ± 0.3
phaC1(wild-type), *EcfabG,* M9	0.7 ± 0.08	10.5 ± 1.5	7.4 ± 1.7	21.7 ± 1.3	38.4 ± 2.9	22.1 ± 1.6	10.4 ± 0.8

[a] Cells were cultivated in M9 medium with 0.25 g L^{-1} of dodecanoate as a carbon source. 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. [b] CDW, cell dry weight. [c] 3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate, 3HO, 3-hydroxyoctonoate; 3HD, 3-hydroxydecanoate; 3HDD, 3-hydroxydodecanoate.

Table 5. Physical Characteristics of SCL-MCL PHA Copolymer Produced Using FabG Enzymes as Monomer Suppliers[a]

relevant genes/source	PHA composition (mol %)[b] 3HB (C4)	3HHx (C6)	3HO (C8)	3HD (C10)	3HDD (C12)	molecular weights[c] M_w ($\times 10^5$)	M_w/M_n	thermal properties[d] T_g (°C)	ΔC_P (J/g)	T_c (°C)	T_m (°C)	ΔH_m (J/g)
R. eutropha P(3HB)	100	ND[e]	ND	ND	ND	4.4	2.7	4	0.7	51	171	52
PhaC1(STQK), *PsfabG*	25.3	20.1	28.5	18.7	7.2	1.2	1.9	−42	0.2		36, 47	0.3

[a] Recombinant *E.coli* LS5218 was cultivated in M9 medium supplemented with 0.2 g L^{-1} dodecanoate as a carbon source. [b] PHA composition determined by GC analysis. 3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanote; 3HO, 3-hydroxyoctanoate; 3HD, 3-hydroxydecanoate. [c] M_n, number-average molecular weight; M_w/M_n, polydispersity index. [d] T_g, glass transition temperature; T_c, crystallization temperature; T_m, melting temperature; ΔH_m, enthalpy of fusion. [e] ND, not detectable.

mids consisted of polymer composed of 25.3 mol % 3HB, 20.1 mol % 3HHx, 28.5 mol % 3HO, 18.7 mol % C10, and 7.2 mol % C12 monomer units. The addition of MCL-monomer units to the PHA copolymer lowered the melting temperature to 47 from 170 °C compared to the P(3HB) homopolymer sample (Table 5). The glass transition temperature (T_g) was also dramatically affected, decreasing from 4 to −42 °C. In addition, the enthalpy of fusion was lowered to 0.3 J/g compared to 52 J/g for P(3HB) homopolymer. These results indicate that the polymer is a SCL-MCL PHA copolymer and that the additional pendant groups within the copolyester dramatically alter the thermal properties of the polymer. These differences in thermal properties may translate to specialized physical properties, leading to the production of biodegradable polymers with a wider array of applications.

Discussion

Previous studies have used several enzyme combinations to produce PHA polymers and copolymers from both related and nonrelated carbon sources (*1*, *21*, *22*). Unlike P(3HB) homopolymer, a stiff and brittle thermoplastic, SCL-MCL PHA copolymers with a high molar percent of 3HB monomer along with a low molar percent of MCL monomer have properties similar to those of polypropylene (*3*). As shown in the study by Fukui et al., an addition of a small amount of MCL monomer units (1.5 mol %) can have a dramatic effect on the melting temperature (*23*). The MCL repeating units are concentrated at the interface region between the crystalline and amorphous phases during the crystallization process, causing unstable and irregular surfaces on the polymer. As a result, the incorporation of even a small molar percent of MCL monomer into a SCL-MCL PHA copolymer will induce a decrease in observed melting temperature (*23*).

Recent studies by Iwata et al. have shown that P(3HB) stretched films could be made with markedly improved mechanical properties as compared to unstretched films, indicating that with further improvement of processing techniques even a SCL P(3HB) homopolymer could have commercial potential (*24*−*26*). Thermal degradation of P(3HB) near its melting temperature (175 °C) presents a major problem in processing. The incorporation of a small amount of MCL repeating units may be useful, since this incorporation will lower the melting temperature away from the degradation temperature.

