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Thiolactomycin Resistance in *Escherichia coli* Is Associated with the Multidrug Resistance Efflux Pump Encoded by *emrAB*

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Thiolactomycin (TLM) and cerulenin are antibiotics that block *Escherichia coli* growth by inhibiting fatty acid biosynthesis at the β-ketoacyl-acyl carrier protein synthase I step. Both TLM and cerulenin trigger the accumulation of intracellular malonyl-coenzyme A coincident with growth inhibition, and the overexpression of synthase I protein confers resistance to both antibiotics. Strain CDM5 was derived as a TLM-resistant mutant but remained sensitive to cerulenin. TLM neither induced malonyl-coenzyme A accumulation nor blocked fatty acid production in vivo; however, the fatty acid synthase activity in extracts from strain CDM5 was sensitive to TLM inhibition. The TLM resistance gene in strain CDM5 was mapped to 57.5 min of the chromosome and was an allele of the *emrB* gene. Disruption of the *emrB* gene converted strain CDM5 to a TLM-sensitive strain, and the overexpression of the *emrAB* operon conferred TLM resistance to sensitive strains. Thus, activation of the *emr* efflux pump is the mechanism for TLM resistance in strain CDM5.

The β-ketoacyl-acyl carrier protein (β-ketoacyl-ACP) synthases are key regulators of dissociated (type II) fatty acid synthase systems typified by *Escherichia coli* (for reviews, see references 6 and 16). β-Ketoacyl-ACP synthase I is required for a critical step in the elongation of unsaturated acyl-ACP, and *fabB* mutants lacking synthase I activity synthesize neither palmitoleic nor *cis*-vaccenic acids and require supplementation with unsaturated fatty acids for growth (29). β-Ketoacyl-ACP synthase II is responsible for the temperature-dependent regulation of fatty acid composition (for a review, see reference 9). Mutants (*fabF*) lacking synthase II activity are deficient in *cis*-vaccenic acid but do not have a growth phenotype (12, 13). β-Ketoacyl-ACP synthase III selectively catalyzes the formation of acetoacetyl-ACP in vitro (19). Synthase III possesses acetyl coenzyme A (acetyl-CoA):ACP transacylase activity (34); however, it is unknown whether synthase III accounts for all of the acetyl transacylase activity. The role of this third condensing enzyme remains to be firmly established, but its position at the beginning of the biosynthetic pathway suggests that it plays a role in governing the rate of fatty acid initiation.

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 (*Saccharomyces cerevisiae* and mammalian) fatty acid synthases (14, 15, 23, 24, 26, 27, 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 type II fatty acid synthase suggests that the β-ketoacyl-ACP synthase and the acetyl-CoA:ACP transacylase activities are the only individual enzymes inhibited by TLM in vitro (24). The findings that malonyl-ACP protects the synthases from TLM inhibition and that they are competitively inhibited with respect to malonyl-ACP (24) are consistent with TLM interacting with the malonyl-ACP site on the condensing enzymes. All three condensing enzymes in *E. coli* are inhibited by TLM both in vivo and in vitro (17). Cerulenin [(2R)(3S)-2,3-epoxy-4-oxo-7,10-dodecadienolyamide] is another fungal antibiotic that inhibits both type I and type II fatty acid biosynthesis systems at the β-ketoacyl-ACP synthase step (7, 37). In contrast to TLM, cerulenin functions by covalently modifying the medium- to long-chain acyl-ACP site of condensing enzymes (20). However, cerulenin does not affect synthase III activity, and thus, it blocks elongation, but not initiation, of fatty acid biosynthesis.

β-Ketoacyl-ACP synthase I is the key intracellular target for TLM and cerulenin action, since the overproduction of synthase I enzyme imparts resistance to both TLM (35) and cerulenin (10). However, strain CDM5 (17), which was selected for TLM resistance (Tlm^r), does not have increased synthase I expression or a TLM-resistant synthase I activity (35), illustrating that high-level TLM resistance can be achieved by a mechanism independent of synthase I. In this article, we show that the activation of a multidrug-resistant efflux pump (*emr*) is responsible for the inability of TLM to inhibit fatty acid biosynthesis in vivo.

MATERIALS AND METHODS

Materials. Sources of supplies were Promega Biotec (restriction endonucleases and other molecular biology reagents), Chugai Pharmaceutical Co. (a generous gift of TLM), Pharmacia (acetyl-CoA and malonyl-CoA), ICN Biochemicals and DuPont New England Nuclear ([1-^{14}C]acetate [specific activity, 56 Ci/mol] and [2-^{14}C]malonyl-CoA [spe-

