

Coexpression of Genetically Engineered 3-Ketoacyl-ACP Synthase III (*fabH*) and Polyhydroxyalkanoate Synthase (*phaC*) Genes Leads to Short-Chain-Length–Medium-Chain-Length Polyhydroxyalkanoate Copolymer Production from Glucose in *Escherichia coli* JM109

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Polyhydroxyalkanoates (PHAs) can be divided into three main types based on the sizes of the monomers incorporated into the polymer. Short-chain-length (SCL) PHAs consist of monomer units of C₃ to C₅, medium-chain-length (MCL) PHAs consist of monomer units of C₆ to C₁₄, and SCL-MCL PHAs consist of monomers ranging in size from C₄ to C₁₄. Although previous studies using recombinant *Escherichia coli* have shown that either SCL or MCL PHA polymers could be produced from glucose, this study presents the first evidence that an SCL-MCL PHA copolymer can be made from glucose in recombinant *E. coli*. The 3-ketoacyl-acyl carrier protein synthase III gene (*fabH*) from *E. coli* was modified by saturation point mutagenesis at the codon encoding amino acid 87 of the FabH protein sequence, and the resulting plasmids were cotransformed with either the pAPAC plasmid, which harbors the *Aeromonas caviae* PHA synthase gene (*phaC*), or the pPPPAC plasmid, which harbors the *Pseudomonas* sp. strain 61-3 PHA synthase gene (*phaC1*), and the abilities of these strains to accumulate PHA from glucose were assessed. It was found that overexpression of several of the mutant *fabH* genes enabled recombinant *E. coli* to induce the production of monomers of C₄ to C₁₀ and subsequently to produce unusual PHA copolymers containing SCL and MCL units. The results indicate that the composition of PHA copolymers may be controlled by the monomer-supplying enzyme and further reinforce the idea that fatty acid biosynthesis may be used to supply monomers for PHA production.

Polyhydroxyalkanoates (PHAs) are polyesters of 3-hydroxyalkanoic acids that are produced as intracellular granules by many different bacteria (29). Bacterial PHA polymers have generated research interest because of their potential use as biodegradable thermoplastics and elastomers; however, the high cost of their production has limited their widespread use (18, 30). Currently, researchers are investigating alternative strategies for PHA production in order to reduce production costs. One way of reducing the costs of PHA production is to use inexpensive growth substrates, such as sugars, and to use recombinant strains of bacteria, such as *Escherichia coli*, to increase the PHA yield (18, 30).

PHAs produced by bacteria consist of three main types: polymers composed of short-chain-length (SCL) monomers, polymers composed of medium-chain-length (MCL) monomers, and polymers composed of SCL-MCL monomers. SCL PHA consists of monomeric subunits 3 to 5 carbons in length, while MCL PHA consists of monomers 6 to 14 carbons in length and SCL-MCL PHA copolymer consists of monomeric subunits 4 to 12 carbons in length. These differences in PHA monomer composition can dramatically affect the properties and qualities of the polymers. SCL PHA homopolymers, such as poly-3-hydroxybutyrate [P(3HB)], form a stiff crystalline material, which leads to brittleness and low extension to break.

This lack of flexibility limits its range of applications. Polymers consisting of only MCL PHA are semicrystalline thermoplastic elastomers, which may have their mechanical properties enhanced by reinforcement with nanocomposite materials and fillers (6–8). However, unlike polymers composed solely of either SCL or MCL monomer units, SCL-MCL PHA copolymers can have a wide range of physical properties, depending on the mole percent composition of the different monomers incorporated into the polymer. SCL-MCL PHA copolymers with low moles percent of SCL monomer units are more elastomeric in nature, while SCL-MCL PHA copolymers, such as a copolymer made of a high mole percent of 3HB and a low mole percent of (*R*)-3-hydroxyhexanoate (3HHx), have been demonstrated to be flexible materials with properties similar to those of polypropylene (20). Because of the potentially wide range of applications for SCL-MCL PHA copolymer, it is desirable to identify methods and metabolic pathways that enable bacteria to produce it.

Although the production of MCL PHA has been successfully accomplished using recombinant *E. coli*, the carbon source for such strains is usually limited to fatty acid or related carbon substrate precursors, such as decanoate and dodecanoate (14, 17, 22). Production of MCL PHA from unrelated carbon sources has proved more difficult to accomplish in *E. coli*, and there are few studies available in which MCL PHA was produced from unrelated carbon sources (15, 25, 26). Two very similar methods utilized the coexpression of either the *E. coli* *tesA* gene, encoding a modified *E. coli* thioesterase, with the PHA synthase gene (*phaC1*) from *Pseudomonas oleovorans*

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(15) or the *Umbellularia californica* thioesterase gene and PHA synthase (26) in *E. coli* grown in the presence of gluconate to accumulate MCL PHA. Coexpression of the *E. coli tesA* gene and the *Pseudomonas oleovorans phaC1* gene led to the accumulation of MCL PHA copolymer composed of C₆, C₈, and C₁₀ monomers (15). Coexpression of the *U. californica* thioesterase gene with the *Ralstonia eutropha phaC* gene and the *Pseudomonas aeruginosa phaC1* gene led to the accumulation of either P(3HB) homopolymer or poly-3-hydroxydecanoate homopolymer, respectively (26). A third method for accumulating MCL PHA from unrelated carbon sources in *E. coli* was by coexpression of the *Pseudomonas putida phaG* gene, encoding a putative transacylase and PHA synthase, and the *P. aeruginosa phaC1* gene in the presence of the enoyl-ACP reductase inhibitor triclosan, which led to the accumulation of 2 to 3% (cellular dry weight) poly-3-hydroxydecanoate homopolymer (25). Although these previous reports describe MCL PHA production from unrelated carbon sources in *E. coli*, there have been no reports regarding the production of SCL-MCL PHA copolymer from unrelated carbon sources in recombinant *E. coli*.

It was previously shown that coexpression of the PHA synthase gene (*phaC*) from *Aeromonas caviae* and the 3-ketoacyl-acyl carrier protein synthase (ACP) III gene (*fabH*) from *E. coli* led to the production of P(3HB) in recombinant *E. coli* grown in the presence of glucose (32). Although these cells were shown to produce P(3HB) homopolymer consisting only of 3HB monomers, this study demonstrated the link between fatty acid biosynthesis and PHA production in recombinant *E. coli* (32).

The FabH protein, or 3-ketoacyl-ACP synthase III, is a member of the β -ketoacyl synthase family of enzymes (5). The primary reaction of the FabH enzyme is the condensation of malonyl-ACP with acetyl-coenzyme A (CoA). It is unique among β -ketoacyl synthase enzymes in that it utilizes acetyl-CoA as a donor and has been shown to have an acetyl-CoA-ACP transacylase activity in vitro (33). Despite the overall similarities in their primary amino acid sequences, the FabH proteins from various bacterial species have been shown to have very different substrate specificities (3, 4, 13). For example, the *E. coli* FabH protein has specificity for carbon substrates 2 to 4 carbons in length, while the *Mycobacterium tuberculosis* FabH protein displays specificity for carbon substrates 10 to 16 carbons in length in vitro (4). The crystal structures of both the *E. coli* FabH protein (5, 23, 24) and the *M. tuberculosis* FabH (28) protein have been determined, and comparison of the primary amino acid sequences and the two crystal structures revealed a potential explanation for the difference in substrate specificity observed between the *E. coli* FabH and the *M. tuberculosis* FabH. In the *E. coli* FabH protein, there is a Phe residue at position 87. This amino acid occupies the end of the predicted substrate-binding pocket and obstructs the binding of straight fatty acid chains longer than four carbons. On the other hand, a Thr residue occupies position 87 in the *M. tuberculosis* FabH protein, and this enzyme displays specificity for substrates of C₈ to C₁₆ lengths (4). The smaller size of Thr87 compared to Phe87 may allow the binding of longer-chain fatty acids and thus produce the observed difference in substrate specificity between the two proteins (28).

