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Inhibition of Fatty Acid Synthesis in *Escherichia coli* in the Absence of Phospholipid Synthesis and Release of Inhibition by Thioesterase Action

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The effects of inhibition of *Escherichia coli* phospholipid synthesis on the accumulation of intermediates of the fatty acid synthetic pathway have been previously investigated with conflicting results. We report construction of an *E. coli* strain that allows valid [^{14}C]acetate labeling of fatty acids under these conditions. In this strain, acetate is a specific precursor of fatty acid synthesis and the intracellular acetate pools are not altered by blockage of phospholipid synthesis. By use of this strain, we show that significant pools of fatty acid synthetic intermediates and free fatty acids accumulate during inhibition of phospholipid synthesis and that the rate of synthesis of these intermediates is 10 to 20% of the rate at which fatty acids are synthesized during normal growth. Free fatty acids of abnormal chain length (e.g., *cis*-13-eicosenoic acid) were found to accumulate in glycerol-starved cultures. Analysis of extracts of [^{35}S]methionine-labeled cells showed that glycerol starvation resulted in the accumulation of several long-chain acyl-acyl carrier protein (ACP) species, with the major species being ACP acylated with *cis*-13-eicosenoic acid. Upon the restoration of phospholipid biosynthesis, the abnormally long-chain acyl-ACPs decreased, consistent with transfer of the acyl groups to phospholipid. The introduction of multicopy plasmids that greatly overproduced either *E. coli* thioesterase I or *E. coli* thioesterase II fully relieved the inhibition of fatty acid synthesis seen upon glycerol starvation, whereas overexpression of ACP had no effect. Thioesterase I overproduction also resulted in disappearance of the long-chain acyl-ACP species. The release of inhibition by thioesterase overproduction, together with the correlation between the inhibition of fatty acid synthesis and the presence of abnormally long-chain acyl-ACPs, suggests with that these acyl-ACP species may act as feedback inhibitors of a key fatty acid synthetic enzyme(s).

In growing cultures of *Escherichia coli*, fatty acid synthesis is tightly coupled to phospholipid synthesis; the intracellular pools of fatty acid synthetic intermediates are very small. This result suggests that fatty acid synthesis is coordinately regulated with or by phospholipid synthesis. Therefore, several groups have performed assays for accumulation of fatty acid biosynthetic intermediates in the absence of phospholipid synthesis. Phospholipid synthesis was blocked by use of mutants to restrict the supply or utilization of *sn*-glycerol 3-phosphate, the precursor required for the first step of phospholipid synthesis. Unfortunately, these experiments have given conflicting results.

The earliest study (13) reported that no fatty acid synthetic intermediates such as free fatty acids (FFA) accumulated. However, the strain used was proficient in β-oxidation; thus, degradation of FFA could explain the lack of accumulation seen. Indeed, when strains (*fadE*) in which β-oxidation was blocked were examined, it was reported that the rate of incorporation of [^{14}C]acetate into FFA by glycerol-starved cells was the same as the rate of incorporation of this precursor into phospholipid when glycerol was supplied (6). However, later work by Nunn et al. (16) showed that these labeling results were complicated by an unexpected shrinkage of the endogenous acetate pool of the glycerol-starved cells. Thus, the specific activities of the cellular acetate pools utilized in fatty acid synthesis differed between the cultures starved of *sn*-glycerol 3-phosphate and the unstarved control cultures. Therefore, equivalent rates of [^{14}C]acetate incorporation did not denote equivalent rates of lipid synthesis. Nunn et al. (16) proceeded to use [^{14}C]succinate as a lipid precursor and reported that fatty acid synthesis was very tightly coupled to phospholipid synthesis; little labeled FFA accumulated in the absence of phospholipid synthesis. However, subsequent work from that laboratory (10, 11) indicated that [^{14}C]succinate pools would expand upon glycerol starvation, giving an underestimation of FFA production.

A plausible explanation for the changes in succinate and acetate pools upon glycerol starvation stems from evidence that FadR, the repressor of the β-oxidation regulon, also downregulates the expression of the glyoxylate operon (11). Since the known regulatory activities of FadR are neutralized by fatty acyl-coenzyme A (CoA) binding (7), it seems probable that FFA are produced and converted to acyl-CoAs via acyl-CoA synthetase. The acyl-CoAs would then neutralize FadR, resulting in increased production of the glyoxylate cycle enzymes. Increased glyoxylate cycle enzyme levels would then result in shrinkage of the acetate pool (16) and expansion of the succinate pool (10, 11). This explanation is probably oversimplified, since the regulation of the glyoxylate cycle by FadR appears indirect; no FadR binding site has been found in the vicinity of the *aceBA* promoter.

For these reasons, the choice of a precursor to measure lipid synthesis during glycerol starvation is problematic. Malonate is not utilized by *E. coli*, and the only direct precursor other than acetate, $^{3}H_2O$, is diluted by the water of the culture medium such that pulse labeling is essentially impossible. An alternative approach was to determine the level of fatty acyl-acyl carrier

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TABLE 1. Bacterial strains used in this study.

