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Effective Enhancement of Short-Chain-Length−Medium-Chain-Length Polyhydroxyalkanoate Copolymer Production by Coexpression of Genetically Engineered 3-Ketoacyl-Acyl-Carrier-Protein Synthase III (*fabH*) and Polyhydroxyalkanoate Synthesis Genes

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Polyhydroxyalkanoates (PHAs) are biodegradable polyesters that have a wide variety of physical properties dependent on the lengths of the pendant groups of the monomer units in the polymer. PHAs composed of mostly short-chain-length (SCL) monomers are often stiff and brittle, whereas PHAs composed of mostly medium-chain-length (MCL) monomers are elastomeric in nature. SCL−MCL PHA copolymers can have properties between the two states, dependent on the ratio of SCL and MCL monomers in the copolymer. It is desirable to elucidate new and low cost ways to produce PHA composed of mostly SCL monomer units with a small mol % of MCL monomers from renewable resources, since this type of SCL−MCL PHA copolymer has superior qualities compared to SCL homopolymer. To address this issue, we have created strains of recombinant *E. coli* capable of producing β-ketothiolase (PhbA) and acetoacetyl-CoA synthase (PhbB) from *Ralstonia eutropha,* genetically engineered 3-ketoacyl-ACP synthase III (FabH) from *Escherichia coli,* and genetically engineered PHA synthases (PhaC) from *Pseudomonas* sp. 61-3 to enhance the production of SCL−MCL PHA copolymers from glucose. The cumulative effect of having two monomer-supplying pathways and genetically engineered PHA synthases resulted in higher accumulated amounts of SCL−MCL PHA copolymer from glucose. Polymers were isolated from two recombinant *E. coli* strains, the first harboring the *phbAB*, *fabH*(F87T), and *phaC1*(SCQM) genes and the second harboring the *phbAB*, *fabH*(F87W), and *phaC1*(SCQM) genes. The thermal and physical properties of the isolated polymers were characterized. It was found that even a very low mol % of MCL monomer in a SCL−MCL PHA copolymer had dramatic effects on the thermal properties of the copolymers.

Introduction

Polyhydroxyalkanoates (PHAs) are 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.[1] 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 of C3−C5 in length. Medium-chain-length (MCL) PHA consists of monomers of C6−C14 in length, and the SCL−MCL PHA copolymer consists of both SCL and MCL subunits. SCL PHAs can be used as thermoplastics. It has been previously shown that poly(3-hydroxybutyrate) homopolymer consisting solely of C4 monomer is stiff, brittle, and difficult to process, whereas SCL−MCL copolymers composed of mostly C4 monomer

with a small amount of C6 monomer have properties similar to polypropylene.[2] The potential environmental benefits associated with PHA polymers, as well as the improved physical properties of SCL−MCL PHA copolymers, point toward the importance of developing new pathways to produce SCL−MCL PHA copolymers.

Difficulties arise in producing SCL−MCL PHA copolymers in vivo due in part to the lack of adequate PHA synthases and monomer supplying enzymes. Our lab has recently used genetic engineering strategies in order to address these problems. A well-studied PHA synthase that has the capacity to incorporate both SCL and MCL monomers into a PHA copolymer chain is the PHA synthase (PhaC1) from *Pseudomonas* sp. 61-3.[3,4] However, the wild-type form of this enzyme has limited reactivity toward C4 substrates. Previous studies conducted in our lab have produced genetically engineered PHA synthase enzymes with higher activity toward C4 monomers.[5,6] In addition, we have demonstrated that coexpression of genetically engineered 3-ketoacyl acyl carrier protein synthase III genes (*fabH*) with

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Table 1. Plasmids Used in This Study

plasmids	relevant phenotype, genotype	source or reference
pGEMC1AB−SCQM	pGEM derivative; *phaC1* (SCQM), *phbAB*, Amp^r, colE1 ori	5
pGEMC1AB−STQK	pGEM derivative; *phaC1* (STQK), *phbAB,,* Amp^r, colE1 ori	5
pBBR1MCS-2	broad host range vector, *lacPOZ'*, Km^r, *mob-*	14
pBBRSCQMAB	pBBR1MCS-2 derivative, *phaC1* (SCQM), *phbAB*	this study
pBBRSTQKAB	pBBR1MCS-2 derivative, *phaC1* (STQK), *phbAB*	this study
pTrcFabH	pTrc99A derivative; Ap^r, P$_{trc}$, *rrnB* T$_1$T$_2$, *ori* (pB*R*322) *lacI^q*wild-type FabH	7
pTrcFabH(F87W)	pTrc99A derivative; Ap^r, P$_{trc}$, *rrnB* T$_1$T$_2$, *ori* (pB*R*322) *lacI^q*, *fabH*(F87W)	7
pTrcFabH(F87T)	pTrc99A derivative; Ap^r, P$_{trc}$, *rrnB* T$_1$T$_2$, *ori* (pB*R*322) *lacI^q*, *fabH*(F87T)	7
pTrcFabH(F87S)	pTrc99A derivative; Ap^r, P$_{trc}$, *rrnB* T$_1$T$_2$, *ori* (pB*R*322) *lacI^q*, *fabH*(F87S)	7
pTrcFabH(F87Y)	pTrc99A derivative; Ap^r, P$_{trc}$, *rrnB* T$_1$T$_2$, *ori* (pB*R*322) *lacI^q*, *fabH*(F87Y)	7
pTrcFabH(F87C)	pTrc99A derivative; Ap^r, P$_{trc}$, *rrnB* T$_1$T$_2$, *ori* (pB*R*322) *lacI^q*, *fabH*(F87C)	7
pTrcFabH(F87I)	pTrc99A derivative; Ap^r, P$_{trc}$, *rrnB* T$_1$T$_2$, *ori* (pB*R*322) *lacI^q*, *fabH*(F87I)	7
pTrcFabH(F87A)	pTrc99A derivative; Ap^r, P$_{trc}$, *rrnB* T$_1$T$_2$, *ori* (pB*R*322) *lacI^q*, *fabH*(F87A)	7
pTrcFabH(F87M)	pTrc99A derivative; Ap^r, P$_{trc}$, *rrnB* T$_1$T$_2$, *ori* (pB*R*322) *lacI^q*, *fabH*(F87M)	7

