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Overproduction of β-Ketoacyl-Acyl Carrier Protein Synthase I Imparts Thiolactomycin Resistance to *Escherichia coli* K-12

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Thiolactomycin [(4S)(2E,5E)-2,4,6-trimethyl-3-hydroxy-2,5,7-octatriene-4-thiolide] (TLM) is a unique antibiotic structure that inhibits dissociated type II fatty acid synthase systems but not the multifunctional type I fatty acid synthases found in mammals. We screened an *Escherichia coli* genomic library for recombinant plasmids that impart TLM resistance to a TLM-sensitive strain of *E. coli* K-12. Nine independent plasmids were isolated, and all possessed a functional β-ketoacyl-acyl carrier protein synthase I gene (*fabB*) based on their restriction enzyme maps and complementation of the temperature-sensitive growth of a *fabB15*(Ts) mutant. A plasmid (pJTB3) was constructed that contained only the *fabB* open reading frame. This plasmid conferred TLM resistance, complemented the *fabB*(Ts) mutation, and directed the overproduction of synthase I activity. TLM selectively inhibited unsaturated fatty acid synthesis in vivo; however, synthase I was not the only TLM target, since supplementation with oleate to circumvent the cellular requirement for an active synthase I did not confer TLM resistance. Overproduction of the FabB protein resulted in TLM-resistant fatty acid biosynthesis in vivo and in vitro. These data show that β-ketoacyl-acyl carrier protein synthase I is a major target for TLM and that increased expression of this condensing enzyme is one mechanism for acquiring TLM resistance. However, extracts from a TLM-resistant mutant (strain CDM5) contained normal levels of TLM-sensitive synthase I activity, illustrating that there are other mechanisms of TLM resistance.

The β-ketoacyl-acyl carrier protein (ACP) synthases are key regulators of dissociated (type II) fatty acid synthase systems typified by the *Escherichia coli* system (for reviews, see references 5 and 28). β-Ketoacyl-ACP synthase I is required for a critical step in the elongation of unsaturated acyl-ACP, and mutants (*fabB*) lacking synthase I activity are unable to synthesize either palmitoleic or *cis*-vaccenic acid and require supplementation with unsaturated fatty acids for growth (6, 30). β-Ketoacyl-ACP synthase II is responsible for the temperature-dependent regulation of fatty acid composition (for a review, see reference 7). Mutants (*fabF*) lacking synthase II activity are deficient in *cis*-vaccenic acid but grow normally (11, 12). β-Ketoacyl-ACP synthase III selectively catalyzes the formation of acetoacetyl-ACP in vitro (17). The role of this third condensing enzyme remains to be established, but its position at the beginning of the biosynthetic pathway suggests that it plays a role in governing the total rate of fatty acid production.

Thiolactomycin [(4S)(2E,5E)-2,4,6-trimethyl-3-hydroxy-2,5,7-octatriene-4-thiolide] (TLM) is a unique antibiotic structure that inhibits type II (bacterial and plant) but not type I (yeast and mammalian) fatty acid synthases (14, 15, 25, 26, 31). The antibiotic is not toxic to mice and affords significant protection against urinary tract and intraperitoneal bacterial infections (23). Understanding the mechanism of TLM action is important to the development of more effective antibiotics that exhibit selective action against type II bacterial fatty acid synthases. An analysis of the individual enzymes of a type II fatty acid synthase system suggests that the β-ketoacyl-ACP synthase and the acetyl-coenzyme A (CoA):ACP transacylase are the only individual enzymes inhibited by TLM in vitro (24). The observations that malonyl-ACP protects the synthases from TLM inhibition and that they are competitively inhibited with respect to malonyl-ACP are consistent with TLM interacting with the malonyl-ACP site on the condensing enzymes rather than with the acyl-ACP site. All three condensing enzymes in *E. coli* are inhibited by TLM both in vivo and in vitro (16). In the present study, we isolated and characterized recombinant plasmids from an *E. coli* genomic library that impart TLM resistance on a sensitive strain to identify relevant TLM targets and to investigate their roles in fatty acid biosynthesis.