These differences in substrate binding specificity may have an effect on the PHA monomer supply in *E. coli*, and it was hypothesized that if the *E. coli* FabH protein had an amino acid residue other than Phe, it would be able to recognize substrates larger than four carbons and subsequently produce MCL 3-hydroxyacyl (3HA) monomers for incorporation into a growing PHA polymer. In order to address whether Phe87 of *E. coli* FabH affects the substrate specificity and monomer supply for PHA, saturation site-directed mutagenesis was performed on the codon encoding Phe87. A series of amino acid substitution mutant expression plasmids were constructed and individually cotransformed into *E. coli* with the expression plasmids harboring PHA synthase genes from either *A. caviae* (9) or *Pseudomonas* sp. strain 61-3 (21). The isolated transformants were assayed for the ability to produce MCL PHA copolymer from glucose. Several recombinant *E. coli* strains harboring a mutant *fabH* gene coexpressed with *phaC* were able to accumulate unusual PHA copolymers containing SCL (C₄) and MCL (C₆ to C₁₀) monomer units, suggesting that the substrate specificity of FabH could be modified on a molecular level and that this specificity is an important regulator of the monomer type incorporated into PHA polymers. Furthermore, this study represents the first report of the production of SCL-MCL PHA copolymers in *E. coli* grown in the presence of an unrelated carbon source.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cultivation conditions. All transformations and experiments were carried out using *E. coli* JM109 {*recA1 endA1 gyrA96 thi-1 hsdR17* ($r_K^- m_K^+$) *supE44 relA1 $\lambda^- lac$ [F' *proAB lacI^q Δ M15]*]} (Takara, Tokyo, Japan) as a host strain, and all strains were grown at 30°C in Luria-Bertani (LB) medium supplemented with glucose to a final concentration of 2 mg ml⁻¹ as a carbon source where appropriate. One hundred micrograms of ampicillin and/or 50 μ g of kanamycin was used for plasmid selection in recombinant *E. coli* strains as appropriate. The plasmids used in this study are listed in Table 1.*

Isolation, analysis, and manipulation of DNA. The DNA sequences of plasmid constructs were confirmed by dye termination cycle sequencing using a Beckman-Coulter CEQ 2000 sequencer. All other genetic techniques were performed as previously described (27).

Cloning of the *E. coli fabH* gene. The *E. coli fabH* gene was amplified from *E. coli* genomic DNA by PCR with the following primers: for the 5' end, 5'-GT GAC TGA GCG TAC ATG TAT ACG AAG-3', where boldface lettering indicates an *Afl*III site engineered into the primer, and for the 3' end, 5'-GAG GAT CCC TAG AAA CGA ACC AGC GCG GAG C-3', where boldface lettering indicates a *Bam*HI site engineered into the primer. The *fabH* PCR product was digested with *Afl*III and *Bam*HI and ligated into the *Nco*I and *Bam*HI sites of the expression plasmid pTrc99A (Amersham Pharmacia). The resulting plasmid was named pTrcFabH, and the insertion of the *fabH* gene was confirmed by digestion with restriction enzymes and DNA sequencing.

Saturation point mutagenesis of Phe87 in the *E. coli fabH* gene. Primers with point mutations in the codon encoding the amino acid at position 87 were made as indicated in Table 2 and were used to amplify the entire pTrcFabH plasmid by PCR using the 3'-5' proofreading enzyme *Pfu*I (Invitrogen). The resultant PCR products were self-ligated and transformed into competent *E. coli* JM109 cells. Transformants were isolated, and the individual plasmids were sequenced to confirm the insertion of the desired point mutation in the *fabH* gene. To confirm that no other point mutations had occurred in regions that could affect the copy number or transcription of the mutant *fabH* genes, the corresponding nucleotide sequences for the promoter, *Lacl*^q binding site, *lacI^q* promoter, *lacI^q* gene, and origin of replication were sequenced on each plasmid. No mutations were found in these regions (data not shown).

PHA production from glucose in recombinant *E. coli*. Confirmed plasmids harboring wild-type or mutant *fabH* genes were cotransformed with either pAPAC or pPPAC into *E. coli* JM109 cells. Transformants were isolated, and the presence of both the pTrcFabH plasmid and either the pAPAC or pPPAC

TABLE 1. Plasmids used in this study

Plasmid	Relevant phenotype	Source or reference
pTrc99A	Expression vector; Ap ^r P _{trc} <i>rrnB</i> T ₁ T ₂ ori (pBR322) <i>lacI</i> ^q	Amersham Pharmacia
pAPAC	pJRDTrc1 derivative; <i>phaC</i> from <i>A. caviae</i> ; Km ^r ; RSF1010 replicon	31
pPPAC	pJRDTrc1 derivative; <i>phaC1</i> from <i>Pseudomonas</i> sp. strain 61-3; Km ^r ; RSF1010 replicon	31
pTrcFabH	pTrc99A derivative; wild-type FabH	This study
pTrcFabH(F87W)	pTrc99A derivative; <i>fabH</i> (F87W)	This study
pTrcFabH(F87T)	pTrc99A derivative; <i>fabH</i> (F87T)	This study
pTrcFabH(F87L)	pTrc99A derivative; <i>fabH</i> (F87L)	This study
pTrcFabH(F87S)	pTrc99A derivative; <i>fabH</i> (F87S)	This study
pTrcFabH(F87Y)	pTrc99A derivative; <i>fabH</i> (F87Y)	This study
pTrcFabH(F87N)	pTrc99A derivative; <i>fabH</i> (F87N)	This study
pTrcFabH(F87C)	pTrc99A derivative; <i>fabH</i> (F87C)	This study
pTrcFabH(F87V)	pTrc99A derivative; <i>fabH</i> (F87V)	This study
pTrcFabH(F87K)	pTrc99A derivative; <i>fabH</i> (F87K)	This study
pTrcFabH(F87P)	pTrc99A derivative; <i>fabH</i> (F87P)	This study
pTrcFabH(F87I)	pTrc99A derivative; <i>fabH</i> (F87I)	This study
pTrcFabH(F87A)	pTrc99A derivative; <i>fabH</i> (F87A)	This study
pTrcFabH(F87D)	pTrc99A derivative; <i>fabH</i> (F87D)	This study
pTrcFabH(F87E)	pTrc99A derivative; <i>fabH</i> (F87E)	This study
pTrcFabH(F87H)	pTrc99A derivative; <i>fabH</i> (F87H)	This study
pTrcFabH(F87M)	pTrc99A derivative; <i>fabH</i> (F87M)	This study
pTrcFabH(F87Q)	pTrc99A derivative; <i>fabH</i> (F87Q)	This study
pTrcFabH(F87G)	pTrc99A derivative; <i>fabH</i> (F87G)	This study
pTrcFabH(F87R)	pTrc99A derivative; <i>fabH</i> (F87R)	This study

plasmid 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 100 ml of LB medium. Cultures were incubated at 30°C and constantly shaken at 150 rpm for 9 h, at which time expression of the *fabH* and *phaC* genes was induced by the addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). The cultures were grown for an additional 3 h, at which time glucose was added to a final concentration of 2 g

ml⁻¹. The cells were allowed to grow for an additional 12 h after glucose addition before being harvested by centrifugation. The PHA contents were determined by gas chromatography (GC) analysis of lyophilized cells