PHA synthase genes (*phaC*) led to the production of SCL−MCL PHA in recombinant *E. coli* grown in the presence of excess glucose.[7] The aim of the current study was to develop and establish metabolic pathways for increased SCL−MCL PHA copolymer production in recombinant *E. coli* from nonrelated carbon sources. To accomplish this, we coexpressed various mutant *fabH* genes in combination with both the (*R*)-3-hydroxybutyrl-CoA monomer supplying enzymes (PhbA and PhbB) and PHA synthases with altered substrate specificity to increase the yield of SCL−MCL PHA copolymer. Furthermore, preliminary physical characterizations of exemplary polymers isolated from *E. coli* were carried out.

Experimental Section

Bacterial Strains, Plasmids, and Cultivation Conditions. All transformations and DNA manipulations were carried out using *E. coli* JM109 [*recA1 endA1 gyrA96 thi-1 hsdR17* (r$_K^-$ m$_K^+$) *supE44 relA1* 1- *lac* [F' *proAB lacI^q* ZDM15] (Takara, Japan). *E. coli* JM109 was also used for production of PHA. Strains were grown at 30 °C in Luria-Bertani (LB) medium supplemented with glucose to a final concentration of 2 mg mL^{-1}. To maintain and select for plasmids within the recombinant *E. coli,* 100 μg of ampicillin and/or 50 μg of kanamycin were used as indicated. The plasmids used in this study are listed in Table 1.

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.[8]

PHA Production from Glucose in Recombinant *E. coli*. Plasmids harboring both genetically engineered *fabH* and *phaC1* genes were transformed into *E. coli* JM109 cells. Transformants were isolated and the presence of the correct plasmids 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 100 mL of LB. Cultures were incubated at 30 °C and constantly shaken at 150 rpm for 5 h at which time expression of the genetically engineered *fabH* genes were induced by the addition of 1 mM IPTG. Cultures 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^{-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.[7]

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 were 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 μm PTFE membrane to remove any residual solid materials. The chloroform was evaporated using a rotary vacuum evaporator (Eyela, 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 × volume of methanol and collected by filtering the solution through a 0.45 μm PTFE 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 a Shimadzu 10A system with RID-10A refractive-index detector with serial columns of ShodexK802 and K806M as described previously.[9] Polystyrene standards with a low polydispersity were used to make a calibration curve.