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MATERIALS AND METHODS

Materials. Sources of supplies were as follows: Promega Biotech, restriction endonucleases and other molecular biology reagents; Chugai Pharmaceutical Co. (Tokyo, Japan), TLM (provided as a generous gift); Pharmacia, acetyl-CoA and malonyl-CoA; ICN Biochemicals and Du Pont, New England Nuclear, [1-^{14}C]acetate (specific activity, 56 Ci/mol) and [2-^{14}C]malonyl-CoA (specific activity, 50.9 Ci/mol); Bio-Rad, electrophoresis supplies. Homogeneous ACP was prepared as described by Rock and Cronan (27), and myristoyl-ACP was synthesized as described by Rock and Garwin (29). Protein was determined by the Bradford method (Bio-Rad) (4). All other materials were reagent grade or better.

Bacterial strains and plasmids. The bacterial strains used in this study were derivatives of *E. coli* K-12. Their characteristics were as follows: strain UB1005, *metB1 relA1 spoT1 gyrA216* λ^- λ^r F^- (3); strain CDM5, a TLM-resistant (Tlm^r) derivative of strain UB1005 (16); strain CY274, *fabB15*(Ts) (provided by J. E. Cronan, Jr.); and strain SJ206, a *recA1 srl*::Tn*10* derivative of strain UB1005. The growth temperature was 37°C. The concentrations of antibiotics used were as follows: tetracycline, 20 μg/ml; ampicillin, 100 μg/ml. Rich medium was composed (per liter) of 10 g of tryptone, 5 g of NaCl, and 1 g of yeast extract, and medium E and M9

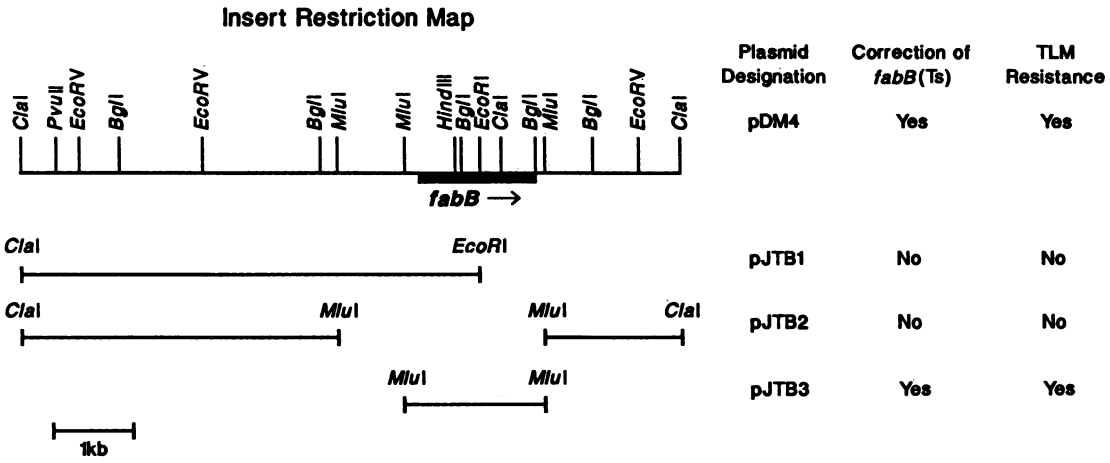

FIG. 1. Restriction maps and TLM resistance of recombinant plasmids. The locations of restriction enzyme cleavage sites, plasmid designations, and the abilities of the plasmids either to correct the *fabB*(Ts) growth phenotype or to confer TLM resistance are shown. In all cases, the indicated fragments were cloned into plasmid pBR322 as described in Materials and Methods.

salts were formulated as described by Miller (22). Minimal medium was prepared by supplementing the salt solutions with glucose (0.4%), methionine (0.01%), and thiamine (0.0005%). The plasmids used in this work are diagrammed in Fig. 1. Plasmid pDM4 (8) was constructed by subcloning a *Cla*I fragment from pLC39-16 into the same site of pBR322 and was supplied by J. E. Cronan, Jr. Complete digestion of pDM4 with *Eco*RI followed by ligation yielded pJTB1. Complete digestion of pDM4 with *Mlu*I followed by ligation yielded pJTB2. Plasmid pJTB3 was constructed by cloning the blunt-ended 1.75-kb *Mlu*I fragment from pDM4 into the *Eco*RV site of pBR322.