GC analysis of PHA compositions. The PHA contents and PHA compositions were determined by GC analysis. Liquid cultures (100 ml) were centrifuged at 5,000 × g for 10 min at 4°C, and the cells were washed twice with ice-cold Milli-Q double-distilled water and lyophilized for a minimum of 48 h. The lyophilized cell

TABLE 2. Primers used in this study

Oligonucleotide sequence ^a	Amino acid ^b	Primer name
CAC GCT TTC CCG AGC GCA GCT	Phe	None (WT)
CAC GCT CTG CCG AGC GCA GCT	Leu	EcfabH(F87L)
CAC GCT TCC CCG AGC GCA GCT	Ser	EcfabH(F87S)
CAC GCT TAC CCG AGC GCA GCT	Tyr	EcfabH(F87Y)
CAC GCT TGC CCG AGC GCA GCT	Cys	EcfabH(F87C)
CAC GCT TGG CCG AGC GCA GCT	Trp	EcfabH(F87W)
CAC GCT CCG CCG AGC GCA GCT	Pro	EcfabH(F87P)
CAC GCT CAC CCG AGC GCA GCT	His	EcfabH(F87H)
CAC GCT CAG CCG AGC GCA GCT	Gln	EcfabH(F87Q)
CAC GCT CGT CCG AGC GCA GCT	Arg	EcfabH(F87R)
CAC GCT ATC CCG AGC GCA GCT	Ile	EcfabH(F87I)
CAC GCT ATG CCG AGC GCA GCT	Met	EcfabH(F87M)
CAC GCT ACC CCG AGC GCA GCT	Thr	EcfabH(F87T)
CAC GCT AAC CCG AGC GCA GCT	Asn	EcfabH(F87N)
CAC GCT AAA CCG AGC GCA GCT	Lys	EcfabH(F87K)
CAC GCT GTG CCG AGC GCA GCT	Val	EcfabH(F87V)
CAC GCT GCG CCG AGC GCA GCT	Ala	EcfabH(F87A)
CAC GCT GAT CCG AGC GCA GCT	Asp	EcfabH(F87D)
CAC GCT GAA CCG AGC GCA GCT	Glu	EcfabH(F87E)
CAC GCT GGC CCG AGC GCA GCT	Gly	EcfabH(F87G)
CGT AGC AGA AGT CGT TGC CAC	Reverse	EcfabH(rev)
TGC TTC TGG CGT CAG GCA GCC ATC G	— ^c	pTrc99F(38)
CGA TGG CTG CCT GAC GCC AGA AGC A	—	pTrc99R(62)
CAC GAG GGA GCT TCC AGG GGG A	—	pTrc99F(2350)
TCA CGT AGC GAT AGC GGA GT	—	pTrc99R(2777)
ACT CCG CTA TCG CTA CGT GA	—	pTrc99F(2767)
CAA ATC GGC GTT TGT CCG CAC TTG	—	L22 REV
ATG GTG GTT AAC GGC GGG ATA TAA C	—	pTrc99R(3920)
CAG ACT GGA GGT GGC AAC GCC AAT CA	—	pTrc99R(3269)

^a Bold face lettering indicates the changed codon. Codon changes were based on *E. coli* codon usage frequency.

^b Amino acid coded for at position 87 of the *E. coli* FabH protein.

^c —, sequencing primers for pTrc99A.

material (30 to 40 mg) was subjected to methanolysis in a methanol-sulfuric acid (85:15) solution. The samples were mixed vigorously for 30 s by vortexing and incubated at 100°C for 140 min. After cooling to room temperature, the water-soluble contents were removed by the addition of 1 ml of sterile double-distilled water and vigorous mixing of the samples by vortexing for 30 s. The aqueous and organic phases were allowed to separate, and the organic phase containing the chloroform-soluble methyl esters was removed with a Pasteur pipette and filtered through a 0.45- μ m-pore-size Millex polytetrafluoroethylene (PTFE) LCR membrane (Millipore) for each sample. A total of 500 μ l of the organic-phase mixture containing the soluble methyl esters and 500 μ l of 0.1% (wt/vol) caprylic acid in methanol were mixed in GC tubes, and these samples were assayed as described previously using a GC17A gas chromatograph with a 30-m column (Shimadzu, Kyoto, Japan) (12).

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 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- μ m-pore-size PTFE 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 hexane. 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 a 10 \times volume of hexane and collected by filtering the solution through a 0.45- μ m-pore-size 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 for polyesters were obtained by GPC analysis using a Shimadzu 10A system with a RID-10A refractive-index detector with serial columns of ShodexK802 and K806 M, as described previously (16).

RESULTS

Effect of coexpression of point mutant *E. coli fabH* and *A. caviae phaC* genes on PHA production in recombinant *E. coli*. To address whether modification of the substrate-binding pocket of *E. coli* FabH could alter the substrate specificity and monomer supply for PHA production in *E. coli*, saturation point mutagenesis was done, in which 19 individual point mutations corresponding to all other amino acids were introduced to the codon encoding amino acid 87 of the *E. coli fabH* gene, as shown in Table 2. The individual mutant pTrcFabH plasmids were cotransformed with pAPAC, which carries the *A. caviae phaC* gene. The PHA accumulation results are summarized in Table 3. As was previously shown, coexpression of the wild-type *fabH* gene with *A. caviae phaC* led to the production of P(3HB) homopolymer composed of C₄ monomers as determined by GC, with a maximum PHA content of 9.8% (cell dry weight). In addition to the *E. coli* strain coexpressing the wild-type *fabH* and *A. caviae phaC* genes, the following 11 *fabH* mutations were capable of supplying monomers for PHA production: F87W, F87T, F87L, F87S, F87Y, F87C, F87V, F87I, F87A, F87M, and F87N (Table 3).

