

**Incomplete Beta Oxidation of  
Unsaturated Fatty Acids Facilitated by  
Thioesterases in *Escherichia coli***

**by**

**Lina Nie**

A dissertation submitted to the Graduate Faculty in Biochemistry in partial  
fulfillment of the requirements for the degree of Doctor of Philosophy

The City University of New York

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**ABSTRACT****Incomplete Beta Oxidation of Unsaturated Fatty Acids Facilitated by  
Thioesterases in *Escherichia coli***

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Lina Nie

Adviser: Professor Horst Schulz

An investigation of thioesterases involved in a novel pathway of  $\beta$ -oxidation, referred to as thioesterase-dependent pathway, led to the identification of a new thioesterase (thioesterase III) that is induced by growth of *E. coli* on oleic acid. This enzyme was partially purified and identified as the *ybaW* gene product by mass spectrometric analysis of tryptic peptides. The *ybaW* gene, which has a putative consensus sequence for binding the fatty acid degradation repressor, was cloned and expressed in *E. coli*. Thioesterase III was shown to be a long-chain acyl-CoA thioesterase that is most active with 3,5-tetradecadienoyl-CoA, a minor metabolite of oleate  $\beta$ -oxidation. Its substrate specificity and induction of expression by fatty acids agree with its proposed function in this pathway by hydrolyzing metabolites of  $\beta$ -oxidation that are resistant to further degradation and that would inhibit the flux through the pathway if allowed to accumulate. This pathway was further studied in *E. coli* with 9-*cis*,11-*trans*-octadecadienoic acid (conjugated linoleic acid) as substrate, which 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  $\beta$ -

oxidation of conjugated linoleic acid to 3,5-dodecadienoyl-CoA, which was hydrolyzed to 3,5-dodecadienoic acid and released from the cell. 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 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 growth even further, but did not abolish it completely. It is concluded that thioesterases II and III function in the partial degradation of conjugated linoleic acid via the thioesterase-dependent pathway of  $\beta$ -oxidation, which provides all energy and carbon precursors required for the growth of *E. coli*.

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**ABBREVIATIONS**

|                               |   |
|-------------------------------|---|
| ACP                           | acyl carrier protein;                                     |
| AP                            | alkaline phosphatase;                                     |
| CLA                           | conjugated linoleic acid;                                 |
| cml                           | chloramphenicol;  |
| cyclodextrin                  | methyl- $\beta$ -cyclodextrin;                            |
| DEAE                          | diethylaminoethyl;  |
| dienoyl-CoA isomerase         | $\Delta^{3,5},\Delta^{2,4}$ -dienoyl-CoA isomerase;       |
| enoyl-CoA isomerase           | $\Delta^3,\Delta^2$ -enoyl-CoA isomerase;                 |
| fadR                          | fatty acid degradation repressor;                         |
| FAO complex                   | multienzyme complex of fatty acid oxidation;              |
| GC/MS                         | gas chromatography in combination with mass spectrometry; |
| HPLC                          | high-performance liquid chromatography;                   |
| IPTG                          | isopropyl-beta-D-thiogalactopyranoside;                   |
| kan                           | kanamycin;  |
| KPi                           | potassium phosphate;                                      |
| MALDI                         | matrix-assisted laser desorption ionization;              |
| MS                            | mass spectrometry;  |
| NTA-Ni <sup>2+</sup> -agarose | nitrilotriacetic acid-Ni <sup>2+</sup> -agarose;          |
| PCR                           | polymerase chain reaction;                                |
| SDS-PAGE                      | sodium dodecylsulfate-polyacrylamide gel electrophoresis; |
| TBS                           | Tris buffered saline;                                     |
| TBST                          | Tris buffered saline with Tween;                          |

TE

thioesterase.

## INTRODUCTION

Fatty acids not only serve as a major source of energy in animals, but also are components of membranes, and precursors of hormones and intracellular messengers. Disorders of fatty acids  $\beta$ -oxidation in humans are associated with many diseases, such as inborn errors of fat metabolism, diabetes (1), cancer (2) and cardiovascular diseases (3,4).