Determination of the MIC of TLM. Strains were streaked and grown on agar plates containing minimal medium E and various concentrations of TLM. For determination of the effect of oleate on cell growth in the absence and presence of TLM, strains were streaked and grown on agar plates containing minimal medium M9, glycerol (0.4%), Brij 58 (0.4%), oleate (0.1%), and various concentrations of TLM. The MIC was defined as the minimal concentration of TLM required to completely inhibit colony formation.

Cloning TLM resistance genes. The *E. coli* genomic library used to isolate TLM-resistant clones was constructed by C. DiRusso (9, 18). *E. coli* chromosomal DNA was partially digested with *Sau*3A and size selected for fragments between 5 and 20 kb. DNA was ligated into the *Bam*HI site of pBR322, and the ligation mix was transformed into strain LE392. Plasmids isolated from a pool of about 5,000 Amp^r colonies were isolated and used as the library. Strain SJ206 (*recA* Tlm^s) was transformed with this plasmid mixture, and Amp^r Tlm^r colonies were selected on minimal medium E containing 200 μM TLM. Twelve Tlm^r colonies were isolated. Plasmids were isolated from each of these individual colonies and used to transform strain SJ206 a second time. Nine of the plasmids conferred both the Tlm^r and Amp^r growth phenotypes, but three of the plasmids transferred only Amp^r and were not investigated further. Plasmid DNA isolation, restriction enzyme digestions, and agarose slab gel electrophoresis were performed as described previously (21).

Preparation of cell extracts. Strains were grown overnight to stationary phase in rich medium (400 ml), and the cells were harvested by centrifugation and washed twice with 0.1 M sodium phosphate (pH 7.0)–5 mM β-mercaptoethanol–1 mM EDTA. Subsequent procedures were carried out at 4°C. The washed cell pellet was resuspended in twice its wet weight of the same buffer and lysed in a French pressure cell at 18,000 lb/in^2. The lysate was centrifuged at 20,000 rpm for 60 min in a JA-20 rotor in a Beckman J2-21 centrifuge, the supernatant fluid was removed and fractionated with ammonium sulfate, and the precipitate formed between 45 and 80% saturation was collected by centrifugation. For the fatty acid synthase assay, the ammonium sulfate precipitate between 0 and 80% was collected. The pellet was dissolved in 2 ml of lysis buffer and dialyzed overnight against the lysis buffer.

β-Ketoacyl-ACP synthase I assay. The standard assay mixture for synthase I contained 47 μM ACP, 1 mM β-mercaptoethanol, 50 μM [2-^{14}C]malonyl-CoA (specific activity, 50.9 μCi/μmol), 110 μM myristoyl-ACP, 0.2 M potassium phosphate (pH 7.0), and 0.1 to 2 μg of protein in a final volume of 20 μl. The ACP, β-mercaptoethanol, and buffer were preincubated at 37°C for 30 min to ensure complete reduction of the ACP. Protein was added last to initiate the reaction, and the reaction mixture was incubated at 37°C for 10 min. When TLM was added, the antibiotic was mixed with the assay components except the protein, which was added last to initiate the reaction. The amount of β-ketohexadecanoyl-ACP formed was determined by hydrolysis of the acyl-ACP with sodium borohydride and extraction of the labelled alcohols into toluene as described by Garwin et al. (10).

Fatty acid synthase assays. The final concentrations of components in the standard assay were 1 mM NADH, 1 mM NADPH, 40 μM acetyl-CoA, 25 μM [2-^{14}C]malonyl-CoA (specific activity, 50.9 μCi/μmol), 15 μM ACP, 1 mM β-mercaptoethanol, 0.1 mM sodium phosphate (pH 7.0), and 0.05 mg of protein per ml (2 μg per assay) in a final volume of 40 μl. Protein was added last to initiate the reaction, and the tubes were incubated at 37°C for 30 min. TLM (5 to 400 μM) was mixed with the other assay components prior to the addition of protein. The reactions were terminated by placement of the assay tubes in an ice slush, and the formation of long-chain acyl-ACP was measured by reduction with sodium borohydride and extraction of the labelled alcohols into toluene (10).