All of the *fabH* mutant strains capable of producing PHA had total PHA contents lower than the PHA accumulated in an *E. coli* strain with pTrcFabH and pAPAC (Table 3). Coexpression of three of the mutant *fabH* genes, F87W, F87V, and F87Q, with the *A. caviae phaC* gene accumulated P(3HB) composed solely of C₄ monomers, in addition to a strain harboring pTrcFabH and pAPAC. However, strains harboring expression plasmids with the eight mutations F87T, F87L, F87S, F87Y, F87C, F87I, F87A, and F87M were able to accumulate SCL-MCL PHA copolymers consisting of various per-

TABLE 3. PHA accumulation in recombinant *E. coli* JM109 strains with *A. caviae phaC*^a

Plasmid (relevant marker)	CDW ^b (g liter ⁻¹)	PHA content (g of PHA accumulated per 100 g of dry cells)	PHA composition (mol%) ^c	
			3 HB (C ₄)	3HHx (C ₆)
pTrcFabH (WT <i>fabH</i>)	1.1 \pm 0.5	9.8 \pm 1.9	100	0
pTrcFabH(F87W)	1.4 \pm 0.1	1.4 \pm 0.4	100	0
pTrcFabH(F87T)	1.2 \pm 0.1	1.8 \pm 0.1	97.2 \pm 0.2	2.8 \pm 0.2
pTrcFabH(F87L)	1.0 \pm 0.2	1.3 \pm 0.4	96.7 \pm 0.02	3.3 \pm 0.02
pTrcFabH(F87S)	1.1 \pm 0.6	4.3 \pm 1.7	96.3 \pm 0.4	3.7 \pm 0.4
pTrcFabH(F87Y)	2.1 \pm 0.5	1.0 \pm 0.08	97.3 \pm 0.3	2.7 \pm 0.3
pTrcFabH(F87N)	1.7 \pm 0.4	ND ^d		
pTrcFabH(F87C)	1.1 \pm 0.07	4.9 \pm 0.2	94.3 \pm 0.8	5.8 \pm 0.8
pTrcFabH(F87V)	1.0 \pm 0.03	0.8 \pm 0.3	100	0
pTrcFabH(F87K)	1.5 \pm 0.7	ND		
pTrcFabH(F87P)	1.4 \pm 0.03	ND		
pTrcFabH(F87I)	1.1 \pm 0.4	4.3 \pm 0.7	97.9 \pm 0.6	2.1 \pm 0.6
pTrcFabH(F87A)	1.4 \pm 0.1	2.5 \pm 1.8	97.5 \pm 0.2	2.5 \pm 0.2
pTrcFabH(F87D)	2.2 \pm 0.2	ND		
pTrcFabH(F87E)	2.1 \pm 0.2	ND		
pTrcFabH(F87H)	1.9 \pm 0.1	ND		
pTrcFabH(F87M)	1.8 \pm 0.01	2.4 \pm 0.9	99.0 \pm 0.9	1.0 \pm 0.9
pTrcFabH(F87Q)	1.8 \pm 0.01	0.8 \pm 0.03	100	0
pTrcFabH(F87G)	1.8 \pm 0.01	ND		
pTrcFabH(F87R)	1.8 \pm 0.6	ND		
pAPAC ^e	1.5 \pm 0.2	ND		

^a Cells were cultivated in LB medium. IPTG and 2 g of glucose ml⁻¹ were added after 9 and 12 h of cultivation, respectively. The results are the averages and the standard deviations of at least three independent experiments. All strains harbored the pAPAC plasmid, which expresses the *A. caviae phaC* gene (32), as well as the pTrcFabH plasmid indicated in the table.

^b CDW, cell dry weight.

^c 3HB, 3-hydroxybutyrate;

^d ND, not detected. The PHA composition was determined by GC after methanolysis of lyophilized cells in the presence of 15% sulfuric acid as described in Materials and Methods.

^e Only the pAPAC plasmid was harbored in this strain.

centages of C₄ and C₆ monomers (Table 3). A control strain of *E. coli* harboring only the pAPAC expression plasmid failed to accumulate PHA. These results suggest that the coexpression of the *fabH* genes and *A. caviae phaC* is essential for PHA production and that overexpression of the *fabH* genes was necessary for the supply of monomers for PHA production; strains expressing the genomic copy of *fabH* alone with the *A. caviae phaC* gene were unable to accumulate detectable levels of PHA. *E. coli* strains harboring plasmids expressing *A. caviae phaC* and the mutation F87N, F87K, F87P, F87D, F87E, F87H, F87G, or F87R in the *fabH* gene also failed to accumulate PHA. These results suggest that the F87N, F87K, F87P, F87D, F87E, F87H, F87G, and F87R mutations inactivate the ability of the FabH protein to act as a monomer supplier for PHA synthesis.

Effect of coexpression of point mutant *E. coli fabH* and *Pseudomonas* sp. strain 61-3 *phaC1* genes on PHA production in recombinant *E. coli*. Although the coexpression of the mutant *E. coli fabH* genes and *A. caviae phaC* led to the production of SCL-MCL PHA, this copolymer was composed solely of 3HB and 3HHx monomers. The observed result may have been influenced by the fact that the *A. caviae* PHA synthase displays strong substrate specificities for C₄ and C₆ monomer units (9). It was unknown whether any of the mutant FabH proteins were capable of generating monomers longer than C₆ for PHA production. In order to investigate whether the ex-

TABLE 4. PHA accumulation in recombinant *E. coli* JM109 strains with *Pseudomonas* sp. strain 61-3 PhaC1^a

Plasmid (relevant marker)	CDW ^b (g liter ⁻¹)	PHA content (wt% of CDW)	PHA composition (mol%) ^c			
			3HB (C ₄)	3HHx (C ₆)	3HO (C ₈)	3HD (C ₁₀)
pTrcFabH (WT <i>fabH</i>)	2.0 ± 0.5	0.8 ± 0.03	96.3 ± 0.7	3.7 ± 0.7	0	0
pTrcFabH(F87W)	2.0 ± 0.9	0.2 ± 0.03	84 ± 0.03	11.3 ± 0.03	4.7 ± 0.03	0
pTrcFabH(F87T)	1.7 ± 0.03	0.8 ± 0.3	76.6 ± 1.6	18.3 ± 0.5	3.8 ± 1.2	1.3 ± 1.1
pTrcFabH(F87L)	1.4 ± 0.2	0.12 ± 0.03	73.5 ± 2.6	26.5 ± 2.6	0	0
pTrcFabH(F87S)	1.2 ± 0.6	0.9 ± 0.4	86.4 ± 3.3	8.8 ± 1.6	3.1 ± 0.9	1.7 ± 0.8
pTrcFabH(F87Y)	1.1 ± 0.6	0.2 ± 0.03	80 ± 0.03	14.4 ± 0.03	5.6 ± 0.03	0
pTrcFabH(F87N)	1.7 ± 0.03	ND ^d				
pTrcFabH(F87C)	2.2 ± 0.03	0.3 ± 0.03	81.2 ± 0.03	15 ± 0.03	3.8 ± 0.03	0
pTrcFabH(F87V)	2.8 ± 0.7	ND				
pTrcFabH(F87K)	1.3 ± 0.3	ND				
pTrcFabH(F87P)	2.0 ± 1.2	ND				
pTrcFabH(F87I)	0.6 ± 0.09	1.7 ± 0.04	92.0 ± 0.5	5.6 ± 0.9	1.3 ± 0.2	1.1 ± 0.2
pTrcFabH(F87A)	1.5 ± 0.5	0.6 ± 0.2	91.1 ± 2.2	7.7 ± 0.8	1.2 ± 1.0	0
pTrcFabH(F87D)	2.5 ± 0.5	ND				
pTrcFabH(F87E)	2.0 ± 0.1	ND				
pTrcFabH(F87H)	2.2 ± 0.3	ND				
pTrcFabH(F87M)	0.3 ± 0.2	2.6 ± 0.6	93.6 ± 0.5	4.4 ± 0.5	1.1 ± 0.1	0.9 ± 0.1
pTrcFabH(F87Q)	2.7 ± 1.0	ND				
pTrcFabH(F87G)	2.3 ± 0.6	ND				
pTrcFabH(F87R)	2.1 ± 0.9	ND				
pPPAC ^e	1.7 ± 0.03	ND				

^a Cells were cultivated in LB medium. IPTG and 2 g of glucose ml⁻¹ were added after 9 and 12 h of cultivation, respectively. The results are the averages and the standard deviations of at least three independent experiments. All strains harbored the pPPAC plasmid, which expresses the *Pseudomonas* sp. strain 61-3 *phaC1* gene (32), as well as the pTrcFabH plasmid indicated in the table.