Strain	Relevant genotype	Source or reference
CDM22	*zhf*::Tn*10* Δ*glpD*	C. M. Murphy
CDM44F	*gpsA*::Kan^r of JC7623	C. M. Murphy
CY14	*metB fadE62 tsx*	6
EM66	*aceA1 zja*::Tn*10*	S. R. Maloy
SJ16	*panD2 zad*::Tn*10* of UB1005	S. Jackowski
UB1005	*metB relA nalA*	D. Clark
BB20-14	*gpsA20 glpD3 glpR2 glpK14 phoA8 tonA22 rel-1*	6
LW3	*fadE* derivative of BB20-14	6
YYC196	Δ*aceEF pps-4 pfl-1 lacZ poxB1*	1
PJ1	*fadL*$^+$ derivative of YYC196	This work
PJ2	*fadE62 zae*::Tn*10* derivative of PJ1	This work
PJ3	Tet^s derivative of PJ2	This work
PJ4	Δ*glpD zhf*::Tn*10* derivative of PJ3	This work
PJ5	Tet^s derivative of PJ4	This work
PJ6	*gltA*::Cm^r derivative of JC7623	This work
PJ7	*gpsA*::Kan^r derivative of PJ5	This work
PJ8	*aceA1 zia*::Tn*10* derivative of PJ7	This work
PJ9	Tet^s derivative of PJ8	This work
PJ20	PJ9 containing plasmid pPJ7	This work
PJ21	PJ9 containing plasmid pPJ6	This work

protein (acyl-ACP) molecules by labeling the protein moiety. Such experiments were done by Rock and Jackowski (19), who reported the accumulation of acyl-ACPs upon glycerol starvation. This result indicated that fatty acid synthesis did continue in the absence of phospholipid synthesis, but it could provide no measure of any FFA produced by hydrolysis of acyl-ACPs. To deal with these constraints, we constructed an *E. coli* strain in which (i) acetate is a specific precursor for lipid synthesis, there being no significant incorporation of acetate into other cellular materials, (ii) there is no endogenous synthesis of acetate, and (iii) the glyoxylate and tricarboxylic acid (TCA) cycles are inoperative in acetate utilization. By use of this strain, we demonstrate that fatty acid synthesis is coupled to phospholipid synthesis and that this coupling can be eliminated by overproduction of either of the thioesterases normally present in *E. coli*.

MATERIALS AND METHODS

Bacterial genetics and DNA manipulations. Transductions with phage P1*vir* and selection of tetracycline-sensitive derivatives of Tn*10*-containing strains were done as previously described (2). Plasmids pPJ6 and pPJ7 were constructed by ligation of the 5.5-kbp *Sal*I citrate utilization fragment of pOH4 (8) into the unique *Sal*I sites of pBR322 and pACYC184, respectively. Plasmid pPJ8 was constructed by ligation of the *Eco*RI-*Pst*I fragment of pMR16 (18) into the polylinker of pHSG575 (24) digested with the same two enzymes.

Media, growth, and labeling conditions. The *E. coli* K-12 strains used in this work are listed in Table 1. All strains were grown on minimal E medium (which contains 0.25% citrate) supplemented with 5 mM acetate, 0.02% glycerol, and 0.05% casein hydrolysate. Succinate (0.4%) was used as the carbon source for strains unable to utilize citrate. When cultures of strain PJ9 derivatives reached early log phase, glycerol starvation was accomplished by filtration followed by several washes of the cells with medium E (6). The cells were resuspended in the supplemented medium lacking glycerol. This cell suspension was then split into two flasks, one of which contained sufficient glycerol to give a final concentration of 0.02%. After growth, turbidity was measured at 540 nm in a Klett colorimeter. At each time point, a 1-ml sample was removed from each culture and labeled with 10 μCi of [^{14}C]acetate (58 mCi/mmol) for 5 min in parallel with the original culture. Incorporation was stopped by dropping the culture sample by pipette into chloroform-methanol (1:2, vol/vol), followed by lipid extraction as previously described (6).

Analysis of radioactive lipids. The incorporation of labeled acetate into lipid was assayed by thin-layer chromatography as previously described (6). The chloroform phase was dried under N_2 and then dissolved in 50 μl of chloroform-methanol (2:1, vol/vol) before being loaded on thin-layer chromatography plates. Silica gel G plates were used to separate neutral lipid and phospholipid. The plates were activated in an 80°C oven for 1 h before sample application. The plates were first developed in petroleum ether-ether-acetic acid (70:30:2, vol/vol/vol) for a distance of 18 cm from the origin and then developed in chloroform-methanol-acetic acid (65:25:8) for a 10-cm distance (6). [1-^{14}C]palmitic acid was used as a standard. Radioactive lipids were quantitated by use of a PhosphorImager densitometer (Molecular Dynamics) as described by the manufacturers.

Fatty acid methyl esters were obtained by esterification by heating lipid samples for 1 h at 80°C with 3% HCl in methanol formed from anhydrous methanol and acetyl chloride (20:1). After an equal volume of water had been added to the reaction mixture, the esters were extracted into petroleum ether for analysis (4). Monoenoic esters were separated from one another (as well as from saturated fatty acids) according to the position of the double bond on thin-layer plates impregnated with 20% $AgNO_3$. The methyl ester samples were applied to activated $AgNO_3$ plates and developed twice in toluene at −20°C (4, 14).

