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Mutations to the active site of 3-ketoacyl-ACP synthase III (FabH) increase polyhydroxyalkanoate biosynthesis in transgenic *Escherichia coli*

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Polyhydroxyalkanoate (PHA) production has been enhanced with engineered 3-ketoacyl-ACP synthase III (FabH) enzymes that accept diverse fatty acyl-ACP substrates and convert them to fatty acyl-CoA substrates for polymerization by PHA synthase enzymes resulting in the production of diverse polymers. Two mutations in the monomer supplying enzyme FabH, His244Ala and the Asn274Ala, were investigated to assess the impact of these mutations on PHA monomer production. PHA production increased more than six-fold with the mutation His244Ala in the FabH enzyme. Engineering of the FabH enzyme for improved PHA monomer supply led to a more productive system for PHA copolymer production.

[**Key words:** Recombinant *Escherichia coli*; Protein engineering; polyhydroxyalkanoates; Bioplastic; 3-Ketoacyl-ACP synthase III; *fabH*]

Polyhydroxyalkanoates (PHAs) are a class of polyesters that can be biologically produced and are completely biodegradable, making them an environmentally friendly alternative to their petroleum based counterparts (1–3). These materials have many applications ranging from use in bulk-commodity plastics to medical applications (1,2,4). The production and use of PHAs are dwarfed by petroleum based plastics due to their limited physical properties and higher cost of production.

The physical properties of PHAs are largely dependent upon monomer composition. PHAs composed of short-chain-length (SCL) monomers, with repeating units 3 to 5 carbons in length, form highly crystalline thermoplastics which are quite brittle. PHAs composed of medium-chain-length (MCL) monomers, with repeating units 6 to 14 carbons in length, form semi-crystalline elastomers which range from tacky to free-flowing (5). PHA copolymers composed of SCL and MCL monomers (SCL–MCL) can possess a wide range of physical properties dependent on the monomer composition (5). Because there is currently not a significant source of these copolymers, few opportunities exist to test them and fully explore their potential applications. Due to the variety of applications that enhanced physical properties of the SCL–MCL PHAs could allow for, developing a cost-effective and efficient biological strategy to produce them would be beneficial in extending the use of PHAs to replace petroleum based plastics.

While pathways for the production of SCL PHAs have been well defined (3,6–8) MCL PHA production is not as well understood. Transgenic production of MCL PHA has been accomplished in *Escherichia coli*, but most of these pathways require fatty acids or oils as carbon sources (9–13). Such pathways rely on β-oxidation, and are not effective at producing PHAs from carbon sources that are initially unrelated to fatty acids, like sugars or CO_2. As such, an alternative model system may lead to lower production cost where SCL–MCL PHAs are produced from simple carbon sources unrelated to fatty acids, by way of fatty acid biosynthesis metabolism. The ability to produce SCL–MCL PHAs from unrelated carbon sources is especially desirable in plants, in order to convert CO_2 directly into polymers photosynthetically in the chloroplast. There are few studies that have shown potential for transgenic production of MCL PHAs from unrelated carbon sources (14–17), and even fewer have produced SCL–MCL copolymers (18–21). Thus, the potential for development in this area is largely unexplored.

One pathway that has successfully generated SCL–MCL PHAs from sugars in recombinant *E. coli* utilizes an engineered 3-ketoacyl-ACP synthase III (FabH) as a MCL-monomer-supplying enzyme (Fig. 1). The pathway makes use of intermediates from fatty acid biosynthesis, which are present in all organisms, giving the pathway potential for introduction to a wide range of organisms, including plants. FabH is a member of the β-ketoacyl synthase family of enzymes. Its native function is the condensation of malonyl-ACP and acyl-CoA units in fatty acid biosynthesis, but it has been demonstrated to have a transacylase activity capable of converting β-ketoacyl-ACP units, intermediates from fatty acid biosynthesis, to β-ketoacyl-CoA units which can be converted into monomer supplying units recognizable by PHA synthase enzymes (19,22–24). Overexpression of *fabH* genes encoding proteins with mutations to amino acid 87 of the binding pocket led to low level MCL PHA monomer supply in transgenic *E. coli* (19). Mutations to the active site, amino acids histidine 244 (H244) and asparagine 274 (N274), have been reported to increase the transacylase activity of the enzyme (22). This transacylase activity is the critical activity in the monomer supplying function of the enzyme, but the effect of these mutations on PHA monomer supplying activity has not been tested.