^b CDW, cell dry weight.

^c 3HB, 3-hydroxybutyrate; 3HO, 3-hydroxyoctanoate; 3HD, 3-hydroxydecanoate.

^d ND, not detected. The PHA composition was determined by GC analysis after methanolysis of lyophilized cells in the presence of 15% sulfuric acid as described in Materials and Methods.

^e Only the pPPAC plasmid was harbored in this strain.

pression of the mutant *E. coli fabH* genes could produce monomers other than 3HB and 3HHx for PHA production, the pTrcFabH plasmids were cotransformed into *E. coli* JM109 with pPPAC. pPPAC is an expression plasmid harboring the *Pseudomonas* sp. strain 61-3 *phaC1* gene (31). Previous studies showed that the *Pseudomonas* sp. strain 61-3 PhaC1 protein was able to produce SCL-MCL PHA copolymers with substrate specificity for monomers consisting of C₄ to C₁₂ (21).

Table 4 shows the results of PHA production in *E. coli* strains transformed with the pTrcFabH plasmids with pPPAC. The results indicate that even the wild-type *fabH* gene coexpressed with pPPAC was able to accumulate a PHA copolymer composed of C₄ and C₆ monomers (Table 4). The coexpression of *Pseudomonas* sp. strain 61-3 *phaC1* and specific mutant *E. coli fabH* genes resulted in the production of SCL-MCL PHA copolymers consisting of monomers of C₄ to C₁₀ as shown in Table 4. All of the pTrcFabH/pPPAC strains accumulated less total polymer than the corresponding pTrcFabH/pAPAC strains. However, the abilities of individual strains to accumulate PHA were similar, and most of the strains harboring *fabH* mutant genes that were able to accumulate PHA with pAPAC were also able to accumulate PHA if cotransformed with pPPAC. The strains harboring *fabH* genes with the eight mutations F87N, F87K, F87P, F87D, F87E, F87H, F87G, and F87R with pPPAC also failed to provide monomers for PHA production when cotransformed with either pAPAC or pPPAC (Tables 3 and 4). A control strain of *E. coli* harboring only pPPAC also failed to accumulate any detectable amounts of PHA, as was the case with an *E. coli* strain harboring only the pAPAC plasmid. These results indicate that the overexpres-

sion of some of the mutant *fabH* genes enabled the recombinant *E. coli* to supply monomers ranging in size from C₄ to C₁₀ for PHA production. The results also imply that some mutations have a deleterious effect on the function of the FabH enzyme.

Physical characteristics of PHA isolated from recombinant *E. coli* strains. The results indicated that coexpression of a wild-type copy of *fabH* and *A. caviae phaC* led to the highest accumulation of PHA, whereas a combination of an *E. coli fabH* gene harboring a Phe87→Cys87 mutation coexpressed with the *A. caviae phaC* gene had the highest content of C₆ monomer (3HHx) (Table 3). Because of the relatively high yield of polymers from these strains, further experiments were carried out to isolate and characterize the physical properties of the polymers produced. The PHA polymers were isolated from a strain of *E. coli* harboring pTrcFabH and pAPAC, as

TABLE 5. Physical characteristics of polymers produced using *fabH* as a monomer supplier^a

Plasmid	PHA composition (mol%) ^b		M _n	M _w	M _w /M _n
	3HB (C ₄)	3HHx (C ₆)			
pTrcFabH	100	0	3.3 × 10 ⁵	1.5 × 10 ⁶	4.3
pTrcFabH(F87C)	93.7	6.3	2.9 × 10 ⁵	1.2 × 10 ⁶	4.3

^a Cells were cultivated in LB medium. IPTG and 10 g of glucose liter⁻¹ were added after 9 and 12 h of cultivation, respectively. All strains harbored the pAPAC plasmid, which expresses the *A. caviae phaC* gene (32), as well as the pTrcFabH plasmid indicated in the table.

^b PHA composition determined by GC analysis. 3HB, 3-hydroxybutyrate.

well as from a strain harboring pTrcFabH(F87C) and pAPAC, for molecular mass analysis via GPC (Table 5). The number-average molecular weight (M_n), weight-average molecular weight (M_w), and polydispersity (M_w/M_n) are similar for the two different polymer isolates. The M_n and M_w are comparable to those of other bacterially produced PHA polymers; however, the polydispersity index is slightly larger than that of other bacterial-polymer isolates (20). This is due to the broad distribution of the molecular masses, which were found to be 9.3×10^3 to 1.4×10^7 Da for the polymer isolated from cells harboring pTrcFabH and pAPAC and 1.2×10^4 to 1.8×10^7 Da for polymer isolated from cells harboring pTrcFabH(F87C) and pAPAC.

DISCUSSION

Unlike petroleum-based plastics, PHAs are environmentally friendly, biodegradable polymers, since they can be easily mineralized to CO_2 and H_2O (30). P(3HB) homopolymer can be produced in many bacterial strains, but it is a highly crystalline, stiff material that is brittle and has limited applications. However, the SCL-MCL PHA copolymers composed of 3HB units with a small amount of 3HA units of C_6 to C_{12} have been demonstrated to be more flexible materials than P(3HB) and thus have better mechanical properties (1, 20), whereas, copolymers consisting solely of MCL PHA are elastomers whose applications may be enhanced by the addition of nanocomposite materials (6, 7). It is likely that SCL-MCL PHA copolymers consisting of low moles percent of 3HA units would have physical characteristics similar to those of MCL PHA copolymers.

In order to develop a metabolic pathway to supply monomers for SCL-MCL PHA copolymer production, we evaluated the effect of saturation site-directed mutagenesis of the codon for Phe87 in the *E. coli fabH* gene on monomer supply for PHA production in recombinant *E. coli*. The data presented here show that coexpression of *E. coli fabH* genes with mutations in the codon for Phe87 with either *A. caviae phaC* or *Pseudomonas* sp. strain 61-3 *phaC1* in recombinant *E. coli* led to the production of SCL-MCL PHA copolymers from glucose. Moreover, these point mutations had dramatic effects on the monomer compositions of the polymers (Tables 3 and 4), which may lead to differences in the physical properties of each polymer.