Fatty acid esters were separated according to chain length by reverse-phase chromatography. Silica gel G plates were activated and impregnated with dodecane (dodecane-hexane; 17:83, vol/vol), spotted with saturated methyl esters, and developed in 100 ml of acetone-acetonitrile (1:1)–7 ml of dodecane (5). These plates separate methyl esters according to chain length, the mobility being inversely proportional to the length of the acyl chain.

Gel electrophoresis. Acyl-ACPs are separated by the chain length of the acyl chain in polyacrylamide gels containing urea (17). Long-chain acyl groups stabilize ACP from complete denaturation by urea; thus, the long-chain species have smaller molecular radii and migrate faster than short-chain acyl-ACPs on these gels (17). Thus, variation of the urea concentration in the gel (up to 5 M) allows acyl-ACPs to be separated by acyl chain length. The general trend is that shorter-chain acyl-ACPs separate in lower concentrations of urea, whereas longer-chain acyl-ACPs separate only at higher urea concentrations (17). [^{35}S]methionine was used to label all ACP species, whereas a derivative of strain PJ20 which carried a *panD* lesion was used for β-[3-^{3}H]alanine labeling of the ACP, acyl-ACP, and CoA pools. [^{14}C]acetate was used to label the acyl chains of the acyl-ACP species. Separation of proteins in the presence of sodium dodecyl sulfate was done as previously described (7).

Materials. Radioactive materials were purchased from ICN, DuPont, and Amersham. Acyl-ACP standards (C_{10} to C_{18}) were made by acyl-ACP synthetase from *Vibrio harveyi* (20). *cis*-13-Eicosenoic acid was synthesized by a 2-carbon-chain elongation of *cis*-11-octadecenoyl-methanesulfonate (Nu Chek Prep) as described by Spener and Mangold (22).

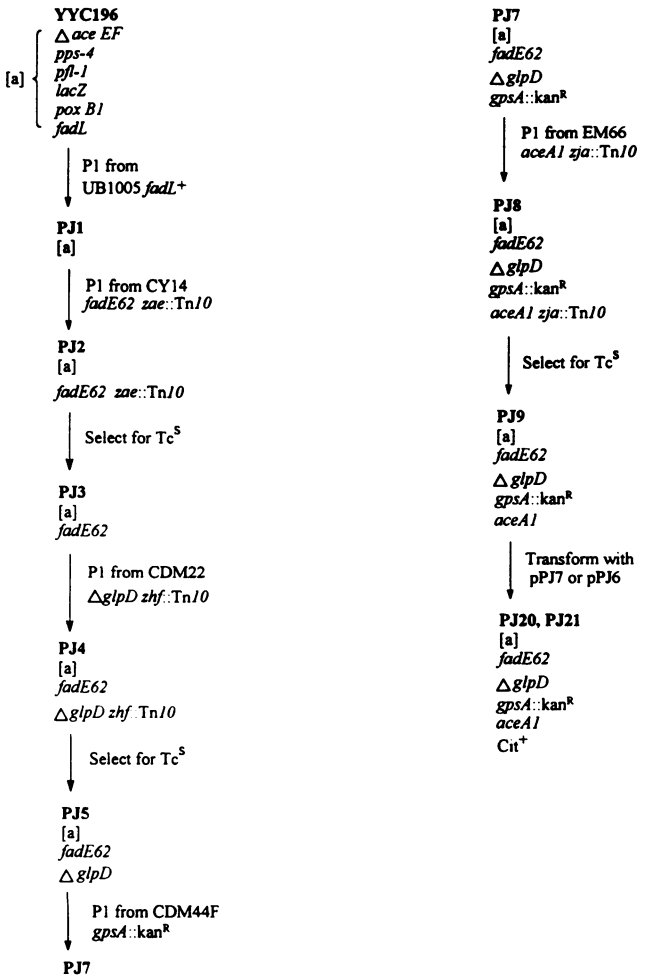

FIG. 1. Steps in the construction of strains PJ20 and PJ21.

RESULTS

Strain design and construction. A series of transductional crosses was used to construct strain PJ9 (Fig. 1). This strain contained mutations that blocked glycerol degradation (*glpD*, to allow efficient glycerol supplementation), glycerol 3-phosphate synthesis (*gpsA*), production of acetate from pyruvate (*aceEF*, *pfl*, and *poxB*), β-oxidation (*fadE*), and function of the glyoxylate cycle (*aceA*) (Table 1). Each of the mutations used was either a known null mutation or a well-characterized point mutation with no detectable residual function. We next intended to block the entry of acetate into the TCA cycle by introduction of a newly constructed citrate synthase (*gltA*) null mutation. However, upon introduction of this null mutation into the multiply mutant strain, the resulting strain grew poorly. In the expectation that poor growth might be due to inefficient supplementation, we then introduced a plasmid-borne citrate transport system (8) into strain PJ9 to allow bypass of the *gltA* lesion by supplementation with citrate (rather than the glutamate normally used). This manipulation failed to increase the growth rate of the *gltA* derivative, but we found that introduction of the citrate transport plasmid into the Glt$^+$ strain and growth in the presence of citrate resulted in blockage of the entry of acetate into the TCA cycle. We believe this situation results from the known inhibition of citrate synthase by citrate (23) and/or inefficient utilization of endogenously produced citrate in the presence of transported citrate. We have no explanation for the poor growth of the *gltA* derivative of strain PJ9. Otherwise, wild-type strains that carry the *gltA* null lesion grow well in the presence of glutamate. We suspect that the highly restricted C_2-C_4 metabolism of the multiply mutant strain PJ9 is responsible.

