β-Ketoacyl-Acyl Carrier Protein Synthase III (FabH) is Essential for Bacterial Fatty Acid Synthesis*

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The abbreviations used are: ACP, acyl carrier protein; ACC, acetyl-CoA carboxylase; KAS, β-ketoacyl-ACP synthase; kb, kilobase pair; IPTG, isopropyl-β-D-thiogalactopyranoside; X-gal, 5-bromo-4-chloro-3-indoyl-β-D-galactoside.
ABSTRACT

β-Ketoacyl-acyl carrier protein (ACP) synthase III (KAS III, also called acetoacetyl-ACP synthase) encoded by the fabH gene is thought to catalyze the first elongation reaction (Claisen condensation) of type II fatty acid synthesis in bacteria and plant plastids. However, direct in vivo evidence that KAS III catalyzes an essential reaction is lacking, since no mutant organism deficient in this activity has been isolated. We report the first bacterial strain lacking KAS III, a fabH mutant constructed in the gram-positive bacterium Lactococcus lactis subsp. lactis IL1403. The mutant strain carries an in-frame deletion of the KAS III active site region and was isolated by gene replacement using a medium supplemented with a source of saturated and unsaturated long-chain fatty acids. The mutant strain is devoid of KAS III activity and fails to grow in the absence of supplementation with exogenous long-chain fatty acids demonstrating that KAS III plays an essential role in cellular metabolism. However, the L. lactis fabH deletion mutant requires only long-chain unsaturated fatty acids for growth, a source of long-chain saturated fatty acids is not required. Since both saturated and unsaturated fatty acids are required for growth when fatty acid synthesis is blocked by biotin starvation (which prevents the synthesis of malonyl-CoA), another pathway for saturated fatty acid synthesis must remain in the fabH deletion strain. Indeed, incorporation of [1-14C]acetate into fatty acids in vivo showed that the fabH mutant retained about 10% of the fatty acid synthetic ability of the wild type strain and that this residual synthetic capacity was preferentially diverted to the saturated branch of the pathway. Moreover, mass spectrometry showed that the fabH mutant retained low levels of palmitic acid upon fatty acid starvation. Derivatives of the fabH deletion mutant strain were isolated that were octanoic acid
auxotrophs consistent with biochemical studies indicating that the major role of FabH is production of short-chain fatty acid primers. We also confirmed the essentiality of FabH in *Escherichia coli* by use of a plasmid-based gene insertion/deletion system. Together these results provide the first genetic evidence demonstrating that FabH conducts the major condensation reaction in the initiation of type II fatty acid biosynthesis in both gram-positive and gram-negative bacteria.
Introduction

Fatty acid biosynthetic pathways are of two classes called types I and II [reviewed in (1,2)]. In the associated or type I fatty acid synthase system, each fatty acid synthetic reaction is catalyzed by a distinct domain of large multifunctional proteins. The dissociated or type II fatty acid synthesis system found in most bacteria and plant plastids consists of a series of discrete proteins, each of which catalyzes an individual reaction of the fatty acid biosynthetic pathway. In some cases, two or more enzymes are able to perform the same chemical reaction, but have differing substrate specificities and physiological functions. The β-ketoacyl-ACP synthases (KAS) provide a good example. All organisms using a type II pathway contain at least two KAS enzymes, KAS II and KAS III (1,2). KAS II is the generic enzyme responsible for the elongations required for synthesis of long-chain fatty acids whereas KAS III is thought to catalyze the first condensation reaction to produce the butyryl thioester of acyl carrier protein (ACP) (1,2). Butyryl-ACP is then thought to act as a substrate for the KAS II-catalyzed elongations that result in long-chain fatty acids (1,2). That is, KAS III is thought to provide a primer for KAS II (and other long-chain KAS isozymes, if present). The in vivo function of KAS II is clearly established since mutants (called fabF) lacking this enzyme have been well studied in Escherichia coli (1,2) and other bacteria (3,4). However the postulated role of KAS III is based only on biochemical analyses, despite considerable efforts no mutant organism lacking this enzyme has been described. Moreover in E. coli which contains KAS III and two long-chain KAS enzymes (KAS I and KAS II), only KAS I appears essential. This premise is based on the observation that overproduction of KAS I, but not KAS III, conferred resistance to thiolactomycin, an antibiotic that inhibits all three KAS isozymes in vitro (5) and fabB mutations that confer thiolactomycin resistance to E. coli have been isolated (6). However, it has not been
shown that the inhibitor is equally effective in vivo. The presence of a significant intracellular pool of acetyl-ACP in *E. coli* (7) is also difficult to reconcile with the postulated role of KAS III since KAS III condenses acetyl-CoA and malonyl-ACP and is inactive with acetyl-ACP (8,9).