Therefore, it is of special interest to develop pathways and enzymes for the production of SCL-MCL PHA copolymers with various MCL monomer compositions. In this study, strains of recombinant *E. coli* LS5218 were used to accumulate SCL-MCL PHA copolymers of different monomer compositions grown in the presence of both related and nonrelated carbon sources (Tables 2 and 3). The results of this study clearly show that the genetic background of the recombinant strain used could directly influence modifications of polymer monomer compositions. *E. coli* LS5218 was originally used for the production of poly-3-hydroxybutyrate-*co*-3-hydroxyvalerate copolymer from glucose and propionate (*15*). This study has shown that the coexpression of either *E. coli* or *Pseudomonas* sp. 61-3 *fabG* genes with *phaC1* enabled *E. coli* LS5218 strain to produce SCL-MCL PHA copolymers from glucose or dodecanoate as a carbon source. For supply of monomers from the β-oxidation pathway, enzymes such as enoyl-CoA hydratase (PhaJ) (*27*) or ketoacyl reductase (FabG) can be used (Figure 1B). Interestingly, expression of the highly active PHA synthase mutants alone in LS5218 leads to the production of a very low amount of P(3HB) accumulation from glucose. This may be due to the constitutive level expression of the β-oxidation pathway in addition to the normal expression of *E. coli fabG* in the cell. It may also be caused by the overproduction of enzymes such as FadB or YfcX, which have been implicated in the production of PHA production from the β-oxidation pathway (*28*).

The 3-ketoacyl-acyl carrier protein reductase (FabG) and 3-ketoacyl-ACP synthase III (FabH) enzymes are important enzymes in the type II fatty acid biosynthetic (FAS II) pathway that is inherent in bacteria and plants (Figure 1A). FabG catalyzes the reduction of 3-ketoacyl-ACP substrates to (*R*)-3-hydroxyacyl-ACP for fatty acid biosynthesis (Figure 1A). However, FabG was found in previous studies to convert 3-ketoacyl-CoA to (*R*)-3-hydroxyacyl-CoA for PHA production in recombinant bacteria (*9*−*11*). These studies were limited to