Based on the results of this study, FabH likely acts as an intermediary for one or more PHA monomer (*R*)-3-hydroxyacyl-CoA-supplying pathways in *E. coli* originating from fatty acid biosynthesis (Fig. 1). The distribution in the mole percent of the specific monomers is likely to be dependent on several factors. One factor is the substrate specificity of the FabH enzyme. Because the *E. coli* wild-type FabH has substrate specificity for two- to four-carbon substrates, its overproduction likely led to the accumulation of acetoacetyl-CoA via a transacylase reaction (Fig. 1, reaction 3), which was converted to (*R*)-3-hydroxybutyryl-CoA by β -ketoacyl-ACP reductase (FabG) (31). Subsequently, (*R*)-3-hydroxybutyryl-CoA could be incorporated into P(3HB) via the PHA synthase (PhaC) (Fig. 1, reaction 5). However, expression of a *fabH* gene with a mutation in the codon encoding amino acid 87 produced an enzyme capable of interacting with substrates of C_4 to C_{10} . The

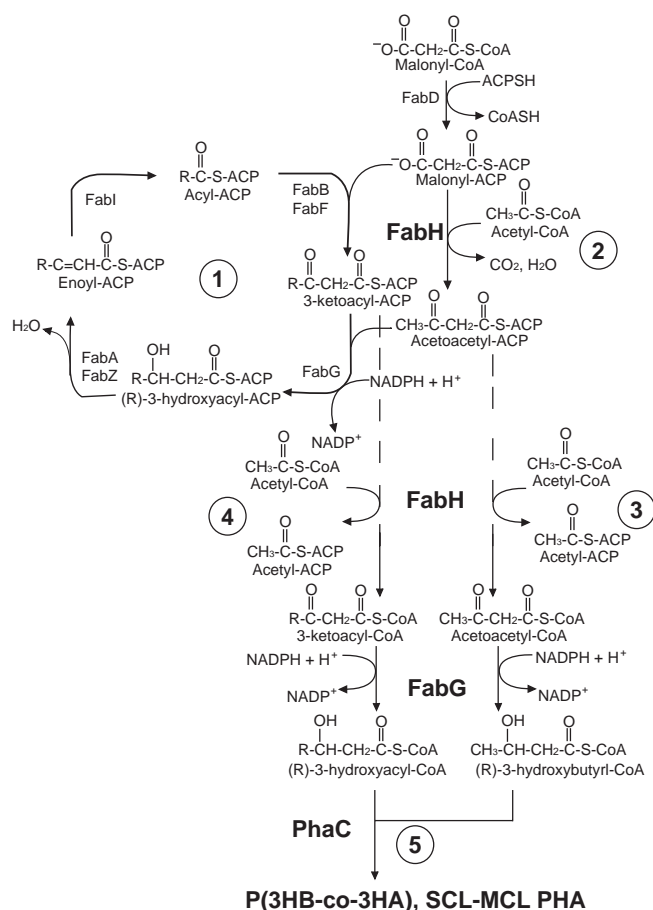


FIG. 1. Proposed pathways for PHA monomer supply from fatty acid biosynthesis in *E. coli* strains overproducing FabH. Enzymes: FabD, malonyl-ACP transacylase; FabH, 3-ketoacyl-ACP synthase III; FabB, 3-ketoacyl-ACP synthase I; FabF, 3-ketoacyl-ACP synthase II; FabG, 3-ketoacyl-ACP reductase; FabA, 3-hydroxydecanoyl-ACP dehydrase; FabZ, 3-hydroxymyristoyl-ACP dehydratase; FabI, enoyl-ACP reductase; PhaC, polyhydroxyalkanoate synthase. The solid lines represent known or dominant reactions, and the dashed lines represent the possible transacylase pathways of the FabH enzyme. Intermediates and cofactors are indicated. (Reaction 1) Fatty acid biosynthesis pathway in *E. coli*. (Reaction 2) The predominant function of FabH is the condensation of malonyl-ACP and acetyl-CoA for fatty acid biosynthesis. (Reaction 3) Overproduced FabH may be capable of a transacylase reaction with its own product, acetoacetyl-ACP, to form acetoacetyl-CoA. Acetoacetyl-CoA can then be converted to (*R*)-3-hydroxybutyryl-CoA by FabG, which in turn could be utilized for PHA production (C_4). (Reaction 4) Overproduced FabH may be able to intercept 3-ketoacyl-ACP substrates from fatty acid biosynthesis (Reaction 1) and convert it to the corresponding CoA form via a transacylase reaction. (Reaction 5) The products from reactions 3 and 4 could be converted to the (*R*)-3-hydroxyacyl-CoA forms by FabG and be incorporated into PHA by PhaC.

modified enzymes would likely be able to recognize both acetoacetyl-ACP and 3-ketoacyl-ACP as substrates, converting them into the equivalent CoA forms, which are then converted to the (*R*)-3-hydroxyacyl-CoA substrates by FabG (Fig. 1, reactions 3 and 4). These substrates are then incorporated into the SCL-MCL PHA copolymer P(3HB-co-3HA) by PhaC (Fig. 1, reaction 5). Although some of the point mutations have changed the number of substrates that FabH can interact with,

TABLE 6. Differences in MCL PHA accumulation from unrelated carbon sources in recombinant *E. coli* strains

Strain (genotype)	PHA content (%) [wt/wt] ^a	Monomer-supplying enzyme	Range of PHA composition (mol%) ^b				Source
			3HB (C ₄)	3HHx (C ₆)	3HO (C ₈)	3HD (C ₁₀)	
JMU193 (<i>fadR::Tn10 fadB64</i>)	0.1–2.0	<i>E. coli</i> TesA	0	21–33	62–71	3–8	15
S17-1 (<i>recA tra proA thi-1</i>)	1.5–3.3	<i>P. putida</i> PhaG ^c	0	0	0	100	25
LS1298 (<i>fadB</i>)	3.2	<i>U. californica</i> Tes	0	0	0	100	26
RS3097 (<i>fadR</i>)	3.4	<i>U. californica</i> Tes	0	0	0	100	26
JM109	0.3–4.9	<i>E. coli</i> FabH ^d	74–99	1–27	1–6	1–2	This study

^a Range of PHA content accumulated by the strains in the particular study.

^b Range of mole percent PHA monomer composition achieved in the particular study.

^c PHA accumulation was done in the presence of the fatty acid biosynthesis inhibitor triclosan.

^d The modified FabH enzymes made in this study.

the specific activities of these enzymes may still favor the utilization of the SCL substrates of C₄ over MCL substrates of C₆ to C₁₀. Therefore, the SCL-MCL PHA copolymers produced by PhaC with FabH mutants have a higher mole percent of C₄ incorporated into the polymer.

A second factor that may determine the mole percent of monomer incorporated into PHA is the availability of the substrate. The main reaction of FabH is the condensation of malonyl-ACP with acetyl-CoA to form acetoacetyl-ACP, which is then used by the fatty acid biosynthesis pathway (Fig. 1, reaction 1). It had been shown previously that overproduction of wild-type FabH led to an accumulation of shorter-chain fatty acids in membrane phospholipids (33). When producing PHA, overproduction of FabH may at first lead to a large increase in the acetoacetyl-ACP pool, which in turn could be converted to acetoacetyl-CoA and eventually to (*R*)-3-hydroxybutyryl-CoA via FabG. This substrate, in the presence of PHA synthase, could be converted to PHA. In the absence of PHA synthase, these compounds may be excreted from the cell. In either case, the enzymatic activity of the overproduced FabH would lead to a decrease in the concentration of substrates available for longer-chain fatty acid biosynthesis. Because a number of cycles of fatty acid biosynthesis would have to be completed before MCL substrates could be produced, the overproduction of FabH could limit the incorporation of MCL monomers of C₆ to C₁₀ into PHA because of the competition with itself to produce SCL substrates of C₄ length. Since incorporation of MCL monomers into PHA would require the overproduced mutant FabH proteins to intercept intermediates of fatty acid biosynthesis, such as 3-ketoacyl-ACP, FabH must also compete with the other enzymes of fatty acid biosynthesis for the substrate. This illustrates another possibility for the lower mole percent composition of MCL monomers of C₆ to C₁₀ in the SCL-MCL PHA copolymers in this study.