Determination of PHA Polymer Composition by Nuclear Magnetic Resonance (NMR). 20 mg of each polymer was dissolved in 1 mL of CDCl$_3$ and subjected to both ^{1}H and ^{13}C NMR analysis. ^{1}H NMR spectra were recorded using a JEOL α-400 spectrometer with a 5.0 μs pulse width (45° pulse angle), 3-s pulse repetition, 7500-Hz spectra width, and 16K data points. For ^{13}C NMR analysis, data were collected using a JEOL ECP-500 spectrometer with a 7.0-μs pulse width (45 ° pulse angle), 5-s pulse repetition,
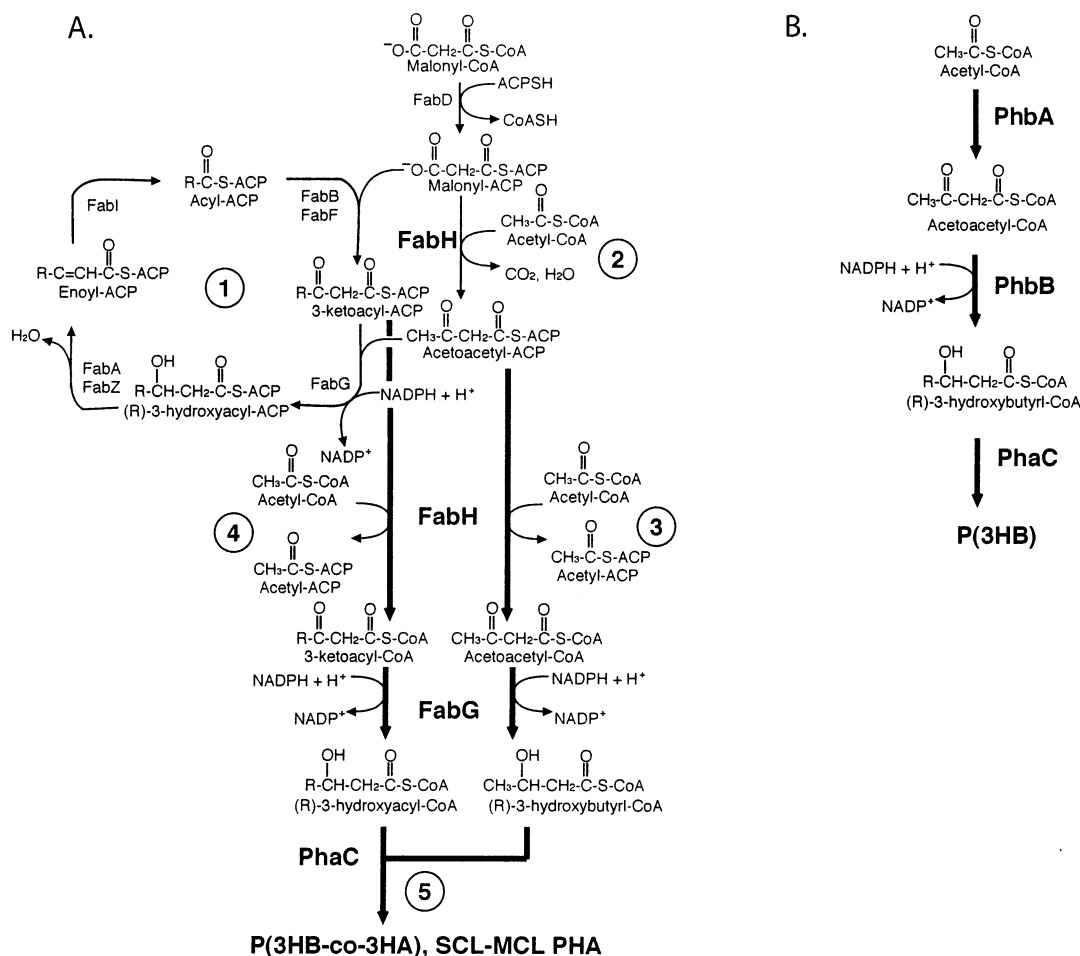
Figure 1. Proposed metabolic pathways for PHA production from glucose in recombinant *E. coli* JM109. A. Production of SCL and MCL monomers from fatty acid biosynthesis via overproduction of genetically engineered FabH proteins and PHA synthase. 1. Fatty acid biosynthesis pathway. 2. FabH catalyzed condensation of malonyl-ACP and acetyl-CoA to form acetoacetyl-ACP. 3 and 4. FabH mediated transacylase reaction. 5. PhaC catalyzed PHA polymerization reaction. B. The production of C4 monomer via the *R. eutropha* PhbAB enzymes.

25 000-Hz spectra width, and 64 K data points. Tetramethylsilane (Me₄Si) 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 Perkin-Elmer Pyris 1 differential scanning calorimeter (DSC) equipped with a liquid-nitrogen-cooling accessory. Data were collected under a nitrogen flow of 20 mL min⁻¹. The polyester samples (ca. 3 mg) were encapsulated in aluminum pans and quenched from the melt at −30 °C and then heated to 200 °C at a rate of 20 °C min⁻¹, during which the heat flow curves were recorded. The observed melting temperatures and enthalpies of fusion were determined from the positions of the endothermic peaks. The glass transition temperatures were taken as the midpoints of the heat capacity change.

Results

Design of SCL−MCL PHA Copolymer Biosynthesis Pathway and Plasmid Construction. For SCL−MCL PHA copolymer biosynthesis from nonrelated carbon sources, the recombinant strains must be equipped with both adequate monomer supplying enzymes and PHA synthases. The proposed metabolic pathways for the production of SCL−

MCL PHA copolymers from glucose in *E. coli* JM109 are illustrated in Figure 1.

For the efficient production of the SCL monomer, (*R*)-3-hydroxybutyrl-CoA, the PhbA and PhbB enzymes were used to convert acetyl-CoA derived from glycolysis to form acetoacetyl-CoA and (*R*)-3-hydroxybutyrl-CoA, respectively. For the production of MCL monomers, (*R*)-3-hydroxyacyl-CoAs, we used genetically engineered FabH. In our previous study, it was shown that the coexpression of *fabH* genes with a point mutation in the codon encoding Phe at position 87 to other amino acids with a PHA synthase enabled recombinant *E. coli* to accumulate SCL−MCL PHA when grown in the presence of excess glucose.[7]

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.[5] 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

