A Novel Paradigm of Fatty Acid β-Oxidation Exemplified by the Thioesterase-Dependent Partial Degradation of Conjugated Linoleic Acid That Fully Supports Growth of *Escherichia coli*†

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ABSTRACT: An alternative pathway of β-oxidation for unsaturated fatty acids was studied in *Escherichia coli*. 9-*cis*,11-*trans*-Octadecadienoic acid (conjugated linoleic acid), a potential substrate of this pathway, was shown to support growth of *E. coli* in the absence of any other carbon source. The identification of 3,5-dodecadienoic acid in the growth medium revealed the partial β-oxidation of conjugated linoleic acid to 3,5-dodecadienoyl-CoA, which was hydrolyzed to 3,5-dodecadienoic acid and released from cells. The involvement of acyl-CoA thioesterases in this process was evaluated by determining the substrate specificity of thioesterase II and comparing it with that of a novel thioesterase (thioesterase III) and by assessing mutant strains devoid of one or both of these thioesterases for growth on conjugated linoleic acid. Both thioesterases were highly active with 3,5-dodecadienoyl-CoA as substrate. A deficiency of either thioesterase decreased the growth rate of cells on conjugated linoleic acid but not on palmitic acid. The absence of both thioesterases reduced the cellular growth in a cumulative manner but did not abolish it. It is concluded that thioesterases II and III and at least one other thioesterase function in the partial degradation of conjugated linoleic acid via the thioesterase-dependent pathway of β-oxidation, which provides all energy and carbon precursors required for the growth of *E. coli*.

Fatty acid β-oxidation provides all energy and all carbon precursors when *Escherichia coli* is grown on oleic acid as the sole carbon source. Ninety percent of oleic acid is degraded to acetyl-CoA via the classical or isomerase-dependent pathway of β-oxidation, which requires Δ^3,Δ^2-enoyl-CoA isomerase (enoyl-CoA isomerase)[1] in addition to the enzymes necessary for the breakdown of saturated fatty acids (*1*). However, approximately 10% of oleic acid is only partially degraded by an alternative pathway that branches off from the classical pathway due to enoyl-CoA isomerase acting on the oleate metabolite 2-*trans*,5-*cis*-tetradecadienoyl-CoA and converting it to 3,5-*cis*-tetradecadienoyl-CoA (*1*). The further β-oxidation of 3,5-*cis*-tetradecadienoyl-CoA is not possible in *E. coli* because the required enzyme, $\Delta^{3,5}$,$\Delta^{2,4}$-dienoyl-CoA isomerase (dienoyl-CoA isomerase) is not present in this organism. Instead, 3,5-*cis*-tetradecadienoyl-CoA is hydrolyzed to 3,5-*cis*-tetradecadienoic acid, which is released into the growth medium (*1*). This alternative breakdown of fatty acids is referred to as thioesterase-dependent pathway of β-oxidation.

Two long-chain acyl-CoA thioesterases, named thioesterases I and II, have been identified in *E. coli* (*2*−*6*) many years ago, and both hydrolyze 3,5-*cis*-tetradecadienoyl-CoA to 3,5-*cis*-tetradecadienoate and CoASH (*1*). However, thioesterase I is a periplasmic enzyme and therefore not positioned to participate in the intracellular hydrolysis of 3,5-*cis*-tetradecadienoyl-CoA (*6*). In contrast, thioesterase II, which is more active with 3,5-*cis*-tetradecadienoyl-CoA than with any other substrate tested, has a cytosolic location and thus could be involved in the disposal of 3,5-*cis*-tetradecadienoyl-CoA that is formed during the β-oxidation of oleic acid. Recently, another long-chain acyl-CoA thioesterase, named thioesterase III, was identified in *E. coli*. This enzyme hydrolyzes 3,5-*cis*-tetradecadienoyl-CoA and consequently might participate in oleate degradation (*7*).

The aim of this study was to evaluate the function and capacity of the thioesterase-dependent pathway of β-oxidation and to determine which thioesterases act in this pathway that ends with the hydrolysis of a partially degraded fatty acyl-CoA and the release of the resultant carboxylic acid from the cell. A surprising observation was that *E. coli* grows

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[1] Abbreviatons: enoyl-CoA isomerase, Δ^3,Δ^2-enoyl-CoA isomerase; dienoyl-CoA isomerase, $\Delta^{3,5}$,$\Delta^{2,4}$-dienoyl-CoA isomerase; conjugated linoleic acid, 9-*cis*,11-*trans*-octadecadienoic acid; 3,5-dodecadienoyl-CoA, 3-*cis*,5-*trans*-dodecadienoyl-CoA; HPLC, high-performance liquid chromatography; CLA, conjugated linoleic acid; thioesterase, acyl-CoA thioesterase; GC/MS, gas chromatography in combination with mass spectrometry; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TE, thioesterase.

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on 9-*cis*,11-*trans*-octadecadienoic acid (conjugated linoleic acid) as the sole carbon source even though this fatty acid only is partially degraded by three cycles of β-oxidation. The final product of its β-oxidation is 3-*cis*,5-*trans*-dodecadienoyl-CoA (3,5-dodecadienoyl-CoA), which is hydrolyzed by several thioesterases to 3,5-dodecadienoic acid that is released into the growth medium.

EXPERIMENTAL PROCEDURES

Materials. CoASH, acetyl-CoA, butyryl-CoA, octanoyl-CoA, decanoyl-CoA, dodecanoyl-CoA, tetradecanoyl-CoA, palmitoyl-CoA, and stearoyl-CoA were purchased from Life Science Resources (Milwaukee, WI). 5-*cis*-Dodecenoic acid was bought from Aldrich while 5-*cis*-tetradecenoic acid was synthesized by Cayman Chemicals (Ann Arbor, MI). 9-*cis*,11-*trans*-Octadecadienoic acid was obtained from Matreya, Inc. (Pleasant Gap, PA). BCl_3−methanol (12%, w/w) was purchased from Supelco (Bellefonte, PA). Burdick & Jackson (Muskegon, MI) was the source of ethyl ether, while hexane was from Fisher Scientific. Protein A−Sepharose CL-4B was obtained from Amersham Biosciences (Piscataway, NJ). Polyacrylamide ready gels, trans-Blot transfer medium, pure nitrocellulose membrane, AP-conjugated goat anti-rabbit antibody, AP-conjugated substrate kit, and hydroxylapatite were purchased from Bio-Rad Laboratories. Methyl-β-cyclodextrin, DEAE-cellulose, oleic acid, and most of the standard biochemicals were obtained from Sigma. A QIAprep spin miniprep kit, QIAGEN genomic tips, and a QIAquick PCR purification kit were bought from QIAGEN Inc. (Valencia, CA). Taq DNA polymerase and 2-long DNA ladder were obtained from New England Biolabs Inc. Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. *E. coli* strain LE392 (*hasR*, *galK*, *trpR*, *metB*, *lacY*) was obtained from the *E. coli* Genetic Stock Center (Yale University) while the creation of strain YR1 (*tesA::kan*^{r} and *tesB::cml*^{r}) that does not express thioesterases I and II has previously been reported (*7*), as have the molecular cloning, overexpression, and purification of thioesterase III (*7*). Antisera to thioesterase II and thioesterase III from *E. coli* were raised in rabbits by Pocono Rabbit Farm and Laboratory, Inc. (Canadensis, PA).