Fatty acid synthesis in vivo. Cells were grown in minimal

medium E at 37°C to a density of 6.5×10^8 cells per ml, and the culture was divided into 1-ml aliquots and placed in tubes containing the indicated concentration of TLM. The cells were labelled in the presence of TLM with 10 μCi of [1-^{14}C]acetate per ml (specific activity, 56 Ci/mol) for 15 min. The cells were harvested by centrifugation and extracted by the method of Bligh and Dyer (2), and fatty acid methyl esters were prepared by using HCl-methanol. The amount of label incorporated into fatty acid was determined by scintillation counting of the methyl ester fraction after extraction into hexane. Argentation thin-layer chromatography was employed to separate saturated from unsaturated fatty acid methyl esters as described by Ulrich et al. (33).

RESULTS

Isolation and characterization of plasmids conferring TLM resistance. The TLM-sensitive strain SJ206 (*recA1*) (MIC, 150 μM) was transformed with an *E. coli* genomic DNA library, and plasmids that conferred the TLM resistance phenotype were selected on plates containing 100 μg of ampicillin per ml and 200 μM TLM. Approximately 300,000 transformants were screened, resulting in the isolation of 12 clones. Transformation of strain SJ206 with the plasmids isolated from these 12 clones confirmed that only nine of the plasmids bestowed both the Tlm^r and Amp^r phenotypes on the recipient strain. The three plasmids that transferred only Amp^r were not investigated further. TLM is a condensing enzyme inhibitor, and we anticipated that the selection was likely to yield condensing enzyme clones. Therefore, the chromosomal inserts were characterized by restriction enzyme mapping. Digestion of the plasmids with *Bgl*I showed that every plasmid contained a 906-bp *Bgl*I fragment that is characteristic of the coding sequence of the β-ketoacyl-ACP synthase I gene (*fabB*) (19). To determine if the plasmids expressed the *fabB* gene, strain CY274 [*fabB*(Ts)] was transformed with each of the plasmids. Strain CY274 is able to grow at 30°C in the absence of a growth supplement, but growth at 42°C requires the presence of oleate to compensate for the lack of a functional synthase I protein (6). All Amp^r tranformants of strain CY274 isolated at 30°C were able to grow in the absence of oleate at 42°C, demonstrating that all the Tlm^r plasmids expressed a functional *fabB* gene product. The MIC for all nine Tlm^r plasmids was greater than 800 μM TLM.

Overexpression of synthase I (*fabB*) imparts TLM resistance. The *fabB* gene has been cloned (8, 19), and a plasmid carrying the *fabB* gene, pDM4 (8), imparted TLM resistance (MIC, >800 μM) when transformed into strain UB1005. Extracts from strain UB1005/pDM4 had a synthase I specific activity of 232 nmol/min/mg of protein compared with 25 nmol/min/mg of protein from strain UB1005, confirming that synthase I activity was overexpressed in strains harboring plasmid pDM4. To establish that the Tlm^r phenotype was due to the *fabB* gene and not to another nearby gene, plasmid pDM4 was mapped and regions of the insert were subcloned into pBR322 (Fig. 1). The complete *fabB* gene was located on a 1.75-kb *Mlu*I fragment that was subcloned into pBR322 to yield pJTB3. The *fabB* gene was the only open reading frame in this fragment which contained approximately 200 bp of DNA upstream of the *fabB* promoter and 175 bp downstream of the stop codon. Plasmid pJTB3 bestowed TLM resistance (MIC, >800 μM) and complemented the *fabB*(Ts) defect in strain CY274 (Fig. 1). Plasmids containing other portions of the insert DNA from pDM4 neither conferred TLM resistance nor corrected the *fabB*(Ts) phenotype (Fig. 1). These data lead to the conclusion that *fabB* was the only gene in plasmid pDM4 required for TLM resistance.