Table 2. PHA Accumulation in Recombinant *E. coli* JM109 Strains from Glucose[a]

relevant genes	cell dry weight (g L^{-1})	PHA content (wt % of CDW)[b]	PHA composition (mol %)[c]		
			3HB (C4)	3HHx (C6)	3HO (C8)
phaC1(STQK), *phbAB*	4.4 ± 0.4	52.6 ± 5.6	100	0	0
phaC1(SCQM), *phbAB*	2.5 ± 0.04	26.9 ± 3.1	100	0	0
phaC1(STQK), *phbAB*, *fabH*(F87T)	2.7 ± 0.3	23.3 ± 0.9	99.9 ± 0.06	0.1 ± 0.06	0
phaC1(SCQM), *phbAB*, *fabH*(F87T)	2.7 ± 0.08	14.9 ± 2.4	99.7 ± 0.1	0.3 ± 0.1	0
phaC1(STQK), *phbAB*, *fabH*(F87S)	3.4 ± 0.2	23.2 ± 1.9	99.9 ± 0.01	0.1 ± 0.01	0
phaC1(SCQM), *phbAB*, *fabH*(F87S)	2.8 ± 0.06	13.1 ± 2.1	99.7 ± 0.03	0.3 ± 0.03	0
phaC1(STQK), *phbAB*, *fabH*(F87W)	3.9 ± 1.2	36.8 ± 6.9	99.8 ± 0.16	0.1 ± 0.03	0.1 ± 0.03
phaC1(SCQM), *phbAB*, *fabH*(F87W)	0.9 ± 0.04	18.8 ± 0.6	99.3 ± 0.11	0.6 ± 0.1	0.1 ± 0.03
phaC1(STQK), *phbAB*, *fabH*(F87A)	3.2 ± 0.4	36.8 ± 1.0	100	0	0
phaC1(SCQM), *phbAB*, *fabH*(F87A)	2.5 ± 0.03	21.5 ± 1.0	100	0	0
phaC1(STQK), *phbAB*, *fabH*(F87C)	3.3 ± 0.2	38.3 ± 1.9	100	0	0
phaC1(SCQM), *phbAB*, *fabH*(F87C)	1.8 ± 0.7	22.7 ± 2.9	100	0	0
phaC1(STQK), *phbAB*, *fabH*(F87I)	3.5 ± 0.1	40.3 ± 3.7	100	0	0
phaC1(SCQM), *phbAB*, *fabH*(F87I)	1.5 ± 0.7	19.7 ± 2.9	100	0	0
phaC1(STQK), *phbAB*, *fabH*(F87L)	3.2 ± 0.1	34.1 ± 5.5	99.9 ± 0.08	0.1 ± 0.08	0
phaC1(SCQM), *phbAB*, *fabH*(F87L)	2.5 ± 0.1	19.4 ± 0.6	99.9 ± 0.04	0.1 ± 0.04	0
phaC1(STQK), *phbAB*, *fabH*(F87M)	3.4 ± 0.2	36.0 ± 2.6	100	0	0
phaC1(SCQM), *phbAB*, *fabH*(F87M)	2.8 ± 0.2	22.1 ± 2.7	100	0	0
phaC1(STQK), *phbAB*, *fabH*(F87Y)	1.9 ± 0.07	38.8 ± 1.6	99.7 ± 0.01	0.3 ± 0.01	0
phaC1(SCQM), *phbAB*, *fabH*(F87Y)	1.5 ± 0.03	27.7 ± 4.4	99.6 ± 0.03	0.4 ± 0.01	0

[a] Cells were cultivated in LB medium. IPTG and 2 g mL^{-1} glucose were added after 5 and 8 h of cultivation, respectively. The results are the averages ± the standard deviations of at least three independent experiments. [b] CDW: cell dry weight. [c] 3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctonoate. The PHA composition was determined by gas chromatography after methanolysis of lyophilized cells in the presence of 15% sulfuric acid as described in the Experimental Section.

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.[6] 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.[5] Furthermore, these mutants also possessed the capacity to produce SCL−MCL PHA copolymer. It was predicted that coexpression of highly active mutant forms of PHA synthases with the *fabH* and *phbAB* genes would enhance SCL−MCL PHA production in *E. coli* JM109.

Two expression plasmids were constructed for the heterologous coexpression of the genetically engineered *phaC1* and *phbAB* genes. The pGEM"C1(STQK)AB and pGEM"C1-(SCQM)AB plasmids were digested with *Bam*HI and *Sac*II and a DNA fragment carrying the genetically engineered *phaC1* gene from *Pseudomonas* sp. 61-3, and the *phbAB* genes from *Ralstonia eutropha*, was ligated into the broad host range plasmid pBBR1-MCS2 between the *Bam*HI and *Sac*II sites to form the broad host range expression plasmids, pBBRSTQKAB and pBBRSCQMAB, respectively. The plasmids were sequenced to verify the correct insertion of the genes. Construction of plasmids containing the genetically engineered *fabH* genes were previously described.[7]

SCL−MCL PHA Copolymer Production from Glucose Is Enhanced by Coexpression of Genetically Engineered *fabH* and PHA Synthesis Genes. In our previous study, we found that overexpression of the following genetically engineered *fabH* genes enabled recombinant *E. coli* to produce medium-chain-length monomers for SCL−MCL PHA copolymer production: F87T, F87S, F87W, F87C,