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TABLE 1. Strains

Strain	Description	Source or reference
BW5660	*srl*::Tn*10*	CGSC[a]
C600	*thr-1 leuB6 lacY1 thi-1 supE44 tonA22* λ^- F^-	CGSC
CDM5	*fabH1 metB1 relA1 spoT1 gyrA216 tlmR* λ^r λ^- F^-	16
Hfr::Tn*10*	Hfr::Tn*10* mapping kit	CGSC
KL164	*nalB4 relA1 thyA24 spoT1 thi-1 deoB13* λ^-	CGSC
KL711	*pyrD34 trp-45 his-68 tyrA2 recA1 thyA33 galK35 malA1 xyl-7 mtl-2 rpsL118 thi-1* λ^- F143 (*tyrA-lysA*)	CGSC
KLE120	*emrB*::Tn*10*(*kan*)	20
LCH47	*argA*::Tn*10 thyA*	Li Hsu
SJ16	*panD2 metB1 relA1 spoT1 gyrA216 zad220*::Tn*10* λ^r λ^- F^-	17
SJ83	*panD2 gshA metB1 relA1 spoT1 gyrA216 srl*::Tn*10* λ^- λ^r	S. Jackowski
SJ168	*recA*::Tn*10*(*cml*) *metB1 relA1 spoT1 gyrA216* λ^r λ^- F^-	P1(GW4212) × UB1005
SJ212	*fabH1 metB1 relA1 spoT1 gyrA216 tlmR* Str^r λ^r λ^- F^-	Str^r of CDM5
SJ251	*metB1 relA1 spoT1 gyrA216 tlmR srl*::Tn*10* λ^r λ^- F^-	This study
SJ256	*thr-1 leuB6 lacY1 thi-1 supE44 tonA21 srl*::Tn*10 tlmR* λ^- F^-	P1(SJ251) × C600
SJ257	*thr-1 leuB6 lacY1 thi-1 supE44 tonA21 thyA argA*::Tn*10 tlmR* λ^- F^-	This study
SJ260	*thr-1 leuB6 lacY1 thi-1 supE44 tonA21 thyA argA*::Tn*10 tlmR* λ^- F143 (*thyA-lysA*)	KL711 × SJ257
SJ261	*emrB*::Tn*10*(*kan*) *metB1 relA1 spoT1 gyrA216* λ^r λ^-	P1(KLE120) × UB1005
UB1005	*metB1 relA1 spoT1 gyrA216* λ^r λ^- F^-	3

[a] CGSC, B. Bachmann, *E. coli* Genetic Stock Center, New Haven, Conn.

cific activity, 50.9 Ci/mol]), Sigma Chemical Co. (cerulenin, malonyl-CoA, succinyl-CoA, acetyl-CoA, and CoA), BMY Chemicals (phosphotransacetylase, acetate kinase, and citrate synthase), and Bio-Rad (Bradford protein assay kit and electrophoresis supplies). Homogeneous ACP was prepared according to the method of Rock and Cronan (28). Malonate decarboxylase was purified from *Pseudomonas ovalis* IAM 1177 as described previously (32). Protein was measured by the Bradford method (4), and cell number was determined spectrophotometrically (36). 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 and are listed in Table 1. The growth temperature was 37°C, and the concentrations (per milliliter) of antibiotics used were as follows: tetracycline, 20 μg; kanamycin, 20 μg; ampicillin, 50 μg. Rich medium was composed of 10 g of tryptone, 5 g of NaCl, and 1 g of yeast extract per liter, and medium E and M9 salts were formulated as described by Miller (22).

The pJTB3 plasmid was a pBR322 derivative that expressed only the β-ketoacyl-ACP synthase I (*fabB*) gene (35). The pEMR2.1 plasmid was a pUC18 derivative that expressed both the *emrA* and the *emrB* genes (21). The pEMR2.6 plasmid was a pUC18 derivative that contained the *emrB* gene, but the EmrB protein was not expressed (21). The pSJ13 plasmid was derived by digesting pEMR2.1 with *Pst*I and religation. The pSJ13 plasmid had approximately 50% of the *emrB* gene deleted, but expressed the *emrA* gene. The pSJ13 plasmid was identical to the pEMR2.2 plasmid (21).

Determination of the MIC of TLM. Strains were streaked and grown on agar plates containing minimal medium E supplemented with glucose (0.4%), casein hydrolysate (0.1%), thiamine (0.0005%), and either 0, 12.5, 25, 50, 100, 200, 400 or 600 μM TLM or 0, 5, 10, 20, 30 or 40 μg of cerulenin per ml. The plates were incubated at 30°C for 16 to 18 h. The MIC was defined as the minimal concentration of TLM or cerulenin required to completely inhibit colony formation.

Quantitation of CoA thioesters. Two methods were used to measure the intracellular pools of CoA-SH, acetyl-CoA, and malonyl-CoA. The primary method used was the acyl-CoA cycling method that measures the amount of acetyl-CoA in the sample (5, 33). Briefly, 1.6 ml of the culture was filtered through a Sartorius 0.45-μm-pore-size cellulose nitrate filter, and the cells were collected. The filters were immediately immersed in 4 ml of ice-cold 0.3 M sulfuric acid in a centrifuge tube. The centrifuge tube was vortex mixed (2,500 rpm) at 4°C for 20 min and then centrifuged at 12,000 rpm (17,000 × *g*) for 5 min. The supernatant was adjusted to pH 6.0 with sodium hydroxide. Malonyl-CoA, acetyl-CoA, and CoA-SH in the neutralized extract were stable for at least 50 h at 4°C (25). The reaction mixture for the acyl-CoA cycling assay contained 50 mM Tris-HCl (pH 7.2), 50 mM malonate, 10 mM ATP, 1.0 U of malonate decarboxylase (EC 2.8.3.3), and cell extracts containing acetyl-CoA and/or malonyl-CoA (2.5 to 80 pmol). The cycling reaction was initiated by the addition of malonate decarboxylase. The mixture was incubated at 30°C for 20 min, and then 1.0 U of acetate kinase (EC 2.7.2.1) was added. After a 20-min incubation, 0.2 ml of 2.5 M neutralized hydroxylamine was added, and the incubation continued for an additional 20 min at 30°C. The reaction was terminated by adding 0.6 ml of 10 mM ferric chloride dissolved in 25 mM trichloroacetic acid and 1 M hydrochloric acid. The A_{540} of acetylhydroxamate was measured. Control over the units of malonate decarboxylase and the length of the first amplification step were the most important factors that determined the sensitivity of the assay. Under the conditions described above, the moles of acetylhydroxamate formed per mole of acetyl-CoA added corresponded to 97,500 cycles per h. Every assay was conducted in duplicate, and all results were expressed as the means of two samples. The intracellular concentrations of malonyl-CoA, acetyl-CoA, and CoA-SH were based on the measurement of dry weight and then converted to micromolar concentrations calculated on the basis of the assumption that the intracellular water volume was 2.7 μl/mg (dry weight) (39).