TABLE 2. Rates of incorporation of [^{14}C]acetate into lipid and protein in the presence and absence of glycerol[a]

Strain	Presence of glycerol	Synthesis (cpm/5 min) into:		Lipid/protein ratio
		Lipid	Protein	
BB20-14	+	6,240	600	10.4
	−	7,800	1,350	5.8
LW3	+	6,780	660	10.3
	−	11,760	2,370	4.9
PJ20	+	17,220	1,260	13.6
	−	9,480	690	13.7

[a] The values given are counts per minute. The symbols + and − denote the presence and absence of glycerol, respectively. The strains were grown to early log phase, starved of glycerol, and labeled with [^{14}C]acetate for 5 min following 2 h of starvation as described in Materials and Methods. Additional leucine (10 μg/ml) was added to the medium. The cell debris pellet was repeatedly extracted with ethanol-ether (3:1, vol/vol) until no radioactivity could be detected in the solvent and was taken as the protein fraction. A turbidity of 30 Klett units equals a cell concentration of 1.5×10^8 cells per ml. The rates are expressed per 1.5×10^8 cells.

The strains used in the labeling studies have the following phenotype: (i) lack of growth on glycerol, fatty acids, or acetate as sole carbon source (due to the *glpD*, *fadE*, and *aceA* lesions, respectively), (ii) requirement for acetate (due to the *aceEF* and *poxB* lesions), (iii) requirement for glycerol or glycerol 3-phosphate (due to the *gpsA* lesion), (iv) lack of growth on fatty acids as the source of acetate (due to the *fadE* lesion), and (v) growth on citrate as sole carbon source (due to the citrate utilization plasmid). Each of these phenotypes was checked at the conclusion of all labeling experiments. The two strains used in this work differed only in the vector portion of the plasmid allowing citrate utilization. The replication origin of the pPJ7 plasmid (present in strain PJ20) is that of pACYC184, whereas plasmid pPJ8 of strain PJ21 has a pBR322 origin.

Specificity and validity of acetate incorporation. Acetate is incorporated into nonlipid cellular constituents by two routes. The direct route is the incorporation of acetyl-CoA in the biosynthesis of leucine. Incorporation of acetate by this route can be prevented by the efficient feedback inhibition and repression of the leucine biosynthetic pathway given by exogenous leucine (25). The second route is indirect, incorporation via the TCA and glyoxylate cycles, giving rise to the biosynthetic precursors α-ketoglutarate, succinate, and oxaloacetate. We blocked this second route by supplementation with citrate, introduction of the *aceA* mutation, and the addition of a mixture of amino acids (casein hydrolysate) to the medium.

The specificity of [^{14}C]acetate incorporation was assessed by assay of incorporation into lipid versus nonlipid (essentially protein) cell components in strain PJ20 grown in the presence of high levels of leucine (cultures supplemented with leucine in addition to that present in the casein hydrolysate). Ratios of lipid to protein incorporation of >13 were obtained for strain PJ20, whereas ratios of ca. 10 were found for other strains (Table 2). More important is that the ratio for strain PJ20 did not change upon glycerol starvation, indicating that the acetate pool was well controlled. In contrast, strain LW3, a strain used

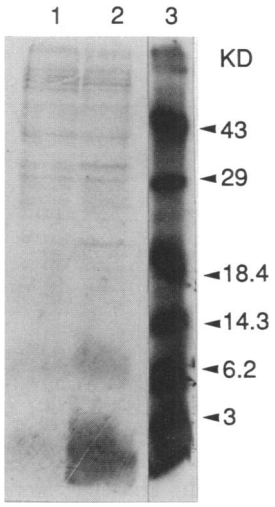

FIG. 2. [^{14}C]acetate incorporation into protein in the presence and absence of glycerol. A culture of PJ20 was grown and starved of glycerol as described in Materials and Methods. After 2 h of starvation, equivalent cell samples were taken from both starved and unstarved groups and labeled for 5 min with [^{14}C]acetate. Cells were harvested by centrifugation and precipitated with 2.5% trichloroacetic acid. The resulting pellets were solubilized and loaded on a sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis gel. The gel was run at 15 mA. Lanes: 1, proteins from cells with no glycerol supplementation; 2, proteins from cells with glycerol supplementation; 3, ^{14}C-labeled protein standards.

in previous experiments (6), incorporated acetate into both protein and lipid at increased rates upon glycerol starvation, reflecting the shrinkage of the acetate pool reported by Nunn et al. (16).