F87A, F87I, F87L, F87M, and F87Y. We co-transformed into *E. coli* JM109 individual plasmids containing the genetically modified *fabH* genes with either the pBBRSTQK-AB or pBBRSCQMAB plasmids. The plasmids were isolated from the recombinant bacteria and were confirmed by restriction digest. Positively identified transformants were then assayed for the ability to produce SCL−MCL PHA copolymer. The results are shown in Table 2. Control strains harboring plasmids expressing either *phaC1*(STQK)*phbAB* or *phaC1*(SCQM)*phbAB* were able to accumulate P(3HB) homopolymer, accumulating 52.6 ± 5.6% and 26.9 ± 3.1% PHA per cell dry weight, respectively. Strains coexpressing the following genetically engineered *E. coli fabH* genes (F87T, F87S, F87W, F87I, F87L, and F87Y) with either the *phaC1*(STQK) or *phaC1*(SCQM) genes were able to produce SCL−MCL PHA copolymer as determined by GC analysis (Table 2). The secondary monomer unit incorporation was very low but differed dependent on the type of genetically engineered *fabH* used as the monomer supplier. These results indicate that the expression of the genetically engineered *fabH* genes enabled recombinant *E. coli* JM109 to produce MCL monomers for SCL−MCL PHA copolymer production from glucose.

Structure of SCL−MCL PHA Copolymers Isolated from Recombinant *E. coli* JM109. Polymers were isolated from the recombinant *E. coli* JM109 strains harboring either the pTrcFabH(F87T) and pBBRSCQMAB plasmids or the pTrcFabH(F87W) and pBBRSCQMAB plasmids. These polymers were chosen for further characterization because they contained the highest relative mol % of secondary and/or tertiary monomer units. 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
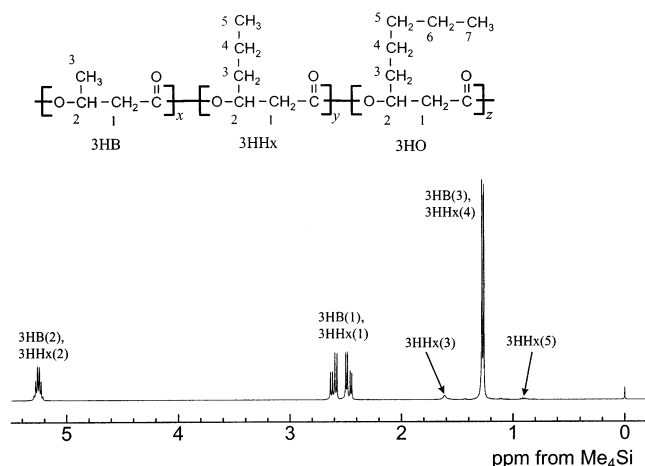
Figure 2. ^{1}H NMR spectrum of polymer isolated from *E. coli* JM109 harboring the pTrcFabH(F87T) and pBBRSCQMAB plasmids grown in the presence of glucose. 3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate. The 3HO fraction was not detectable by ^{1}H NMR but was confirmed by ^{13}C NMR (Figure 3).
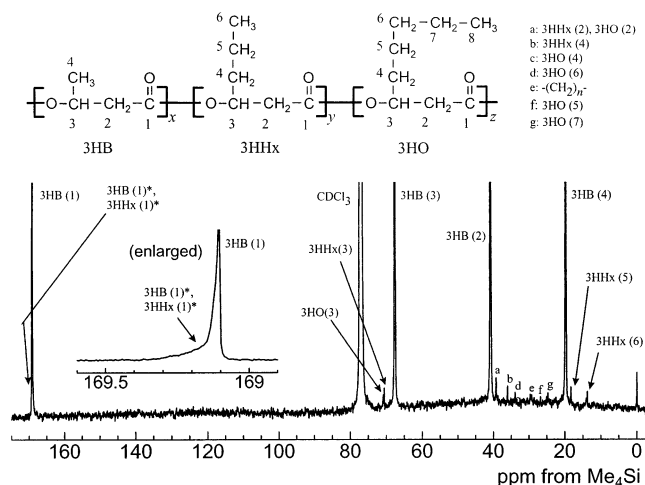

Figure 3. ^{13}C NMR spectrum of polymer isolated from *E. coli* JM109 harboring the pTrcFabH(F87T) and pBBRSCQMAB plasmids grown in the presence of glucose together with expanded carbonyl carbon resonances; 3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate.

Figure 4. ^{1}H NMR spectrum of polymer isolated from *E. coli* JM109 harboring the pTrcFabH(F87W) and pBBRSCQMAB plasmids grown in the presence of glucose. 3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate. The 3HO fraction was not detectable by ^{1}H NMR but was confirmed by ^{13}C NMR (Figure 5).