The goal of the present study was to elucidate the pathways involved in the initiation of fatty acid synthesis and to clarify the physiological role of FabH. Despite numerous attempts we have failed to obtain *fabH* mutants of *E. coli* and *Salmonella enterica* Serovar Typhimurium. A difficulty in these gram-negative bacteria is that a low level of de novo fatty acid biosynthesis is required to produce the acyl-ACP precursors of lipid A (10-12) and thus null mutant strains lacking essential fatty acid synthetic genes cannot be isolated. We therefore turned to a gram-positive bacterium, *Lactococcus lactis* (gram-positive bacteria lack lipid A). *L. lactis* is one of the best-characterized lactic acid bacteria and the genome of *L. lactis* subsp. *lactis* IL1403 has been sequenced (13). Lactic acid bacteria require biotin for growth and it has been reported that oleate or a derivative of oleate such as Tween 80 can functionally replace biotin (14-17). Biotin is the required cofactor of acetyl-CoA carboxylase (ACC), the essential enzyme carrying out the first committed step of fatty acid biosynthesis (18). The observation that biotin-free media (containing aspartate to bypass the loss of pyruvate carboxylase, a second biotin-requiring enzyme) cannot support the growth of lactic acid bacteria and that exogenous fatty acids (or Tween 80) functionally replace biotin can be explained by a lack of ACC activity that results blockage of fatty acid synthesis. Since exogenous fatty acids have biotin-replacing capability in other lactic acid bacteria (14-17), we hypothesized that null mutants lacking essential fatty acid synthetic genes should grow with exogenous fatty acid supplementation unlike the case in gram-negative bacteria (10-12). In this paper, we report the isolation of the first *fabH* null mutant obtained in any organism by gene replacement in *L. lactis* subsp. *lactis*. We also demonstrated
the essentiality of KAS III in *E. coli* and conducted biochemical characterizations of the *L. lactis fabH* mutant in order to elucidate the physiological roles of KAS III.
EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—The bacterial strains and plasmids used in this work are listed in Table 1. All bacterial strains are derivatives of *E. coli* K-12 or *Salmonella enterica* Serovar Typhimurium LT2 except *L. lactis* subsp. *lactis* strain IL1403. Strain CL66 containing a *fabH* point mutation R271K (presumably resulting from a prior PCR amplification) was used as a template for PCR amplification of the *S. enterica fabH*. Plasmid pCL49 was obtained by insertion of the 2 kb *plsX* (truncated) plus *fabH* PCR product of *S. enterica* chromosomal DNA (amplified with primers SalS-N and SalH-C, Table 2) into pCR2.1 (Invitrogen). Plasmid pORI280 which contains an erythromycin resistance gene, the origin of lactococcal replication of plasmid pWV01, and the *E. coli* β-galactosidase gene expressed under lactococcal promoter P32 was used as the vector for gene replacement (19). Plasmid pCL58 was constructed by insertion of the 1.2 kb *fabH* PCR product of *L. lactis* chromosome DNA (amplified with primers LacH-P and LacH-C, Table 2) into pCR2.1. Plasmid pCL61 containing the complete *L. lactis fabH* gene was derived by insertion of the 1.2 kb *BamHI-XbaI fabH* fragment of pCL58 into pK18 (20) cut with the same enzymes. Plasmid pCL62 containing the in-frame *fabH* deletion was constructed by digestion of pCL61 with *BcgI* followed by formation of blunt ends by treatment with T4 DNA polymerase plus the 4 dNTPs followed by ligation with T4 DNA ligase. To ensure that the expected *fabH* in-frame deletion had been made, the deletion region was checked by sequencing. Plasmid pCL66 was obtained by insertion of the 1.2 kb *BamHI-XbaI* fragment (containing the in-frame *fabH* deletion) of pCL62 into pORI280 cut with the same enzymes. All primers (Table 2) used for PCR in this work were synthesized by the Genetic Engineering Facility, University of Illinois at Urbana-Champaign.
Media and Culture Conditions—*E. coli* and *S. enterica* cultures were grown in rich broth (RB) (21), Luria-Bertani (LB) (22) liquid medium or on agar plates made of RB or LB. *L. lactis* cultures were grown in GM17 medium (Difco) or on GM17 agar plates (19). Antibiotics were added at the following concentrations (in μg/ml): kanamycin, 50; ampicillin, 100; spectinomycin, 100; tetracycline, 12; and erythromycin, 150. Single copy integrants of *E. coli* were selected using a mixture spectinomycin and streptomycin at 17.5 μg/ml each. Single copy integrants of *L. lactis* were selected using erythromycin at 5 μg/ml. Final concentrations of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), 0.2% arabinose, 0.4% glucose, 0.005% fucose, 0.3 mg/ml avidin, 0.1% Tween-40, 0.1% Tween 80, 0.02% oleate, 0.02% octanoic acid, 0.02% *cis*-vaccinate, and 0.2% Tergitol NP-40 (a metabolically inert detergent used to solublize fatty acids) were obtained by appropriate supplementation of the media.

The minimal medium contained medium E and 4% Vitamin-Free Casamino Acids plus the following (final concentrations in mM) major components: glucose, 100; sodium acetate, 30; asparagines, 1.6; glutamine, 10; tryptophan, 1; sodium chloride, 50; ammonium chloride, 9.5; potassium sulfate, 0.28; potassium phosphate, 1.3 mM; and Tricine, 4. The minor components (final concentrations in μM) were: calcium chloride, 0.5; magnesium chloride, 520; ferrous sulfate, 10; ammonium molybdate, 0.006; boric acid, 0.8; 0.006; cobalt chloride, 0.06; cupric sulfate, 0.02; manganous chloride, 0.16; zinc sulfate, 0.02; biotin, 0.8; pyridoxal, 20; folic acid, 4.6; riboflavin, 5.2; niacinamide, 16; thiamine, 6; and pantothenate, 4. The pantothenate was replaced with the radioactive species for labeling studies. The fatty acid supplement consisted of (final concentrations) 0.1% of Tween 40 and Tween 80 plus 0.02% oleic acid.
CRIM Plasmid Integration—Ligation mixtures containing EcoRI-digested pAH144 and the 2 kb S. enterica fabH EcoRI fragment of pCL49 were transformed into both strain UB1005 and into strain DY330 carrying the CRIM helper plasmid pAH69 and transformants resistant to both spectinomycin and streptomycin were selected. The resulting S. enterica fabH integrants CL81 (from UB1005) and CL106 (from DY330) were verified by PCR using primers SalS-N, SalH-C (Table 2) and the copy number of the integrants were checked by use of primers P1, P2, P3, and P4 (21).

Construction of an E. coli fabH Deletion Mutation in the Presence of S. enterica fabH—Linear DNA fragments carrying a kanamycin resistance cassette flanked by the FRT sites were amplified by PCR from pKD13 using primers 1 and 2 (Table 2). These primers were homologous at the 3’ end to priming sequences in pKD13 and contained 40 base 5’ end extensions homologous to E. coli fabH. The respective 1.4 kb PCR products were purified, treated with DpnI, and then transformed into CL106, the strain harboring a λ prophage which contains the recombination genes exo, bet, and gam under control of a temperature-sensitive λcl-repressor (23). The resulting strain CL110 which contained a replacement of the E. coli fabH gene by the kanamycin cassette was then transduced by phage P1 into strain CL81 with selection for kanamycin resistance. Strain CL111 carrying the E. coli fabH deletion and a copy of the S. enterica fabH integrated into the chromosomal attHK022 site was obtained and verified by PCR using primers SalS-N and SalH-C (Table 2).

Construction of the L. lactis fabH Null Deletion Mutant—The replacement of the L. lactis fabH gene was conducted according to the method of Leenhouts et al. (19) (Fig. 1). Plasmid pCL66 containing an in-frame fabH deletion was transformed into strain IL1403 followed by selection on GSM17E5 agar plates (19) supplemented with 0.1% Tween 40, 0.1% Tween 80, and 0.02%
oleate at 30°C. These transformants were then plated on GM17E plates containing 5-bromo-4-chloro-3-indoyl-β-D-galactoside (X-gal) plates supplemented with fatty acids as above and blue colonies were isolated. The integrant strain was grown in GM17 medium with fatty acid supplementation and the overnight culture was diluted 10⁶ times and 2 µl was inoculated into 2 ml of GM17 medium containing fatty acids and grown overnight at 30°C. Dilutions of the overnight culture were plated on GM17 plus X-gal supplemented with fatty acids and screened for white colonies. All white colonies were then PCR amplified using primers LacH-P and LacH-C (Table 2) and the resulting PCR products were digested with either BcgI or HpaI. Strains having PCR fragments digested by HpaI, but not by BcgI, were candidate fabH deletion mutants. The PCR products of these strains were then cloned into plasmid pCR2.1 and checked by sequencing. One of these strains, CL112, had the expected sequence and was subjected to Southern blot analysis.