Synthesis of Substrates and Metabolites. Oleoyl-CoA, 5-*cis*-dodecenoyl-CoA, and 5-*cis*-tetradecenoyl-CoA were synthesized from oleic acid, 5-*cis*-dodecenoic acid, and 5-*cis*-tetradecenoic acid, respectively, by the mixed anhydride method as described by Fong and Schulz (*8*). 3,5-*cis*-Tetradecadienoyl-CoA and 3,5-*cis*-dodecadienoyl-CoA were prepared from the corresponding 5-*cis*-enoyl-CoA thioesters by dehydrogenation with acyl-CoA oxidase as previously described (*1*). 2-*trans*,5-*cis*-Tetradecadienoyl-CoA (*9*), 2-*trans*-tetradecenoyl-CoA (*1*), L-3-hydroxytetradecanoyl-CoA (*1*), 3-*trans*-tetradecenoyl-CoA (*10*), and 3-ketohexadecanoyl-CoA (*11*) were synthesized by published procedures. All acyl-CoA thioesters were analyzed and purified by reverse-phase high-performance liquid chromatography (HPLC) on a Waters μBondapak C_{18} column (30 cm × 3.9 mm), which was attached to a Waters gradient HPLC system. The absorbance of the eluate was monitored at 254 nm. Separation of long-chain acyl-CoAs was achieved by linearly increasing the acetonitrile/water (9:1 v/v) content of the 50 mM ammonium phosphate elution buffer (pH 5.5) from 40% to 70% in 20 min at a flow rate of 2 mL/min.

Bacterial Growth Conditions and Preparation of Bacterial Extracts. E. coli cells were grown on LB medium from single colonies. The initial culture was diluted 5-fold into M9 minimal medium containing 1% (w/v) tryptone, 2 mM $MgSO_4$, 10 μM $CaCl_2$, 1 μM $FeCl_3$, and additionally oleic acid (0.1% v/v) plus 0.4% Triton X-100. After cultures had grown at 37 °C under shaking at 200 rpm to an absorbance of 1 at 600 nm, they were diluted 20 times into the same basic growth medium that contained either glucose (0.1% w/v), oleic acid (0.1% v/v), palmitic acid (0.1% w/v), or conjugated linoleic acid (CLA) (0.1% v/v) as the sole carbon source. Fatty acids were solubilized with cyclodextrin at a 1:6 molar ratio or with 0.4% Triton X-100 that also was added to the glucose-containing medium. Growth curves were obtained by measuring the change in absorbance at 600 nm. For the preparation of cell extracts, cultures were harvested at an absorbance of 1 at 600 nm by centrifugation at 2300*g* for 30 min at 4 °C. Cell pellets were washed twice with M9 minimal medium and stored at −80 °C. Cell pastes were suspended in two to three times their volume of 0.1 M KP_i (pH 7.0) containing 10% glycerol and were sonicated for a total of 2 min (10 s × 12) at 0 °C and centrifuged at 33000*g* for 1 h at 4 °C. The resultant supernatants were collected for enzyme and protein assays.

Separation of E. coli Acyl-CoA Thioesterases (Thioesterases) on DEAE-cellulose. E. coli cell pellets (1.5 g) grown on either glucose, oleate, or CLA were suspended in 0.1 M KP_i (pH 7.0) containing 10% glycerol, sonicated for a total of 2 min (10 s × 12) at 0 °C, and centrifuged at 33000*g* for 1 h at 4 °C. The resultant supernatants were dialyzed overnight against 0.02 M Tris-HCl (pH 7.8) containing 10% glycerol and applied to a DEAE-cellulose column (15 × 1.0 cm) previously equilibrated with dialysis buffer. The column was then developed with a linear gradient made up of 125 mL each of 0.02 M Tris-HCl (pH 7.8) containing either 50 mM NaCl or 350 mM NaCl. Fractions were assayed for thioesterase activity with myristoyl-CoA as substrate, and those with high activities were pooled, concentrated, and stored at −80 °C.

Purification of E. coli Thioesterase II. E. coli cells harboring pUC *tesB* (*5*) were grown overnight at 37 °C in LB medium containing 50 μg/mL ampicillin. The initial culture was diluted 50-fold into the same medium, and this culture was grown to an absorbance of 1 at 600 nm. Isopropyl β-D-thiogalactopyranoside was added to a final concentration of 1 mM, and the culture was incubated at 37 °C for 4 h. Cells were harvested by centrifugation at 3000*g* for 10 min and washed with 10 volumes of 0.1 M KP_i (pH 8) and stored at −80 °C. One gram of cell paste was suspended in 2 mL of 20 mM KP_i (pH 8) containing 1 mM phenylmethanesulfonyl fluoride and 1 mM benzamidine, homogenized by sonication for a total of 2 min (20 s × 6) at 0 °C, and centrifuged at 33000*g* for 1 h at 4 °C. The supernatant was collected and brought to 50% saturation with $(NH_4)_2SO_4$ and then centrifuged at 10000*g* for 10 min. The precipitated protein was dissolved in a minimal volume of 20 mM Tris-HCl (pH 7.8) containing 10% glycerol and loaded onto a DEAE-cellulose column (8 × 2.5 cm) equilibrated with the dissolving buffer. The column was developed with a linear gradient made up of 500 mL each of 0.02 M Tris-HCl (pH 7.8) containing either 50 mM NaCl or 500 mM NaCl.

Fractions were assayed for thioesterase activity, and those with high activities were pooled and stored at −80 °C.

Enzyme and Protein Assays. Thioesterases were assayed by measuring the release of CoASH from acyl-CoAs with Ellman's reagent (*12*). A standard assay mixture contained 0.175 M KP_i (pH 8.0), 0.2 mM 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), and 20 μM acyl-CoA. The progress of the reaction was determined spectrophotometrically at 412 nm, and rates were calculated using an extinction coefficient of 13600 M^{-1} cm^{-1}. A kinetic analysis of purified thioesterase II was performed with several long-chain acyl-CoAs. Kinetic constants (V_{max}, K_m) were obtained by nonlinear curve fitting using the Sigma Plot 2000 program. When long-chain substrates (>C14) were used, bovine serum albumin (1 mg/mL) was included in the assay mixture. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of substrate to product in 1 min. Protein concentrations were determined by the dye-binding assay as described by Bradford (*13*) with bovine serum albumin as standard.

