Only One of the Two Annotated *Lactococcus lactis fabG* Genes Encodes a Functional β-Ketoacyl−Acyl Carrier Protein Reductase†

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ABSTRACT: The small genome of the Gram-positive bacterium *Lactococcus lactis* ssp. *lactis* IL1403 contains two genes that encode proteins annotated as homologues of *Escherichia coli* β-hydroxyacyl−acyl carrier protein (ACP) reductase. *E. coli fabG* encodes β-ketoacyl−acyl carrier protein (ACP) reductase, the enzyme responsible for the first reductive step of the fatty acid synthetic cycle. Both of the *L. lactis* genes are adjacent to (and predicted to be cotranscribed with) other genes that encode proteins having homology to known fatty acid synthetic enzymes. Such relationships have often been used to strengthen annotations based on sequence alignments. Annotation in the case of β-ketoacyl-ACP reductase is particularily problematic because the protein is a member of a vast protein family, the short-chain alcohol dehydrogenase/reductase (SDR) family. The recent isolation of an *E. coli fabG* mutant strain encoding a conditionally active β-ketoacyl-ACP reductase allowed physiological and biochemical testing of the putative *L. lactis* homologues. We report that expression of only one of the two *L. lactis* proteins (that annotated as FabG1) allows growth of the *E. coli fabG* strain under nonpermissive conditions and restores in vitro fatty acid synthetic ability to extracts of the mutant strain. Therefore, like *E. coli*, *L. lactis* has a single β-ketoacyl-ACP reductase active with substrates of all fatty acid chain lengths. The second protein (annotated as FabG2), although inactive in fatty acid synthesis both in vivo and in vitro, was highly active in reduction of the model substrate, β-ketobutyryl-CoA. As expected from work on the *E. coli* enzyme, the FabG1 β-ketobutyryl-CoA reductase activity was inhibited by ACP (which blocks access to the active site) whereas the activity of FabG2 was unaffected by the presence of ACP. These results seem to be an example of a gene duplication event followed by divergence of one copy of the gene to encode a protein having a new function.

β-Ketoacyl−acyl carrier protein reductase catalyzes the first of the two reductive steps of the fatty acid synthetic cycle (*1*−*4*). In the type II fatty acid synthetic pathway the intermediates are covalently linked by a thioester bond to the prosthetic group of acyl carrier protein (ACP).[1] In *Escherichia coli* the elongation cycle begins with a Claisen condensation reaction catalyzed by one of the three β-ketoacyl-ACP synthases (FabB, FabF, or FabH) that adds two carbons to the C_N acyl chain of an acyl-ACP. The resulting C_{N+2} β-ketoacyl-ACP is reduced by the NADPH-dependent β-ketoacyl-ACP reductase to yield a β-hydroxyacyl-ACP, which is then dehydrated by a β-hydroxyacyl-ACP dehydrase (either FabA or FabZ) to produce enoyl-ACP. Finally, an NADH-dependent enoyl-ACP reductase (FabI) reduces enoyl-ACP to give a C_{N+2} acyl-ACP, the substrate for the next elongation cycle. Elongation ceases when the acyl-ACP attains the chain length required for acylation of phospholipid or lipid A precursors.

Recently, this laboratory reported the first mutants in *fabG*, the gene encoding β-ketoacyl-ACP reductase (*5*). Mutants were isolated in both *Escherichia coli* and *Salmonella enterica* serovar *Typhimurium*. The *E. coli* mutant strain and the mutant enzyme were characterized in detail. The *fabG* strain is temperature-sensitive and fails to grow or synthesize fatty acids at 42 °C. The mutant β-ketoacyl-ACP reductase activity is extremely labile both to temperature and to fractionation and handling (*5*). It should be noted that FabG is a member of a very large family of enzymes, the short-chain alcohol dehydrogenase/reductase (SDR) family (*6*, *7*), which carry out a wide variety of reduction and dehydrogenase reactions using NADH or NADPH. Due to the abundance of SDR proteins we have argued that annotation of *fabG* genes can be problematic and that various annotators of the *E. coli* and other bacterial genomes have probably assigned β-ketoacyl-ACP reductase activity to open reading frames that are unlikely to possess this enzyme activity (*3*, *5*). We have now tested our argument by use of the *E. coli fabG*(Ts) mutant.

Assignments of functions to the proteins of bacteria and archaea are often aided by gene location because enzymes

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[1] Abbreviations: ACP, acyl carrier protein; ORF, open reading frame; acyl-ACP, acyl carrier protein with the fatty acyl chain attached to the prosthetic group thiol via a thioester bond; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; SDR, short-chain alcohol dehydrogenase/reductase; IPTG, isopropyl β-D-thiogalactopyranoside; PCR, polymerase chain reaction; *fabG*(Ts), a temperature-sensitive mutation of the *fabG* gene.