KAS III Assay—Acetoacetyl-ACP synthase activity was assayed according to Tsay et al. (5). The assay mixtures contained 0.1 M sodium phosphate, pH 7.0, 1 mM 2-mercaptoethanol, 70 μM malonyl-CoA, 45 μM [1-14C]acetyl-CoA (specific activity, 55 mCi/mmol), 265 μM ACP, 1.1 mM cerulenin, and 25, 50, or 100 μg of extract protein from either strain IL1403 or strain CL112 (ΔfabH) in a final volume of 80 µl. The mixture of ACP, sodium phosphate, and 2-mercaptoethanol was preincubated at 37°C for 30 min to obtain complete reduction of ACP. The remaining components excepting protein were then added. The reaction was initiated by addition of protein and incubated at 37°C for 15 min. The assay mixture was then pipetted onto a 3MM filter disc. The discs were washed successively with 10, 5, and 1% ice-cold trichloroacetic acid at 20 ml/filter. The filters were then dried and counted in 4 ml of liquid scintillation fluor solution.
Synthesis of [3-3H]Pantothenate—E. coli strain AT1371 carrying plasmid pEC (24) was grown overnight at 37°C in 2 L of LB medium containing IPTG (70 µg/ml) and ampicillin. Cells were centrifuged, resuspended in 10 ml of TD buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM DTT), and disrupted in a French pressure cell at 18,000 psi. The lysate was centrifuged in a JA-20 rotor at 16,000 rpm at 4°C for 1 h to remove cell debris. A protein fraction obtained by adding ammonium sulfate to the supernatant to 60% of saturation was collected. The protein pellet was dissolved in 5 ml of TD buffer and dialyzed overnight at 4°C against 2 L of the same buffer.

Synthesis of [3H]pantothenate was performed according to Cronan (25). The pantothenate synthetase reaction contained 5 mM D-pantoic acid, 10 mM ATP (potassium salt), 10 mM MgSO₄, 100 mM Tris-HCl, (pH 10) 100 mM NH₄Cl, 0.3 mM β-[3-3H]alanine (specific activity, 60 Ci/mmol), and 90 µl crude extract of pantothenate synthetase in a final volume of 100 µl.

The mixture was incubated at 25°C for 28 h and then applied to a 0.6 x 2.5-cm column of AG 50W-X8 (hydrogen form) ion exchange resin. [3-3H]Pantothenate was eluted in 81% yield with 1 ml of H₂O, evaporated under a stream of nitrogen, and dissolved in the minimal medium given above (with pantothenate omitted) and sterilized by filtration.

In Vivo Labeling of ACP Pools—Strains IL1403 and CL112 (Δ fabH) were grown at 30°C for 72 h in the [3H]pantothenate-containing minimal medium described above. The cells were harvested by centrifugation and the cells were washed 3 times with cold M9 medium, and lysed on ice by the method of Clewell and Helinski (26). The lysate was centrifuged to sediment the DNA and supernatant fluid was fractionated on a 15% PAGE gel containing 2.5 M urea at 4°C. The ACP species were deuterated by treating the lysate with 1 mM dithiothreitol, pH 8.0 at 37°C for 2 h and analyzed on the same gel. The gels were then subjected to fluorography.
Phospholipid Extraction—Phospholipid extraction was accomplished as described by Bligh and Dyer ([27]). Strains IL1403 and CL112 were grown in 5 ml of GM17 and 5 μCi sodium-[1-14C]acetate supplemented with 0.1% Tween 40 and 0.1% Tween 80 at 30°C to log or stationary phase. For analysis of fatty acid compositions by mass spectroscopy the strains were grown with cis-vaccenate (0.02%) supplementation of either GM17 medium or the minimal medium given above. Biotin limitation was obtained by adding 0.3 mg/ml of avidin to a culture of strain IL1403 grown to log phase under the above medium. In fatty acid starvation experiments the cultures were grown to a density of 10^8 cells/ml, washed with M17 medium 3 times, resuspended in GM17 medium without addition of exogenous fatty acids, and grown for 7 h at 30°C. The cultures were then centrifuged and washed three times with cold M17 medium and the cell pellets were suspended in 6 ml of methanol-chloroform (2:1, v/v) followed by shaking for one hour at 30°C. Cell debris was removed by centrifugation and the supernatant was transferred to a fresh tube. The organic and aqueous phases were separated by addition of 2 ml each of water and chloroform. The mixture was then vortexed and centrifuged. The upper aqueous layer was removed and the lower organic layer washed twice with an equal volume of 2 M KCl. After a final wash with an equal volume of water, the solvent was evaporated under a stream of nitrogen. The phospholipids were then transesterified to form methyl esters or hydrolyzed to generate free fatty acids as described below.

Fatty acid analyses. — Base-catalysed transesterification to form methyl esters was modified from the procedure of Christie (29). Phospholipids were dissolved in 1.2 ml of dry methanol containing 0.2 ml of 25% sodium methoxide in methanol (Aldrich). After 15 min at room temperature 1.2 ml of 2 N HCl was added and the fatty acid methyl esters were extracted into 1.2 ml of petroleum ester. The aqueous layer was extracted twice more and the organic layers were
combined and evaporated under a stream of nitrogen. Saturated and unsaturated fatty acid methyl esters were separated on silver nitrate thin layer plates as described by Morris and Wharry (28). Analtech Silica Gel GHL, 20% AgNO₃ TLC plates were developed with toluene at -20°C and dried before samples application. Fatty acid methyl esters were dissolved in a small volume of hexane and applied to the plate which was then developed twice with toluene at -20°C. The chromatogram was dried and fatty acid methyl esters were detected by autoradiography.

Free fatty acids were recovered by hydrolysis of phospholipids dissolved in 2 ml of 1N KOH in ethanol-water (1:1, v/v) and incubated at 42°C for 1 h (29). The mixture was twice extracted with 2 ml of hexane which was then discarded. The reactions were acidified with concentrated HCl to pH 2 and the fatty acids were twice extracted into 2 ml of petroleum ether-ethyl ether (1:1, v/v). The pooled organic fractions were combined and evaporated under a stream of nitrogen. The free fatty acids were suspended in a small volume of hexane-ethyl ether (2:1, v/v) and applied to an Analtech Silica Gel G plate which was developed with a mobile phase of ethyl ether, hexane, and acetic acid (30:70:2). The plate was dried and subjected to autoradiography. The plate was also exposed to a Molecular Dynamics Phosphorimager screen for quantitation.