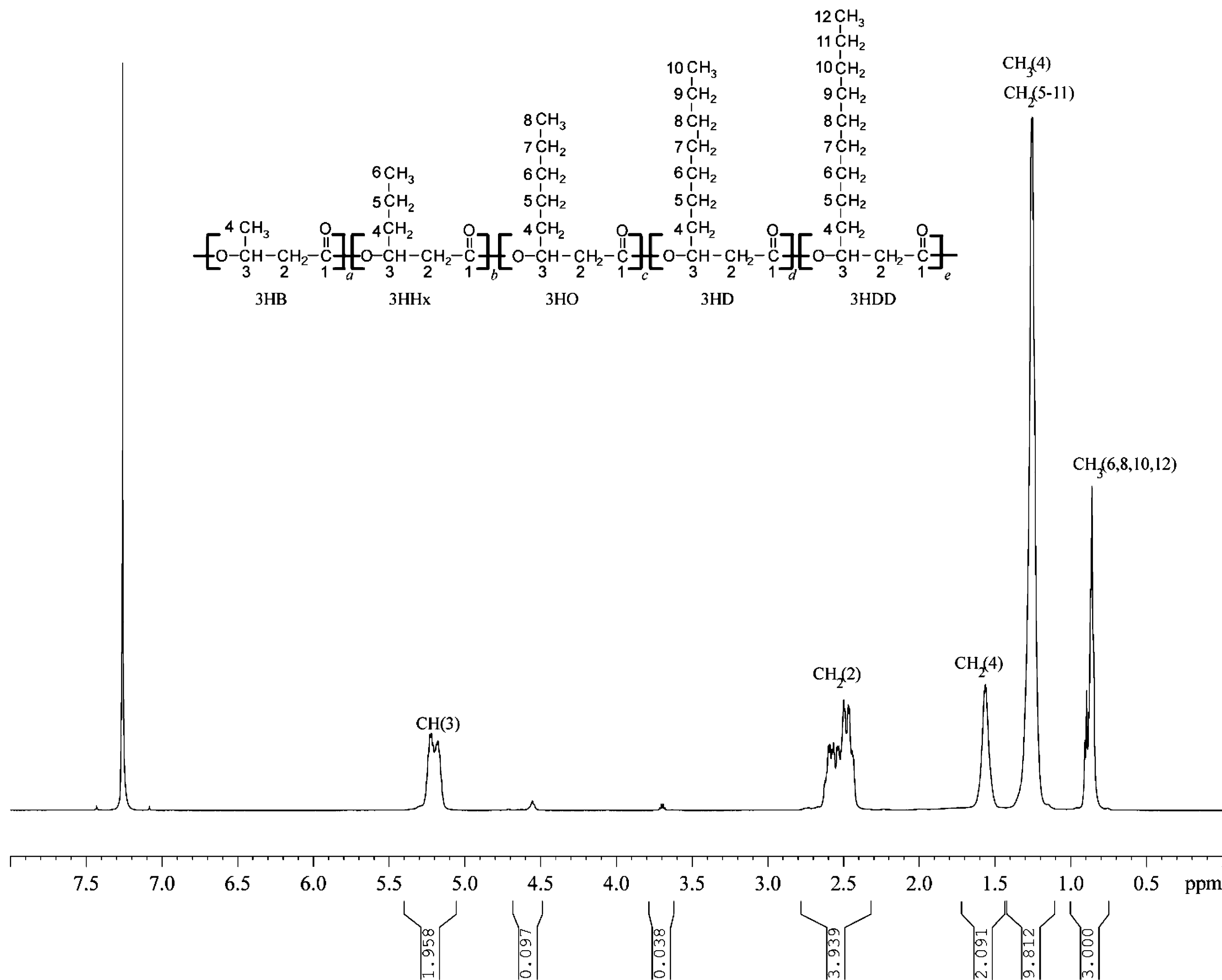

Figure 2. ^{1}H NMR spectra of SCL-MCL PHA copolymer isolated from *E. coli* LS5218 cultivated on dodecanoate: 3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate; 3HD, 3-hydroxydecanoate; 3HDD, 3-hydroxydodecanote. Chemical shifts for methine, methyline, and methyl groups are indicated.

the production of PHA from related carbon sources (fatty acids) and utilized the β-oxidation pathway (Figure 1B). In a previous study (*7*), it was shown that the FabG enzyme could also be used in PHA monomer supply from nonrelated carbon sources via the fatty acid biosynthetic pathway in *E. coli* JM109 (Figure 1A), through which intermediates from the fatty acid biosynthesis pathway would be first converted to 3-ketoacyl-CoA forms from 3-ketoacyl-acyl-carrier-protein forms by engineered FabH enzymes and that these 3-ketoacyl-CoA substrates would be converted to 3-hydroxyacyl substrates by FabG. The current study demonstrated for the first time, the enhancement of SCL-MCL PHA copolymer production from both related and nonrelated carbon sources 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 in *E. coli* LS5218. *E. coli* LS5218 has a constitutively active β-oxidation pathway and the constant production of enzymes in this pathway coupled with the coexpression of FabG, FabH and PHA synthase genes results in the production of PHA copolymers with different ratios of PHA comonomers.

Because FabG enzymes from different organisms have different substrate specificities (*29*), it may be possible to design new metabolic pathways in *E. coli* capable of producing SCL-MCL PHA copolymers with specific compositions if coexpressed with engineered *phaC1* and *fabH* genes. The *E. coli* FabG protein has a broad substrate specificity but was shown to have slightly higher substrate specificity for the longer MCL monomers (*7*). The *Mycobacterium tuberculosis* FabG enzyme recognizes much longer chain length substrates and thus could be used to supply longer chain length monomers (*30*). The crystal structures of the *E. coli* FabG (*31*, *32*) and *M. tuberculosis* FabG (*30*) enzymes are known and may allow for the rational design of a PHA monomer-supplying enzyme in a manner similar to that taken with FabH (*6*). Therefore, through specific point mutations and gene shuffling experiments, it may be possible to rationally design enzymes with different substrate specificities for PHA polymer production. In addition, our lab has used in vitro evolutionary systems to engineer the PHA synthases with new specific activities used in this study (*16*, *18*, *33*). This in vitro evolutionary system can be used to evolve monomer-supplying enzymes such as *fabG* for increased PHA production and/or new substrate specificities. Such experiments are currently underway.