A third factor determining the PHA composition is likely to be the specificity of the PHA synthase. It has been shown that the *A. caviae* PhaC protein has specificity for (*R*)-3-hydroxyacyl-CoA substrates of C₄ to C₆ lengths (9). Although the *Pseudomonas* sp. strain 61-3 PhaC1 is capable of incorporating substrates of C₄ and C₆ lengths into a PHA polymer, it has a preferred specificity for substrates of C₈ to C₁₂ lengths (19, 21). Based on the mole percent compositions of the polymers produced by the various strains shown in Tables 3 and 4, it appears that all of the FabH proteins capable of producing monomers for PHA production do so with a specificity of C₄ > C₆ > C₈ > C₁₀ > C₁₂. Therefore, the differences between the amounts

of PHA accumulated in cells expressing the *A. caviae* phaC genes with the mutant fabH genes and the amount of PHA accumulated in cells expressing the *Pseudomonas* sp. strain 61-3 phaC1 gene with the mutant fabH genes shown in Tables 3 and 4 reflect the differences in the specific activities of the two PHA synthases, as well as the differences in the activities of the various mutant FabH proteins.

Saturation point mutagenesis at amino acid position 87 of FabH led to a broadening of substrate specificity and monomer-supplying capacity for PHA synthesis. In strains of *E. coli* overexpressing mutant fabH and PHA synthase (*phaC*) genes that could accumulate PHA, the point mutations at amino acid 87 have likely changed the binding-pocket size while leaving the transacylase function of the enzyme intact, thus accounting for the broadening of substrate specificity and the ability to provide monomers for SCL-MCL PHA production. However, in strains of *E. coli* overexpressing mutant fabH and phaC genes that failed to accumulate PHA, it is not known whether this resulted from the inactivation of the enzyme or from the lack of specificity of the PHA synthase for monomers produced by these enzymes. Previously, it was shown that *M. tuberculosis* FabH has specificity for substrates of C₈ to C₁₆ in vitro (4). If the FabH mutants generated by this study created monomers with longer carbon backbones (more than C₁₂), they might fail to be incorporated into a growing PHA chain because of the specificities and limitations of the PHA synthases used in this study.

In previous studies, MCL PHA copolymer produced from glucose in *E. coli* consisted solely of MCL monomers with various yields (15, 25, 26) (Table 6). Furthermore, these studies had to use *E. coli* strains that were inhibited in the β -oxidation pathway in the cases where thioesterases were used as monomer-supplying enzymes, or they had to apply triclosan, an inhibitor of the fatty acid biosynthesis pathway, in order to generate MCL PHA from unrelated carbon sources. The present study used only JM109, a typical laboratory cloning strain of *E. coli*, to generate MCL PHA and is the first report to show that SCL-MCL PHA copolymer can be made in recombinant *E. coli* grown in the presence of glucose.

Although higher levels of SCL-MCL PHA production have been observed in native organisms with the inherent ability to produce SCL-MCL PHA, the molecular weights of the polymers accumulated were much lower (20) than those achieved in the present study. Because *E. coli* does not have a PHA depolymerase, the molecular weights of polymers produced in recombinant strains may be higher than those of polymers

produced by native organisms (18). The molecular weights of microbially produced PHAs are important, since PHA polymer of high molecular weight has been shown to have improved mechanical properties (2).

Glucose and related sugars are cheaper than fatty acids as a carbon source for PHA production, but sugars are comparable in price to plant oils, which can also be used as a carbon source for PHA production (10). However, in order to use plant oils as a carbon source for PHA production, an intact β -oxidation pathway must be present within the host organism. The PHA-producing pathway presented here derives monomers from fatty acid biosynthesis rather than from the β -oxidation pathway and thus does not need to use strains with inhibited β -oxidation pathways or expensive inhibitors of fatty acid biosynthesis in order to produce SCL-MCL PHA copolymer. The ubiquity of fatty acid biosynthesis reactions in all organisms makes this new pathway highly desirable, as it may be possible to transfer the system to other organisms, such as photosynthetic organisms, to further reduce the costs of the carbon source. Studies of these possibilities are under way. In addition, because of the likely codependence between FabG and FabH necessary for monomer supply for PHA, studies are under way in our laboratory to determine whether coexpression of *fabG* and *fabH* would further change yields and/or compositions of PHAs in bacteria. Further mutagenesis studies of fatty acid biosynthesis and PHA synthase genes may allow us to precisely control the compositions of PHA copolymers and lead to commercial applicability for these microbially produced biodegradable polymers.

In conclusion, we have engineered a new pathway for SCL-MCL PHA copolymer accumulation from unrelated carbon sources in *E. coli*. By making subtle changes in the substrate-binding pocket of FabH, we have been able to engineer new substrate specificities for the enzyme that result in different and specific monomer compositions of C₄ to C₁₀ for SCL-MCL PHA copolymers. Because FabH proteins from other bacterial species have substrate specificities different from those of *E. coli* FabH (3, 4, 11, 13), they may be able to supply other monomers and different ratios of monomers for incorporation into PHA polymers produced in recombinant *E. coli*. It may be possible to modify the substrate specificities and activities of FabH via additional mutation. There are at least two other residues (Arg196 and Leu191) in the *E. coli* FabH protein besides F87 that may limit the size of the substrate-binding pocket, based on a comparison of the crystal structures of *E. coli* FabH and *M. tuberculosis* FabH (28), and saturation point mutagenesis of these residues may lead to production of FabH enzymes with different monomer specificities and activities. The modified substrate specificities obtained from further mutation could result in PHA copolymers with monomer mole percent distributions different from those achieved in this study. Furthermore, if FabH enzymes with enhanced activities were generated by further mutation, it might be possible to produce recombinant strains capable of accumulating larger amounts of PHA.