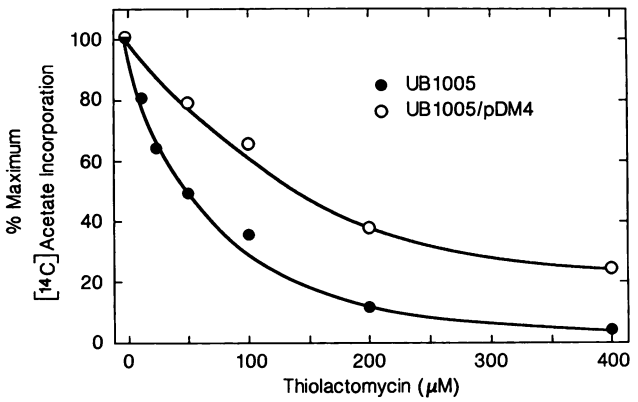

FIG. 2. Overproduction of β-ketoacyl-ACP synthase I shifts the dose-response curve for the inhibition of fatty acid biosynthesis by TLM in vivo. Strains UB1005 and UB1005/pDM4 were grown to a density of 6.5×10^8 cells per ml and treated with the indicated concentration of TLM. The cells were then pulse-labelled with [1-^{14}C]acetate for 15 min, and the incorporation of label into the fatty acid fraction was determined as described in Materials and Methods. Strain UB1005 incorporated a maximum of 1,430 cpm of [1-^{14}C]acetate per 10^8 cells, and strain UB1005/pDM4 incorporated 2,960 cpm of [1-^{14}C]acetate per 10^8 cells.

Selective inhibition of unsaturated fatty acid biosynthesis by TLM in vivo. The effect of TLM on the composition of fatty acids synthesized in sensitive and resistant strains was determined by pulse-labelling cells treated with different concentrations of TLM with [1-^{14}C]acetate. TLM effectively inhibited the total incorporation of [1-^{14}C]acetate into fatty acids in strain UB1005, with 50% inhibition occurring at 50 μM TLM (Fig. 2). The addition of TLM concentrations of 200 μM or higher virtually abolished [1-^{14}C]acetate incorporation. The presence of plasmid pDM4 in strain UB1005 shifted the TLM dose-response curve to the right (Fig. 2). Fifty percent inhibition in strain UB1005/pDM4 occurred at 150 μM TLM, and there were significant amounts of fatty acids synthesized at 400 μM. Unsaturated fatty acid biosynthesis was selectively inhibited by TLM. Between 0 and 50 μM TLM, the proportion of unsaturated fatty acids dropped from 70 to 40% and there was a corresponding increase in the percentage of saturated fatty acids, indicating that TLM selectively inhibits unsaturated fatty acid formation (Fig. 3). In contrast, TLM did not reduce the proportion of unsaturated fatty acid produced by strain UB1005/pDM4, illustrating that the overproduction of synthase I activity reverses the inhibition of unsaturated fatty acid biosynthesis (Fig. 3). These data support the hypothesis that β-ketoacyl-ACP synthase I is a major target for the inhibition of fatty acid biosynthesis by TLM in vivo. However, synthase I is not the only enzyme inhibited by TLM, because the addition of oleate to the medium to compensate for the inactivation of synthase I did not increase the MIC of TLM for strain UB1005 (MIC, 150 μM).

TLM inhibition of fatty acid synthase in vitro. Extracts from strains UB1005 and UB1005/pDM4 were prepared, and the ability of TLM to inhibit the fatty acid synthase reaction in vitro was determined (Fig. 4). The formation of long-chain fatty acids was much more sensitive to TLM inhibition in the in vitro system than in the in vivo experiments. In cell