The rate of incorporation of [^{14}C]acetate into protein was greater in the presence of glycerol than in the absence of the supplement (Table 2). This result seemed in conflict with previous work showing that protein synthesis continues normally following glycerol starvation (12). However, these comparisons are complicated by the cellular lipoproteins. The acyl donor of acylated proteins is phospholipid (26); thus, labeling of lipoprotein acyl groups proceeded in glycerol-supplemented cells but was blocked in glycerol-starved cells. Hence, a portion of the label in the protein fraction of glycerol-supplemented cells was due to incorporation into lipid followed by attachment to protein. Previous assays of protein synthesis following glycerol starvation (12) used [^{3}H]leucine incorporation, which would not differentially label lipoproteins.

In order to determine the level of incorporation into lipoprotein, we separated the proteins of cultures labeled in the presence and absence of glycerol by gel electrophoresis (Fig. 2). These cultures were grown with lower levels of leucine (the amount present in casein hydrolysate) to allow a greater incorporation into protein. As expected, the low-molecular-weight lipoproteins were preferentially labeled in the glycerol-supplemented cultures. The identity of these characteristically diffuse bands was confirmed by loss of label upon hydroxylamine treatment of the gel (data not shown), which removes the two acyl chains linked by ester bonds (but not the amide-linked fatty acid [26]). Therefore, we attribute the additional labeling observed in the presence of glycerol (Table 2) to lipoprotein synthesis. Note that the intensities of labeling of the high-molecular-weight (nonacylated) proteins in the presence and absence of glycerol (Fig. 2) were essentially

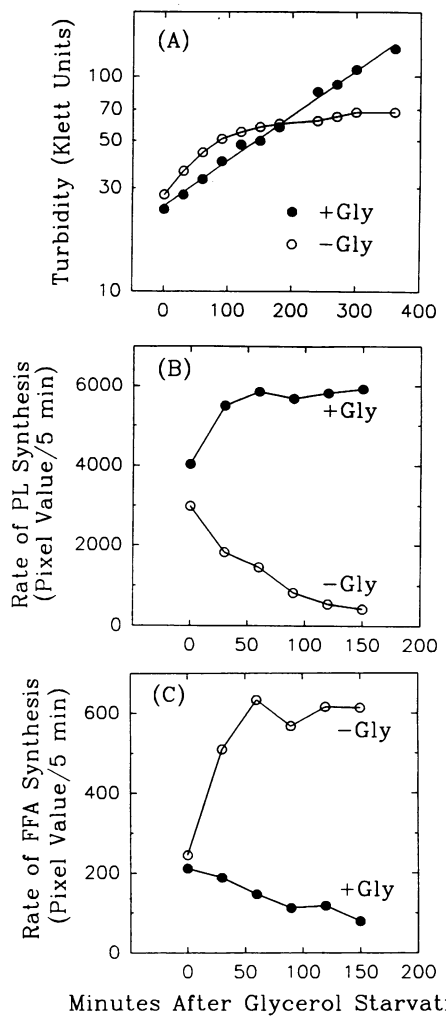

FIG. 3. Growth (A), phospholipid (PL) synthesis (B), and FFA synthesis (C) in the presence and absence of glycerol. Cells of strain PJ20 growing exponentially in glycerol-supplemented medium were filtered, washed, and resuspended in the same medium lacking glycerol (Gly) (see Materials and Methods). The culture was split into two flasks, and one flask was supplemented with glycerol; the other half remained unsupplemented. The cultures were shaken at 37°C, and samples were taken at the indicated times. One-milliliter samples were removed from both cultures and added to a tube containing 10 μCi of [^{14}C]acetate (58 mCi/mmol). After 5 min of incubation, 6 ml of methanol-chloroform (2:1, vol/vol) was added to quench incorporation. The extracted lipid was separated into PL and FFA fractions by thin-layer chromatography (see Materials and Methods). A turbidity of 30 Klett units equaled a cell concentration of 1.5×10^8/ml. The rates are expressed per 5×10^6 cells. All quantitation of radioactivity was done by PhosphorImager densitometry. The pixel value is an arbitrary unit employed by the PhosphorImager quantitation system and is linear with radioactivity of >5 orders of magnitude. +Gly, glycerol-supplemented culture; −Gly, glycerol-starved culture.

identical (as detected by phosphor storage technology), indicating that the acetate pools did not change upon glycerol starvation.

FFA accumulation following glycerol starvation. Strains PJ20 and PJ21 permitted valid [^{14}C]acetate labeling of lipids in both normally growing cultures and cultures in which phospholipid synthesis was blocked. As expected from prior work (6), glycerol starvation of these strains resulted in a cessation of

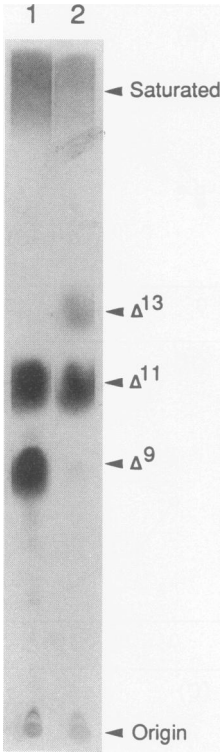

FIG. 4. Analysis of fatty acids from FFA and phospholipid fractions. A culture of strain PJ20 was starved of glycerol for 2 h, and samples were labeled with [^{14}C]acetate as described in Materials and Methods. The FFA fraction and the phospholipid fatty acid moieties were methylated, and the esters were separated by argentation chromatography. Lane 1, phospholipid fatty acids; lane 2, FFA fraction. Identification was done by cochromatography with authentic unlabeled standards which were detected by spraying with dichlorofluoroscein (4).