Phospholipid samples were subjected to cone voltage degradation mass spectrometry (CVD-MS) analysis performed on a Micromass Quattro I at the Mass Spectrometry Laboratory, School of Chemical Sciences, University of Illinois at Urbana-Champaign. The cone voltage of 90V resulted in complete degradation of phospholipids to their component fatty acids.
RESULTS

Construction of a L. lactis fabH Null Deletion Mutant—Based on the genomic sequence (13) we deleted the region encoding the fabH active site by digestion of the coding sequence with BcgI followed by resection of the ends with T4 DNA polymerase and ligation. These manipulations produced an in-frame deletion of a 36 bp fragment encoding the KAS III active site cysteine plus neighboring residues and also generated a new HpaI site (Fig. 1A). The plasmid was then introduced into L. lactis IL1403 and cointegrants were selected (Fig. 1B). Upon resolution of the cointegrates the white colonies could result from chromosomal retention of either the restored wild type allele or of the fabH null deletion allele (Fig. 1B). The colonies that retained the fabH deletion allele were readily identified via digestion of PCR products with either BcgI or HpaI. The PCR products of 140 white colonies were digested and 45 of these were cut by HpaI but not by BcgI, indicating they contained the fabH deletion allele. All of the colonies giving HpaI-sensitive PCR products required fatty acid supplementation for growth whereas the 95 colonies giving HpaI-resistant PCR products grew in the absence of fatty acid supplementation. The PCR products of three of the fatty acid auxotrophic colonies were then cloned and upon sequencing the expected deletion (Fig. 1A) was found in all three products. One of these strains, strain CL112, was submitted to Southern blot analysis and was found to have the expected chromosomal map (30) and this was the strain studied in the experiments reported below.

The fabH Mutant Requires only Unsaturated Fatty Acids for Growth—Strain CL112 (ΔfabH) possessed a phenotype in that growth occurred only upon supplementation with exogenous fatty acids (Fig. 2) indicating that fabH is essential for L. lactis fatty acid synthesis. It should be noted that CL112 grew (albeit extremely poorly) in GM17 lacking exogenous fatty acid.
supplementation indicating that GM17 might contain a low level of fatty acids. Interestingly, CL112 grew well when supplemented with one of a variety of unsaturated fatty acids such as oleate, \textit{cis}-vaccenate (C18:1), or palmitoleate (C16:1) (data not shown). It was found that 20 µg/ml of oleate was capable of maintaining the growth of strain CL112 in the absence of supplementation with a source of saturated fatty acids. Since \textit{L. lactis} requires both saturated and unsaturated fatty acids when grown with limited biotin (see below) it seemed clear that another route of saturated fatty acid synthesis existed. This was demonstrated by [1-\textsuperscript{14}C]acetate labeling of fatty acids and mass spectrometric analyses of fatty acid contents (see below).

\textit{The \textit{fabH} Mutant Retains 10\% of the Normal Fatty Acid Biosynthetic Ability.} \textit{De novo} fatty acid synthesis was assayed by incorporation of labeled acetate into phospholipids of the wild-type strain IL1403 and strain CL112 (\textit{Δ}\textit{fabH}). The phospholipid acyl chains were transesterified to their methyl esters or hydrolyzed to free fatty acids and the products analyzed by thin layer chromatography (Fig. 3). Strain IL1403 utilized [1-\textsuperscript{14}C]acetate to produce labeled saturated fatty acids (mainly palmitate) plus the unsaturated fatty acids palmitoleate (C16:1) and \textit{cis}-vaccenate (C18:1) (Fig. 3) indicating that the wild-type strain of \textit{L. lactis} has the expected fatty acid synthetic ability. In contrast strain CL112 synthesized only low levels of fatty acids (Fig. 3) and these were all saturated species indicating that the \textit{fabH} null deletion mutant retained residual fatty acid synthetic ability. Upon fatty acid starvation, higher levels of radioactive phospholipid fatty acid moieties (assayed as fatty acid methyl esters) were found in both strains consistent with the higher cell densities (Fig. 3). Strain IL1403 grown in GM17 supplemented with Tween 80 and avidin produced very low levels of radioactive phospholipid fatty acid moieties (Fig. 3), indicating that most fatty acid synthesis had been blocked by biotin limitation. Biotin limited cultures required supplementation both saturated and unsaturated fatty acids for full growth,
although the organism was able to grow for several generations with only oleic acid (data not shown).

To quantitatively compare the fatty acid synthetic abilities of the two strains, the levels of [1-\textsuperscript{14}C]acetate incorporated into fatty acids per cell were calculated by comparing the values for radioactive fatty acids obtained by scintillation counting of the silica gel scraped from the plates with the values obtained from the same thin layer plates by phosphorimager measurements. In several experiments the rate of incorporation of labeled acetate into fatty acids in the wild-type strain IL1403 was about 10 to 20 times the rate in strain CL112 (\textit{Δ}fab\textit{H}) indicating that the \textit{fabH} null deletion mutant retained about 5\% to 10\% of the fatty acid biosynthetic ability of the wild-type (Table 3). The residual fatty acid synthetic ability of strain CL112 provides a source of saturated fatty acids and explains why the \textit{fabH} null deletion mutant requires only unsaturated fatty acids for growth. Upon fatty acid starvation, both strains IL1403 and CL112 incorporated about 3-fold greater levels of radioactive acetate into lipids compared to cells grown with fatty acid supplementation. These data suggest the possibility that exogenous fatty acids might trigger down-regulation of fatty acid synthesis. Another possibility is that the exogenous fatty acids might compete with endogenously synthesized fatty acids for incorporation into phospholipids. Strain IL1403 grown in GM17 supplemented with avidin and \textit{cis}-vaccenate showed a rate of radioactive acetate incorporation into fatty acids about one-fifth that seen in strain CL112 (Fig. 3B, Table 3) indicating that residual fatty acid synthesis remained. A possible explanation for this residual synthetic ability was that a portion of the avidin might have been digested by the many proteases excreted by this organism thereby releasing traces of biotin to support low levels of fatty acid biosynthesis.
Analysis of Fatty Acid Contents—To assess the fatty acid compositions of strain IL1403 and CL112, the total phospholipids from cultures of each strain were subjected to electrospray ionization mass spectrometry (31,32) as described in Experimental Procedures. In cultures grown in medium GM17 with cis-vaccenate supplementation, strains IL1403 and CL112 had similar fatty acid compositions (Table 4). However, upon starvation for fatty acids the unsaturated fatty acid (C18:1) content decreased dramatically in strain CL112 and only saturated fatty acids remained (Table 4), indicating that blockage of cell growth was due to the lack of unsaturated fatty acids. Cells grown in GM17 medium supplemented with oleate alone or with all fatty acids (Tween 40 plus Tween 80) had fatty acid contents similar to those given in Table 4 (data not shown). These data indicate that when the flux through the fatty acid synthetic pathway is limited the intermediates of the fatty acid pathway are preferentially utilized for saturated species. These data are consistent with the finding that the fabH mutant grows with unsaturated fatty acids as sole supplement (Fig. 3).