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of a metabolic pathways are frequently encoded by operons or clusters of cotranscribed genes (*8*−*13*). Given an open reading frame (ORF) with reasonable homology to *E. coli* FabG chromosomal location provides a logical criterion for annotating an SDR-encoding gene as a putative *fabG* gene (*3*). Genes adjacent to genes that encode homologues of other enzymes of the pathway of interest are often considered stronger candidates than genes that lack such "location-aided annotation" (*8*−*13*). In some bacteria, such as *Lactococcus lactis* sp. *lactis* IL1403, two *fabG* genes have been annotated (*14*, *15*). Both of the *L. lactis fabG* genes are adjacent to genes encoding putative fatty acid synthetic proteins, and thus the annotations of both genes are strengthened by their genomic locations. In *L. lactis* the *fabG1* gene lies within a large cluster of genes that encode homologues of all of the genes required for saturated fatty acid synthesis in *E. coli*. The *fabG2* gene is far removed from *fabG1* on the chromosome and is located immediately upstream of a gene encoding a homologue of *Streptococcus pneumoniae* FabK, a known enoyl-ACP reductase (*16*). In both cases the *fabG* gene is oriented such that it could be cotranscribed with the neighboring annotated fatty acid synthetic gene(s), and such cotranscription has been predicted (www.tigr.org/tigr-scripts/operons/). Therefore, both *fabG* genes not only encode proteins having sufficient homology (>33% identity) with *E. coli* FabG to be readily annotated as β-ketoacyl-ACP reductase genes but seem particularly strong candidates due to their chromosomal locations. However, *E. coli* contains only a single FabG, and hence it is unclear why *L. lactis* should require a second gene. We report complementation studies using the *E. coli fabG* mutant as well as in vitro studies of the purified proteins that indicate that only one of the *L. lactis* genes, *fabG1*, encodes a functional β-ketoacyl-ACP reductase. The protein encoded by the other gene, *fabG2*, had activity with the model reductase substrate, β-ketobutyryl-CoA (also called acetoacetyl-CoA), but lacked the ability to support fatty acid synthesis both in vivo and in vitro.

EXPERIMENTAL PROCEDURES

Materials. The sources of materials were American Radiolabeled Chemicals Inc., [2-^{14}C]malonyl-CoA (specific activity of 57 mCi/mmol); Amersham, L-[^{35}S]methionine; Sigma, cerulenin and acetyl-CoA; New England Biolabs, molecular biology reagents; Novagen, pET28b(−); Qiagen, QIAprepSpin miniprep kit and Ni^{2+}−agarose column; and Bio 101, G′nome DNA kit.

Construction of Plasmids. The genes were amplified from genomic DNA obtained from strain *L. lactis* IL1403 (the strain of known genome sequence). The primers of the 5′-end of the *fabG* genes created novel restriction sites for *Nco*I at the N-terminal methionine (*fabG1*, 5′-CTCATATCTTAAACCATGGAAATTAAAAATA; *fabG2*, 5′-AAGCAATG). The primers at the 3′-end of *fabG* genes placed a *Pst*I site downstream of the stop codon (*fabG1*, 5′-GCACTAACTGCAGCATAACC; *fabG2*, 5′-GCAGCTACTACTGCAGCATTAG). The polymerase chain reaction was performed with Taq DNA polymerase, and the products were ligated into the TA cloning vector pCR2.1 followed by transformation into *E. coli* OneShot cells (Invitrogen). Following overnight growth, plasmids were isolated, and the *fabG* genes were sequenced by the Genetic Engineering Facility, University of Illinois at Urbana-Champaign, and found to have the sequences expected (*14*, *15*). These plasmids, pHW64 (*fabG1*) and pHW65 (*fabG2*), were then digested with *Nco*I and *Pst*I, and the appropriate fragments were isolated and ligated into pBAD24 (*17*) digested with the same enzymes to give plasmids pHW60 (*fabG1*) and pHW61 (*fabG2*). The QuikChange kit (Stratagene) was used to construct plasmids pHW66 (*fabG1*) and pHW67 (*fabG2*) containing *Nde*I sites that overlapped the initiation methionine codons of each gene. The *Nde*I site primers for pHW64 (*fabG1*) were 5′-GAATTCGCCCTTCTCATATCTTAACATATGGAAATTAAAAATAAAAATG and 5′-CATTTTTATTTTTAATTTCCATATGTTAAGATATGAGAAGGGCGAATTC. The *Nde*I primers for pHW65 (*fabG2*) were 5′-GAATTCGCCCTTCCTAAAAGGCATATGGAAGCAATGAACAACGAAC and 5′-GTTCGTTTGTTCATTGCTTCCATATGCCTTTTAGGAAGGGCGAATTC. Plasmids pHW66 and pHW67 were then digested with *Nde*I and *Bam*HI, and the appropriate fragments were ligated to pET28b digested with the same enzyme to give plasmids pHW62 (*fabG1*) and pHW63 (*fabG2*), respectively.

Expression and Purification of His-Tagged Proteins. Plasmids pHW62 and pHW63 were each transformed into BL21(DE3) (*18*). To assay expression of *fabG1* and *fabG2* in *E. coli*, the proteins were selectively labeled with [^{35}S]-methionine and separated by SDS−PAGE (*18*). An overnight culture was diluted (1:50) into 5 mL of fresh LB medium and grown at 37 °C for 4 h. Isopropyl β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 1 mM, and the culture continued to grow for 30 min. Rifampicin (0.2 mg/mL) and [^{35}S]methionine (3−5 μCi/mL) were then added, and the incubation was continued for 30 min. The cells from 0.1 mL of the cultures were harvested and the proteins separated on 12% SDS−PAGE gels. The destained gels were dried, and the labeled proteins were detected by autoradiography. To purify FabG1 and FabG2, metal-chelate affinity chromatography was used as previously done for *E. coli* FabG (*19*). An overnight culture of BL21-(DE3) carrying pHW62 or pHW63 was diluted 100-fold into 500 mL of LB medium and grown at 37 °C for 4 h. IPTG (1 mM) was then added, and growth was continued for 3 h at 30 °C. The cultures were centrifuged, and the cells were resuspended in 5 mL of lysis buffer [20 mM Tris-HCl (pH 7.4), 300 mM NaCl, 10 mM imidazole] and lysed by passage through a French pressure cell at 18000 lb/in.^{2}. The lysate was centrifuged in a 29000*g* at 4 °C for 1 h to remove cell debris. The supernatant was applied to a Ni^{2+}−agarose column (Qiagen) and washed with 20 mM imidazole in metal-chelate affinity chromatography buffer [20 mM Tris-HCl (pH 7.4), 300 mM NaCl, 1 mM β-mercaptoethanol]. His-tagged proteins were eluted with 200 mM imidazole in the same buffer and then dialyzed overnight at 4 °C against 4 L of buffer [20 mM Tris-HCl (pH 7.4), 300 mM, NaCl, 1 mM β-mercaptoethanol]. The purified proteins were stored at −80 °C. Electrospray mass spectrometry of the purified proteins was done as previously described (*20*).