Separate determination of malonyl-CoA in the cell extract was conducted after the enzymatic conversion of acetyl-CoA to citrate and CoA-SH by using citrate synthase (EC 4.1.3.7). The reaction mixture contained 50 mM Tris-HCl

(pH 7.2), 10 mM magnesium sulfate, 1 mM 2-mercaptoethanol, 2 mM oxaloacetate, and 1.32 U of citrate synthase in a total volume of 1.0 ml. The reaction mixture was incubated for 20 min at 30°C, and the reaction was terminated by placing the reaction tube in an ice slush. CoA-SH was measured following its enzymatic conversion to acetyl-CoA by using phosphotransacetylase (EC 2.3.1.8). The reaction mixture contained 50 mM Tris-HCl (pH 7.2), 10 mM magnesium sulfate, 1 mM 2-mercaptoethanol, 10 mM ammonium sulfate, 0.1 mM acetylphosphate, 1.0 U of phosphotransacetylase, and cell extracts containing CoA-SH in a total volume of 1.0 ml. The reaction mixture was incubated at 30°C for 20 min, and the reaction was terminated by filtering the mixture through a Millipore UFP1 LGC ultrafilter to remove phosphotransacetylase.

A second method was used to verify the results obtained with the acetyl-CoA cycling method. Strain SJ16 (*panD*) was labeled with β-[3-^{3}H]alanine to selectively label the CoA pool (18). The distribution of CoA thioesters was determined before and after cerulenin treatment by using a modification of the high-pressure liquid chromatography (HPLC) method of DeBuysere and Olson (8) as described in detail by Vallari et al. (36). Recovery of CoA derivatives from the column was approximately 70%.

Preparation of cell extracts for enzyme assays. 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, and 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 by using a JA-20 rotor in a Beckman J2-21 centrifuge, the supernatant fluid was removed and adjusted to 80% ammonium sulfate, and the precipitate formed was collected by centrifugation. The pellet was dissolved in 2 ml of lysis buffer and dialyzed overnight against the lysis buffer.

Fatty acid synthase assays. The final concentrations of components in the standard assay were as follows: 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 incubations were terminated by placing 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 labeled alcohols into toluene (11).

Fatty acid synthesis in vivo. Cells were grown in minimal medium E supplemented with glucose (0.4%), casein hydrolysate (0.1%), and thiamine (0.0005%) at 37°C to a density of 6.5 × 10^8 cells per ml, and the culture was divided into 1-ml aliquots, which were placed in tubes containing the indicated concentration of TLM. The cells were labeled 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 acids was determined by scintillation counting of the methyl ester fraction after extraction into hexane.

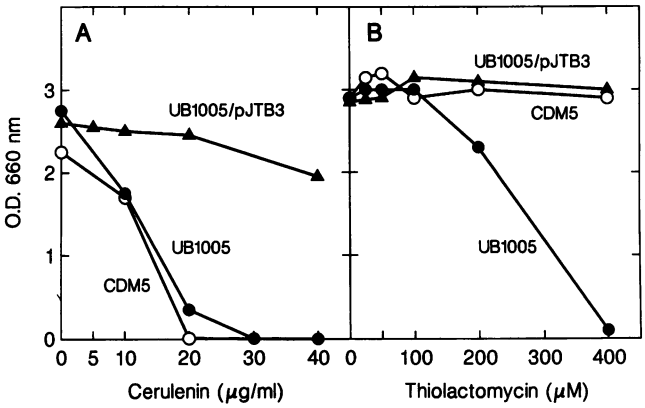

FIG. 1. Growth of strains UB1005, CDM5, and UB1005/pJTB3 in the presence of TLM (B) or cerulenin (A). Cells were inoculated into 5 ml of minimal medium E containing glucose, thiamine, and methionine and grown to a density of 5.4 × 10^5 cells per ml, and then the indicated concentrations of drugs were added and cultures were incubated for 20 h at 30°C. Cell growth was measured by optical density (O.D.) at 660 nm.

RESULTS

Effects of TLM and cerulenin on cell growth. The abilities of TLM and cerulenin to inhibit the growth of strains UB1005, CDM5, and UB1005/pJTB3 were compared (Fig. 1). Growth of the wild-type strain UB1005 was inhibited by both cerulenin and TLM. Strain UB1005/pJTB3 was resistant to growth inhibition by both cerulenin and TLM. Strains harboring the pJTB3 plasmid overproduce the *fabB* gene product. Therefore, this result was consistent with the hypothesis that the predominant intracellular target for TLM and cerulenin was β-ketoacyl-ACP synthase I (10, 35). Strain CDM5 was selected as a TLM-resistant strain (Tlm^r); however, strain CDM5 remained sensitive to growth inhibition by cerulenin. These data suggested the existence of a mechanism for TLM resistance that was independent of cerulenin resistance and β-ketoacyl-ACP synthase I.