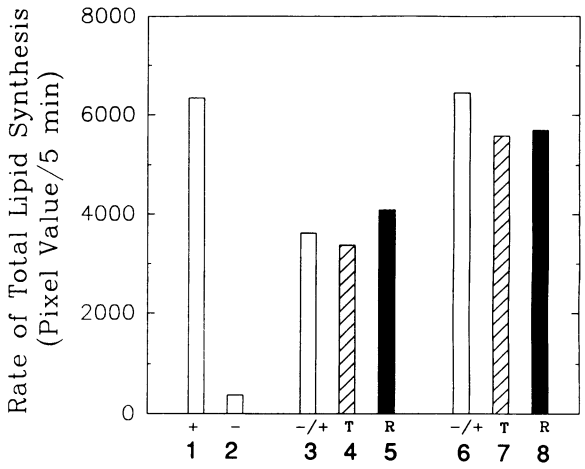

FIG. 5. Restoration of fatty acid and phospholipid synthesis in glycerol-starved cells by glycerol restoration. A culture of strain PJ20 was starved of glycerol as described in Materials and Methods. After 2 h of starvation, 1-ml samples from both unstarved (column 1) and starved (column 2) cultures were labeled with [^{14}C]acetate. The remainder of the starved culture was divided into three flasks. No antibiotic was added to the first flask (columns 3 and 6). Tetracycline (T) was added to the second flask to a final concentration of 50 μg/ml (lanes 4 and 7), and rifampin (R) was added to the third flask to a final concentration of 100 μg/ml (lanes 5 and 8). The antibiotics were incubated with cell cultures for 10 min prior to glycerol restoration. Following restoration of glycerol (0.02% final concentration), samples were taken and labeled for 5 min with [^{14}C]acetate. For columns 3 through 5, samples were taken immediately after glycerol restoration; for columns 6 through 8, samples were taken 5 min after glycerol restoration. Lipids were analyzed as described in Materials and Methods. The rates of total lipid synthesis are expressed per 5×10^6 cells.

turbidity increase after ca. 100 min of starvation (Fig. 3A) and the rate of phospholipid synthesis began to decrease immediately upon resuspension in the absence of glycerol (Fig. 3B). As the phospholipid synthetic rate decreased, the rate of FFA synthesis increased until it reached about 15% of the rate of incorporation into phospholipid observed in the presence of glycerol (Fig. 3C). (We found that the rates of FFA synthesis in starved cells relative to the rates of phospholipid synthesis in unstarved cells varied from 10 to 20% in various experiments.)

These results indicated that fatty acid and phospholipid syntheses were tightly, but not completely, coupled; fatty acid synthesis continued at a low rate, giving an appreciable accumulation of FFA. The FFA fraction consisted largely of unsaturated species with an unusually long chain length distribution, as shown by argentation chromatography (Fig. 4) and reverse-phase chromatography (data not shown). This switch to longer chain lengths was observed previously (6). Notably, *cis*-13-eicosenoic acid, a fatty acid not normally found in *E. coli*, accumulated in glycerol-starved cells (Fig. 4).

Lipid synthesis resumed rapidly upon restoration of glycerol and had returned to a normal rate by 5 min after restoration (Fig. 5). The resumption of lipid synthesis was not inhibited by addition of either tetracycline or rifampin, indicating that no new protein or RNA synthesis was required for resumption of phospholipid synthesis (Fig. 5).

ACP and acyl-ACP pools. Rock and Jackowski (19) reported the accumulation of acyl-ACP species upon glycerol starvation but were unable to identify the acyl groups with the methods then available. Post-Beitenmiller et al. (17) have recently reported an improved separation of ACP and its acyl species by gel electrophoresis in the presence of various concentrations of urea. By use of this electrophoretic system, we found that extracts of [^{35}S]methionine-labeled glycerol-starved and unstarved cells showed the accumulation of several long-chain acyl-ACP species, with the major species being ACP acylated with *cis*-13-eicosenoic acid (Fig. 6). The acyl group was identified by labeling the acyl-ACPs with [^{14}C]acetate rather than [^{35}S]methionine. The acetate-labeled acyl-ACP band was excised from the gel and treated with sodium methoxide to form the methyl ester of the acyl group, after which extraction of the ester and analysis by argentation chromatography were performed (data not shown).

Upon restoration of phospholipid synthesis (supplementation with glycerol), all of the long-chain acyl-ACP species decreased ca. 50% in 5 min, a result consistent with transfer of the acyl groups into phospholipid (data not shown). As shown in Fig. 6, the levels of nonacylated ACP were similar in starved and unstarved cells. Similar results were obtained with cells labeled with β-[^{3}H]alanine, a precursor of the ACP prosthetic group (data not shown). We also assayed [^{14}C]acetate-labeled extracts of starved and unstarved cells for accumulation of long-chain acyl-CoAs (9) and were unable to detect the presence of these compounds in either strain (data not shown).