In Vitro Fatty Acid Synthesis Assay. The in vitro fatty acid synthesis extracts were prepared as described by Heath and Rock (*19*). Derivatives of strain CL115 (*panD*) and the *panD fabG*(Ts) strain CL104 carrying either pHW60 or pHW61 were cultured in 500 mL of LB medium, and arabinose (0.01%) was added. The cultures were grown at 30 °C to

late log phase. The cultures were then centrifuged and the cells resuspended in 5 mL of lysis buffer [0.1 M sodium phosphate (pH 7.0), 5 mM β-mercaptoethanol, 1 mM EDTA], and a lysate was prepared as described above. Ammonium sulfate was added to the supernatant to 45% of saturation and the precipitated protein was removed by centrifugation for 30 min at 12000*g*. Additional ammonium sulfate was added to the supernatant to give 80% of saturation, and the precipitated protein was collected by centrifugation. The protein pellet was dissolved in 2 mL of lysis buffer and dialyzed for 5 h at 4 °C against 2 L of the same buffer. Protein concentrations were determined by the Bradford assay with bovine serum albumin as the standard. The fatty acid synthesis assay was performed according to the method of Jackowski and Rock (*21*). The assay mixtures contained 0.1 M LiCl, 0.1 M sodium phosphate (pH 7.0), 1 mM β-mercaptoethanol, 50 μM acetyl-CoA, 0.175 mM NADH, 0.149 mM NADPH, 54 μM ACP, 30 μg of protein extract from strain CL115 or CL104 carrying different plasmids (pHW60, pHW61, or pBAD24), and (when added) 1 μg of purified His-tagged FabG1 or FabG2 in a final volume of 40 μL. Cerulenin, when present, was added to a final concentration of 1 mM. The reaction mixtures were incubated for 5 min at room temperature to allow cerulenin to inactivate FabB and FabF, and [2-^{14}C]malonyl-CoA (specific activity of 57 mCi/mmol) was then added (45 μM final concentration) to initiate fatty acid synthesis. After incubation at 37 °C for 10 min the reactions were stopped by placing the tubes in ice slush. Samples of mixtures were mixed with gel loading buffer and analyzed by conformationally sensitive gel electrophoresis (*22*) on 15% polyacrylamide gels containing 2.5 M urea (*23*) for approximately 3 h at 4 °C. The gels were fixed, soaked in Enlightning (DuPont), dried, and exposed to X-ray film.

Determination of Fatty Acid Compositions. The cultures of CL115 and CL104 carrying pHW60 were grown at 42 °C in RB medium overnight. Cultures (5 mL) were harvested and washed three times with RB. The phospholipids were extracted for 1 h with 6 mL of chloroform−methanol (2:1 v/v) at room temperature followed by centrifugation. The resulting supernatant was added to 2 mL of water and 2 mL of chloroform, and the solution was mixed and centrifuged. The upper (aqueous) phase was removed, and an equal volume of 2 M KCl was added, followed by mixing and centrifugation. The top KCl phase was removed, and an equal volume of water was added, followed by mixing and centrifugation. The resulting bottom organic phase was dried under a stream of nitrogen in a fume hood. Collision-induced dissociation electrospray mass spectrometry (CID ES-MS) was performed on a VG Quattro instrument by using the negative ion mode. Samples were dissolved in 1:2 (v/v) chloroform−methanol. Mass spectra were acquired with a cone voltage of 50 V over the *m*/*z* range 650−800 in 1 s. In-source collusion-induced dissociation was achieved by increasing the cone voltage to 150 V, with the quadrupole being scanned from 100 to 400 mass units in 1 s. The values for any cyclopropane fatty acids present were added to the values for the unsaturated species from they were derived. For analysis of radioactive fatty acids in the reactions of cell-free fatty acid synthesis, the acyl-ACPs were converted to methyl esters or free fatty acids (*24*). The methyl ester samples were separated by argentation thin-layer chromatography (*24*) whereas the free fatty acids were separated by reverse-phase chromatography (*25*).

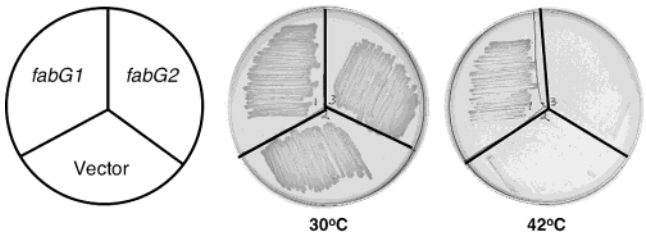

FIGURE 1: Growth of the *E. coli fabG*(Ts) strain CL104 carrying either *fabG1*, *fabG2*, or the vector pBAD24. The medium was RB agar supplemented with 0.01% arabinose. The temperatures of growth are given below the plates. No growth occurred at 42 °C without arabinose supplementation.