Due to concerns that medium GM17 might contain small amounts of fatty acids, strains IL1403 and CL112 were grown in a chemically defined minimal medium containing all required nutrients plus cis-vaccenate. In the absence of exogenous saturated fatty acids, the fatty acid composition of the wild-type strain IL1403 was 65% saturated fatty acids and 35% unsaturated fatty acids whereas the fatty acid content of CL112 (∆fabH) was 28% saturated fatty acids and 72% unsaturated fatty acids (Table 4B). These results confirm that the residual fatty acid synthesis in CL112 was directed to saturated fatty acids.

The ∆fabH mutant is Devoid of KAS III Activity—The possibility remained that despite loss of the active site cysteine the protein encoded by the fabH deletion allele retained enzymatic activity. We therefore assayed extracts of the mutant strain for KAS III activity using a
modification of the acetoacetyl-ACP synthase assay of Tsay et al. (5). The activity of strain CL112 (ΔfabH) (0.0006 nmol of acetoacetyl-ACP formed per min/mg protein) was less than 1% of that of the wild-type strain IL1403 (0.067 nmol of acetoacetyl-ACP formed per min/mg protein) (Fig. 4). Moreover, this putative trace of activity failed to increase with increased extract protein concentration (Fig. 4) and thus was not valid. Therefore, we believe that CL112 extracts are devoid of FabH activity. The *L. lactis* wild-type strain IL1403 crude extract had a level of acetoacetyl-ACP synthase activity about three-fold greater than that of *E. coli* wild-type strain extracts (5).

**ACP and Acyl-ACP Levels**—The fatty acid synthetic genes of *L. lactis* (13) are clustered into what appears to be a series of operons as found in the *E. coli fab* cluster (21). The first gene of the cluster is *fabH* and the next is *acpA*, the gene encoding ACP. We designed the *fabH* deletion to be in-frame in order to avoid a polar effect on the transcription of downstream genes. We also expected that mutants in *fabH* would not be polar on *acpA* expression, since there is a sufficient space between the genes for a dedicated *acpA* promoter. The *acpA* gene was expected to have its own promoter [as does *E. coli acpP*, (21)] because ACP is required in much larger molar quantities than the other fatty acid synthetic proteins. However, the possibility remained that the Δ*fabH* mutation somehow decreased expression of downstream genes and the block in fatty acid synthesis was due to depletion of a protein encoded by a downstream gene rather than to KAS III deficiency per se. To test if the *fabH* deletion mutation had a polar effect upon expression of the downstream genes we determined the levels of ACP by labeling cultures with [3-3H]pantothenate, a precursor of the 4’phosphopantotheine prosthetic group of ACP. *L. lactis* is a natural pantothenate auxotroph and the requirement is not satisfied by β-alanine. Hence we converted β-[3-3H]alanine to [3-3H]pantothenate by use of *E. coli* pantothenate synthetase and
used the product to label the ACP pools of strains IL1403 and CL112 in a defined minimal medium (Fig. 5). The overall ACP levels were essentially identical in the wild type and mutant strains demonstrating that the \textit{fabH} deletion mutation did not affect expression of downstream genes. Analysis of the composition of the ACP pools on 2.5 M urea gels showed that acetyl-ACP and holo-ACP were major species (acetyl-ACP co-migrates with holo-ACP on these gels (4)). The strains had similar ACP compositions in that acetyl-ACP, holo-ACP, and traces of acyl-ACPs were present in samples lacking dithiothreitol (DTT) treatment whereas treatment of the samples with DTT gave only partial deacylation of the acyl-ACP species (Fig. 5). Although qualitatively similar, the levels of acyl-ACPs were lower in \textit{L. lactis} than in \textit{E. coli} presumably due to the slower growth rate of \textit{L. lactis} under the conditions of labeling.

\textbf{Bypass Mutants of the \textDelta fabH Strain That Grow with Octanoic Acid Supplementation—}The \textit{fabH} null deletion mutant was supplemented with different fatty acids (C4, C6, C8, or C10) as sole fatty acid sources to test if these acids would support growth. Only octanoic acid gave any growth response and the growth observed was very feeble in that formation of small colonies required two weeks of incubation. However, faster growing colonies arose in the background of small colonies and these fast growing strains still required fatty acids for growth. These faster growing strains grew well upon supplementation with octanoic acid (Fig. 6), although not grow as well as the wild-type strain IL1403. The isolation of these \textDelta fabH strains in which the loss of KAS III can be bypassed with octanoic acid indicates that the physiological role of FabH is for production of short-chain fatty acids.

\textbf{KAS III is an Essential Enzyme in \textit{E. coli}—}Our results with \textit{L. lactis} suggested that our prior failures to isolate \textit{fabH} mutants of \textit{E. coli} or \textit{S. enterica} were probably due to the lack of sufficient residual fatty acid synthesis for synthesis of the lipid A, an essential component of the
outer membrane, although polar effects on transcription of downstream genes could also have precluded success. In order to definitively test if KAS III is an essential gene in both gram-positive and gram-negative bacteria we used the CRIM (Conditional-Replication, Integration, and Modular) plasmids of Haldimann and Wanner (33) to test the essentiality of *fabH* in *E. coli*. We used this system of plasmids to insert a second copy of *fabH* that encoded a functional KAS III into the *E. coli* chromosome such that it could readily be removed. We then disrupted the *fabH* gene of the *fab* cluster by insertion of a kanamycin resistance cassette through homologous recombination and tested this strain and its parent for survival upon loss of the second copy. The second *fabH* copy was the gene from *S. enterica* Serovar Typhimurium. *S. enterica* is a close relative of *E. coli* and the fatty acid genes are known to be essentially identical at the protein level and fully interchangeable between the two species (21). However, the genes differ sufficiently at the nucleotide level (mainly due to changes in the wobble positions of the genetic code) that homologous recombination does not occur between homologous genes. We used the *S. enterica fabH* copy to avoid disruption of the second copy rather than the *fab* cluster copy upon homologous recombination catalyzed insertion of the kanamycin resistance cassette. The *S. enterica fabH* was inserted into the chromosomal attachment site (*attHK022*) of phage HK022 and the insertion was tagged with a cassette encoding resistance to spectinomycin and streptomycin.