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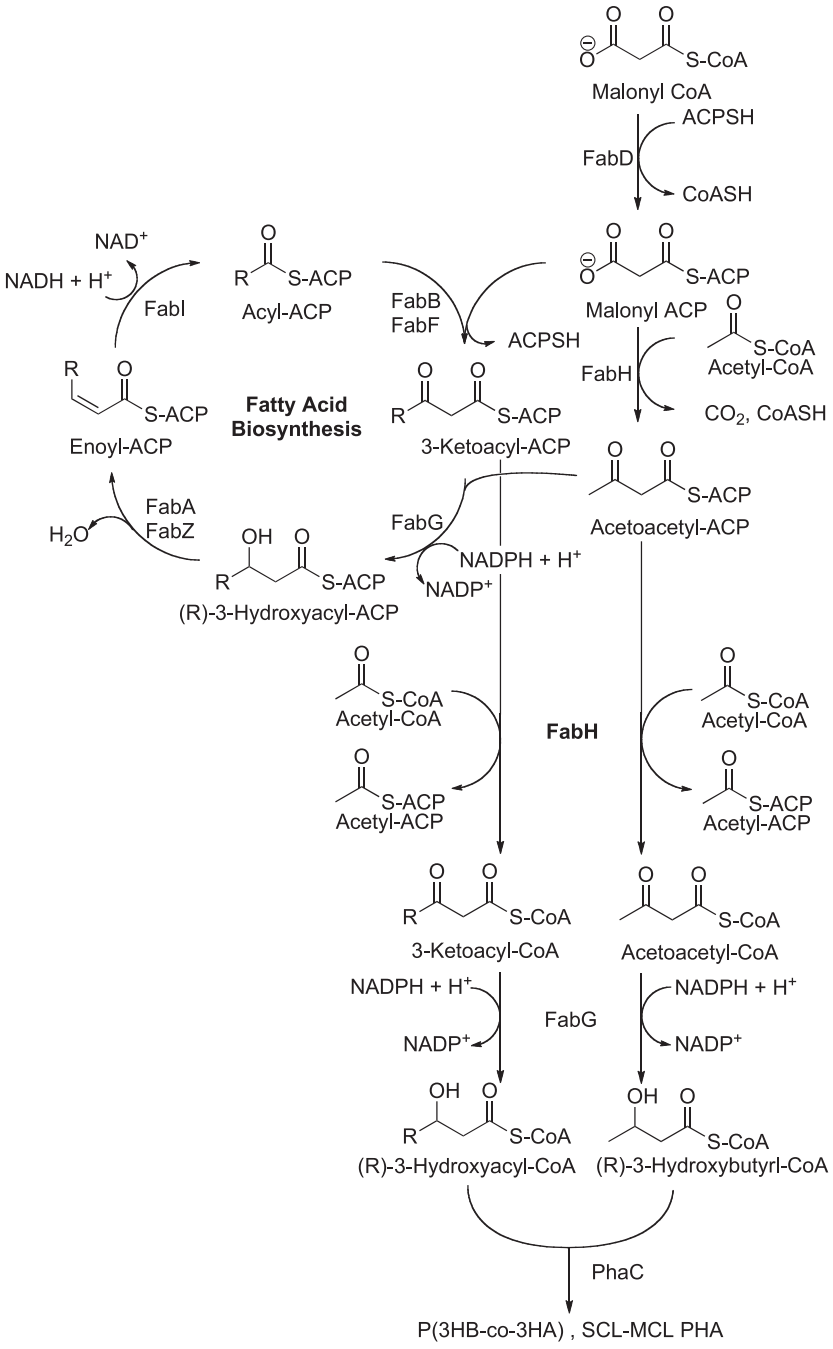

FIG. 1. Metabolic pathway for PHA production from glucose in recombinant *E. coli* adapted from Nomura et al. (19). 3-ketoacyl-ACP intermediates from fatty acid biosynthesis are converted to 3-ketoacyl-CoA by the engineered FabH (shown in bold) to produce medium-chain-length monomers. The native FabH continues the normal function condensing acetyl-CoA and malonyl-ACP into acetoacetyl-ACP, which can be converted to acetoacetyl-CoA by the engineered FabH and processed to produce short-chain-length monomers. The monomers are assembled into a polymer by the PHA synthase (PhaC).

In this work we improved the PHA monomer supplying efficiency of the *E. coli* FabH enzyme for PHA production in transgenic *E. coli* through introduction of specific mutations to the active site of the enzyme.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cultivation conditions Competent cell preparation, transformation, and plasmid DNA isolation were preformed according to common protocols (25) using *E. coli* JM109 (Promega, USA). Plasmids were isolated from *E. coli* cells by alkaline lysis with sodium dodecylsulfate. Transgenic production of PHA was performed in *E. coli* K-12 MG1655. Bacterial strains were grown in LB medium supplemented with glucose as described below. To maintain and select for plasmids within recombinant *E. coli*, 100 μg L^{-1} of ampicillin was used. Plasmids and strains used in this study are listed in Table 1.

Construction and analysis of plasmids All protocols were carried out according manufacturer's instructions as outlined below. Primers were purchased from Invitrogen (USA) and all enzymes were purchased from Promega (USA). The pTrc99A plasmids containing the wild type and F87 mutant *fabH* genes were obtained from a previous study by Nomura et al. (19). Site-directed mutagenesis for introduction of active site mutations was performed with the QuickChange kit (Stratagene, USA). Briefly, complimentary pairs of oligonucleotide primers were designed which contained the modified codon of interest and annealed to the same sequence on opposite strands of the plasmid (Table 2). These primers were used in a polymerase chain reaction (PCR) with *Pfu* polymerase and *Dam* methylated pTrcFabH plasmids as the template to generate copies of the plasmid with the desired mutation. The PCR products were then treated with *Dpn*I to digest the methylated template. The *Dpn*I treated PCR product was then used to transform *E. coli*, and