Identification of Fatty Acid Metabolites in the Bacterial Growth Medium. E. coli cells were grown to midlogarithmic phase in M9 medium containing conjugated linoleic acid (0.1% v/v) solubilized with cyclodextrin at a 1:6 molar ratio. Cells were separated from the growth medium by centrifugation at 2300*g* for 30 min at 4 °C. The supernatant was acidified to pH 1−2 with 2 N H_2SO_4 and then extracted three times with 100 mL of ether each. The organic phase was extracted with aqueous sodium bicarbonate. After acidifying the aqueous phase with 2 N H_2SO_4, it was extracted three times with 8 mL of ether each. The combined ether extracts were dried over anhydrous sodium sulfate, and the residual material, after removal of drying agent by filtration and ether by evaporation under a stream of N_2, was methylated by reacting it with 2 mL of BCl_3−methanol (12% w/w) for 10 min at 60 °C. After the reaction mixture was allowed to cool down, 1 mL of H_2O and 1 mL of hexane were added. The organic layer was carefully removed and dried over anhydrous sodium sulfate. The residue after the removal of sodium sulfate and evaporation of hexane was dissolved in a minimal volume of anhydrous ethanol. This fraction, which contained the methyl esters of fatty acids that were present in the growth medium, was analyzed by gas chromatography in combination with mass spectrometry (GC/MS). For the purpose of identifying 3,5-dodecanoate, an aqueous solution of 20 nmol of 3,5-dodecadienoyl-CoA and 20 nmol of *n*-pentadecanoyl-CoA (internal standard) was hydrolyzed by reacting it with 4 N KOH at 25 °C for 1 h. The reaction mixture was acidified to pH 1−2 with 2 N H_2SO_4 and extracted three times with 8 mL of ether each. The extracted fatty acids were converted to their methyl esters as described above. Aliquots of 1 μL of the fatty acid methyl esters were injected at 250 °C into a GC/MS instrument (Shimadzu Scientific Instruments) consisting of a gas chromatograph (model GC-17A) interphased with a mass spectrometer (QP-5000) and equipped with a capillary column (30 m, inner diameter 0.25 mm, film thickness 0.25 μm, EC-5; Alltech Associates Inc., Deerfield, IL). The oven temperature was raised from 100 to 230 °C at 5 °C/min to 300 °C at 20 °C/min and then held constant for 6 min. The mass spectrometer served as a detector and was operated at 280 °C.

Construction of E. coli Strains with ybaW or ybaW/tesB Deletion(s). Strains containing a deletion of the *ybaW* gene (Δ*ybaW*) or deletions of the *ybaW* and *tesB* genes (Δ*ybaW*/Δ*tesB*) were constructed by the method of Datsenko and Wanner (*14*). Primers of 50 bp were synthesized, which were homologous to regions adjacent to the *ybaW* gene encoding thioesterase III (*7*) or *tesB* gene and to regions of the template plasmid pKD3 that carried the chloramphenicol resistance gene. These primers were used to amplify by the polymerase chain reaction (PCR) the chloramphenicol resistance gene of plasmid pKD3 (*14*) flanked by 30-bp sequences corresponding to upstream and downstream regions of the gene of interest. This product was transformed into the parent strain, which carried a temperature-sensitive arabinose-λ inducible Red helper plasmid (*14*). Transformants were selected on LB medium containing 20 μg/mL chloramphenicol, and temperature-sensitive plasmids were cured at 37 °C. The *ybaW* or *tesB* insertional mutants were further converted to deletion mutants with the aid of a helper plasmid expressing the FLP recombinase, which acted on the FLP recognition target sequences flanking the chloramphenicol resistance gene. All strains were colony-purified at 43 °C and tested for loss of antibiotic resistance and loss of the FLP helper plasmid. The gene deletions were confirmed by PCR amplification of the relevant locus DNA sequence and by immunoblotting using antisera raised against thioesterase II and thioesterase III.

Identification of Thioesterases II and III in Wild-Type and Mutant E. coli Cells. One gram of *E. coli* cell pellet grown on oleate was suspended in 2 mL of 0.1 M KP_i (pH 7.0) containing 10% glycerol, sonicated for a total of 2 min (10 s × 12) at 0 °C, and centrifuged at 33000*g* for 1 h at 4 °C. The resultant supernatant was dialyzed against 0.02 M Tris-HCl (pH 7.8) containing 10% glycerol and applied to a DEAE-cellulose column (1.5 × 2.5 cm) previously equilibrated with dialysis buffer. The column was washed with 35 mL of 0.02 M Tris-HCl (pH 7.8) containing 50 mM NaCl and 10% glycerol and then eluted with 30 mL of 0.02 M Tris-HCl (pH 7.8) containing 250 mM NaCl and 10% glycerol. The eluate was concentrated and stored at −80 °C for immunoprecipitation. Mixtures of 50 μL of protein A−Sepharose CL-4B agarose, 50 μL of primary antiserum (rabbit anti-*E. coli* thioesterase II or anti-*E. coli* thioesterase III antiserum), and 300 μL of washing buffer containing 20 mM Tris-HCl (pH 7.8), 150 mM NaCl, 0.1% (w/v) Triton X-100, and 10% (w/v) glycerol were incubated in a microcentrifuge tube for 1 h at 4 °C under gentle shaking. The sample was centrifuged at 10000*g* for 1 min, and after careful removal of the supernatant, the beads were washed with 1 mL of washing buffer for at least three times. One milliliter of *E. coli* cell extract after partial purification on DEAE-cellulose was added to the tube, and this mixture was incubated for 1.5 h at 4 °C. The immunoprecipitated complex was collected by centrifugation at 10000*g* for 1 min and washed five times with 1 mL of washing buffer. After the last wash, the pellet was suspended in 50 μL of sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) sample buffer to a final concentration of 1× sample buffer and then vortexed and heated at 95 °C for 10 min. After centrifugation for 1 min at 10000*g* at room temperature, the solubilized immunoprecipitate was collected and kept frozen until used for SDS−PAGE or immunoblot-