Two isogenic strains were constructed. Strain CL81 contained a single copy of *S. enterica fabH* inserted at the *attHK022* site of strain UB1005 plus the wild type *fab* cluster copy, whereas strain CL111 contained the *S enterica fabH* insertion and the *fab* cluster copy disrupted by the kanamycin cassette. Therefore strain CL81 contained two functional copies of *fabH* whereas strain CL111 had only a single functional copy, the *S. enterica fabH* inserted at the
attHK022 site. We then tested the viability of both strains upon removal of the *S. enterica fabH* gene. Two removal methods were used. In the first the excisionase and integrase genes of phage HK022 were expressed from plasmid pAH83 (33). Expression of the phage genes resulted in excision of the spectinomycin-streptomycin resistant plasmid containing *S. enterica fabH*. Since the plasmid is unable to replicate in this strain background, it is lost during subsequent growth cycles. In the presence of pAH83, 178 colonies sensitive to spectinomycin and streptomycin were found among the 406 colonies of strain CL81 scored. In contrast none of the 411 scored colonies of strain CL111 were sensitive to spectinomycin-streptomycin. That is, the *S. enterica fabH* was readily removed from a strain carrying a second functional copy of the gene, but could not be removed from strain CL111 in which the *S. enterica* gene is the only functional *fabH*. The straightforward interpretation of this result is that *fabH* is an essential gene. We have confirmed this result by removing the *S. enterica fabH* through homologous recombination with DNA fragments introduced by phage P1-mediated transduction. Phage P1 stocks grown on two strains (34) each of which carries a tetracycline-resistant Tn10 element inserted to *attHK022*. Strains CAG12094 and CAG18466 carry insertions at min 21.6 and min 22.9 of the *E. coli* chromosome, respectively (35), whereas *attHK022* is located at min 22.7 (33). Given these close genetic linkages we expected that many of the recombinant colonies that inherited a Tn10 element would lose the integrated *S. enterica fabH* spectinomycin-streptomycin resistant plasmid. Therefore if the recipient strain remained viable, we expected that a high proportion of the tetracycline-resistant recombinants would be sensitive to spectinomycin-streptomycin. When the P1 lysate was grown on strain CAG12094, 86% of the 512 tetracycline-resistant recombinants of strain CL81 were sensitive to spectinomycin-streptomycin whereas none of the 166 transductants of strain CL111 were sensitive to spectinomycin-streptomycin.
Similar results were obtained with P1 lysate grown on CAG18466. In that case 84% of the 516 tetracycline-resistant transductants of strain CL81 were sensitive to spectinomycin-streptomycin whereas none of the 236 tetracycline-resistant transductants of CL111 were sensitive to spectinomycin-streptomycin. These data together with the excision data demonstrate that fabH is required for cell growth.
DISCUSSION

We have demonstrated that fabH (hence KAS III) function is essential for growth in *L. lactis* subsp. *lactis* IL1403 and *E. coli* K-12. The *L. lactis* deletion mutant is devoid of KAS III activity yet retained 5-10% of the normal fatty acid synthetic capacity. The obvious candidate for the *L. lactis* enzyme catalyzing the residual elongation reactions is FabF (KAS II), the only recognizable KAS isozyme remaining in the ΔfabH strain. KAS II catalyzes the condensation of an acyl-ACP (rather than the acetyl-CoA used by KAS III) with malonyl-ACP (1,2) (Fig. 7). Thus, if KAS II is to catalyze initiation of a new acyl chain a source of acetyl-ACP is required. Since KAS III is the only known acetyl-CoA:ACP transacylase (1,2), transacylation cannot provide a source of acetyl-ACP in the ΔfabH strain. The only known source of acetyl-ACP is KAS-catalyzed decarboxylation of malonyl-ACP (7,36) and it therefore seems that in the ΔfabH strain KAS II must generate its acetyl-ACP substrate. Indeed, a comparatively large acetyl-ACP pool is found in *E. coli* (7). Heath and Rock (7) concluded that acetyl-ACP was a by-product of fatty acid synthesis rather than an active intermediate in fatty acid initiation. Our results indicate that this conclusion should be re-evaluated, although the hypothesis that KAS II is responsible for the residual fatty acid synthesis of the ΔfabH strain remains to be verified by construction and characterization of an *L. lactis* strain missing both KAS isozymes. It should be noted that although KAS II seems able to initiate fatty acid synthesis, it may not play this role when KAS III is functional since the rate of malonyl-ACP decarboxylation is much slower than that of the elongation reaction (36) and thus should be suppressed when the enzyme is provided with a suitable acyl-ACP substrate. It seems probable that in the ΔfabH strain, the acyl-ACP supply is limiting such that a portion of the KAS II can catalyze malonyl-ACP decarboxylation. In *E. coli* the size of the acetyl-CoA pool varies with growth conditions, but seems likely to always greatly
exceed the pool of acetyl-ACP (37-39). Hence, it seems of advantage that KAS III catalyze the bulk of the initiation reactions (Fig. 7).