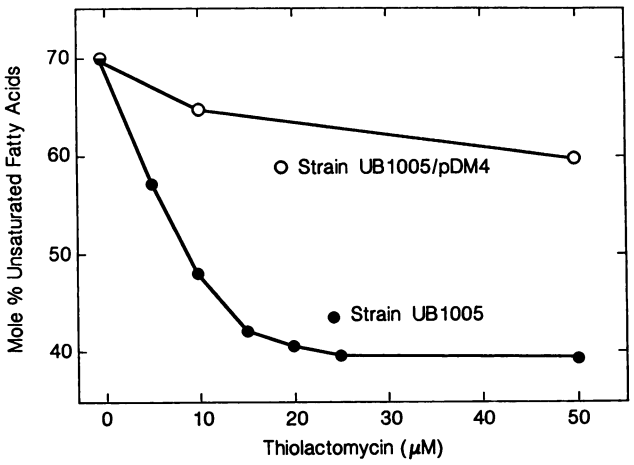

FIG. 3. TLM selectively inhibits unsaturated fatty acid synthesis in vivo. Strains UB1005 and UB1005/pDM4 were grown to a density of 6.5×10^8 cells per ml and treated with the indicated concentration of TLM. The cells were then pulse-labelled with [1-^{14}C]acetate for 15 min as described in the legend to Fig. 2, fatty acid methyl esters were prepared, and the amounts of labelled unsaturated and saturated fatty acids were determined by argentation thin-layer chromatography as described in Materials and Methods.

extracts, 50% inhibition occured at 10 μM TLM (Fig. 4) compared with 50 μM TLM in whole cells (Fig. 2). Extracts from strain UB1005/pDM4 were resistant to TLM inhibition of long-chain acyl-ACP formation (Fig. 4). These data corroborate the conclusion that increased cellular content of β-ketoacyl-ACP synthase I activity results in a TLM-resistant type II fatty acid synthase system.

Biochemical characterization of β-ketoacyl-ACP synthase I activity in strain CDM5. We examined β-ketoacyl-ACP synthase I activity in extracts from strain CDM5, a TLM-resistant derivative of strain UB1005 (16) to determine if either increased expression of the normal synthase I or a mutation that results in a synthase I refractory to TLM inhibition contributed to the TLM-resistant-growth phenotype. A comparison of the specific activities of synthase I in extracts from strains UB1005 (MIC, 150 μM) and CDM5 (MIC, >800 μM) showed no difference in the levels of synthase I activity (Fig. 5, inset). In addition, there was no difference in the susceptibility of synthase I to TLM inhibition in either strain UB1005 or strain CDM5 (Fig. 5). There was no difference in the activity or resistance of synthase I in a TLM-resistant strain.

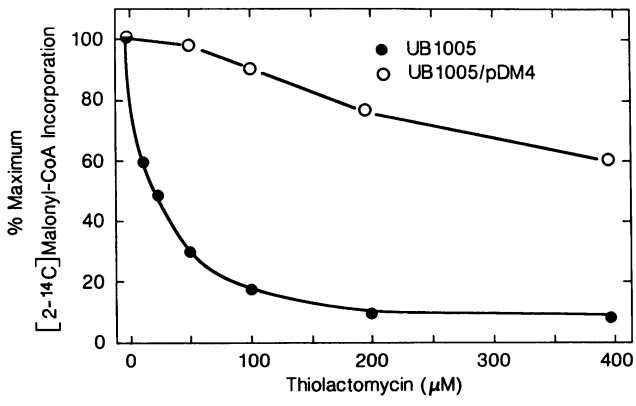

FIG. 4. Overproduction of β-ketoacyl-ACP synthase I imparts TLM resistance to fatty acid biosynthesis in vitro. Extracts from strain UB1005 or strain UB1005/pDM4 were prepared and assayed for fatty acid synthase activity in the presence of the indicated concentrations of TLM as described in Materials and Methods. The strain UB1005 extracts (2 μg of protein per assay) incorporated 25,947 cpm of [2-^{14}C]malonyl-CoA per assay into long-chain fatty acids; extracts (2 μg of protein per assay) from strain UB1005/pDM4 incorporated 25,141 cpm of [2-^{14}C]malonyl-CoA.