Fatty acid biosynthesis in TLM-sensitive and -resistant strains. The ability of TLM to inhibit fatty acid synthesis in vivo was determined for each of the strains (Fig. 2). [^{14}C]acetate incorporation into fatty acids was not significantly inhibited by 400 μM TLM in strain CDM5 (Tlm^r). In contrast, 400 μM TLM almost completely blocked [^{14}C]acetate incorporation into fatty acids in the wild-type strain UB1005. Although the MIC for TLM was significantly higher in strain UB1005/pJTB3, TLM progressively suppressed [^{14}C]acetate incorporation, reaching a maximum of 80% inhibition at 400 μM TLM.

Extracts from strains UB1005, CDM5, and UB1005/pJTB3 were prepared, and the ability of TLM to inhibit the fatty acid synthase reaction in vitro was determined (Fig. 3). Extracts from strain UB1005/pJTB3 possessed a fatty acid synthase activity that was refractory to inhibition by TLM. In contrast, long-chain fatty acid formation in extracts from both strains UB1005 and CDM5 was inhibited by TLM to the same extent. Since TLM was reported to be a competitive inhibitor with respect to malonyl-CoA, we determined the TLM dose-response curve in the presence of increasing concentrations of malonyl-CoA in the assay. These experiments confirmed the results of Nishida et al. (24) and showed that higher concentrations of malonyl-CoA protected the fatty acid synthase from TLM inhibition (data not shown). In

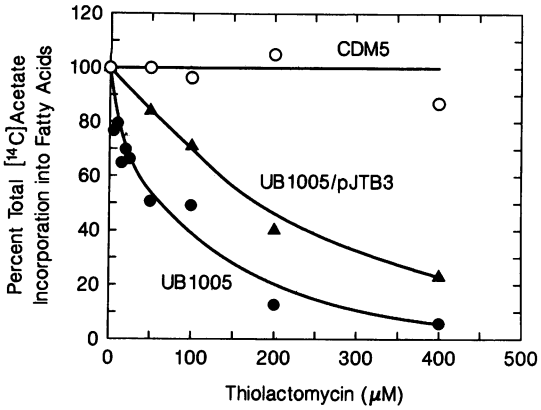

FIG. 2. Fatty acid biosynthesis in strain CDM5 was refractory to TLM inhibition. Strains CDM5, UB1005, and UB1005/pJTB3 were grown to a density of 6.5 × 10^8 cells per ml and treated with the indicated concentrations of TLM for 10 min. The cells were then labeled with [1-^{14}C]acetate for 15 min, and the incorporation of label into the fatty acid fraction was determined as described in Materials and Methods.

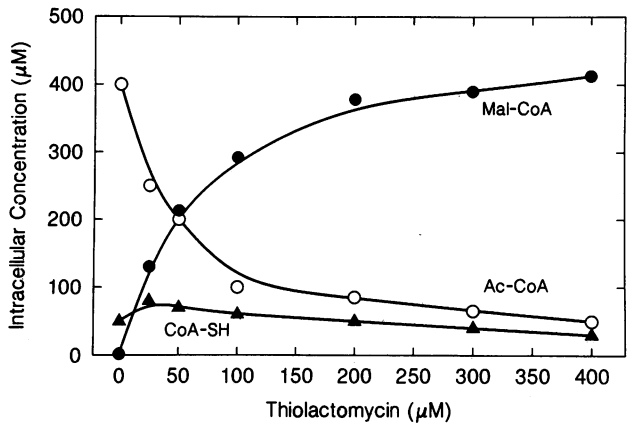

FIG. 4. Effect of TLM on the composition of the CoA thioester pool in strain UB1005. Strain UB1005 was grown to a density of 6.8 × 10^8 cells per ml at 37°C. At this cell density, aliquots (10 ml each) of the culture were transferred to other flasks containing different concentrations of TLM. After 15 min at 37°C, aliquots (1.6 ml each) were removed and extracted for analysis of the CoA pool composition. Acetyl-CoA, malonyl-CoA, and CoA-SH were assayed by the acyl-CoA cycling method described in Materials and Methods.

summary, the fatty acid synthase from strain CDM5 was resistant to TLM inhibition in vivo (Fig. 2) but was sensitive to TLM inhibition in vitro (Fig. 3).