The key observation that led to the discovery of KAS III was the accumulation of short-chain acyl-ACPs in cerulenein-treated extracts of *E. coli* (8). Under these conditions octanoyl-ACP was the predominant acyl-ACP species implying that *in vivo* the role of KAS III is to produce short-chain acyl-ACPs that act as primers for the long-chain KAS isozymes (8). The isolation of derivative of the Δ*fabH* strain that allowed bypass of the mutation by octanoic acid supplementation clearly demonstrates that this hypothesis is correct. The enzymatic route by which octanic acid provides a bypass of KAS III deficiency is unknown, but several plausible mechanisms can be proposed. First, the activity or specificity of an *L. lactis* acyl-CoA synthetase (of which *L. lactis* has two candidates) could have been altered by mutation to produce levels of octanoyl-CoA sufficient for physiological function of the acyl-CoA:ACP transacylation activity of KAS II (36). Another KAS II-related possibility is that the substrate specificity of the KAS II transacylation activity (36) has been altered to favor octanoyl-CoA. The final possibility is that *L. lactis* contains an acyl-ACP synthetase such as that found in the luminescent bacterium *Vibrio harveyi* (40,41) that ligates fatty acids to ACP and this enzyme has been mutationally modified to better utilize octanoate. These seem reasonable hypotheses, although it is unclear why analogous mutations would not allow growth on hexanoate or butyrate (those strains that grow well on octanoate fail to grow on C4, C6, or C10 acids). Growth was not expected (or observed) on decanoic acid because the locations of the double bonds of the essential unsaturated fatty acids indicate that they are introduced by dehydration of 3-hydroxydecanoyl-ACP (42), a precursor to the postulated decanoyl-ACP formed from exogeneous decanoate.
Revill et al. (43) have reported that KAS III is essential for growth in *Streptomyces coelicolor* A3(2) by use a strategy formally analogous to that we used in *E. coli*. However, the interpretation of this result in this bacterium is much less straightforward than in the bacteria we have studied since *S. coelicolor* has four genes that encode KAS III homologues. The reason for this diversity of KAS III genes is presumably the unusually wide range of fatty acids synthesized by *S. coelicolor*. In addition to the even-numbered straight-chain fatty acids found in *E. coli* and *L. lactis*, *S. coelicolor* contains a wide array of unusual terminally branched acids plus straight-chain acids having odd numbers of carbon atoms. Synthesis of these acids require primers other than acetyl-CoA and use of these primers requires special KAS III isozymes. Choi and coworkers (44) have shown that *E. coli* KAS III is inactive with the primers used in synthesis of terminally branched chain fatty acids by *Bacillus subtilis* whereas the two *B. subtilis* KAS III iso-enzymes readily use these CoA thioesters, albeit with differing preferences. For these reasons it is unclear whether the *fabH* gene studied in *S. coelicolor* is required for fatty acid synthesis per se or only for synthesis of an essential branched or odd-chain length fatty acid.
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| Strains or Plasmid | Relevant characteristics | Sources or references |
|--------------------|--------------------------|-----------------------|
| **E. coli**        |                          |                       |
| MG1655             | wild type                | Lab collection        |
| UB1005             | F^metB1 relA1 spoT1 gyrA216 λ^rλ^- | Lab collection |
| DY330              | W3110 ΔlacU169 gal490 λcI857 Δ(cro-bioA) | (23) |
| CAG12094           | MG1655, zcb-3059::Tn10   | (34,35)               |
| CAG18466           | MG1655, zcc-282::Tn10    | (34,35)               |
| CL81               | UB1005, attHK022::(plsX'fabH; aadA) | This work |
| CL106              | DY330, attHK022::(plsX'fabH; aadA) | This work |
| CL110              | DY330, attHK022::(plsX'fabH; aadA) fabH::kan | This work |
| CL111              | CL81, fabH::kan          | This work |
| BMH71-81           | F^ lacI^qΔ(lacZ)M15 proA^B^Δ/(lac-proAB) thi glnV | Lab collection |
| AT1371             | DH5α/pEC, P_lac panC on pEC | (24) |
| **S. enterica Serovar Typhimurium** |  |
|---|---|
| CL66 | LT2, *fabF::kan, fabH* (R271K) | This work |

| **L. lactis ssp. lactis** |  |
|---|---|
| IL1403 | Wild type | (13) |
| CL112 | IL1403, in-frame *fabH* deletion | This study |

| **Plasmids** |  |
|---|---|
| pAH144 | Plasmid dependent upon *pir* in host, R6K *γori*, *attPHK022, Spc* *Str* | (33) |
| pAH69 | CRIM helper plasmid, *amp, oriR101, IntHK022* | (33) |
| pAH83 | CRIM helper plasmid, *amp, oriR101, Xis and IntHK022* | (33) |
| pKD13 | Template plasmid, *amp, FRT-flanked kan* | (45) |
| pCL49 | Insertion of the 2 kb *plsX' fabH* PCR product of *S. enterica* strain CL66 (amplified with primers SalS-N and SalH-C) into pCR2.1 | This work |
| pORI280 | Plasmid dependent upon *RepA* in host, ori of pWV01, P<sub>32</sub>, *lacZ, Em'* | (19) |
| pK18 | Cloning vector | (20) |
| pCL58    | Insertion of the 1.2 kb *fabH* PCR product of *L. lactis* chromosome DNA (amplified with primers LacH-P and LacH-C) into pCR2.1 | This study |
|----------|----------------------------------------------------------------------------------------------------------------------------|------------|
| pCL61    | Insertion of the 1.2 kp *BamHI*-*XbaI* *fabH* fragment of pCL58 into pK18 cut with the same enzymes                           | This study |
| pCL62    | pCL61 was digested with *BglI* followed by blunt ending with T4 DNA polymerase + 4 dNTPs and then ligated                    | This study |
| pCL66    | Insertion of the 1.2-kb *BamHI*-*XbaI* fragment (containing an in-frame *fabH* deletion) of pCL62 into pORI280 cut with *BamHI* and *XbaI* | This study |
Table 2. PCR Primers used in this study

| Primer   | Sequence (5’-3’)                                      |
|----------|------------------------------------------------------|
| Primer 1 | GCCACATTGCCGCGCCAAACGAAACCCTTCAACCATGGTTCCGGGATCCGTCGACCTGCAGT |
| Primer 2 | CGCCCCAGATTTCACGTATTGATCGGCTACGCTTAATGCATGTGTAGGCTGGAGCTGCTTTC |
| SalS-N   | ACGCTAATTCGCAGCTCACT                                    |
| SalH-C   | ACAAATGCAAATTGCAGTCAT                                    |
| LacH-P   | TCAATCGATTTAGAAGATAAGGGA                                  |
| LacH-C   | GAAAAACTTGTAAACTTTGAAAGC                                  |
| P1<sup>a</sup> | GGAATCAATGCCTGAGTG                                        |
| P2<sup>a</sup> | ACTTAACGGCTGACATGG                                         |
| P3<sup>a</sup> | ACGAGTATCGAGATGGCA                                        |
| P4<sup>a</sup> | GGCATCAACAGCACATTC                                        |

<sup>a</sup> Primers P1, P2, P3, and P4 are from reference (34).
Table 3. Incorporation of [1-\(^{14}\)C]acetate into lipids

| Strains (growth condition) | nmol [1-\(^{14}\)C]acetate incorporated/OD\(_{600}\) | Experiment 1 | Experiment 2 | Experiment 3 |
|---------------------------|----------------------------------|-------------|-------------|-------------|
| IL1403 (log)              | 0.40                             | 0.60        | 0.51        |
| IL1403 (stationary)       | 0.41                             | –           | –           |
| CL112 (log)               | 0.05                             | 0.03        | 0.05        |
| CL112 (stationary)        | 0.02                             | –           | –           |
| IL1403 (fatty acid starvation) | 1.27                              | 1.13        | –           |
| CL112 (fatty acid starvation) | 0.14                              | 0.10        | –           |
| IL1403 (avidina)          | –                                | –           | 0.01        |

\(a\) Strain IL1403 was grown to log phase (experiment 3) or stationary phase (experiment 4) in GM17 medium supplemented with 0.3 mg/ml avidin and Tween 80 as described in Experimental Procedures.
Table 4. Fatty acid compositions of mutant and wild type strains

| Strain  | Growth Medium | Fatty Acid Supplementa | Cellular Fatty Acids (percent by weight) |
|---------|---------------|------------------------|------------------------------------------|
|         |               |                        | C14:0 | C16:0 | C18:0 | C18:1 |
| IL1403  | GM17          | +                      | 2.7   | 11.7  | 22.6  | 63.0  |
| CL112   | GM17          | +                      | 3.2   | 14.2  | 32.3  | 50.3  |
| CL112   | GM17          | -                      | 4.0   | 32.8  | 57.2  | 6.0   |
| IL1403  | Minimal       | +                      | 3.6   | 19.5  | 41.3  | 35.6  |
| CL112   | Minimal       | +                      | <0.3  | 8.2   | 19.8  | 72.0  |

a The fatty acid supplement was 0.02% cis-vaccenate. Fatty acid starvation was done as described in Experimental Procedures.
Figure legends

Fig. 1. Construction of the fabH deletion allele. Panel A. Partial nucleotide and amino acid sequence of the fabH gene in L. lactis where *C represents the essential active site cysteine of FabH. The 36 bp deletion generated by BglI digestion and T4 DNA polymerase resection is shown.