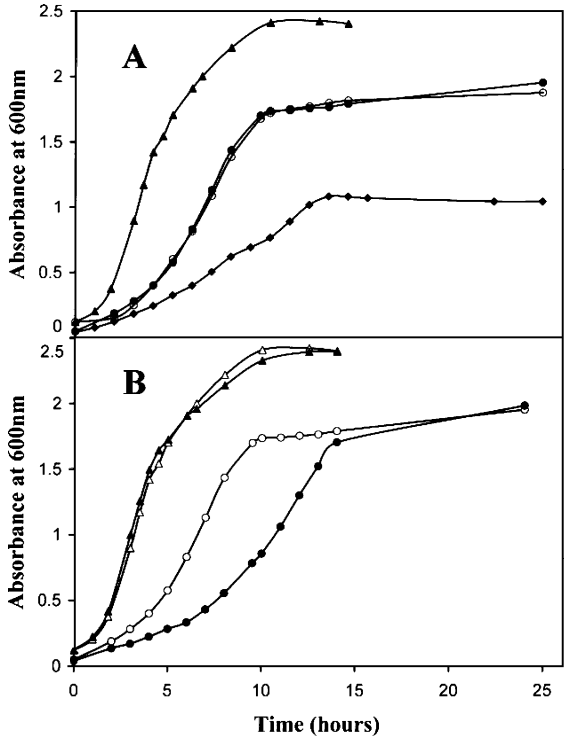


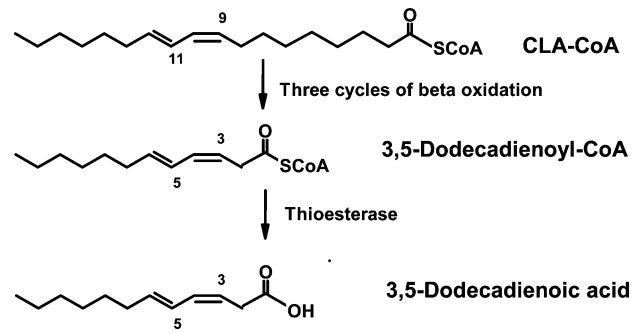
FIGURE 1: Growth of *E. coli* on glucose or a fatty acid as the sole carbon source. (A) Wild-type *E. coli* grown on (▲) glucose, (●) oleic acid, (○) palmitic acid, and (◆) conjugated linoleic acid. (B) Wild-type *E. coli* grown on (Δ) glucose, (▲) strain YR1 (*tesA/tesB* mutant) grown on glucose, (○) wild-type *E. coli* grown on oleate, and (●) strain YR1 (*tesA/tesB* mutant) grown on oleate.

ting. Proteins were subjected to SDS−PAGE as described by Laemmli (*15*) and transferred from the gels to nitrocellulose membranes by use of a TransBlot SD semidry electrophoretic transfer cell system. The membrane was blocked with 5% nonfat milk in Tris-buffered saline (TBS) and incubated with rabbit antiserum raised against *E. coli* thioesterase III or *E. coli* thioesterase II (diluted 500-fold) in 5% bovine serum albumin in TBS. After three washes with TBS containing Tween-20, the membrane was incubated with AP-conjugated goat anti-rabbit antibody at a 1:3000 dilution in 5% bovine serum albumin in TBS and developed with AP-conjugated substrate until bands appeared on the membrane.

RESULTS

Growth of E. coli on Fatty Acids as the Sole Carbon Source. Growth of *E. coli* is fully supported by β-oxidation of oleic acid even though a fraction of this fatty acid is only partially degraded (*1*). 3,5-Tetradecadienoyl-CoA, a metabolite of oleic acid that is resistant to further β-oxidation, is hydrolyzed, presumably by a long-chain acyl-CoA thioesterase, and the resultant acid is released into the growth medium (*1*). In an attempt to evaluate the function of thioesterases in the β-oxidation of fatty acids in *E. coli*, the growth behavior of wild-type *E. coli* (strain LE392) was compared with the growth behavior of strain YR1 that is devoid of thioesterases I and II. As shown in Figure 1A, wild-type *E. coli* grew very well on glucose and grew slower, but equally well on oleic acid or palmitic acid. The growth behavior of the thioesterase double mutant YR1 was the same as the parental strain when glucose was the carbon source, whereas

Scheme 1: Proposed Pathway for the β-Oxidation of Conjugated Linoleic Acid in *E. coli*[a]



[a] Abbreviation: CLA-CoA, conjugated linoleoyl-CoA.

it grew slower than wild-type *E. coli* on oleate (Figure 1B). The observation that *E. coli* can grow on oleic acid in the absence of thioesterases I and II and the identification of 3,5-tetradecadienoic acid in the growth medium of strain YR1 grown on oleate (*7*) suggest that at least one additional long-chain acyl-CoA thioesterase is present in this organism and functions in the thioesterase-dependent pathway of β-oxidation.

β-Oxidation of Conjugated Linoleic Acid by E. coli. To gain a better understanding of the function of thioesterases in the degradation of unsaturated fatty acids, we studied the growth of *E. coli* on CLA. As shown in Figure 1A, wild-type *E. coli* grew on CLA as the only carbon source, even though it grew slower on CLA than on oleic acid and it grew to a lower cell density. According to the hypothetical pathway for the β-oxidation of CLA, shown in Scheme 1, this fatty acid, after conversion to its CoA thioester, passes three times through the β-oxidation cycle to yield 3,5-dodecadienoyl-CoA that is assumed to be resistant to further β-oxidation because of the absence of dienoyl-CoA isomerase from *E. coli* (*1*). Disposal of 3,5-dodecadienoyl-CoA might be facilitated by its hydrolysis to 3,5-dodecadienoic acid followed by the release of the latter metabolite into the growth medium. If CLA is degraded in *E. coli* via the thioesterase-dependent pathway (see Scheme 1), only 3 mol of acetyl-CoA would be generated per mole of CLA compared to 7 and 8 mol of acetyl-CoA formed per mole of palmitic acid and oleic acid, respectively. The lower density of cultures grown to stationary phase on CLA as compared to cultures grown on oleate or palmitate (see Figure 1A) agrees with a reduced yield of acetyl-CoA due to the partial β-oxidation of CLA. Proof for the operation of the pathway shown in Scheme 1 would be the identification of 3,5-dodecadienoic acid in the medium in which wild-type *E. coli* was grown with CLA as the sole carbon source. To test for the presence of this compound, the acidic fraction of the growth medium was extracted, converted to methyl esters, and analyzed by GC/MS. The most prominent peak in the GC spectrum was observed at an elution time of close to 14 min (see Figure 2A) in a region where methyl 3,5-dodecadienoate is expected to be eluted. An expanded spectrum of the region between 13 and 18 min is shown in Figure 2B with the most prominent peak (peak 1) centered at 13.7 min. The mass spectrum of the material corresponding to peak 1 is shown in Figure 2C. A mass-to-charge ratio of 210 for the molecular ion agrees with that of methyl 3,5-