Effect of TLM on the intracellular pools of malonyl-CoA. The inhibition of β-ketoacyl-ACP synthase may lead to the elevation of malonyl-CoA concentrations, which in turn may protect the synthase from TLM inhibition. Therefore, we determined whether the intracellular concentration of malonyl-CoA was altered by TLM treatment and whether elevated malonyl-CoA could be associated with TLM resistance in strain CDM5. As anticipated on the basis of our previous analysis of the CoA thioester pools in *E. coli* (35), acetyl-CoA is the most abundant CoA thioester in strain UB1005 growing in glucose minimal medium (Fig. 4). Treatment of the cells with TLM resulted in a dose-dependent decrease in acetyl-CoA levels and a concomitant increase in the content of malonyl-CoA (Fig. 4). CoA-SH levels did not change appreciably following exposure to TLM. We also examined the change in the CoA pool composition in cells treated with cerulenin and found that malonyl-CoA increased at the expense of acetyl-CoA (data not shown). To verify the results obtained with the acyl-CoA cycling method, we labeled strain SJ16 (*panD*) with β-[3-^{3}H]alanine to uniformly label the CoA pool, treated the cells with cerulenin for different times, and quantitated the changes in the CoA pool composition by reversed-phase HPLC (36). These results were in agreement with the data obtained with the acyl-CoA cycling method and showed an accumulation of malonyl-CoA at the expense of acetyl-CoA (data not shown). The only CoA pool component detected that was not measured by the acyl-CoA cycling method was succinyl-CoA, which accounted for 6% of the total CoA pool (36). A comparison of the effects of TLM on the malonyl-CoA levels in strains UB1005, CDM5, and UB1005/pJTB3 showed that the malonyl-CoA concentration increased in strains UB1005 and UB1005/pJTB3 but TLM did not significantly increase the malonyl-CoA pool in strain CDM5 (Fig. 5). Therefore, the accumulation of malonyl-CoA in TLM-treated cells was consistent with the inhibition of β-ketoacyl-ACP synthase activity by the antibiotic, but there was no correlation between the level of malonyl-CoA and the resistance phenotype of the strains.

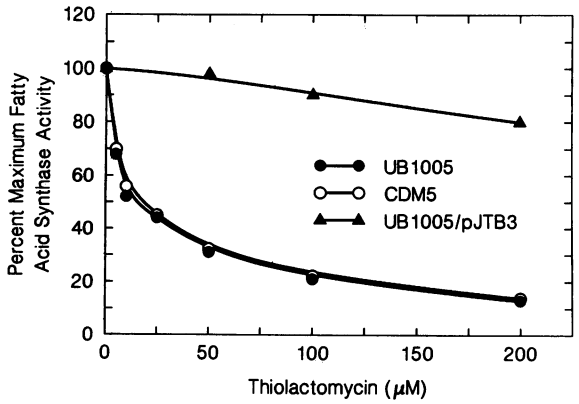

FIG. 3. The fatty acid synthase from strain CDM5 was sensitive to TLM inhibition in vitro. Extracts from strains UB1005, CDM5, and UB1005/pJTB3 were prepared and assayed for fatty acid synthase activity in the presence of the indicated concentrations of TLM as described in Materials and Methods.

Genetic map position of the *tlmR* gene. The location of the *tlmR* gene on the *E. coli* chromosome was determined by a series of interrupted mating experiments between strain CDM5 (*gyrA tlmR* F$^-$) or strain SJ212 (an Str^r derivative of strain CDM5) and the collection of Hfr donor strains carrying specific Tn*10* insertions as described by Wanner (38). Tetracycline- and nalidixic acid- or streptomycin-resistant exconjugates were selected and screened for sensitivity to TLM. Only three Hfr strains (BW5660 [84.8%], BW6163 [19.3%], and BW6169 [2.5%]) were able to convert the Tlm^r phenotype of strain CDM5 to a Tlm^s phenotype. These data placed the *tlmR* allele between 50 and 60 min of the chromosome. A series of P1 transductions with markers in this region localized the *tlmR* gene to 57.5 min on the chromosome. Cotransduction frequencies with *recA*::Tn*10*(*cml*) (35%) and *srl*::Tn*10* (24%) indicated that *tlmR* was located close to *nalB*. The *gshA* gene lies in this region and is

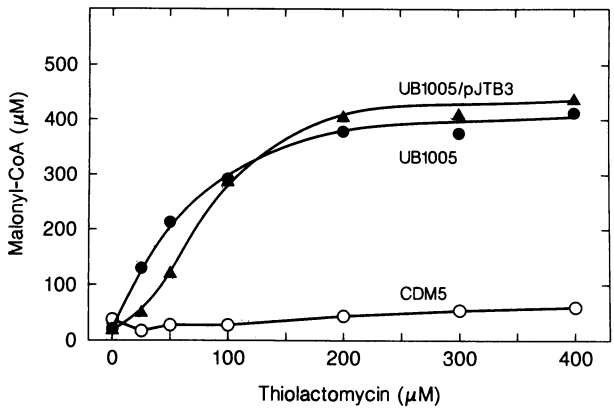

FIG. 5. Comparison of the TLM effects on the intracellular malonyl-CoA concentration in strains UB1005, CDM5, and UB1005/pJTB3. Cells were grown in glucose minimal medium to a density of 6.8×10^8 cells per ml at 37°C, treated with TLM, and assayed as described in the legend to Fig. 4.

responsible for carrying out the first reaction of glutathione biosynthesis (1). TLM is a thiolactone and is inactivated by treatment with thiol-reducing agents. However, on the basis of the diamide test for cellular sulfhydryl content (1), strain CDM5 possessed the same level of intracellular sulfhydryls as its Tlm^s parent, indicating that mutations at the *gshA* locus were not responsible for the *tlmR* phenotype. Strain KL164 (*nalB4*) was obtained from the *E. coli* Genetic Stock Center, and analysis of its TLM growth phenotype showed that this strain was also Tlm^r. To determine whether TLM and nalidixic acid resistances were due to the same mutation, P1 phage grown on strain KL164 was introduced into strain C600 and recombinants were selected for Nal^r and screened for Tlm^r. These data show that Tlm^r and Nal^r were not due to the same mutation but could be genetically resolved. However, the 96% cotransduction frequency obtained in this experiment indicated that the mutations that confer these two phenotypes are closely linked. This conclusion was verified with a three-point cross using *srl*::Tn*10* as the selectable marker. These data confirmed that *nalR* and *tlmR* were separate, but closely linked, mutations.