Other Factors Influencing Monomer Supply in PHA Production. Although engineered enzymes are an important aspect of SCL-MCL PHA copolymer production, the type of strain used for PHA production is also important. The major difference between these *E. coli* JM109 and LS5218 strains is

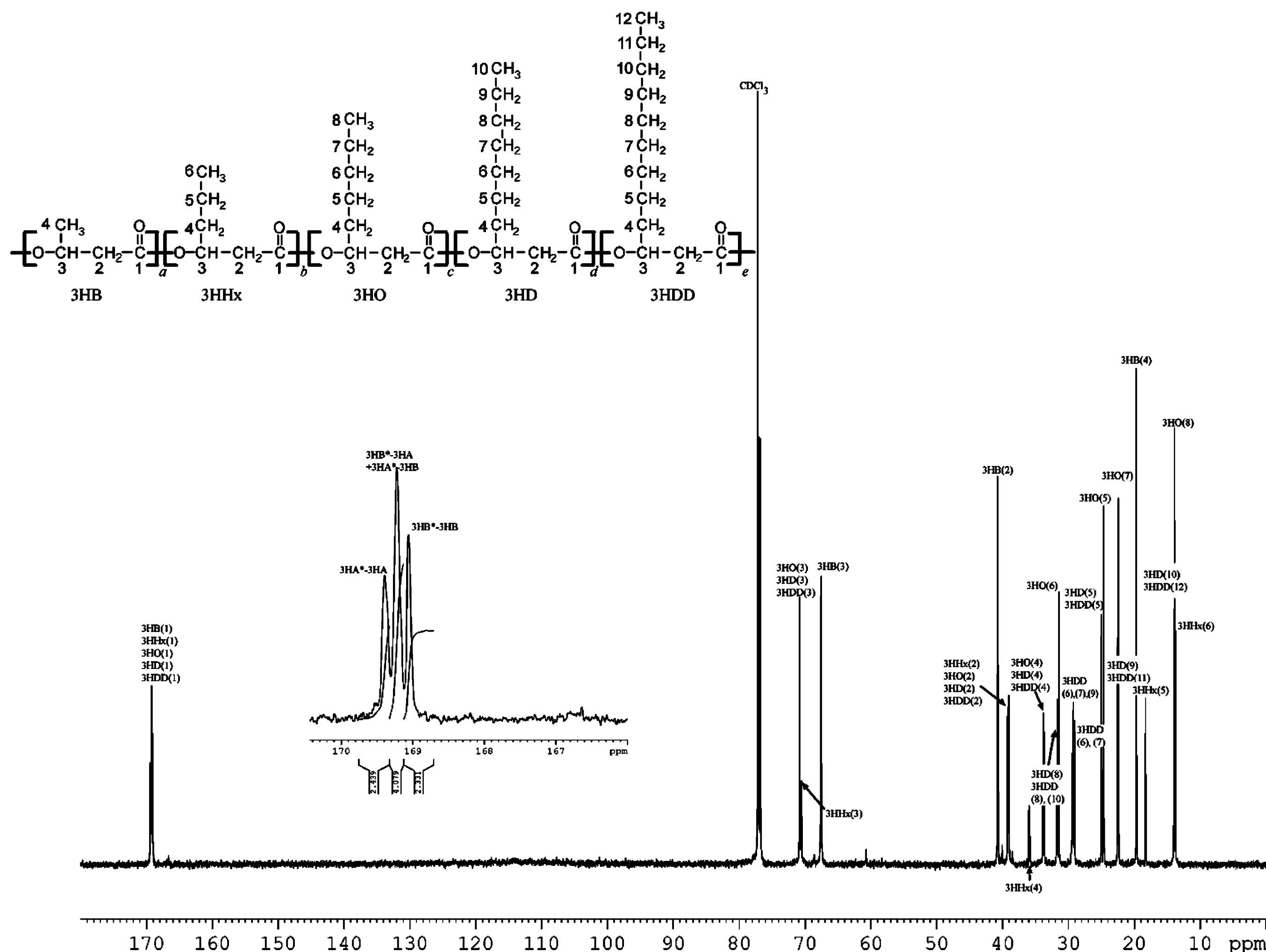

Figure 3. ^{13}C NMR spectra of PHA isolated from *E. coli* LS5218 cultivated on dodecanoate with expanded carbonyl carbon resonances: 3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate; 3HD, 3-hydroxydecanoate; 3HDD, 3-hydroxydodecanote. 3HA denotes 3-hydroxyalkanoates containing from 6 to 12 carbon atoms. The assignments of the carbon atoms are indicated.