Mitochondria and peroxisomes are two subcellular organelles that have the capacity to degrade fatty acids by the sequential removal of two-carbon units, a process called  $\beta$ -oxidation. In animal cells, fatty acids are degraded in both mitochondria and peroxisomes. Very long-chain fatty acids and methyl-branched carboxylic acids such as pristanic acid, dicarboxylic acids and hydroxylated cholestanic acids (5), all of which are poor substrates of mitochondrial  $\beta$ -oxidation, are partially degraded in peroxisomes and then excreted or completely degraded in mitochondria. Thus, in animals the biological role of the peroxisomal  $\beta$ -oxidation system is to partially degrade fatty acids that are prevented from entering mitochondria. However in lower eukaryotes, such as yeast, fatty acid  $\beta$ -oxidation only occurs in peroxisomes and results in the complete degradation of fatty acids. The degradation of fatty acids requires their prior conversion to fatty acyl-CoA thioesters in an ATP-dependent reaction catalyzed by acyl-CoA synthetases. The  $\beta$ -oxidation of fatty acyl-CoA thioesters proceeds through four sequential reactions that are catalyzed by distinct enzymes: the oxidation of a fatty acyl-CoA to a 2-enoyl-CoA; the hydration of the 2-enoyl-CoA to 3-hydroxyacyl-CoA; the dehydrogenation of the 3-hydroxyacyl-CoA to a 3-ketoacyl-CoA; the thiolysis of the 3-

ketoacyl-CoA to acetyl-CoA and a fatty acyl-CoA shortened by a two-carbon unit. In mitochondria, long-chain fatty acids are first partially degraded by the membrane-bound, very long-chain acyl-CoA dehydrogenase and trifunctional  $\beta$ -oxidation complex that contains the long-chain and medium-chain activities of enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase (see Fig. 1) (6). Chain-shortened fatty acyl-CoAs are completely degraded by the matrix system of soluble  $\beta$ -oxidation enzymes that have a preference for medium- and short-chain fatty acyl-CoAs. The corresponding enzymes of  $\beta$ -oxidation in peroxisomes are acyl-CoA oxidase, multifunctional enzyme 1 (MFE 1) / multifunctional enzyme 2 (MFE 2), 3-keto-acyl-CoA thiolase 1 and SCP-thiolase. Both MFE 1 and MFE 2 contain enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities but they form and act on 3-hydroxyacyl-CoA intermediates with C and D configuration, respectively. Additionally, MFE 1 contains  $\Delta^3, \Delta^2$ -enoyl-CoA isomerase (enoyl-CoA isomerase).

The degradation of fatty acids has also been studied in prokaryotes. In *E. coli*, activated fatty acids are dehydrogenated by acyl-CoA dehydrogenase to 2-*trans*-enoyl-CoA and then completely degraded by a multienzyme complex of fatty acid oxidation (FAO complex). This complex consists of two  $\alpha$ - and  $\beta$ -subunits each that form an  $\alpha_2\beta_2$  structure. The large  $\alpha$ -subunit contains the activities of enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, 3-hydroxyacyl-CoA epimerase and enoyl-CoA isomerase (7) while the smaller  $\beta$ -subunit contains of 3-ketoacyl-CoA thiolase (8). Only one additional auxiliary enzyme, 2,4-dienoyl-CoA reductase, is required for the metabolism of unsaturated fatty acids with even-numbered double bonds.

Fatty acids are predominantly unbranched and have an even number of carbon atoms. Most are between 12 and 22 carbons long. Saturated fatty acids predominate in solid animal fat such as butter, while vegetable oils contain more unsaturated fatty acids than saturated ones. More than half of them are unsaturated or polyunsaturated, which are required in the human diet, but cannot be synthesized by the body and must be obtained from food. Therefore, an in-depth understanding of the  $\beta$ -oxidation of unsaturated and polyunsaturated fatty acids is important for studying and interpreting abnormalities of fatty acid metabolism in humans.  $\beta$ -Oxidation of unsaturated fatty acids, which usually contain *cis* double bonds, requires the assistance of additional enzymes besides the enzymes necessary for  $\beta$ -oxidation of saturated fatty acids. When double bonds are closer to the  $\beta$ -carbon of the fatty acyl-CoA residue due to chain shortening (9), enoyl-CoA isomerase, 2,4-dienoyl-CoA reductase and  $\Delta^{3,5},\Delta^{2,4}$ -dienoyl-CoA isomerase (dienoyl-CoA isomerase) participate in the metabolism of double bonds. The degradation of unsaturated fatty acids with double bonds extending from even-numbered position is outlined in Scheme 1.

Oleic acid is a monounsaturated fatty acid that contains a chain consisting of 18 carbon atoms with a double bond between carbons 9 and 10. It occurs naturally in greater quantities than any other unsaturated fatty acid. It has been demonstrated that odd-numbered double bonds such as the 9-*cis* double bond, which is present in oleic acid and many other dietary fatty acids, can be degraded in animals by  $\beta$ -oxidation either via the well-established isomerase-dependent pathway or the reductase-dependent pathway