To determine whether *tlmR* was dominant or recessive, F143 carrying the region of the chromosome including *tlmR* was introduced into strain SJ257 and the resulting partial diploid (strain SJ260) was tested for TLM resistance. Strain SJ260 remained resistant to TLM (Table 2), illustrating that *tlmR* was a dominant mutation. We also examined the TLM resistance of strain SJ83 (*gshA*). This strain lacks the major intracellular sulfhydryl, glutathione. Strain SJ83 was more sensitive to TLM growth inhibition than the wild-type parent strain UB1005 (Table 2), demonstrating that intracellular glutathione afforded some protection against TLM growth inhibition.

TABLE 2. Growth inhibition by TLM and cerulenin

Strain	MIC	
	TLM (μM)	Cerulenin (μg/ml)
UB1005 (Tlm^s)	150	20
CDM5 (*tlmR*)	>600	20
UB1005/pBR322	150	20
UB1005/pJTB3	>600	80
UB1005/pEMR2.1	>600	10
UB1005/pEMR2.6	200	20
UB1005/pSJ13	25	20
SJ83 (*gshA*)	25	20
SJ260 (F143)	>600	20
SJ261 [*emrB*::Tn*10*(*kan*)]	25	20

TLM resistance is associated with the *emrAB* operon. Lomovskaya and Lewis (21) described a multidrug resistance efflux pump (*emrAB*) located at 57.5 min on the chromosome that catalyzes the efflux of structurally unrelated, moderately hydrophobic drugs. Two approaches were used to establish the involvement of *emr* in TLM resistance. First, Tlm^s strains were transformed with plasmids that overexpress the *emrAB* operon (Table 2). Plasmids containing the complete *emrAB* operon conferred TLM resistance to sensitive strains. However, plasmids carrying deletions in the *emrAB* operon did not result in a Tlm^r phenotype. To determine the linkage between *emrB* and *tlmR*, P1 phage grown on strain KLE120 [*emrB*::Tn*10*(*kan*)] was transduced into strain CDM5 and kanamycin-resistant recombinants were selected. There were no Kan^r Tlm^r recombinants found, suggesting that *emrB* and *tlmR* are the same gene. In fact, strain SJ261 [*emrB*::Tn*10*(*kan*)] was more sensitive to TLM growth inhibition than strain UB1005 (Table 2), indicating that the wild-type expression level of *emr* was sufficient to confer some degree of resistance. Cerulenin resistance was not altered by either deletion or overexpression of the *emr* pump.

DISCUSSION

Our results point to the activation of a TLM efflux pump as the major factor in acquired TLM resistance in strain CDM5. Two lines of evidence suggest that strain CDM5 is unable to accumulate sufficient intracellular concentrations of TLM to inhibit the β-ketoacyl-ACP synthases. First, fatty acid synthesis in vivo is highly resistant to TLM, whereas the fatty acid synthase activity in vitro is sensitive to TLM inhibition (Fig. 2 and 3). Second, TLM triggers the accumulation of malonyl-CoA both in the TLM-sensitive parental strain UB1005 and in the UB1005/pJTB3 strain, which is TLM-resistant because of the overproduction of the major intracellular TLM target, β-ketoacyl-ACP synthase I (Fig. 4 and 5). Although the competitive inhibition of β-ketoacyl-ACP synthases in vitro by TLM (24) indicates that elevated intracellular levels of malonyl-CoA may protect against antibiotic inhibition of fatty acid synthesis, we found no correlation between malonyl-CoA levels and TLM resistance or sensitivity. The inability of strain CDM5 to accumulate sufficient TLM to inhibit fatty acid formation could be due to a mutation in a transport system or pore that is responsible for the uptake of the drug or to the activation of an efflux pump that maintains the intracellular concentrations of the drug at a low level. Introducing a wild-type *tlmR* gene into a TLM-resistant strain by using F143 did not convert the Tlm^r to a Tlm^s phenotype, indicating that the *tlmR* allele is dominant. This finding suggests that resistance is not due to the inactivation of an uptake system, since uptake activity should have been restored in the partial diploid strain SJ260. These genetic and physiological experiments are consistent with the *tlmR* mutation resulting in the activation of an extrusion system rather than the inactivation of an uptake system.