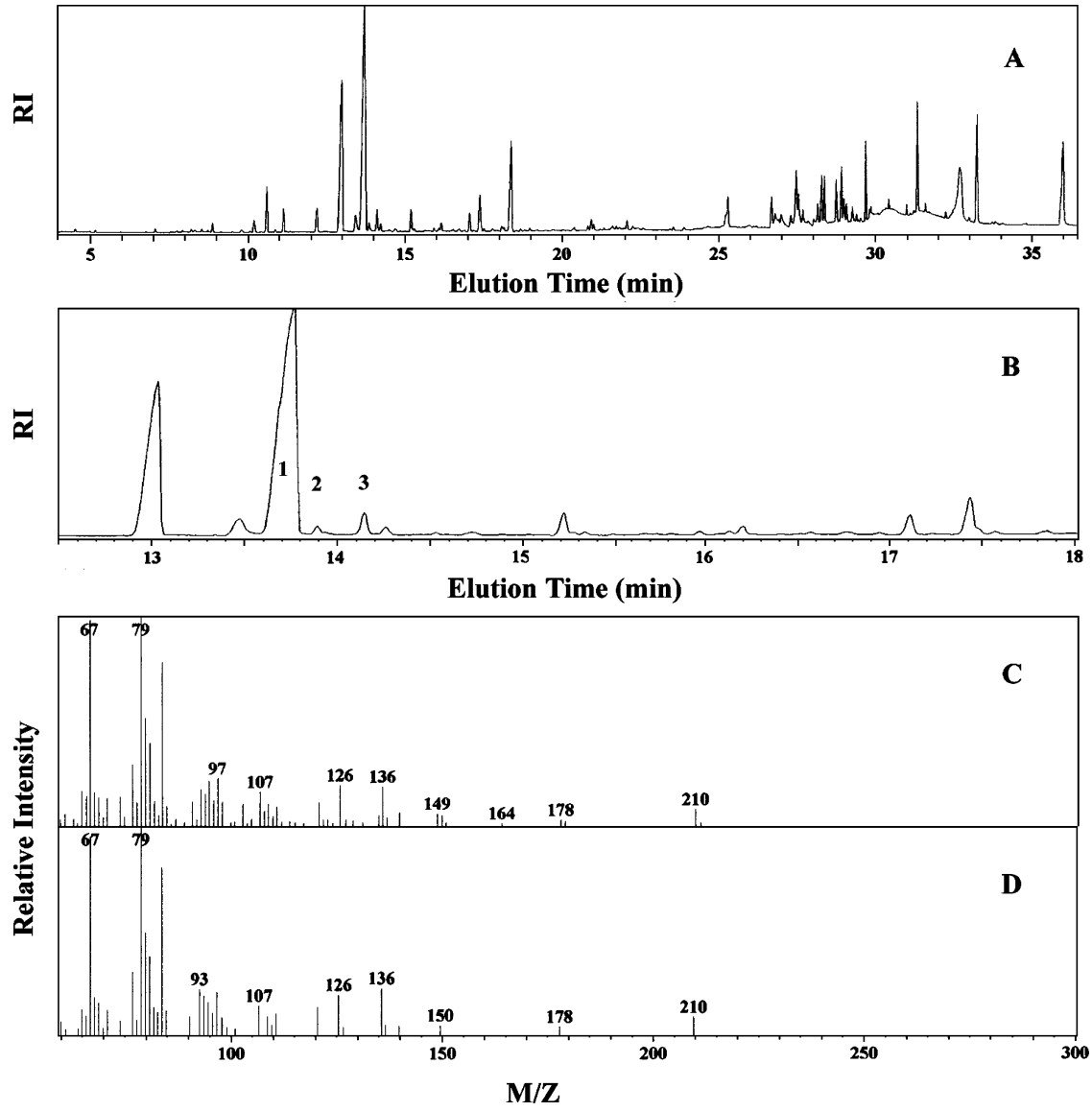


FIGURE 2: Identification of 3,5-dodecadienoic acid in the growth medium of wild-type *E. coli* cells grown on conjugated linoleic acid. (A) Gas chromatogram of the methyl esters of the acidic fraction extracted from the growth medium of wild-type *E. coli* cells grown to midlogarithmic phase. (B) Region of the gas chromatogram shown in panel A where methyl 3,5-dodecadienoate would be eluted. Peaks marked 1 through 3 have molecular ions with mass-to-charge ratios (*m*/*z*) of 210. (C) Mass spectrum of the material that gave rise to peak 1 in panel B. (D) Mass spectrum of the major peak of methyl 3,5-dodecadienoate prepared from 3,5-dodecadienoyl-CoA.

dodecadienoate. Furthermore, the mass spectrum shown in Figure 2C is virtually identical with the spectrum of authentic methyl 3,5-dodecadienoate (Figure 2D) that was prepared by hydrolyzing synthetic 3,5-dodecadienoyl-CoA. Materials corresponding to the small peaks 2 and 3 also yielded mass spectra that were virtually identical to that shown in Figure 2D. It is likely that these materials are the methyl esters of stereoisomers of 3-*cis*,5-*trans*-dodecadienoic acid, which is the main metabolite of 9-*cis*,11-*trans*-octadecadienoic acid. The same products were identified when *E. coli* strain YR1, which is devoid of thioesterases I and II, was grown on CLA, whereas growth of *E. coli* on either oleic acid or palmitic acid did not yield 3,5-dodecadienoic acid (data not shown). The identification of 3,5-dodecadienoic acid as the major acidic compound in the growth medium proves that the β-oxidation of CLA proceeds by the thioesterase-dependent pathway shown in Scheme 1.

Long-Chain Acyl-CoA Thioesterases in E. coli. The conclusion that CLA is degraded in *E. coli* via the thioesterase-dependent pathway of β-oxidation raised the question as to which thioesterase(s) might be responsible for the hydrolysis of 3,5-dodecadienoyl-CoA. Of the two well-known long-chain acyl-CoA thioesterases in *E. coli* (*6*), only thioesterase II could be involved because thioesterase I is a periplasmic protein (*6*). The substrate specificity of purified thioesterase II was determined with various acyl-CoAs that represent the spectrum of intermediates formed during fatty acid β-oxidation. The data shown in Figure 3A agrees with the potential function of this enzyme in the thioesterase-dependent pathway of β-oxidation because it is active with medium-chain and long-chain acyl-CoAs even if a hydroxyl residue, a keto group, or a double bond is present close to the thioester function. The best substrate, however, is 3,5-tetradecadienoyl-CoA, the terminal metabolite of oleate β-oxidation via the thioesterase-dependent pathway, while 3,5-dodecadienoyl-CoA, the end product of CLA β-oxidation, is a good substrate. The disposal of these two intermediates of β-oxidation in *E. coli* requires their thioesterase-catalyzed