(10). As shown in Scheme 2, oleoyl-CoA (I) is chain-shortened to 5-*cis*-tetradecenoyl-CoA (II) by two cycles of  $\beta$ -oxidation. 2-*trans*, 5-*cis*-Tetradecadienoyl-CoA (III) is produced by the dehydrogenation of compound II by long-chain acyl-CoA dehydrogenase (11). After completing the cycle of  $\beta$ -oxidation, the resultant 3-*cis*-dodecenoyl-CoA (VI) is isomerized to 2-*trans*-dodecenoyl-CoA (VII) and finally completely degraded via the  $\beta$ -oxidation spiral. In this isomerase-dependent pathway, only enoyl-CoA isomerase is required as an auxiliary enzyme. However in mitochondria, oleic acid is also degraded by another pathway, a four-step reaction sequence named reductase-dependent pathway (10). 3,5-*cis*-Tetradecadienoyl-CoA (XI), which is formed from 2-*trans*,5-*cis*-tetradecadienoyl-CoA by enoyl-CoA isomerase, is converted to 2,4-*trans*-tetradecadienoyl-CoA (XII) by yet another auxiliary enzyme, dienoyl-CoA isomerase (12). Intermediate XII is reduced in an NADPH-dependent reaction catalyzed by 2, 4-dienoyl-CoA reductase to 3-*trans*-tetradecaenoyl-CoA (XIII), which is converted by enoyl-CoA isomerase to 2-*trans*-tetradecaenoyl-CoA (XIV) that is a substrate of the  $\beta$ -oxidation spiral and can be completely degraded by this process. Strictly speaking, the reductase-dependent pathway should be called dienoyl-CoA-isomerase-dependent pathway. A study with rat heart mitochondria demonstrated that more than 80% of oleate  $\beta$ -oxidation occurs via the classical isomerase-dependent pathway, whereas the reductase-dependent pathway is the minor pathway (13). However, the reductase-dependent pathway is essential for the degradation of 3, 5-*cis*-tetradecadienoyl-CoA, which is formed from the oleate metabolite 2-*trans*,5-*cis*-teradecadienoyl-CoA by enoyl-CoA isomerase that functions in the isomerase-dependent pathway. Without this pathway, 3, 5-dienoyl-CoAs would most likely accumulate and impair the oxidative function of

mitochondria because of a decline of free CoA and possibly because of an inhibition of some of the enzymes of  $\beta$ -oxidation. It was concluded that both pathways are essential for the degradation of unsaturated fatty acids with odd-numbered double bonds. The isomerase-dependent pathway facilitates the major flux through  $\beta$ -oxidation while the reductase-dependent pathway prevents the accumulation of an otherwise undegradable metabolite (13).

A study of oleate  $\beta$ -oxidation in *E. coli* demonstrated that 90% of the 2-*trans*, 5-*cis*-tetradecadienoyl-CoA was degraded via the isomerase-dependent pathway, while approximately 10% was converted to 3,5-tetradecadienoyl-CoA (14). However, no dienoyl-CoA isomerase was detected in *E. coli*, which is essential for the reductase-dependent pathway. It was observed that 3, 5-tetradecadienoyl-CoA (XI), if allowed to accumulate, would strongly inhibit  $\beta$ -oxidation of oleate. This intermediate is hydrolyzed by a thioesterase and the resultant 3, 5-tetradecadienoic acid is released into the growth medium (see Scheme 3). This observation provides evidence for a novel function of a thioesterase in  $\beta$ -oxidation.

In contrast to mammalian peroxisomes, where various fatty and carboxylic acids are partially degraded by a  $\beta$ -oxidation system, mitochondrial  $\beta$ -oxidation facilitates the complete degradation of fatty acids to acetyl-CoA without the accumulation of intermediates (6). Thus, acyl-CoA intermediates are usually not hydrolyzed by thioesterases that are present in mitochondria and therefore partially degraded fatty acids are normally not released from the organelle. The situation is similar in prokaryotic

organism as for example *E. coli*. However, when fatty acid oxidation is impaired by the inhibition or deficiency of a  $\beta$ -oxidation enzyme, partially degraded fatty acids, often in the form of acylcarnitines, are excreted from mitochondria (15,16). However, cases of incomplete degradation in mitochondrial or prokaryotic organism have been observed, that involve the release of partially degraded fatty acids without having a negative impact on the energy metabolism. One case is the partial degradation of elaidic acid, a *trans* fatty acid, in rat mitochondria, which is based on an earlier observation that perfusion of rat hearts with elaidic acids, but not with oleic acid, resulted in the appearance of 5-tetradecenoic acid in the perfusion medium without compromising the performance of the heart (17). A detailed analysis of elaidate  $\beta$ -oxidation in rat mitochondria revealed the accumulation of 5-*trans*-tetradecenoyl-CoA in the mitochondrial matrix because it was poorly acted upon by long-chain acyl-CoA dehydrogenase (18). Due to its accumulation, 5-*trans*-tetradecenoyl-CoA was hydrolyzed to 5-*trans*-tetradecenoic acid by thioesterase and also converted to 5-*trans*-tetradecenoylcarnitine by carnitine palmitoyltransferase. Both products were released from mitochondria.