the constitutive expression of the genes involved in the β-oxidation of fatty acids (Figure 4D). In LS5218, the coexpression of *fabG* genes alone with *phaC1* genes led to production of the highest levels of (12−13% of cell dry weight) of PHA that was much higher than that of JM109 (0.02−0.14% of cell dry weight). In LS5218, a large pool of acetyl-CoA produced by glycolysis combined with the overproduction of FadA and YfcY enzymes may result in a reversal of the β-oxidation pathway, converting acetyl-CoA to acetoacetyl-CoA. In the presence of FabG, acetoacetyl-CoA is converted to 3HB-CoA, which is easily converted to P(3HB) in the presence of a PHA synthase, thus explaining the increased PHA production from glucose in LS5218 strains coexpressing *fabG* and *phaC1* genes as compared to JM109 strains coexpressing the same genes.

In addition, In JM109, introduction of *fabH* and *fabG* both positively affected PHA production (*7*). However, in LS5218, the coexpression of these genes with PHA synthase decreased overall PHA content as compared to expression of *fabG* with any of the PHA synthase genes (Tables 2 and 3), although overexpression of these genes resulted in copolymer production from a nonrelated carbon source. The decrease in SCL-MCL PHA production was due to the competitive metabolic pathways for the 3-ketoacyl-CoA PHA precursors (Figure 4A,B). The overproduction of FabH(F87T) increased the production of 3-ketoacyl-CoA, which accounts for the broad range substrate production (Figure 4C). However, 3-ketoacyl-CoA is a substrate for the β-oxidation pathway, and so there is a competition between the synthesis of *R*-3HA-CoA mediated by FabG and the breakdown of 3-ketoacyl-CoA by the β-oxidation pathway (Figure 4). In JM109, expression of the genes involved in the β-oxidation is regulated, so that the 3-ketoacyl-CoA intermediates generated by FabH and *R*-3-HA-CoA intermediates generated by FabG could accumulate for use as MCL PHA monomers (Figure 4C) thus, in JM109 overproduction of FabH and FabG with a PHA synthase increased PHA content (*7*). On the other hand, in LS5218, the constitutive expression and use of fatty acid intermediates should lead to a decrease in SCL-MCL PHA content, since intermediates generated by the overproduction of FabH(F87T) and FabG can be used by several other competitive pathways. First, overexpression of the *fabH* and *fabG* genes causes increased flux through the fatty acid biosynthetic pathway (Figure 4AB) as compared to the overexpression of *fabG* alone. This diverts acetyl-CoA away from the path shown in Figure 4D where the overproduced FadA catalyzes the conversion of acetyl-CoA to acetoacetyl-CoA, which is then converted to the 3HB-CoA substrate for P(3HB) production. When *fabH* and *fabG* genes are coexpressed in LS5218, this again increases flux through the fatty acid biosynthetic pathway (Figure 4A,B) although 3-ketoacyl-CoA could be made, the constitutive expression of the β-oxidation pathway enzymes leads to yet another competitive pathway (Figure 4C), thus depleting the amount of *R*-3HA-CoA for PHA biosynthesis (Figure 4C).