RESULTS

Complementation of an E. coli fabG Mutant with L. lactis fabG Genes. To test the putative β-ketoacyl-ACP reductase functions of *L. lactis* FabG1 and FabG2, we cloned the *fabG1* and *fabG2* genes into the *E. coli* pBAD24 vector to obtain plasmids pHW60 (*fabG1*) and pHW61 (*fabG2*), respectively. Transcription of the *L. lactis* genes proceeded from the arabinose-regulated *araBAD* promoter in these plasmids. The vector also supplied the ribosome binding site necessary for translational initiation. These plasmids were then transformed into the *E. coli fabG*(Ts) strain CL104 (*5*). The resulting transformants were tested for growth at 42 °C on RB plates in the presence of arabinose (Figure 1). Only the derivative of strain CL104 that carried pHW60 grew at 42 °C, the nonpermissive temperature of the *fabG*(Ts) strain. This result indicated that *fabG1* could replace the function of *E. coli fabG* whereas *fabG2* could not. We also compared the fatty acid compositions of wild-type strain CL115 and mutant strain CL104 carrying pHW60 (*fabG1*) when grown at 42 °C (Tables 1 and 2) and found that the content and composition of fatty acids in 42 °C grown cultures of strain CL104 carrying pHW60 were similar to those of the wild-type strain CL115. Note that in these experiments it remained possible that the failure of *fabG2* to complement growth of strain CL104 was due to poor gene expression in *E. coli*. However, other data (see below) precluded this explanation.

Analysis of Fatty Acid Synthesis in Vitro. Although *L. lactis fabG2* failed to complement the *E. coli fabG* mutant, it remained possible that the protein has β-ketoacyl-ACP reductase activity but that the level of FabG2 expression was too low to support growth of the host. To test the activity of FabG2 by a different means, we prepared cell-free extracts of the wild-type *E. coli* strain CL115 and derivatives of the *fabG*(Ts) strain CL104 carrying FabG1- or FabG2-encoding plasmids and assayed fatty acid synthesis in vitro. Note that the mutant enzyme is inactivated by the ammonium sulfate treatment used to prepare the extracts (*5*). The reactions containing acetyl-CoA, [2-^{14}C]malonyl-CoA, NADPH, NADH, and ACP were conducted as described in Experimental Procedures. Identification of the acyl-ACP species was based on their relative electrophoretic migration rates. Extracts of the wild-type strain CL115 and the *fabG*(Ts) strain CL104 carrying pHW60 (*fabG1*) incorporated [2-^{14}C]malonyl-CoA into long-chain acyl-ACP species (Figure 2A, lanes 1 and 3) whereas extracts of strain CL104 carrying pHW61(*fabG2*)

Table 1: Bacterial Strains and Plasmids

strain or plasmid	relevant genotype	ref[a]
strains		
CL115	*panD*, Cm^{rr}	*5*
CL104	*fabG*(Ts) *panD*, Cm^r, Tet^r, Km^r	*5*
HW60	CL104 carrying pHW60, Amp^r	
HW61	CL104 carrying pHW61, Amp^r	
HW62	CL104 carrying pBAD24, Amp^r	
BL21(DE3)	T7 RNA polymerase expression	*18*
plasmids		
pBAD24	expression vector, Amp^r	*17*
pCR2.1	cloning vector, Km^r, Amp^r	
pET28b(−)	expression vector, T7 promoter, Km^r	
pHW64	*fabG1* in pCR2.1, *Nco*I site overlaps initiation codon	
pHW65	*fabG2* in pCR2.1, *Nco*I site overlaps initiation codon	
pHW66	*fabG1* in pCR2.1, *Nde*I site overlaps initiation codon	
pHW67	*fabG2* in pCR2.1, *Nde*I site overlaps initiation codon	
pHW60	*fabG1* from pHW64 in pBAD24	
pHW61	*fabG2* from pHW64 in pBAD24	
pHW62	*fabG1* from pHW66 in pET28b(+)	
pHW63	*fabG2* from pHW66 in pET28b(−)	

[a] Those entries lacking a reference are described in Experimental Procedures.

Table 2: Fatty Acid Compositions of the Extractable Lipids of Strains Grown at 42 °C[a]

strain	composition (% by wt)			
	C14:0	C16:1	C16:0	C18:1
CL115	14.4	45.8	32.4	7.3
CL104 (pHW60)	8.4	57.4	32.3	2.0

[a] Plasmid pHW60 expresses *L. lactis* FabG1. C14:0, C16:1, C16:0, and C18:1 denote myrisitic, palmitoleic, palmitic, and *cis*-vaccenic acids, respectively. The cyclopropane fatty acids present were included with their monoenoic precursors.

and CL104 carrying the pBAD24 vector failed to incorporate [2-^{14}C]malonyl-CoA into fatty acyl-ACP species (Figure 2A, lanes 5 and 7). The only products formed were malonyl-ACP and/or acetyl-ACP. (Acetyl-ACP, which comigrates with holo-ACP and malonyl-ACP on 2.5 M urea gels, is formed by decarboxylation of malonyl-ACP.) We also tested the ability of FabG2 to perform the first reductive step of fatty acid synthesis, the reduction of β-ketobutyryl-ACP to β-hydroxybutyryl-ACP by addition of cerulenin, an antibiotic that blocks activity of the long-chain β-ketoacyl-ACP synthases I and II but which has no effect on β-ketoacyl-ACP synthase III (an enzyme specific for short-chain substrates) (*1*, *3*). Upon addition of cerulenin to these extracts accumulation of butyryl-ACP was seen in the extracts of the wild-type strain CL115 and strain CL104 carrying pHW60 (*fabG1*) (Figure 2A, lanes 2 and 4). In contrast, the only products seen in extracts of strain CL104 carrying pHW61 (*fabG2*) or carrying vector pBAD24 were low levels of butyryl-ACP [due to the residual β-ketoacyl-ACP reductase activity of the *fabG*(Ts) extracts] plus acetyl-ACP or malonyl-ACP (Figure 2A, lanes 6 and 8). (β-Ketobutyryl-ACP does not accumulate since it is unstable under the conditions of electrophoresis.)