DISCUSSION

Our data show that β-ketoacyl-ACP synthase I is a major TLM target and that increased expression of this condensing enzyme is one mechanism for acquiring TLM resistance. This conclusion leads to the hypothesis that when synthase I is overexpressed, the other two condensing enzymes are not needed. Unlike synthase I, synthase II (*fabF* gene product) is not essential for the growth of *E. coli* (12). Inactivation of synthase II by the introduction of the *fabF1* mutation into strains UB1005 and CDM5 did not alter the TLM sensitivity or resistance of the strains (data not shown). Overproduction of synthase II would not be expected to impart TLM resistance because it is unlikely to circumvent the synthase I requirement for unsaturated fatty acid biosynthesis. However, direct demonstration of this point awaits cloning and overexpression of synthase II. In contrast, synthase III appears to be essential because it catalyzes the condensation of acetyl-CoA and malonyl-ACP to initiate fatty acid biosynthesis, but it is incapable of elongation of acyl-ACPs longer than six carbons (17). Recently, we have cloned the synthase III gene (*fabH*), and the presence of a plasmid carrying the *fabH* gene results in a 20-fold overproduction of synthase III activity (32). Introduction of this plasmid into strain UB1005 did not impart TLM resistance to this sensitive strain (data not shown), providing direct evidence that synthase III overproduction is not a mechanism for TLM resistance.

There are two generally accepted pathways for the initiation of fatty acid biosynthesis (Fig. 6). First, acetyl-CoA is transacylated to ACP by acetyl transacylase and is then condensed with malonyl-ACP by either synthase I or II to form acetoacetyl-ACP. The acetyl transacylase step is required since synthases I and II cannot utilize acetyl-CoA (13). The second route to acetoacetyl-ACP is the condensation of acetyl-CoA with malonyl-ACP catalyzed by synthase III. TLM blocks initiation by inhibiting acetyl transacylase (24) and all three β-ketoacyl-ACP synthases (16, 24). However, synthase I may be the only enzyme absolutely required for fatty acid biosynthesis. In the absence of a suitable acyl-ACP acceptor, synthase I will slowly catalyze malonyl-ACP decarboxylation to form acetyl-ACP (1). We postulate that overproduction of synthase I allows this reaction pathway to bypass both the acetyl transacylase and synthase III routes to the formation of acetoacetyl-ACP (Fig. 6). This bypass mechanism is easily demonstrated in vitro, where acetyl-CoA, in contrast to malonyl-CoA, is not absolutely required for fatty acid synthase activity in cell extracts (20). Thus, the overproduction of synthase I is proposed to circumvent the priming step in fatty acid biosynthesis, making synthase I the only necessary condensing enzyme.

Increased β-ketoacyl-ACP synthase I activity is not the only mechanism for TLM resistance, nor is synthase I the only TLM target. Synthase I activity is not increased nor is the enzyme refractory to TLM inhibition in the TLM-

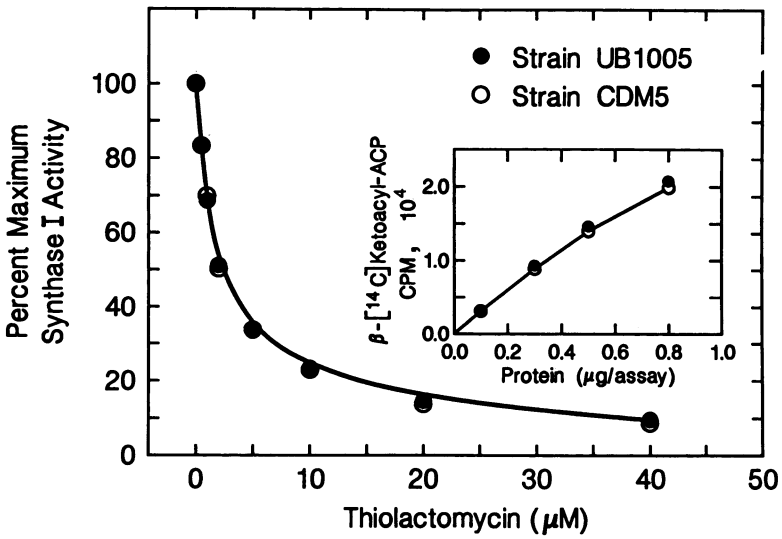

FIG. 5. Comparison of β-ketoacyl-ACP synthase I activity in strain UB1005 and its TLM-resistant derivative, strain CDM5. Extracts (0.5 μg of protein per assay) from strains UB1005 and CDM5 were assayed for synthase I activity in the presence of the indicated concentrations of TLM as described in Materials and Methods. Extracts from both strains UB1005 and CDM5 possessed the same specific activity in the β-ketoacyl-ACP synthase I assay (inset).