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REFERENCES

- Abe, H., and Y. Doi. 2002. Side-chain effect of second monomer units on crystalline morphology, thermal properties, and enzymatic degradability for random copolyesters of (*R*)-3-hydroxybutyric acid with (*R*)-3-hydroxyalkanoic acids. *Biomacromolecules* **3**:133–138.
- Aoyagi, Y., Y. Doi, and T. Iwata. 2003. Mechanical properties and highly ordered structure of ultra-high-molecular-weight poly[(*R*)-3-hydroxybutyrate] films: effects of annealing and two-step drawing. *Polym. Degrad. Stab.* **79**:209–216.
- Choi, K. H., R. J. Heath, and C. O. Rock. 2000. β -Ketoacyl-acyl carrier protein synthase III (FabH) is a determining factor in branched-chain fatty acid biosynthesis. *J. Bacteriol.* **182**:365–370.
- Choi, K. H., L. Kremer, G. S. Besra, and C. O. Rock. 2000. Identification and substrate specificity of β -ketoacyl (acyl carrier protein) synthase III (mtFabH) from *Mycobacterium tuberculosis*. *J. Biol. Chem.* **275**:28201–28207.
- Davies, C., R. J. Heath, S. W. White, and C. O. Rock. 2000. The 1.8 Å crystal structure and active-site architecture of β -ketoacyl-acyl carrier protein synthase III (FabH) from *Escherichia coli*. *Structure Fold Des.* **8**:185–195.
- Dubief, D., E. Samain, and A. Dufresne. 1999. Polysaccharide microcrystals reinforced amorphous poly(β -hydroxyoctanoate) nanocomposite materials. *Macromolecules* **32**:5765–5771.
- Dufresne, A., M. B. Kellerhals, and B. Witholt. 1999. Transcrystallization in mcl-PHAs/cellulose whiskers composites. *Macromolecules* **32**:7396–7401.
- Dufresne, A., and E. Samain. 1998. Preparation and characterization of a poly(β -hydroxyoctanoate) latex produced by *Pseudomonas oleovorans*. *Macromolecules* **31**:6426–6433.
- Fukui, T., and Y. Doi. 1997. Cloning and analysis of the poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) biosynthesis genes of *Aeromonas caviae*. *J. Bacteriol.* **179**:4821–4830.
- Fukui, T., and Y. Doi. 1998. Efficient production of polyhydroxyalkanoates from plant oils by *Alcaligenes eutrophus* and its recombinant strain. *Appl. Microbiol. Biotechnol.* **49**:333–336.
- Han, L., S. Lobo, and K. A. Reynolds. 1998. Characterization of the β -ketoacyl-acyl carrier protein synthase III from *Streptomyces glaucescens* and its role in initiation of fatty acid biosynthesis. *J. Bacteriol.* **180**:4481–4486.
- Kato, M., H. J. Bao, C. K. Kang, T. Fukui, and Y. Doi. 1996. Production of a novel copolymer of 3-hydroxybutyric acid and medium-chain-length 3-hydroxyalkanoic acids by *Pseudomonas* sp. 61–3 from sugar. *Appl. Microbiol. Biotechnol.* **45**:363–370.
- Khandekar, S. S., D. R. Gentry, G. S. Van Aller, P. Warren, H. Xiang, C. Silverman, M. L. Doyle, P. A. Chambers, A. K. Konstantinidis, M. Brandt, R. A. Daines, and J. T. Lonsdale. 2001. Identification, substrate specificity, and inhibition of the *Streptococcus pneumoniae* β -ketoacyl-acyl carrier protein synthase III (FabH). *J. Biol. Chem.* **276**:30024–30030.
- Kichise, T., S. Taguchi, and Y. Doi. 2002. Enhanced accumulation and changed monomer composition in polyhydroxyalkanoate (PHA) copolyester by in vitro evolution of *Aeromonas caviae* PHA synthase. *Appl. Environ. Microbiol.* **68**:2411–2419.
- Klinke, S., Q. Ren, B. Witholt, and B. Kessler. 1999. Production of medium-chain-length poly(3-hydroxyalkanoates) from gluconate by recombinant *Escherichia coli*. *Appl. Environ. Microbiol.* **65**:540–548.
- Kusaka, S., H. Abe, S. Y. Lee, and Y. Doi. 1997. Molecular mass of poly[(*R*)-3-hydroxybutyric acid] produced in a recombinant *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **47**:140–143.
- Langenbach, S., B. H. Rehm, and A. Steinbuchel. 1997. Functional expression of the PHA synthase gene *phaCI* from *Pseudomonas aeruginosa* in *Escherichia coli* results in poly(3-hydroxyalkanoate) synthesis. *FEMS Microbiol. Lett.* **150**:303–309.
- Lee, S. Y. 1996. Bacterial polyhydroxyalkanoates. *Biotechnol. Bioeng.* **49**:1–14.
- Matsumoto, K., M. Matsusaki, K. Taguchi, M. Seki, and Y. Doi. 2002. Isolation and characterization of polyhydroxyalkanoates inclusions and their associated proteins in *Pseudomonas* sp. 61–3. *Biomacromolecules* **3**:787–792.
- Matsusaki, H., H. Abe, and Y. Doi. 2000. Biosynthesis and properties of poly(3-hydroxybutyrate-co-3-hydroxyalkanoates) by recombinant strains of *Pseudomonas* sp. 61–3. *Biomacromolecules* **1**:17–22.
- Matsusaki, H., S. Manji, K. Taguchi, M. Kato, T. Fukui, and Y. Doi. 1998. Cloning and molecular analysis of the poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyalkanoate) biosynthesis genes in *Pseudomonas* sp. strain 61–3. *J. Bacteriol.* **180**:6459–6467.
- Park, S. J., J. P. Park, and S. Y. Lee. 2002. Metabolic engineering of *Escherichia coli* for the production of medium-chain-length polyhydroxyalkanoates rich in specific monomers. *FEMS Microbiol. Lett.* **214**:217–222.
- Qiu, X., C. A. Janson, A. K. Konstantinidis, S. Nwagwu, C. Silverman, W. W. Smith, S. Khandekar, J. Lonsdale, and S. S. Abdel-Meguid. 1999. Crystal structure of β -ketoacyl-acyl carrier protein synthase III. A key condensing enzyme in bacterial fatty acid biosynthesis. *J. Biol. Chem.* **274**:36465–36471.
- Qiu, X., C. A. Janson, W. W. Smith, M. Head, J. Lonsdale, and A. K. Konstantinidis. 2001. Refined structures of beta-ketoacyl-acyl carrier protein synthase III. *J. Mol. Biol.* **307**:341–356.
- Rehm, B. H., T. A. Mitsky, and A. Steinbuchel. 2001. Role of fatty acid de

- novo biosynthesis in polyhydroxyalkanoic acid (PHA) and rhamnolipid synthesis by pseudomonads: establishment of the transacylase (PhaG)-mediated pathway for PHA biosynthesis in *Escherichia coli*. *Appl. Environ. Microbiol.* **67**:3102–3109.
26. **Rehm, B. H., and A. Steinbuchel.** 2001. Heterologous expression of the acyl-acyl carrier protein thioesterase gene from the plant *Umbellularia californica* mediates polyhydroxyalkanoate biosynthesis in recombinant *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **55**:205–209.
 27. **Sambrook, J., E. Fritsch, and T. Maniatis.** 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 28. **Scarsdale, J. N., G. Kazanina, X. He, K. A. Reynolds, and H. T. Wright.** 2001. Crystal structure of the *Mycobacterium tuberculosis* β -ketoacyl-acyl carrier protein synthase III. *J. Biol. Chem.* **276**:20516–20522.
 29. **Steinbuchel, A., and H. Silke.** 2001. Biochemical and molecular basis of microbial synthesis of polyhydroxyalkanoates in microorganisms. *Adv. Biochem. Eng. Biotechnol.* **71**:81–123.
 30. **Sudesh, K., H. Abe, and Y. Doi.** 2000. Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Prog. Polym. Sci.* **25**:1503–1555.
 31. **Taguchi, K., Y. Aoyagi, H. Matsusaki, T. Fukui, and Y. Doi.** 1999. Co-expression of 3-ketoacyl-ACP reductase and polyhydroxyalkanoate synthase genes induces PHA production in *Escherichia coli* HB101 strain. *FEMS Microbiol. Lett.* **176**:183–190.
 32. **Taguchi, K., Y. Aoyagi, H. Matsusaki, T. Fukui, and Y. Doi.** 1999. Over-expression of 3-ketoacyl-ACP synthase III or malonyl-CoA-ACP transacylase gene induces monomer supply for polyhydroxybutyrate production in *Escherichia coli* HB101. *Biotechnol. Lett.* **21**:579–584.
 33. **Tsay, J. T., W. Oh, T. J. Larson, S. Jackowski, and C. O. Rock.** 1992. Isolation and characterization of the β -ketoacyl-acyl carrier protein synthase III gene (*fabH*) from *Escherichia coli* K-12. *J. Biol. Chem.* **267**:6807–6814.