Expression of L. lactis FabG Proteins in E. coli and Their Purification. To further test the function of the *L. lactis* FabG proteins, we cloned the two *fabG* genes into the pET28 expression vector. These plasmids, pHW62 (*fabG1*) and pHW63 (*fabG2*), were transformed into strain BL21(DE3). The dependence of *L. lactis fabG* transcription on T7 RNA polymerase allowed assay of expression of the encoded proteins by labeling cultures with [^{35}S]methionine after addition of rifampicin to block synthesis of proteins encoded by the chromosome (*18*). Plasmids that carried the His-tagged *fabG1* and *fabG2* genes expressed [^{35}S]methionine-labeled proteins with apparent masses of 25 and 24 kDa, respectively, on SDS−PAGE (Figure 3A) whereas the expected values for the His-tagged proteins were 27.6 and 29.3 kDa, respectively. Both proteins were readily purified to essential homogeneity from nonradioactive cultures by nickel-chelate chromatography and had the same apparent masses as those seen in the radioactive labeling experiment (Figure 3B). The discrepancies between the expected and observed SDS−PAGE molecular weight values can be attributed to somewhat atypical gel mobilities of the FabG proteins because mass spectroscopy of the purified His-tagged proteins gave masses of 27599.7 and 29354.0 for FabG1 and FabG2, respectively. These values were in good agreement with the calculated values (given the expected posttranslational removal of the N-terminal methionine residues) of 27630.6 and 29358.6 for FabG1 and FabG2, respectively. The fact that the FabG1 and FabG2 proteins were produced at similar abundances in *E. coli* from the same ribosome binding site (the sites of pET28b and pBAD234 are identical) argues that the failure of *L. lactis fabG2* expression to rescue growth of the *E. coli fabG*(Ts) strain at the nonpermissive temperature (Figure 1) cannot be attributed to a lack of protein expression. This was further tested by adding the purified FabG1 and FabG2 proteins to the in vitro fatty acid synthesis system prepared from the *E. coli fabG*(Ts) strain CL104.

Correction of the Fatty Acid Synthesis Defect in Extracts of the fabG(Ts) Mutant with Purified L. lactis FabG Proteins.

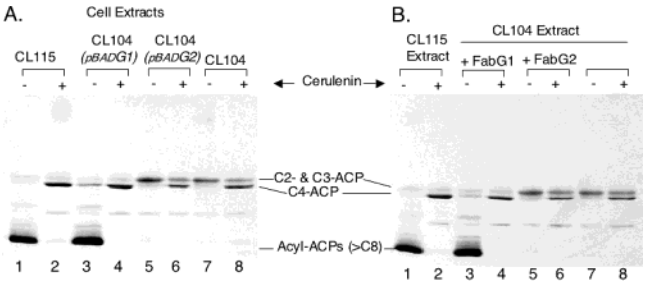

FIGURE 2: Products of in vitro synthesis of *E. coli* extracts containing *L. lactis* FabG1 or FabG2. (A) In vitro fatty acid synthesis products of extracts of derivatives of *E. coli* strain CL104 *fabG*(Ts) carrying pHW60 (*fabG1*) or pHW61 (*fabG2*) encoding the *L. lactis* FabG homologues, designated on the figure as *pBADG1* and *pBADG2*, respectively. Extracts of the wild-type strain CL115 and the mutant strain CL104 were analyzed in parallel. C2- and C3-ACP denotes a mixture of acetyl-ACP and malonyl-ACP whereas C4 denotes butyryl-ACP. Cerulenin was added as shown. (B) In vitro fatty acid synthesis products of an extract of *E. coli* strain CL104 *fabG*(Ts) supplemented with purified preparations of the His-tagged forms of FabG1 or FabG2 or left unsupplemented as given. An unsupplemented extract of the wild-type strain CL115 was analyzed in parallel. The autoradiograms of 2.5 M urea−PAGE gels are shown. Although the cultures from which the extracts were prepared were grown at 30 °C (the permissive temperature), the mutant β-ketoacyl-ACP reductase encoded by the *fabG*(Ts) gene lacked activity due to its lability during the ammonium sulfate treatment used to prepare the fatty acid synthesis extracts (*5*).

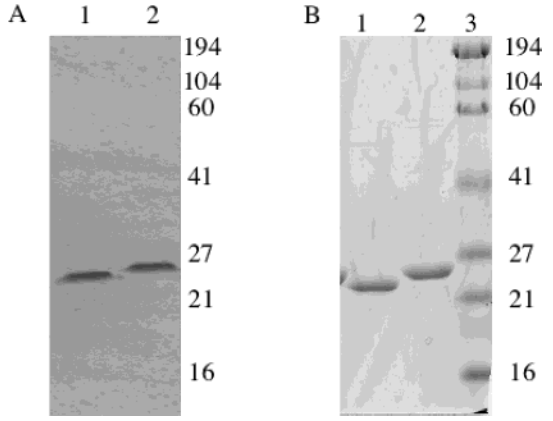

FIGURE 3: Expression of FabG1 and FabG2 in *E. coli*. (A) Plasmids with a T7 promoter upstream of FabG1 and FabG2 were used to express the proteins which were selectively labeled with [^{35}S]-methionine in the presence of rifampicin. The proteins were separated by SDS−PAGE and visualized by autoradiography as described in Experimental Procedures. Lanes: 1, pHW62 (*fabG1*); 2, pHW63 (*fabG2*). (B) Analysis of the purified FabG proteins with detection by staining with Coomassie Blue. Lanes: 1, FabG1; 2, FabG2. The positions of molecular mass markers (Bio-Rad, Richmond, CA) are shown. The markers were (from top to bottom) myosin, β-galactosidase, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme, and (not shown) aprotinin.