Figure 5. ^{13}C NMR spectrum of polymer isolated from *E. coli* JM109 harboring the pTrcFabH(F87W) and pBBRSCQMAB plasmids grown in the presence of glucose together with expanded carbonyl carbon resonances; 3HB, 3-hydroxybutyrate; 3HHx, 3-hydroxyhexanoate; 3HO, 3-hydroxyoctanoate.

the ^{1}H NMR spectra. Additional information for tertiary (C8) 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.,[10] the degree of randomness for P(3HB-*co*-3HH) was determined based on peak intensities for four carbonyl resonance lines (i.e., 3HB monomer next to 3HB, 3HB monomer next to 3HH, 3HH monomer next to 3HB, and 3HH monomer next to 3HH). However, the randomness value could not be estimated using this method since the 3HH resonance line adjacent to the 3HB resonance was too small to be observed clearly. Accordingly, we compared the experimentally obtained spectra and simulated spectra in which random distribution was assumed.[11] The R-factor was found to be 0.998 (near 1.000) as determined by the least-squares method, in which the experimentally obtained spectra and the simulated spectra were compared.
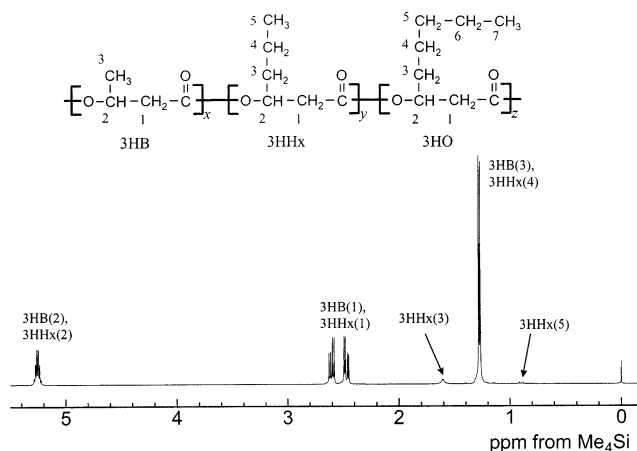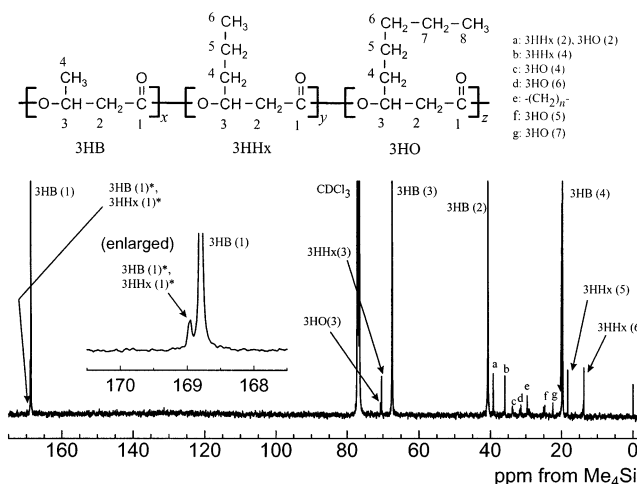
This result indicates that the polymer isolated from this strain was a random copolymer. The ^{1}H NMR and ^{13}C NMR spectra of polymer isolated from *E. coli* JM109 harboring the pTrcFabH(F87T) and pBBRSCQMAB plasmids revealed that the polymer was composed of 98.88 mol % 3HB, 0.92 mol % 3HHx, and 0.20 mol % 3HO. These values were similar to those obtained by GC analysis (Table 2).

The ^{1}H NMR and ^{13}C NMR spectra for the polymer isolated from *E. coli* harboring the pTrcFabH(F87W) and pBBRSCQMAB plasmids revealed that the polymer consisted of 98.74 mol % 3HB, 0.98 mol % 3HHx, and 0.28 mol % 3HO (Figures 4 and 5). These values were also similar to those obtained by GC analysis. As was the case with the polymer isolated from recombinant *E. coli* harboring the pTrcFabH(F87T) and pBBRSCQMAB plasmids, no 3HO was detected by ^{1}H NMR (Figure 4). However, the ^{13}C NMR spectrum revealed the presence of the 3HO monomer within the SCL−MCL PHA copolymer. The degree of randomness

Table 3. Thermal Properties of SCL−MCL PHA Copolymers Produced from Glucose with Recombinant *E. coli* JM109[a]

| | PHA composition (mol %)[b] | | | thermal properties[c] | | | | molecular weights[d] | |
relevant genes	3HB (C4)	3HHx (C6)	3HO (C8)	T_g (°C)	T_c (°C)	T_m (°C)	ΔH_m (J/g)	M_n	M_w/M_n
phbCAB	100			0	50	170	52	5.0×10^5	2.2
fabH (F87T), *phaC1(SCQM),phbAB*	98.88	0.92	0.20	−1	59	155, 167	47	1.9×10^5	2.4
fabH (F87W), *phaC1(SCQM), phbAB*	98.74	0.98	0.28	−3	58	141, 157	41	2.7×10^5	2.4