Effects of thioesterase overexpression. The abnormally long-chain acyl-ACP molecules that accumulated in cells in which

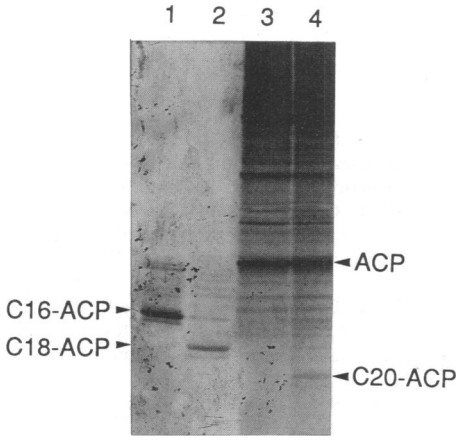

FIG. 6. Compositions of the acyl-ACP pool upon glycerol starvation. A culture of strain PJ21 was grown in E minimal media with all the required supplements and 10 μCi of [^{35}S]methionine per ml (1,074 Ci/mmol) to early log phase and starved of glycerol as described in Materials and Methods. After 2 h of starvation, 1-ml samples were taken from both starved and unstarved cells and cells were harvested by centrifugation. The cells were washed with medium E, resuspended, and precipitated with 2.5% TCA. The precipitate was resuspended in resuspension buffer (1). The gel contained 5 M urea and was run at 15°C and 20 mA. Lanes 1 and 2, acyl-ACP standards; lane 3, glycerol supplementation; lane 4, no glycerol supplementation.

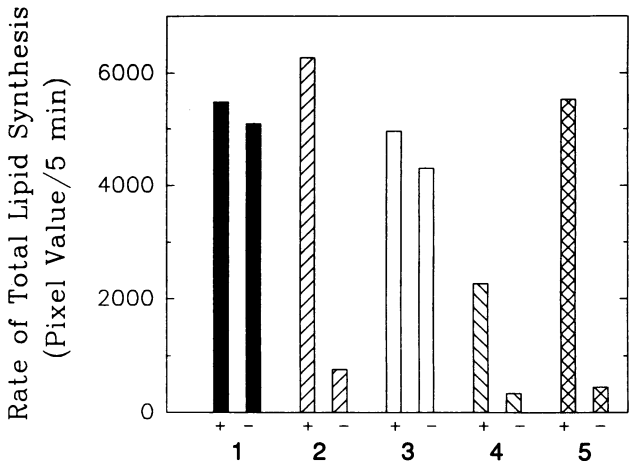

FIG. 7. Effects of overexpression of thioesterase I, thioesterase II, or ACP on the rate of lipid synthesis. The data are arranged in pairs of columns representing the cultures with (+) and without (−) glycerol. Strain PJ20/pHC61 (the plasmid encodes thioesterase I) (column pair 1), strain PJ20/pHC64 (the plasmid encodes an internally deleted inactive thioesterase I) (column pair 2), strain PJ20/pUC120*tesB* (the plasmid encodes thioesterase II) (column pair 3), strain PJ21 (column pair 4), and strain PJ21/pHSG575 (vector plasmid) (column pair 5) were grown to early log phase and starved of glycerol as described in Materials and Methods. Following 2 h of starvation, samples were taken and labeled with [^{14}C]acetate for 5 min. Lipids were extracted and analyzed as described in Materials and Methods. The rates are expressed per 5×10^6 cells.

phospholipid synthesis was blocked seemed reasonable candidates as inhibitors of fatty acid synthesis. If so, we expected that hydrolysis of these compounds should release inhibition. To test this hypothesis, we introduced plasmids that carried either *tesA* or *tesB*, genes which encode thioesterases I and II, respectively. The plasmids used overproduce the encoded thioesterase >100-fold (references 2 and 15 and data not shown). Introduction of either the *tesA* plasmid pHC61 (2) or the *tesB* plasmid pUC120*tesB* (15) into strain PJ20 gave strains in which lipid synthesis continued at essentially the same rate in both the presence and absence of glycerol (Fig. 7). All of the labeled lipid accumulated in the glycerol-starved cells was FFA with the chain length distribution characteristic of starved cells, except that *cis*-13-eicosenoic acid was not found (data not shown). Overexpression of thioesterase I also eliminated the accumulation of long-chain acyl-ACP species (Fig. 8). These effects were specific to the thioesterase overproduction plasmids. Introduction of plasmids that gave overproduction of ACP, 3-ketoacyl-ACP synthase I, 3-ketoacyl-ACP synthase III, or 3-hydroxydecanoyl-ACP dehydrase (the products of the *acpP*, *fabB*, *fabH*, and *fabA* genes, respectively) gave no relief of inhibition (Fig. 7 and data not shown). It should be noted that although thioesterase I is a periplasmic protein (2), upon 100-fold overproduction about half of the activity is cytosolic, presumably because of titration of the protein export apparatus (3).