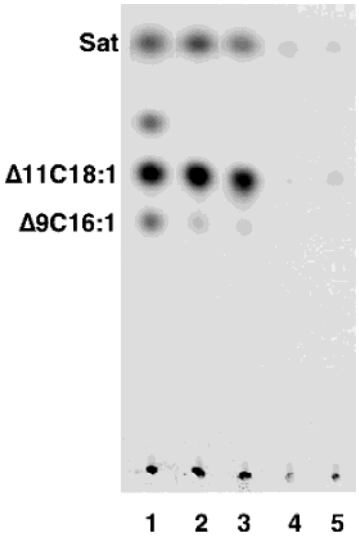

FIGURE 4: Analysis of the fatty acids synthesized in vitro by extracts of the *E. coli fabG*(Ts) strain CL104 supplemented with FabG1 and/or FabG2. Lanes: 1, products formed by the extract of the wild-type strain CL115; 2−5, products formed by the CL104 extract supplemented with lane 2, both FabG1 and FabG2; 3, FabG1; 4, FabG2; 5, no supplement. The reactions were conducted as in Figure 2B and Experimental Procedures. The acyl-ACPs were hydrolyzed, and the resulting fatty acids were converted to their methyl esters, which were analyzed by argentation thin-layer chromatography. An autoradiogram of the chromatographic plate is shown. For unknown reasons the ratio of unsaturated to saturated fatty acids synthesized in vitro is greater than that seen in vivo. The migration positions of the fatty acid species are shown. The designations are as follows: Sat, saturated fatty acids; C16Δ9, palmitoleic (*cis*-9-hexadecenoic) acid; C18Δ11, *cis*-vaccenic (*cis*-11-octadecenoic) acid. The spot running between the saturated and *cis*-vaccenic (*cis*-11-octadecenoic) acid spots of lane 1 is probably *cis*-13-eicosenoic acid.

Extracts of the *fabG*(Ts) strain CL104 failed to assimilate [2-^{14}C]malonyl-CoA into the elongation cycle (Figure 2A). We added purified FabG1 or FabG2 to the CL104 extracts and assayed in vitro synthesis of fatty acyl-ACPs (Figure 2B). As expected, the addition of purified FabG1 protein to the extracts of strain CL104 allowed the synthesis of long-chain acyl-ACPs and the accumulation (in the presence of cerulenin) of butyryl-ACP. In contrast, the addition of purified FabG2 protein failed to correct the fatty acid synthesis defect of the strain CL104 extracts (Figure 2B). The availability of the purified proteins also allowed us to test the possibility that FabG2 might work in concert with FabG1 to give a different spectrum of acyl species than that seen with FabG alone. We therefore added purified FabG1 and FabG2 to the extracts of strain CL104 and analyzed the compositions of the fatty acids synthesized in vitro. When supplemented with FabG1 or with FabG1 plus FabG2, the extract of strain CL104 formed high levels of C12, C14, and C16 fatty acids, and both saturated and unsaturated species were present (Figure 4, lanes 3 and 4). As expected, only traces of fatty acids were formed in unsupplemented strain CL104 extracts or in those supplemented with FabG2 (Figure 4, lanes 5 and 6). Since addition of FabG2 to FabG1-supplemented extracts had no effect on the rate of fatty acid synthesis or the composition of the products synthesized, no fatty acid synthetic role for FabG2 could be demonstrated.

FabG2 Catalyzes β-Ketobutyryl-CoA Reduction. Zhang and co-workers (*26*) recently reported several mutant *E. coli* FabG proteins that were inactive in reduction of β-ketobutyryl-ACP but which retained reductase activity with the model substrate, β-ketobutyryl-CoA. These mutant proteins were constructed to test the hypothesis that two arginine residues, R129 and R172, were important in binding the ACP moiety of the physiological substrate. Upon alignment of FabG1 and FabG2 with *E. coli* FabG we found that FabG2 lacked both arginine residues whereas FabG1 retained both residues (Figure 5). This raised the possibility that the lack of activity of FabG2 in fatty acid synthesis might be specifically due to an inability to bind the ACP moiety. If so, FabG2 might retain the ability to reduce β-ketobutyryl-CoA. This was readily shown to be the case (Figure 6). FabG2 was a potent β-ketobutyryl-CoA reductase having a V_{max} of 158 μmol min^{-1} (mg of protein)$^{-1}$, a value more than 4-fold greater than that of FabG1 [V_{max} of 36 μmol min^{-1} (mg of protein)$^{-1}$]. However, the K_m value for FabG2 with β-ketobutyryl-CoA as the substrate (4.06 mM) was 13-fold higher than that of FabG1 (0.29 mM).

The activity of FabG2 in this model reaction provided a means to test the ability of the protein to bind ACP. This assay was based on the finding of Zhang et al. (*26*) that ACP inhibited β-ketobutyryl-CoA reduction by *E. coli* FabG through occlusion of the reductase catalytic site. Addition of ACP inhibited β-ketobutyryl-CoA reduction by FabG1 as expected from the *E. coli* FabG data (Figure 7). In contrast, ACP addition had no effect on reduction of β-ketobutyryl-CoA by FabG (Figure 7). Therefore, we conclude that FabG2 is inactive in fatty acid synthesis due to a failure to bind β-ketobutyryl-ACPs rather than to an incompetent catalytic site.

Attempts To Convert FabG2 to a β-Ketoacyl-ACP Reductase. Inspection of the alignments (Figure 5) of FabG2 with FabG1 and *E. coli* FabG led to the straightforward hypothesis that mutagenesis of *fabG2* to produce mutant proteins containing the two arginine residues targeted in the investigations of the *E. coli* enzyme might bestow both ACP