The cloned *emrB* gene of *E. coli* belongs to a family of genes encoding membrane translocases that include multi-

drug-resistant proteins of gram-positive bacteria (e.g., QacA [30]) and pumps that protect cells from individual antibiotics (e.g., TetA [40]). EmrB has a typical structure for an integral membrane transporter, possessing 14 putative membrane-spanning α-helices. The *emrB* gene is expressed as a component of the *emrAB* operon, which has been characterized as a multidrug resistance efflux pump (21). The expression of both the *emrA* and the *emrB* genes is required for pump activity. One feature that distinguishes *emrAB* from other members of the gene family is that it confers resistance to structurally unrelated but moderately hydrophobic compounds. TLM falls within this general classification, and the association of Tlm^r with the *emr* efflux pump was confirmed by our finding that *tlmR* and *emrB* are genetically indistinguishable. On the other hand, cerulenin is more hydrophobic than TLM, and overexpression of the *emr* pump does not confer resistance to this antibiotic (Table 2). Overproduction of the *emrAB* genes via the introduction of a multicopy plasmid leads to the TLM resistance phenotype (Table 2) and, as reported previously (21), resistance to a number of other hydrophobic drugs such as nalidixic acid. However, our data suggest that the mutation creating Tlm^r in strain CDM5 does not result in the overexpression of the *emrAB* genes, since cells can be Tlm^r without being resistant to other pump drugs such as nalidixic acid (strain SJ256). The *nalB4* mutation is another example of activation of the *emrAB* efflux pump toward a specific drug. Strains that are *nalB4* but are sensitive to TLM were derived. Genetic crosses between Tlm^r and Nal^r strains show that the mutations that give rise to these phenotypes are not identical, although the cotransduction frequencies indicate that they may both lie within the *emrAB* operon. It seems reasonable to conclude that the *nalB4* and *tlmR* mutations represent alterations in the efflux pump structure that permit enhanced efflux of the particular drug used in the selection. Comparison of the sequences of *nalB4* and *tlmR* DNA with the wild-type *emrAB* sequence will be required to verify this hypothesis.

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REFERENCES

1. **Apontoweil, P., and W. Berends.** 1975. Isolation and initial characterization of glutathione-deficient mutants of *Escherichia coli* K12. Biochim. Biophys. Acta **399:**10–22.
2. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37:**911–917.
3. **Booth, B. R.** 1980. Cell surface proteins of *E. coli*. Biochem. Biophys. Res. Commun. **94:**1029–1036.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
5. **Chohnan, S., and Y. Takamura.** 1991. A simple micromethod for measurement of CoASH and its use in measuring the intracellular levels of CoASH and short chain acyl-CoAs in *Escherichia coli* K12 cells. Agric. Biol. Chem. **55:**87–94.
6. **Cronan, J. E., Jr., and C. O. Rock.** 1987. Biosynthesis of membrane lipids, p. 474–497. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
7. **D'Agnolo, G., I. S. Rosenfeld, J. Awaya, S. Omura, and P. R. Vagelos.** 1973. Inhibition of fatty acid biosynthesis by the antibiotic cerulenin. Specific inactivation of β-ketoacyl-acyl carrier protein synthetase. Biochim. Biophys. Acta **326:**155–166.
8. **DeBuysere, M. S., and M. S. Olson.** 1983. The analysis of acyl-coenzyme A derivatives by reverse-phase high performance liquid chromatography. Anal. Biochem. **133:**373–379.
9. **de Mendoza, D., and J. E. Cronan, Jr.** 1983. Thermal regulation of membrane lipid fluidity in bacteria. Trends Biochem. Sci. **8:**49–52.
10. **de Mendoza, D., A. Klages Ulrich, and J. E. Cronan, Jr.** 1983. Thermal regulation of membrane fluidity in *Escherichia coli*. Effects of overproduction of β-ketoacyl-acyl carrier protein synthase I. J. Biol. Chem. **258:**2098–2101.
11. **Garwin, J. L., A. L. Klages, and J. E. Cronan, Jr.** 1980. Structural, enzymatic, and genetic studies of β-ketoacyl-acyl carrier protein synthases I and II of *Escherichia coli*. J. Biol. Chem. **255:**11949–11956.
12. **Garwin, J. L., A. L. Klages, and J. E. Cronan, Jr.** 1980. β-Ketoacyl-acyl carrier protein synthase II of *Escherichia coli*. Evidence for function in the thermal regulation of fatty acid synthesis. J. Biol. Chem. **255:**3263–3265.
13. **Gelmann, E. P., and J. E. Cronan, Jr.** 1972. Mutant of *Escherichia coli* deficient in the synthesis of *cis*-vaccenic acid. J. Bacteriol. **112:**381–387.
14. **Hayashi, T., O. Yamamoto, H. Sasaki, A. Kawaguchi, and H. Okazaki.** 1983. Mechanism of action of the antibiotic thiolactomycin inhibition of fatty acid synthesis of *Escherichia coli*. Biochem. Biophys. Res. Commun. **115:**1108–1113.
15. **Hayashi, T., O. Yamamoto, H. Sasaki, and H. Okazaki.** 1984. Inhibition of fatty acid synthesis by the antibiotic thiolactomycin. J. Antibiot. **37:**1456–1461.
16. **Jackowski, S., J. E. Cronan, Jr., and C. O. Rock.** 1991. Lipid metabolism in procaryotes, p. 43–85. *In* D. E. Vance and J. E. Vance (ed.), Biochemistry of lipids, lipoproteins and membranes. Elsevier Science Publishers, Amsterdam.
17. **Jackowski, S., C. M. Murphy, J. E. Cronan, Jr., and C. O. Rock.** 1989. Acetoacetyl-acyl carrier protein synthase. A target for the antibiotic thiolactomycin. J. Biol. Chem. **264:**7624–7629.
18. **Jackowski, S., and C. O. Rock.** 1981. Regulation of coenzyme A biosynthesis. J. Bacteriol. **148:**926–932.
19. **Jackowski, S., and C. O. Rock.** 1987. Acetoacetyl-acyl carrier protein synthase, a potential regulator of fatty acid biosynthesis in bacteria. J. Biol. Chem. **262:**7927–7931.
20. **Kauppinen, S., M. Siggaard-Anderson, and P. van Wettstein-Knowles.** 1988. β-Ketoacyl-ACP synthase I of *Escherichia coli*: nucleotide sequence of the *fabB* gene and identification of the cerulenin binding residue. Carlsberg Res. Commun. **53:**357–370.
21. **Lomovskaya, O., and K. Lewis.** 1992. *emr*, an *Escherichia coli* locus for multidrug resistance. Proc. Natl. Acad. Sci. USA **89:**8938–8942.
22. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
23. **Miyakawa, S., K. Suzuki, T. Noto, Y. Harada, and H. Okazaki.** 1982. Thiolactomycin, a new antibiotic. IV. Biological properties and chemotherapeutic activity in mice. J. Antibiot. **35:**411–419.
24. **Nishida, I., A. Kawaguchi, and M. Yamada.** 1986. Effect of thiolactomycin on the individual enzymes of fatty acid synthase in *Escherichia coli*. J. Biochem. **99:**1447–1454.
25. **Nomura, G., Y. Yoshida, and Y. Takamura.** 1988. Measurement of intracellular acetyl-CoA and malonyl-CoA in bacteria and yeast using acyl-CoA cycling. Agric. Biol. Chem. **52:**843–844.
26. **Noto, T., S. Miyakawa, H. Oishi, H. Endo, and H. Okazaki.** 1982. Thiolactomycin, a new antibiotic. III. *In vitro* antibacterial activity. J. Antibiot. **35:**401–410.
27. **Oishi, H., T. Noto, H. Sasaki, K. Suzuki, T. Hayashi, H. Okazaki, K. Ando, and M. Sawada.** 1982. Thiolactomycin, a new antibiotic. I. Taxonomy of the producing organism, fermentation and biological properties. J. Antibiot. **35:**391–395.