DISCUSSION

Strains PJ20 and PJ21 were constructed to permit valid acetate labeling of cells under conditions of phospholipid synthesis inhibition. By use of these strains, we demonstrated that fatty acid synthesis is coupled to phospholipid synthesis, but the coupling is not complete. Fatty acid synthesis continues at about 15% of the normal rate resulting in FFA formation. The FFA accumulated in glycerol-starved cells were of abnormally long chain length, and acids of the same chain lengths were also found in thioester linkage to ACP. However, one fatty acid, *cis*-13-eicosenoic acid, was found as a major acyl-ACP species, although it was only a minor component of the FFA fraction. The increased chain length of the FFA in comparison with those normally found in *E. coli* results from blocking of phospholipid synthesis (6). The chain lengths

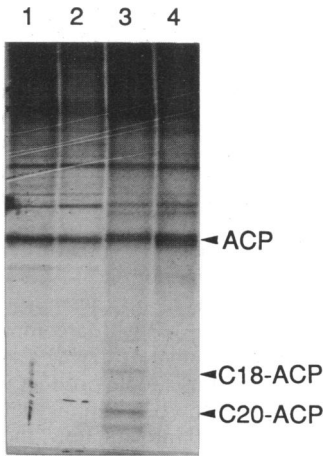

FIG. 8. Effects of thioesterase I expression on long-chain acyl-ACP accumulation. Strain PJ20/pHC61 (the plasmid encodes thioesterase I) (lanes 1 and 2) and strain PJ20/pHC64 (the plasmid encodes an internally deleted thioesterase I) (lanes 3 and 4) were starved of glycerol and labeled with [^{35}S]methionine as described in the legend to Fig. 6. Samples were prepared, loaded on a 5 M urea gel, and run at 15°C and 20 mA as described in Materials and Methods. Lanes 1 and 3, no glycerol supplementation; lanes 2 and 4, glycerol supplementation.

found in the phospholipids are the result of competition between the elongation reactions of fatty acid synthesis and acyl transfer into phospholipid. Thus, in the absence of phospholipid synthesis, extra cycles of chain elongation take place, resulting in abnormally long acyl chains (6).

Overexpression of either of the *E. coli* thioesterases results in relief of the inhibition of fatty acid synthesis engendered by glycerol starvation and also eliminated the accumulation of acyl-ACP species and the synthesis of fatty acids of abnormal length. These results argue that the inhibition of fatty acid synthesis may be caused by the accumulation of acyl-ACP species. Restoration of fatty acid synthesis by thioesterase overproduction could result from either loss of the acyl-ACP per se or the increase in ACP concentration resulting from the cleavage of the acyl group. We favor the former explanation, since the ACP pools of the starved cells were not significantly depleted (Fig. 6) and overproduction of ACP failed to relieve inhibition of fatty acid synthesis (Fig. 7).

Acyl-CoAs synthesized from the liberated FFA by acyl-CoA synthetase might also act as feedback inhibitors of fatty acid synthesis, but this seems unlikely because (i) we were unable to detect accumulation of acyl-CoAs during blockage of phospholipid synthesis; (ii) the *E. coli* thioesterases hydrolyze acyl-CoAs much more rapidly than acyl-ACPs (21), and thus the normal cellular levels of these enzymes are sufficient to preclude acyl-CoA accumulation; and (iii) recent work (3) has shown that FFA produced by *E. coli* are found in the medium (rather than within the cells), so that FFA are largely unavailable for acyl-CoA synthetase action.

The most straightforward model for the inhibition of fatty acid synthesis upon blockage of phospholipid synthesis is that long-chain acyl-ACP species accumulate and inhibit a key fatty acid synthetic enzyme(s). Thioesterase overproduction cleaves the long-chain acyl-ACP species, resulting in relief of the inhibition. Long-chain acyl-ACPs seem more likely than short-chain acyl-ACPs to be the inhibitory species, since we observe accumulation of only long-chain species and thioesterase I, which efficiently relieves inhibition, is unable to cleave short-chain ($<C_{12}$) acyl thioesters (2). If a single acyl-ACP species is the inhibitor, the strongest candidate would be *cis*-13-eicosenoyl-ACP, the product of the elongation of *cis*-vaccenic acid. *cis*-13-Eicosenoic acid is markedly enriched in the acyl-ACP pool relative to the FFA fraction and thus seems resistant to hydrolysis at the levels of thioesterase normally present in *E. coli*. The accumulation of this abnormally long unsaturated fatty acid could provide a signal that fatty acid synthesis is ahead of the utilization of acyl-ACP in phospholipid synthesis and provide a homeostatic mechanism to slow fatty acid synthesis.

The identity of the fatty acid synthetic enzyme(s) that is inhibited by the putative inhibitory acyl-ACP is not clear. The enoyl reductase step seems to be the most straightforward, since its product is an acyl-ACP with a completed acyl group (rather than an acyl intermediate carrying a keto, hydroxy, or *trans*-2-enoic group) and thus simple product inhibition of this enzyme could give the observed coupling between fatty acid and phospholipid synthesis. Unfortunately, the gene(s) encoding enoyl reductase activity has not yet been identified. It seems likely that identification of the inhibited enzyme will require an in vitro system that accurately reflects in vivo metabolism and/or the isolation of mutants refractory to inhibition.

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