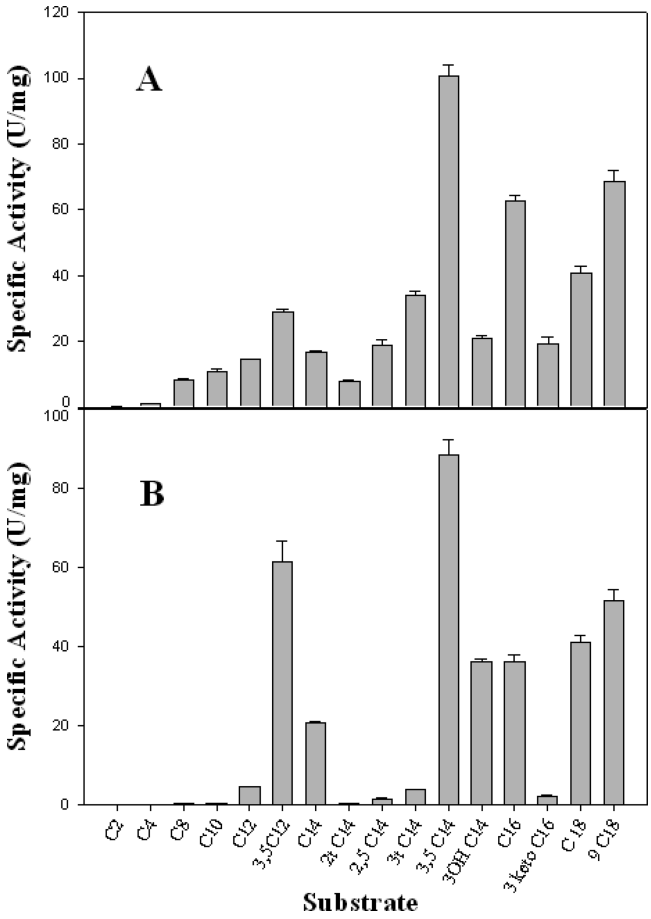


FIGURE 3: Substrate specificities of *E. coli* thioesterases: (A) purified thioesterase II; (B) purified thioesterase III. Thioesterase activities were determined at acyl-CoA concentrations of 20 μM with the following: C2, acetyl-CoA; C4, butyryl-CoA; C8, octanoyl-CoA; C10, decanoyl-CoA; C12, dodecanoyl-CoA; 3,5C12, 3,5-*cis*-dodecadienoyl-CoA; C14, tetradecanoyl-CoA (myristoyl-CoA); 2tC14, 2-*trans*-tetradecenoyl-CoA; 2,5C14, 2-*trans*,5-*cis*-tetradecadienoyl-CoA; 3tC14, 3-*trans*-tetradecenoyl-CoA; 3,5C14, 3,5-*cis*-tetradecadienoyl-CoA; 3OHC14, 3-hydroxytetradecanoyl-CoA; C16, hexadecanoyl-CoA (palmitoyl-CoA); 3ketoC16, 3-ketohexadecanoyl-CoA; C18, octadecanoyl-CoA (stearoyl-CoA); 9C18, 9-*cis*-octadecenoyl-CoA (oleoyl-CoA). Values of enzyme activity are means of three or four determinations ± SD. Data shown in panel B are from ref *7*.

hydrolyses. A kinetic analysis of thioesterase II with several good substrates yielded similar K_m values of 9 ± 1, 6 ± 1, 13 ± 3, and 7 ± 1 μM for dodecanoyl-CoA, 3,5-dodecadienoyl-CoA, tetradecanoyl-CoA, and 3,5-tetradecadienoyl-CoA, respectively. Consequently, the spectrum of specific activities determined at a fixed substrate concentration (see Figure 3) reflects the ratio of k_{cat} values. An inhibition of the enzyme by long-chain substrates, which was minimized by the inclusion of bovine serum albumin in the assay mixture, interfered with the accurate determination of kinetic parameters for the hydrolysis of long-chain acyl-CoAs.

When an extract of *E. coli* strain YR1, which contained neither thioesterase I nor thioesterase II, was assayed for thioesterase activity with myristoyl-CoA as substrate, a low level of activity was detected in cells grown on oleate as sole carbon source (see Table 1). Although the myristoyl-CoA thioesterase activity of the YR1 strain was much lower than the activity of the wild-type cells, this level of activity apparently was sufficient to support growth of the mutant

Table 1: Thioesterase Activities in Extracts from Wild-Type *E. coli* Cells and Strain YR1 with Deletion Mutations in *tesA* and *tesB* Encoding Thioesterase I and II, Respectively

	thioesterase activity (milliunits/mg)	
growth medium	wild type (LE 392)	mutant (YR1)
LB	21.5 ± 1.6	≥0
glucose	26.2 ± 1.7	0.5 ± 0.03
oleate	32.6 ± 2.3	1.8 ± 0.1

on oleate, albeit at a slower rate than growth of wild-type cells (see Figure 1B). The myristoyl-CoA thioesterase activity in YR1 cells supports the idea that one or more long-chain thioesterases are present in *E. coli* besides thioesterases I and II. When the soluble proteins extracted from *E. coli* YR1 cells were separated on DEAE-cellulose, two peaks of myristoyl-CoA thioesterase activity were detected in extracts of glucose-grown cells and three peaks of activity in extracts of cells grown on either oleate or CLA (see Figure 4). The thioesterase corresponding to peak 3 in Figure 4B, which was eluted ahead of where thioesterase II would have emerged, if present, was identified as a novel thioesterase that is encoded by the *ybaW* gene. The purification, identification, and molecular cloning of this thioesterase, named thioesterase III, has recently been described (*7*). The substrate specificity of thioesterase III, shown in Figure 3B, proved this enzyme to be a long-chain specific thioesterase that is most active with 3,5-tetradecadienoyl-CoA and 3,5-dodecadienoyl-CoA, which are hydrolyzed during growth of *E. coli* on oleic acid and CLA, respectively. A comparison of the substrate profiles of thioesterase II and III leads to

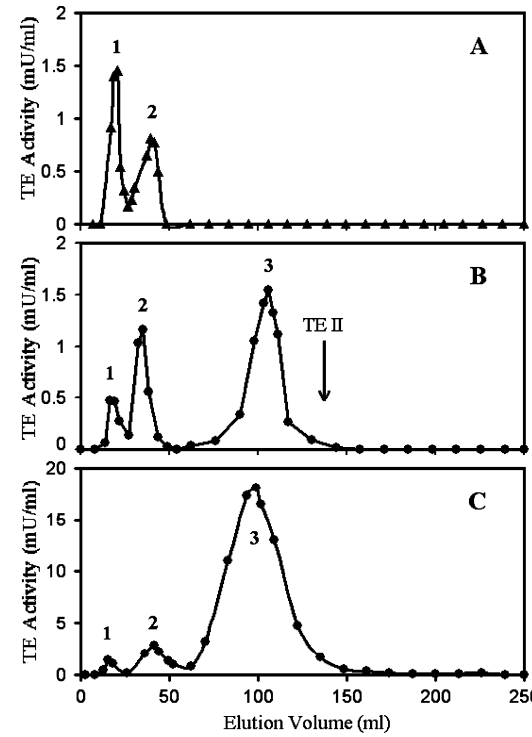


FIGURE 4: Induction of a thioesterase by growth of *E. coli* on oleate. Soluble extracts of strain YR1 (*tesA/tesB* mutant) cells grown on either glucose (panel A) or oleate (panel B) or conjugated linoleic acid (panel C) as the sole carbon source were subjected to chromatography on DEAE-cellulose. Fractions were assayed for thioesterase with 20 μM tetradecanoyl-CoA (myristoyl-CoA) as substrate. The arrow in panel B indicates the position where thioesterase II would have been eluted, if present.