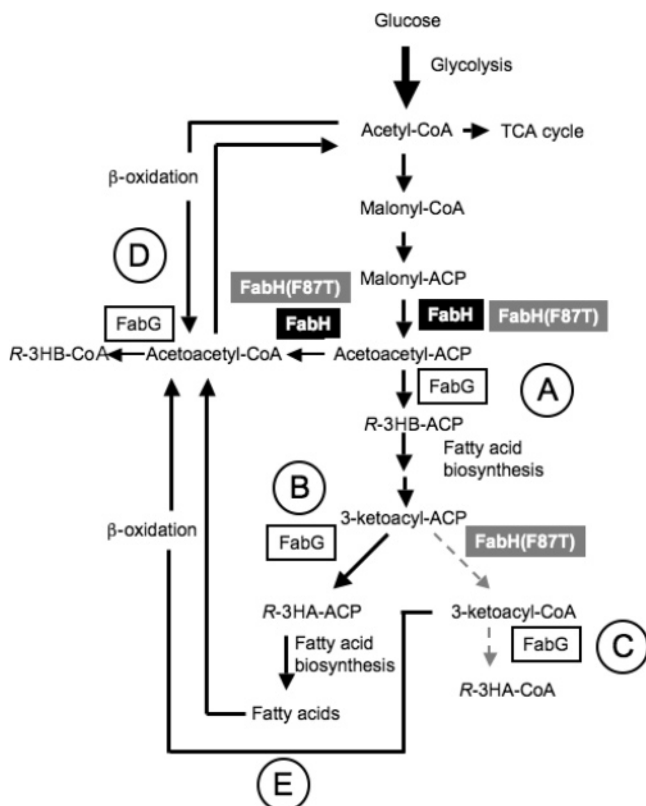

Figure 4. Competitive pathways for PHA production from a nonrelated carbon source in *E. coli* LS5218. Relevant enzymes and substrates are as indicated in figure. 3HB-ACP: 3-hydroxybutyryl-acyl carrier protein; 3HB-CoA: 3-hydroxybutyrl-CoA; *R*-3HA-ACP: *R*-3-hydroxyacyl-acyl carrier protein; *R*-3HA-CoA: *R*-3-hydroxyacyl-CoA. (A) Overproduction of FabH /FabH(F87T) and FabG increases overall flux through the fatty acid biosynthetic pathway. (B) FabG overproduction can lead to an increase in 3HA-ACP. (C) FabG overproduction can reduce 3-ketoacyl-CoA to *R*-3HA-CoA (medium-chain-length monomer for PHA production). (D) Overproduced FadA or YfcY from the β-oxidation pathway may lead to the production of acetoacetyl-CoA from acetyl-CoA produced via glycolysis. (E) Overproduced β-oxidation pathway enzymes can degrade 3-ketoacyl-CoA produced by overproduced FabH(F87T).

In LS5218, the coexpression of *fabG* genes with *phaC1* genes led to production of the highest levels of (12−13% of cell dry weight) of PHA when grown in the presence of excess glucose as a carbon source (Tables 2 and 3). However, the coexpression of *fabG* and *phaC1* genes accumulated much lower amounts (0.02−0.14% of cell dry weight) of P(3HB) homopolymer in *E. coli* JM109 (*7*). Again the genetic background differences of these two strains explains this apparent discrepancy in PHA accumulation. The *fadR* mutation of LS5218 causes elevated levels of expression of genes for all of the enzymes involved in β-oxidative pathways in *E. coli*, resulting in increased production of these enzymes. When LS5218 is grown in the presence of excess glucose, it results in a large acetyl-CoA pool (Figure 4). Combined with the overproduction of FadA and YfcY enzymes, this results in a reversal of the β-oxidation pathway, converting acetyl-CoA to acetoacetyl-CoA (Figure 4D). In the presence of FabG, acetoacetyl-CoA is converted to 3HB-CoA, which is easily converted to P(3HB) in the presence of a PHA synthase, thus explaining the increased PHA production from glucose in LS5218 strains coexpressing *fabG* and *phaC1* genes as compared to JM109 strains coexpressing the same genes.

Increasing the yield and decreasing costs are two very important aspects of PHA production that must be considered for the future production of these biodegradable polyesters. The use of engineered enzymes and strains with novel metabolic pathways could lead to economical production of PHA copolymers. Another important factor is the ability to control the molecular composition of the polymers produced by biological systems. By doing so, the number of uses and applications for such polymers may be greatly expanded. Although high yields of P(3HB) homopolymer and SCL PHA copolymers have been produced from nonrelated carbon sources (*1*), similar high yields have yet to be achieved for SCL-MCL PHA copolymers. Protein engineering techniques that have be successfully applied to PHA synthase genes (for review see ref 34) could be applied to the *fabH* and *fabG* genes to increase their activity and alter their substrate specificity. The current study has shown that FabG plays an important role in enhancing the yield and determining the monomer composition of SCL-MCL PHA copolymers produced in recombinant *E. coli* from both related and nonrelated carbon sources. 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 improved production of biodegradable polyesters.

Acknowledgment

The authors thank D. Kiemle from Analytical and Technical Services (AT&S) at SUNY-ESF for his assistance with NMR analysis. This work was supported by a grant from the New Energy and Industrial Technology Development Organization (NEDO) 06D49501D to K.M. and C.T.N., and T.E.E. and A.S.A. were supported by undergraduate fellowships from a Minority International Research Training Grant (NIH/NCMHHD MHIRT MD00148013/Ortiz).

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Received September 3, 2007. Accepted December 18, 2007.

BP070303Y