28. **Rock, C. O., and J. E. Cronan, Jr.** 1980. Improved purification of acyl carrier protein. Anal. Biochem. **102:**362–364.
29. **Rosenfeld, I. S., G. D'Agnolo, and P. R. Vagelos.** 1975. Synthesis of unsaturated fatty acids and the lesion in *fabB* mutants. J. Biol. Chem. **248:**2452–2460.
30. **Rouch, D. A., D. S. Cram, D. DiBerardino, T. G. Littlejohn, and R. A. Skurray.** 1990. Efflux-mediated antiseptic resistance gene *qacA* from *Staphylococcus aureus*: common ancestry with tetracycline- and sugar-transport proteins. Mol. Microbiol. **4:**2051–2062.
31. **Sasaki, H., H. Oishi, T. Hayashi, I. Matsuura, K. Ando, and M. Sawada.** 1982. Thiolactomycin, a new antibiotic. II. Structure elucidation. J. Antibiot. **35:**396–400.
32. **Takamura, Y., and Y. Kitayama.** 1981. Purification and some properties of malonate decarboxylase from *Pseudomonas ovalis*, an oligomeric enzyme with bifunctional properties. Biochem. Int. **3:**483–491.
33. **Takamura, Y., Y. Kitayama, A. Arakawa, S. Yamanaka, M. Tosaki, and Y. Ogawa.** 1985. Malonyl-CoA:acetyl-CoA cycling. A new micromethod for determination of acyl-CoAs with malonate decarboxylase. Biochim. Biophys. Acta **834:**1–7.
34. **Tsay, J. T., W. Oh, T. J. Larson, S. Jackowski, and C. O. Rock.** 1992. Isolation and characterization of the β-ketoacyl-acyl carrier protein synthase III gene (*fabH*) from *Escherichia coli* K-12. J. Biol. Chem. **267:**6807–6814.
35. **Tsay, J. T., C. O. Rock, and S. Jackowski.** 1992. Overproduction of β-ketoacyl-acyl carrier protein synthase I imparts thiolactomycin resistance to *Escherichia coli* K-12. J. Bacteriol. **174:**508–513.
36. **Vallari, D. S., S. Jackowski, and C. O. Rock.** 1987. Regulation of pantothenate kinase by coenzyme A and its thioesters. J. Biol. Chem. **262:**2468–2471.
37. **Vance, D. E., I. Goldberg, O. Mitsuhashi, K. Bloch, S. Omura, and S. Nomura.** 1972. Inhibition of fatty acid synthetases by the antibiotic cerulenin. Biochem. Biophys. Res. Commun. **48:**649–656.
38. **Wanner, B. L.** 1986. Novel regulatory mutants of the phosphate regulon in *Escherichia coli* K-12. J. Bacteriol. **191:**39–58.
39. **Winkler, H. H., and T. H. Wilson.** 1966. The role of energy coupling in the transport of β-galactosides by *Escherichia coli*. J. Biol. Chem. **241:**2200–2211.
40. **Yamaguchi, A., N. Ono, T. Akasaka, T. Noumi, and T. Sawai.** 1990. Metal-tetracycline/H^+ antiporter of *Escherichia coli* encoded by a transposon, Tn*10*. The role of the conserved dipeptide, Ser65-Asp66, in tetracycline transport. J. Biol. Chem. **265:**15525–15530.