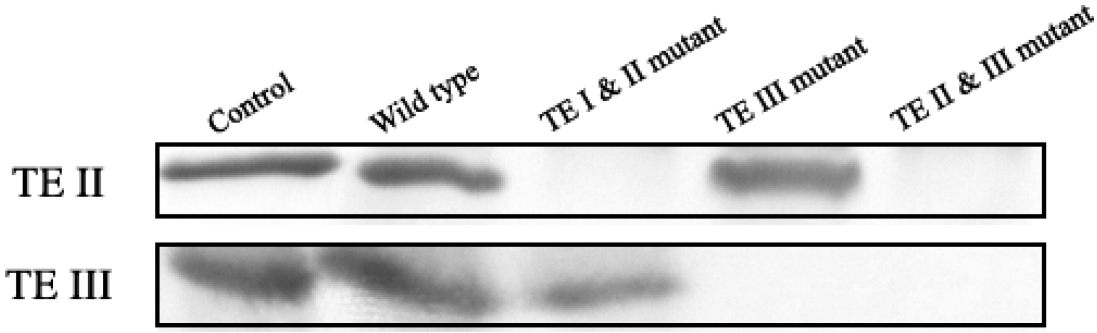


FIGURE 5: Immunoblots of thioesterases II and III present in wild-type *E. coli* and thioesterase mutant cells. Thioesterases II and III present in cell extracts were partially purified by chromatography on DEAE-cellulose and immunoprecipitation before being subjected to immunoblotting. For experimental details see Experimental Procedures. Key: control, purified thioesterase II or III; wild type, wild-type *E. coli*; TE I & II mutant, mutant with deletions in the *tesA* and *tesB* genes encoding thioesterase I and II, respectively; TE III mutant, mutant with a deletion in the *ybaW* gene encoding thioesterase III, TE II & III mutant, mutant with deletions in *tesB* and *ybaW* genes encoding thioesterase II and III, respectively. Abbreviations: TE II, thioesterase II; TE III, thioesterase III.

the conclusion that both enzymes could function in the thioesterase-dependent pathways of oleate and CLA β-oxidation. Noteworthy is the observation that the activity of thioesterase III was 20 times higher in cells grown on CLA than in cells grown on oleate (compare peaks 3 shown Figure 4B,C). Thus, the level of expression of this thioesterase varies greatly depending on the fatty acid present in the growth medium.

Growth of E. coli Thioesterase Mutants on Palmitate or CLA. The identification of thioesterase III raised the question as to whether this enzyme alone or together with thioesterase II operates in the thioesterase-catalyzed β-oxidation of CLA. To address this question, mutants with deleted thioesterase genes were generated. Specifically, a mutant with a deleted *ybaW* gene encoding thioesterase III (TE III mutant) and a mutant with deleted *ybaW*/*tesB* genes encoding thioesterase II and III (TE II and III mutant) were created. The successful deletion of the two thioesterase genes was confirmed by immunoblotting. Shown in Figure 5 are immunoblots that were obtained with thioesterase preparations that had been partially purified by column chromatography of *E. coli* cell extracts on DEAE-cellulose followed by immunoprecipitation of the two thioesterases with antisera raised against them. The absence of thioesterase II from the TE I and II mutant, of thioesterase III from the TE III mutant, and of thioesterases II and III from the TE II and III mutant is demonstrated. Deletion of the thioesterase genes was also demonstrated by the absence of products after amplification of the *ybaW* gene or *tesB* gene by PCR (data not shown). Growths of the TE III mutant, the TE II and III mutant, and TE I and II mutant were compared with the growth of the wild-type (parental) strain on either palmitic acid or CLA as sole carbon source. The growth curves shown in Figure 6A demonstrate that growth on palmitic acid was not affected by the absence of either thioesterase II or thioesterase III. The slower growth of the TE I and II mutant was most likely due to the absence of thioesterase I that is encoded by the *tesA* gene because the absence of thioesterases II and III did not cause any growth defect. With CLA as the carbon source a very different result was observed. Growth was slower in the absence of thioesterase III but was further reduced when both thioesterases II and III were eliminated (see Figure 6B). The growth impairment attributable to the absence of thioesterase II is most likely smaller than the growth inhibition observed with the thioesterase I and II double mutant on CLA (see Figure 6B). This conclusion is supported by the growth inhibition observed with the thioesterase I and II double mutant but not with the thioesterase II and III double mutant on palmitate. Hence a thioesterase I mutation seems to negatively affect the growth on fatty acids in a manner that is not limited to the function of thioesterases in the β-oxidation of unsaturated fatty acids like CLA. Together, the results support the idea that both thioesterases II and III contribute to the hydrolysis of the CLA metabolite 3,5-dodecadienoyl-CoA but that other thioesterases, possibly those corresponding to peaks 1 and/or 2 in Figure 4, also function in the thioesterase-dependent pathway of β-oxidation in *E. coli.*

DISCUSSION

β-Oxidation of fatty acids in *E. coli* is carried out by a simple system of enzymes, which in part are organized as a multienzyme complex (*16*). Expression of the β-oxidation enzymes is induced by long-chain fatty acids that provide all energy and all carbon precursors when *E. coli* is grown on fatty acids as the sole carbon source. The simplicity of this β-oxidation system and the ease of generating mutants make *E. coli* an ideal organism to study the degradation of different fatty acids and to assess the existence, significance, and capacity of alternative pathways of β-oxidation.

We previously have reported that β-oxidation of oleic acid in *E. coli* proceeds by a major pathway and a minor pathway; the major pathway is the classical or isomerase-dependent pathway that accounts for 90% of oleate degradation, whereas 10% of oleate is degraded by the alternative or thioesterase-dependent pathway (*1*). 3,5-Tetradecadienoyl-CoA, a metabolite of the alternative pathway, cannot be further degraded by β-oxidation because the required enzyme, dienoyl-CoA isomerase, is not present in *E. coli*. Instead, 3,5-tetradecadienoyl-CoA is hydrolyzed, and the resultant 3,5-tetradecadienoic acid is released into the growth medium. This observation raised the question if growth of *E. coli* can be supported by a fatty acid that only is partially degraded via the thioesterase-dependent pathway. Is the thioesterase activity in *E. coli* high enough to permit a rapid flux through such pathway without being so high that β-oxidation is inhibited by the hydrolysis of the fatty acyl-CoA substrate and/or its degradable metabolites? To address these questions, CLA was tested as a carbon source for growth of *E. coli.*