[a] Cells were cultivated in LB medium. IPTG and 10 g L^{-1} glucose were added after 5 and 8 h of cultivation, respectively. [b] PHA composition determined by NMR analysis, 3HB, 3-hydroxybutyrate, 3HHx,3-hydroxyhexanote, 3HO, 3-hydroxyoctanoate. [c] T_g, glass transition temperature; T_c, crystallization temperature; T_m, melting temperature; ΔH_m, enthalpy of fusion. [d] M_n, number-average molecular weight; M_w/M_n, polydispersity index.
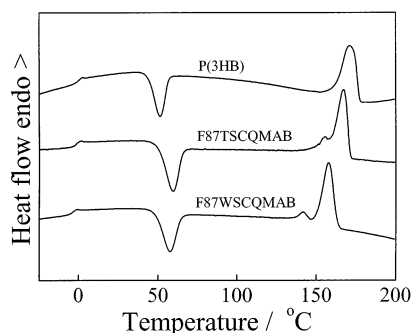

Figure 6. DSC thermograms of melt-quenched samples isolated from *E. coli* JM109 harboring the pTrcFabH(F87T) and pBBRSCQMAB plasmids (F87TSCQMAB), pTrcFabH(F87W) and pBBRSCQMAB plasmids (F87WSCQMAB), and P(3HB) homopolymer. The heating rate for each sample was 20 °C/min.

of the monomer side chains of the polymer isolated from the *E. coli* strain harboring the pTrcFabH(F87W) and pBBRSCQMAB plasmids was determined in a manner similar to the polymer isolated from the *E. coli* strain harboring the pTrcFabH(F87T) and pBBRSCQMAB plasmids. It was found that the polymer isolated from the *E. coli* harboring the pTrcFabH(F87W) and pBBRSCQMAB plasmids was a random copolymer (Figure 5).

Characterization of Thermal Properties of SCL−MCL PHA Copolymer Produced by Recombinant *E. coli* JM109 from Glucose. The thermal properties of the polymers isolated from recombinant *E. coli* harboring either the pTrcFabH(F87T) and pBBRSCQMAB plasmids or the pTrcFabH(F87W) and pBBRSCQMAB plasmids were determined by differential scanning calorimetry (DSC) analysis and compared with the thermal properties of P(3HB) homopolymer. All data were determined from the endotherm profiles (Figure 6). For the SCL−MCL PHA copolymers, 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, whereas the higher temperature melting peaks arose from the recrystallization during the heating process. The thermal properties for each polymer are summarized in Table 3. As determined by NMR analysis, the SCL−MCL PHA copolymer isolated from the recombinant *E. coli* strain harboring pTrcFabH(F87T) and pB-BRSCQMAB plasmids consisted of polymer composed of 98.88 mol % 3HB, 0.92 mol % 3HHx, and 0.20 mol % 3HO monomer units as determined by NMR analysis. The addition of 0.92 mol % 3HHx and 0.20 mol % 3HO monomer units to the PHA polymer lowered the melting temperature to 150 °C from 170 °C compared to the P(3HB) sample (Table 3).

In addition, the enthalpy of fusion was lowered to 47 J/g compared to 52 J/g for P(3HB) homopolymer.

Polymer isolated from the recombinant *E. coli* strain harboring the pTrcFabH(F87W) and pBBRSCQMAB plasmids consisted of 98.74 mol % 3HB, 0.98 mol % 3HHx, and 0.28 mol % 3HO monomers as determined by NMR analysis. The addition of 0.98 mol % 3HHx and 0.28 mol % 3HO monomers to the SCL−MCL PHA copolymer further reduced the melting temperature to 141°C compared to 170 °C for the P(3HB) homopolymer (Table 3). These results indicate that the addition a very small amount of secondary (C6) and tertiary (C8) MCL monomers to PHA copolymers caused dramatic changes to the thermal properties of the polymer.

Discussion

The efficient production of PHA copolymers with improved physical and mechanical properties is of great importance for the development of their use and practical application as biodegradable plastics. This study combined the expression of two different monomer-supplying pathways, with genetically engineered PHA synthase enzymes to improve the yield of SCL−MCL PHA copolymer produced from nonrelated carbon sources in *E. coli*.

For the monomer supplying pathways, the *R. eutropha* PhbA and PhbB enzymes were used to enhance the production of (*R*)-3-hydroxybutyrl-CoA for PHA biosynthesis. These enzymes are well characterized and have been used previously to supply (*R*)-3-hydroxybutyrl-CoA for PHA production.[5] For the supply of MCL monomers, we used genetically engineered 3-ketoacyl-ACP synthase III (FabH) proteins that had been modified at amino acid 87. The genetically modified FabH proteins were characterized previously and found to produce low amounts of SCL and MCL monomers for PHA copolymer production.[7] The natural reaction of the FabH protein is the condensation of acetyl-CoA and malonyl-ACP to form acetoacetyl-ACP (Figure 1); however, it is the very low transacylase activity (~200-fold less)[12] that allows FabH to convert the ACP forms of the substrate to the −CoA forms of the substrate that can be incorporated into the growing polymer chain by PHA synthase. However, the amount of PHA produced by the overproduction of FabH alone results in a very low amount of SCL−MCL PHA produced.[7] The results of the current study show that the coexpression of genes for these two different monomer supplying pathways (Figure 1) enabled and enhanced the production of SCL and MCL

monomers for PHA production from glucose in recombinant *E. coli* JM109.