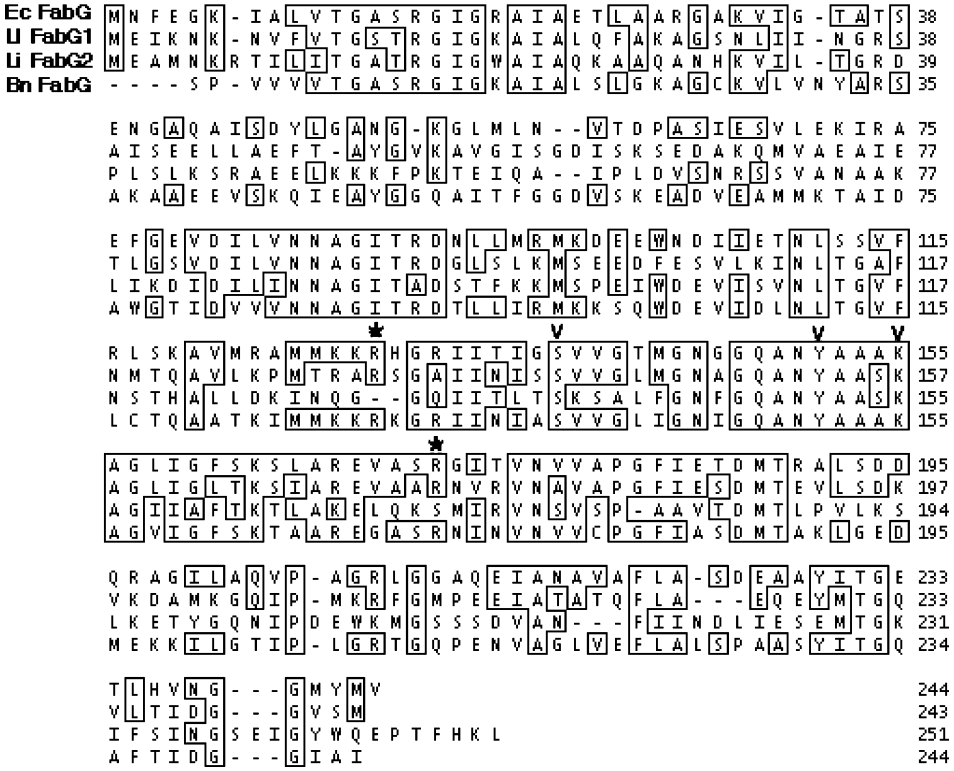

FIGURE 5: Alignment of FabG1 and FabG2 with the β-ketoacyl-ACP reductases of known structure, the *E. coli* FabG, and *B. napus* proteins. The FabG sequences are denoted as Ec, Ll 1, Ll 2, and Bn, respectively, and identical residues are boxed. The two arginine residues studied by Zhang et al. (*26*) are highlighted by asterisks and the catalytic triad (*27*, *28*) by the **V** symbols. The alignment was done by aligning identical residues with Clustal W.

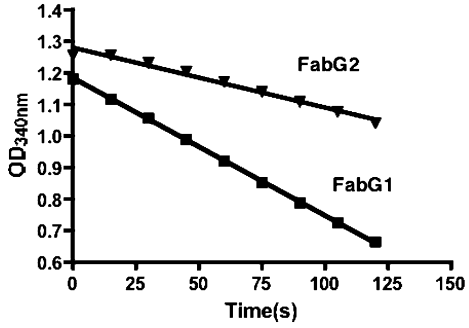

FIGURE 6: Reduction of β-ketobutyryl-CoA catalyzed by FabG1 or FabG2. The reaction mixture (*33*) contained 0.5 mM β-ketobutyryl-CoA (acetoacetyl-CoA), 0.2 mM NADPH, 0.1 M sodium phosphate buffer (pH 7.4), and 10 μg of a purified His-tagged FabG protein in a volume of 0.5 mL. A blank cuvette lacking enzyme was continuously subtracted from the experimental cuvettes. The reaction was followed by oxidation of NADPH. The lower activity of FabG2 relative to FabG1 is due to the fact that the acetoacetyl-CoA concentration was 10-fold below the saturating concentration for FabG2 whereas FabG1 was essentially saturated with substrate.

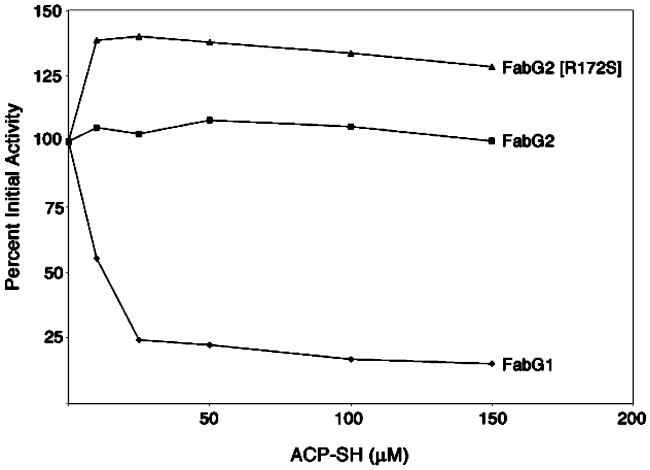

FIGURE 7: ACP inhibition of β-ketobutyryl-CoA reduction by *L. lactis* FabG1 and FabG2. The experiments were conducted as described by Zhang et al. (*26*). Symbols: circles, FabG1; squares, FabG2; triangles, the R172S mutant of FabG2. ACPSH denotes the reduced form of ACP.

binding and β-ketobutyryl-ACP reductase activity. We therefore constructed genes encoding three mutant proteins, one with a S172R mutation, one with an insertion of two residues (R plus S) between residues 130 and 131, and a third having both mutations. The arginine substitution is the equivalent of *E. coli* FabG R172 whereas the R plus S insertion introduces the equivalent of *E. coli* FabG R129. The genes encoding the three mutant proteins were cloned into vector pBAD24 and tested for complementation of the *E. coli fabG*(Ts) mutant strain. None of the three mutant genes allowed growth of the strain at the nonpermissive temperature (data not shown). To further investigate the mutant proteins, we moved the encoding genes to expression vector pET28 in order to obtain high levels of the His-tagged proteins. The S172R protein was produced in soluble form and had normal β-ketobutyryl-CoA activity (29.1 μmol min^{-1} mg^{-1}) whereas the two proteins containing the insertion of R and S were obtained only as inclusion bodies, that is, as inactive aggregates. We therefore purified the two insertion mutant proteins under denaturing conditions and attempted to fold the proteins from urea solutions. Although the protocol resulted in recovery of a wild-type protein preparation having a β-ketobutyryl-CoA activity essentially identical to those obtained when purified under native conditions, both

of the mutant proteins precipitated at urea concentrations <1 M and had no detectable activity.