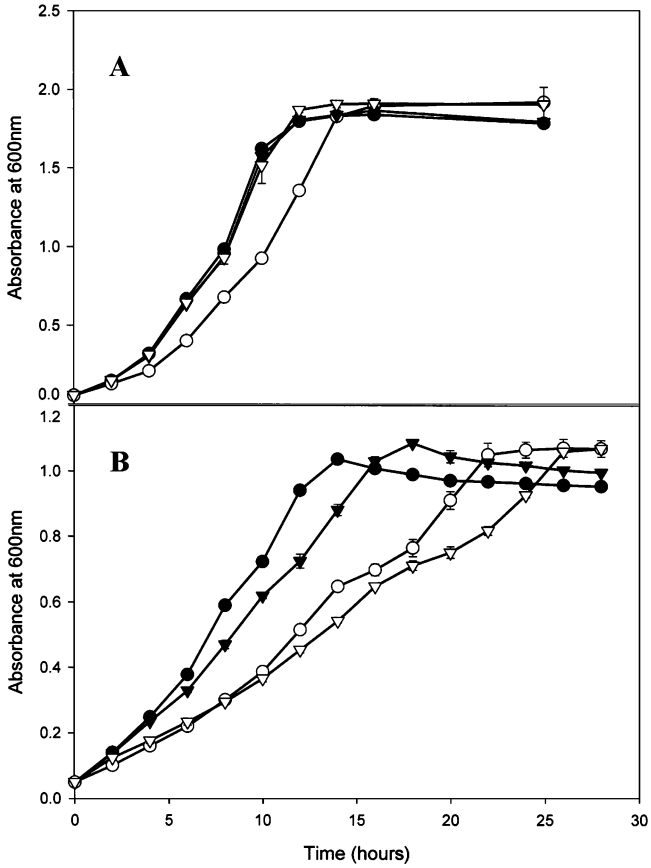


FIGURE 6: Growth curves of wild-type *E. coli* and thioesterase mutants on palmitic acid (A) or conjugated linoleic acid (B): (●) wild-type *E. coli*; (○) TE I & II mutant; (▼) TE III mutant; (▽) TE II & III mutant. Values of absorbance at 600 nm are means of three samples ± SD. For explanation of mutants, see legend to Figure 5.

CLA was chosen because it is thought to be chain shortened by three cycles of β-oxidation to yield 3,5-dodecadienoyl-CoA that cannot be degraded any further because of the absence of dienoyl-CoA isomerase from *E. coli.* 3,5-Dodecadienoyl-CoA might then be hydrolyzed to 3,5-dodecadienoic acid, which would be released into the growth medium. The data show that *E. coli* grows on CLA as the sole carbon source, even though the growth is slower than on oleic acid and proceeds to a lower cell density. Furthermore, 3,5-dodecadienoic acid, the expected final product of CLA β-oxidation in *E. coli*, was identified in the growth medium, thus confirming the predicted pathway of β-oxidation for CLA. The reason for *E. coli* growing slower on CLA than on oleate could be the reduced production of acetyl-CoA from the former fatty acid but also could be due to the slower degradation of CLA than oleic acid. It is conceivable that the hydrolysis of 3,5-dodecadienoyl-CoA limits the rate of CLA β-oxidation, but it is also possible that other steps of the pathway are slowed down because of the presence of two conjugated double bonds in CLA. However, the growth impairment observed with the thioesterase mutants suggests that the hydrolysis of 3,5-dodecadienoyl-CoA limits the rate of the thioesterase-dependent pathway of CLA β-oxidation, at least in the absence of one of the several thioesterases involved in the process. The reduced cell density at stationary phase is most likely due to the more than 50% decrease in acetyl-CoA production when oleic acid is replaced by an equimolar amount of CLA as carbon source.

The least known aspect of the novel thioesterase-dependent pathway of β-oxidation is the intracellular hydrolysis of a terminal fatty acyl-CoA and the release of the resultant fatty acid into the growth medium. The hydrolytic enzymes catalyzing such reactions are acyl-CoA thioesterases that would have to be active with medium-chain and long-chain acyl-CoAs to function in CLA and oleate β-oxidation. At least three such enzymes, thioesterases I, II, and III, are known to exist in *E. coli*, and even though thioesterases I and II have been studied for a long time, their functions remain unknown (*5, 6*). It is unlikely that thioesterase I functions in the thioesterase-dependent pathway of β-oxidation because of its periplasmic location. However, thioesterases II and III might catalyze the hydrolysis of intracellular acyl-CoAs like 3,5-tetradecadienoyl-CoA and 3,5-dodecadienoyl-CoA, because both are highly active with these two compounds as substrates. An evaluation of several thioesterase mutants of *E. coli* demonstrated that deficiencies of thioesterases II and III caused the organism to grow slower on CLA but not on palmitic acid. Moreover, the absence of both thioesterases II and III impaired the growth more than a deficiency of either thioesterase alone. These observations support the conclusion that not one specific thioesterase, e.g., thioesterase III, is responsible for the terminal hydrolytic step of the pathway but that several thioesterases cooperate in catalyzing this reaction. In fact, the observed growth of *E. coli* in the absence of thioesterases II and III suggests that at least one or more additional thioesterases participate in this process.

Altogether, this work demonstrates the operation of a novel pathway of β-oxidation, named thioesterase-dependent pathway, in *E. coli*, which allows the organism to grow on a fatty acid that is only partially degraded. The key reaction of this pathway is the hydrolysis of a terminal fatty acyl-CoA by a group of thioesterases that regenerate free CoA fast enough to meet the continuous needs of β-oxidation and other metabolic pathways for this cofactor and that yield a partially degraded fatty acid, which is released into the growth medium. The hydrolysis of long-chain acyl-CoAs also prevents an inhibition of β-oxidation by metabolites, which was observed when 3,5-tetradecadienoyl-CoA was allowed to accumulate (*1*).

This study prompts the question of whether a thioesterase-dependent pathway of β-oxidation also operates in higher organisms. The answer is a qualified yes based on a recent study that demonstrated the hydrolysis of a small percentage of 5-*trans*-tetradecenoyl-CoA, a metabolite of elaidic acid, in rat liver mitochondria (*17*). Another fraction of 5-*trans*-tetradecenoyl-CoA was converted to 5-*trans*-tetradecenoyl-carnitine. Both the hydrolysis of 5-*trans*-tetradecenoyl-CoA and its conversion to the carnitine derivative were attributed to an elevated concentration of 5-*trans*-tetradecenoyl-CoA in mitochondria, which occurred without compromising mitochondrial respiration. The maximal capacity of the thioesterase-dependent pathway of mitochondrial β-oxidation has not been determined but might be limited by the ability of the organism to dispose of the product. If the product of this pathway is a long-chain fatty acid that is released into the circulation, it will remain bound to serum albumin until it has been metabolized by incorporation into lipids or by

conversion to a more water-soluble product that can be excreted as a solute. Future studies of β-oxidation in mitochondria should consider the possibility of β-oxidation proceeding via a thioesterase-dependent pathway even if the capacity of such pathway is limited.

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