resistant strain CDM5 (Fig. 5), illustrating that another mutation is responsible for the high level of TLM resistance in this strain. Both the β-ketoacyl-ACP synthase III and acetyl-CoA:ACP transacylase activities in extracts from strain CDM5 are refractory to TLM inhibition in vitro (16). The specific activity of synthase III in extracts from strain CDM5 was 10-fold lower than in extracts from its TLM-sensitive parent, resulting in a marked defect in the ability of strain CDM5 to incorporate acetyl-CoA into fatty acids in vitro. Thus, the mutation in strain CDM5 results in the production of a β-ketoacyl-ACP synthase III activity that is resistant to TLM but is much less stable to in vitro manipulations than the wild-type enzyme. These observations suggest that mutations in synthase III contribute to acquired TLM resistance. However, 50% inhibition of synthase III activity by TLM occurs at about 200 μM (16), whereas 50% inhibition of synthase I activity occurs at 2 μM TLM (Fig. 5), so it is not clear how the elongation reactions of fatty acid synthesis are protected from TLM inactivation in strain CDM5. These data point to an additional, uncharacterized

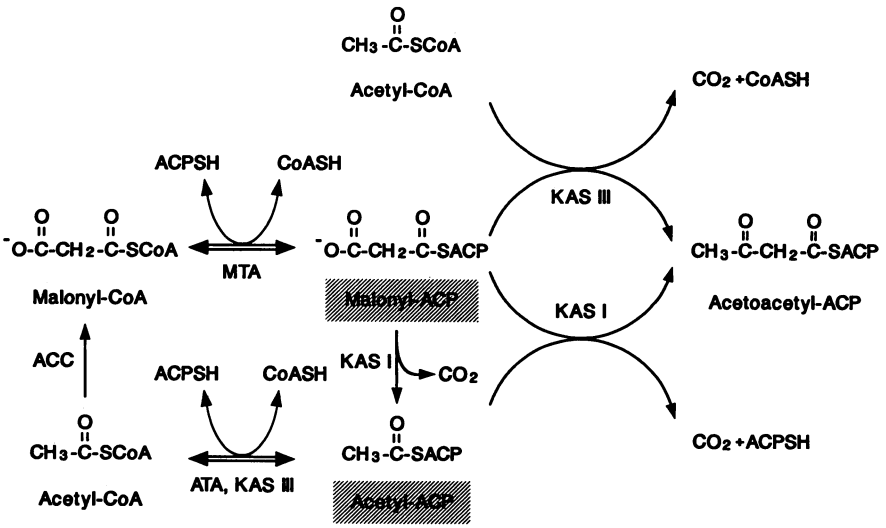

FIG. 6. Pathways for initiation of fatty acid biosynthesis. There are three potential mechanisms for the formation of acetoacetyl-ACP in *E. coli*. First, β-ketoacyl-ACP synthase III catalyzes the condensation of acetyl-CoA with malonyl-ACP. In the second pathway, the acetate moiety is first transferred from acetyl-CoA to acetyl-ACP by either acetyl-CoA:ACP transacylase or β-ketoacyl-ACP synthase III. The acetyl-ACP is then condensed with malonyl-ACP by β-ketoacyl-ACP synthase I. The third pathway is the decarboxylation of malonyl-ACP by synthase I to form acetyl-ACP, which is subsequently condensed with malonyl-ACP by synthase I. Synthase I is the only condensing enzyme required for the initiation of fatty acid biosynthesis by the third pathway. Abbreviations: ACC, acetyl-CoA carboxylase; MTA, malonyl-CoA:ACP transacylase; ATA, acetyl-CoA:ACP transacylase; KAS I, β-ketoacyl-ACP synthase I; and KAS III, β-ketoacyl-ACP synthase III.

mutation in strain CDM5 that is the major determinant of the high-level TLM-resistant-growth phenotype.

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