DISCUSSION

The *L. lactis* genome contains two genes annotated as encoding β-ketoacyl-ACP reductases (*14*, *15*). The *fabG1* gene encodes a protein that is 46% identical to *E. coli* FabG whereas the *fabG2*-encoded protein is 33% identical to *E. coli* FabG [the *L. lactis* FabG1 and FabG2 proteins are 38% identical (Figure 5)]. All of the active site residues defined by X-ray crystal structures of *E. coli* FabG (*27*, *28*) and a β-ketoacyl-ACP reductase from the plant *Brassica napus* (*29*, *30*) plus the residues that interact with NADPH (*27*) are conserved in the two *L. lactis* proteins. Moreover, both of the putative *fabG* genes are adjacent to genes that probably encode other fatty acid synthetic proteins. The long stretch of amino acid residues responsible for the low percentage identity between *E. coli* FabG and FabG2 (*E. coli* residues 38−77) aligns no better with FabG1 or with the plant FabG than with FabG2 (although this protein segment is involved in NADPH binding, most interactions are with backbone amide groups and hence are insensitive to residue substitutions). Despite the strong sequence conservation elsewhere in these proteins only the *L. lactis fabG1* gene could replace the function of *E. coli fabG* and only FabG1 was active in supporting in vitro fatty acid synthesis. Why does FabG2 fail to function in fatty acid biosynthesis? We believe the protein is inactive because it is unable to efficiently bind β-ketoacyl-ACP substrates. This hypothesis is based on the report of Zhang et al. (*26*) that appeared during the course of our investigations. These workers dramatically and specifically decreased the activity of *E. coli* FabG for β-ketobutyryl-ACP by mutagenesis of two arginine residues, Arg-129 and Arg-172. These residues were chosen due to their locations adjacent to the entrance to the *fabG* active site plus the fact that ACP is an extraordinarily acidic protein. *L. lactis* FabG1 contains both Arg residues whereas FabG2 has neither. In FabG2 a two-residue gap appears at the location of Arg-129 whereas Arg-172 is replaced by serine, a substitution that would be expected to give a significant loss of activity (*26*). The two-residue deletion would not only remove the charged arginine side chain but might also distort the structure of the protein. Indeed, attempts using various threading programs to model the FabG2 sequence on the *E. coli* FabG template failed because FabG2 was rejected due to steric considerations in the protein segment containing the two-residue deletion. It is clear that FabG2 retains the catalytic site residues necessary for reductase activity because the enzyme rapidly reduces the model substrate, β-ketobutyryl-CoA. Moreover, β-ketobutyryl-CoA reduction by *E. coli* FabG is inhibited by ACP which inhibits by blocking access to the active site (*26*). FabG1 was similarly inhibited by ACP, but FabG2 was not indicating an inability to bind ACP. However, we were unable to convert FabG2 to a β-ketobutyryl-ACP reductase by introduction of the key arginine residues. One of the three mutant proteins constructed, S172R, retained solubility and β-ketobutyryl-CoA reductase activity but was unable to restore growth to the *E. coli fabG*(Ts) mutant strain at the nonpermissive temperature. When highly expressed, the other two mutant proteins were inactive and insoluble, suggesting that the two-residue insertion had disrupted protein folding. Therefore, differences between FabG2 and FabG1 other than the arginine residues tested are responsible for the lack of FabG2 function in fatty acid synthesis.

The most straightforward interpretation of our data is that FabG2 was formerly a bona fide β-ketoacyl-ACP reductase but because of redundancy with FabG1 has been (or is being) derivatized to reduce or oxidize a different substrate. Recent work on yeast genomes provides a framework in which to consider our findings. Keelis and co-workers (*31*) showed that the present genome of *Saccharomyces cerevisiae* arose by genome duplication followed by loss of most of the duplicated genes. For the remaining duplicate genes one copy evolves slowly and retains the ancestral function whereas the other copy loses the original function and evolves rapidly to derive a new function. *L. lactis* has an unusually large number of duplicated genes for a free-living organism with a small genome. This bacterium has more duplicated genes than *E. coli*, which has a genome size twice that of *L. lactis* and 2- and 10-fold more than two organisms (*Neisseria meningitides* and *Thermotoga maritima*, respectively) having *L. lactis*-sized genome (*12*). Therefore, gene derivation may be an ongoing process in this organism. Our finding that FabG2 is not a β-ketoacyl-ACP reductase shows that gene location is no litmus for prediction of function.

The most clear-cut function for FabG2 would be to act as a β-hydroxyacyl-CoA dehydrogenase such as those functioning in β-oxidative fatty acid degradation pathways. However, the *L. lactis* genome encodes only one of the standard β-oxidation enzymes, an acyl-CoA synthetase (*14*, *15*). For example, the organism lacks a gene encoding a thiolase (a highly conserved protein readily identified by sequence comparisons). Therefore, it seems that no enzymes would be available to provide the substrates (or use the products) of a degradative β-hydroxyacyl-CoA dehydrogenase. Moreover, consistent with the lack of a β-oxidation system *L. lactis* (unlike *E. coli*) does not shorten exogenously supplied fatty acids prior to their incorporation into phospholipids (*32*). On the basis of our data we recommend that *fabG1* be designated *fabG* and the *fabG2* designation be discarded and replaced by the original ORF designation.

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