Panel B. The gene disruption required two steps. First, plasmid pCL66 carrying an in-frame fabH deletion was transformed into strain IL1403 with selection for erythromycin resistance. Since plasmid pCL66 lacks repA, the gene required for plasmid replication, this plasmid must integrate into chromosome via homologous recombination to give erythromycin-resistant colonies. The recombination event may occur within either sequence 1 or within sequence 2 (for simplicity only the outcome of recombination via sequence 1 is shown). Integrant colonies will stain blue on fatty acid supplemented GM17E5Xgal plates (19) due to the vector lacZ gene. Blue colonies are then grown in liquid culture for many generations to allow resolution of the cointegrate and then screened for white colonies on GM17Xgal medium supplemented with fatty acids to detect colonies in which excision of the vector had occurred by homologous recombination between the two copies of fabH. Recombinational resolution of the cointegrate results either in reversion to the wild type allele through crossover within sequence 1 or in replacement with the mutant allele through crossover within sequence 2. The gray box represents the active site of the fabH gene.
Fig. 2. Growth of strains IL1403 (WT) and CL112 (ΔfabH) with or without exogenous fatty acids. Strain CL112 failed to grow without fatty acid supplementation. The exogenous fatty acids supplied were 0.1% Tween 40, 0.1% Tween 80, and 0.02% oleate. The growth of strain CL112 with oleate as sole supplement was identical to that seen in the fatty acid supplemented plate.

Fig. 3. Lipid synthesis in strains IL1403 and CL112 (ΔfabH). Lipid synthesis was measured by the incorporation of [1-14C]acetate (5 μCi/ml) into lipids after conversion to fatty acid methyl esters or free fatty acids as described in Experimental Procedures. Panel A. The autoradiograph of an argentation thin layer chromatographic separation of the fatty acid methyl esters is shown. Lanes 1 and 3, IL1403; Lanes 2 and 4, CL112. In the samples of lanes 1 and 2 the cells were harvested at the late log phase whereas in lanes 3 and 4, the cultures were grown to a density of 10^8 cells/ml. The cells were harvested by centrifugation, washed, and resuspended in GM17 lacking fatty acid supplementation. The cultures were then labeled during growth at 30°C for 7 h and processed.

Panel B. Saturated and unsaturated fatty acids comigrate in this chromatographic system. Lanes 1, 2, 5, 8, and 9 are strain IL1403 samples whereas lanes 3, 4, 6, and 7 are samples from strain CL112. The samples of lanes 1, 3, and 7 were obtained from cells harvested at the log phase whereas in the samples of lanes 2, 4, and 8 the cells were from stationary phase overnight cultures. The cultures of lanes 5 and 6 were starved for exogenous fatty acids as described in Experimental Procedures and then labeled. The sample of lane 9 is
strain IL1403 grown to log phase in GM17 supplemented with 0.3 mg avidin and 0.1% Tween 80.

Fig. 4. Assay of KAS III activity in cell-free extracts. Acetoacetyl-ACP synthase assays were conducted as described in Experimental Procedures. The squares represent the strain IL1403 extract whereas the diamonds denote the strain CL112 extract. Note that the synthase activity of strain CL112 did not depend on protein concentration.

Fig. 5. The compositions of the ACP pools of the wild type and the fabH mutant strains. Strain IL1403 and CL112 (∆fabH) were grown at 30°C for 72 h in the minimal medium containing [3H]pantothenate (specific activity, 60 Ci/mmol) and all required nutrients as listed in Experimental Procedures except pantothenate. The acyl-ACP intermediates were extracted and dithiothreitol (1 mM, pH 8.0) was added to the samples of the even numbered lanes in order to produce nonesterfied ACPs. The products were then analyzed on 15% acrylamide gels containing 2.5 M urea. The gels were exposed to fluorography (Experimental Procedures). Lanes 1 and 2 are strain IL1403 samples whereas lanes 3 and 4 are strain CY112 samples.

Fig. 6. Octanoic acid dependent growth of ∆fabH bypass mutants. Growth of strains IL1403, CL112 (∆fabH), and CL114 a ∆fabH bypass derivative on GM17 medium supplemented with long-chain fatty acids or octanoic acid.
Fig. 7. Possible pathways for the initiation of fatty acid biosynthesis in *L. lactis*. KAS III condenses malonyl-ACP with acetyl-CoA to generate acetoacetyl-ACP in the primary initiation pathway of fatty acid synthesis. Other reactions involved in the initiation reactions are shown. We propose that KAS II is responsible for the residual fatty acid synthesis in the *fabH* mutant that lacks KAS III. This pathway would proceed by decarboxylation of malonyl-ACP to give acetyl-ACP and then condensation of these two acyl-ACPs to give acetoacetyl-ACP. Abbreviations: ACC, acetyl-CoA carboxylase; MTA, malonyl-CoA:ACP transacylase; KAS II, ß-ketoacyl-ACP synthase II; KAS III, ß-ketoacyl-ACP synthase III.
Fig. 1
Fig. 2.

Plus Fatty Acids

No Fatty Acids
Fig. 3.

- Saturated (C16:0)
- cis-Vaccenate (C18:1)
- Palmitoleate (C16:1)

Origin
Extract protein (μg) vs. [\(^{14}\text{C}\)]Acetoacetyl-ACP (CPM x 10\(^{-3}\))

Fig. 4

|   | IL1403 | CL112 (\(\Delta fabH\)) |
|---|--------|-------------------------|
|   | -      | +                       |
| - | DTT    | -                       |
| + |        | +                       |

Holo-ACP + Acetyl-ACP
Acyl-ACP

Fig. 5.
Fig. 6.
Fig. 7.
β-Ketoacyl-Acyl carrier protein synthase III (FabH) is essential for bacterial fatty acid synthesis

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