Previous studies have shown that genetically engineered *Pseudomonas* sp. 61-3 PHA synthases had higher specific activities toward C4 substrates and were capable of incorporating a higher mol % of C4 monomers into PHA polymer than the wild-type PHA synthase.[5,6] To increase the yield and incorporate a higher mol % of C4 into SCL−MCL PHA copolymer, we utilized two genetically engineered *Pseudomonas* sp. 61-3 PHA synthases that were able to produce the highest amount of P(3HB) homopolymer in recombinant *E. coli* from glucose.[5] The first enzyme, PhaC1(STQK), had the Ser at 325 changed to Thr and Gln at 481 changed to Lys. These amino acid substitutions result in an enzyme with a much higher activity toward the C4 monomer and lowered activity toward a MCL monomer (C10) than the wild-type enzyme.[6] The second PHA synthase used in this study, PhaC1(SCQM), had the Ser at 325 changed to Cys and Gln at 481 changed to Met. Based on the results from this study, although this enzyme is capable of incorporating C4 into the PHA polymer, it has a higher affinity toward medium-chain-length substrates compared to the PhaC1(STQK) enzyme. These differences in the specific activity toward the various substrates may also result in the differences in the mol % and type of secondary MCL monomer units incorporated in the SCL−MCL PHA copolymers isolated and characterized in this study.

By using two different monomer supplying pathways and highly active genetically engineered PHA synthases, the yield of SCL−MCL PHA copolymer produced from glucose in recombinant *E. coli* JM109 could be drastically increased compared to our previous study,[7] with a marked increase in the mol % of C4 monomer incorporated into the random copolymer due to the expression of the *R. eutropha phbAB* genes and thus the increase in (*R*)-3-hydroxybutyrl-CoA. In addition, the monomer composition of the PHA polymer could be altered if either the monomer-supplying enzymes or the PHA synthase used were changed (Table 1). Further alteration of either the PHA synthases or the monomer supplying enzymes by genetic engineering may lead to the production of enzymes capable of making SCL−MCL PHA copolymers of very different monomer compositions, and thus very different physical properties. The amount of secondary (C6) and tertiary (C8) monomers incorporated into the SCL−MCL PHA copolymer in this study was very small as determined by both GC and NMR analysis (Table 1 and Figure 2). However, even the addition of small amounts of secondary and tertiary monomers into a P(3HB) polymer caused the melting temperature to be reduced from 170 to 155 °C with little change to the glass transition temperature (Table 3). With additional monomers incorporated into the SCL−MCL PHA copolymer, the melting temperature was further reduced (Table 3). These results are similar to those obtained by Fukui et al.,[13] but are remarkable, because in this study it was shown that less than 1.5 mol % of secondary (C6) and tertiary (C8) monomer units caused similar dramatic changes to the thermal properties of the polymers. Unlike the study by Fukui et al.,[13] the SCL−MCL copolymers obtained from this study had C8 monomers incorporated into

the copolymer in addition to the C4 and C6 monomers that make up the backbone of the polyester.

It is clear from Figure 1 that the native FabG protein must be involved in the production of SCL−MCL PHA copolymer. It has also been observed that coexpression of the *fabH* and *fabG* genes in recombinant *E. coli* will enhance SCL−MCL PHA copolymer production from glucose (data not shown). It will be interesting to examine whether coexpression of *fabG, fabH,* and *phbAB* will further enhance the production of SCL−MCL PHA copolymer from glucose in recombinant *E. coli.* These studies are currently underway.

The observed changes in the thermal properties of the polymers obtained in this study were likely caused by the additional exclusion of the longer pendant groups from the core of the P(3HB) crystalline structure. The addition of pendant groups longer than C6 to the polymer dramatically effects this exclusionary action during the crystallization process, resulting in a decrease in the melting temperature of the polymer. It can be predicted from these results that both higher mol % of MCL monomer units and/or longer pendant groups drive these changes in the thermal properties of SCL−MCL PHA copolymers. These differences in thermal properties may translate to other material properties such as enhanced elasticity, thus improving the SCL−MCL PHA copolymers for a wider array of industrial uses. By coexpressing various monomer supplying enzymes with genetically engineered PHA synthases, we may be able to create polymers with specific and desired properties dependent on the activities and specificities of the enzymes utilized.

Conclusions

In this study, genetically engineered PHA synthases and two different monomer-supplying pathways were used to enhance the production of SCL−MCL PHA copolymers from glucose in *E. coli* JM109. The SCL−MCL PHA copolymers from this study had very different thermal properties compared to P(3HB) homopolymer, which, and these differences in thermal properties may lead to enhanced physical characteristics. Further enhancement of enzyme activity via genetic engineering may lead to increased production of SCL−MCL PHA copolymers and eventually to their commercial production.

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