Thioesterases are ubiquitous enzymes that hydrolyze acyl derivatives of coenzyme A (CoA), acyl carrier protein (ACP), and other thiol containing compounds including proteins and peptides that contain cysteine residues. The best understood function of a thioesterase is in mammalian fatty acid synthesis where the enzyme terminates the process of chain elongation by hydrolysis of fatty acyl-ACP. The proposed function of this thioesterase is supported by its existence as an integral part of the mammalian fatty acid synthetase, which is a multienzyme complex. The search for

an enzyme with a similar function in *E. coli* led to the identification and characterization of two long-chain acyl-CoA thioesterases referred to as thioesterases I and II (19-21). However, both thioesterases were shown to hydrolyze long-chain acyl-ACP much more slowly than the corresponding CoA derivatives (22) and hence are unlikely to function in fatty acid synthesis. Work done by Ying Ren in this laboratory suggested that thioesterase II is responsible for hydrolyzing 3,5-*cis*-tetradecadienoyl-CoA. Thioesterase I, which is encoded by the *tesA* gene, was identified as a periplasmic enzyme with a molecular weight at 22,000 Da (20,23). However, thioesterase II, which is encoded by the *tesB* gene (21), is a 122 kDa cytosolic homotetramer and therefore is positioned to hydrolyze fatty acyl-CoAs located in the cytoplasm. Secondly, the growth on oleate induces the expression of thioesterase II but not that of the type I enzyme (see Fig. 2) (14). Thirdly, the substrate profiles of a crude preparation of thioesterase II (see Fig. 3B) (14), which was induced by growth of *E. coli* on oleate, and of purified recombinant thioesterase II (see Fig. 4A), were almost identical. Both of them revealed that thioesterase II has a preference for long-chain fatty acyl-CoAs. Its highest activity was observed with 3, 5-*cis*-tetradecadienoyl-CoA as substrate. The hypothesis that thioesterase II may be the enzyme responsible for the hydrolysis of 3, 5-*cis*-tetradecadienoyl-CoA also agrees with the kinetic data (see Table 1) that were determined with the purified thioesterase II and myristoyl-CoA, 3, 5-*cis*-tetradecadienoyl-CoA and palmitoyl-CoA as substrates. This data revealed that the enzyme is most active with 3, 5-tetradecadienoyl-coA as substrate.

In an attempt to further evaluate the function of thioesterases in the  $\beta$ -oxidation of fatty acids in *E. coli*, the growth behaviors of wild-type *E. coli* and *E. coli* strain YR1 that is devoid of thioesterases I and II were compared by Yin Ren. As shown in Fig. 5A, 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 it grew slower than wild-type *E. coli* on oleate (Fig. 5B) but achieved the same cell density. This observation prompted the idea that thioesterase II either is not the only enzyme to hydrolyze 3,5-*cis*-tetradecadienoyl-CoA or perhaps does not act on this metabolite at all.

The specific aim of this project was to evaluate the function and capacity of the alternative pathway of  $\beta$ -oxidation in *E. coli* and to determine which thioesterase or 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. An additional aim was the identification and characterization of a novel thioesterase, ultimately named thioesterase III, which was detected in an *E. coli* strain able to grow on oleic acid despite the absence of thioesterases I and II.

## EXPERIMENTAL PROCEDURES

*Materials* – CoASH, acetyl-CoA, butyryl-CoA, octanoyl-CoA, decanoyl-CoA, dodecanoyl-CoA, tetradecanoyl-CoA, palmitoyl-CoA, stearoyl-CoA and isopropyl-beta-D-thiogalactopyranoside (IPTG) 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 Chemical (Ann Arbor, MI). 9-*cis*,11-*trans*-Octadecadienoic acid (conjugated linoleic acid) was obtained from Matreya, Inc. (Pleasant Gap, PA). BCl<sub>3</sub>-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. Sep-Pak C<sub>18</sub> cartridges used for concentrating acyl-CoAs and  $\mu$ Bondapak C<sub>18</sub> columns (30 cm x 3.9 mm) for high-performance liquid chromatography (HPLC) were purchased from Waters Associates (Milford, MA). Protein A-Sepharose<sup>TM</sup> CL-4B was purchased from Amersham Biosciences (Piscataway, NJ). Polyacrylamide ready gels, protein assay dye reagent, trans-Blot® transfer medium pure nitrocellulose membrane, alkaline phosphatase (AP)-conjugated goat anti-rabbit antibody, AP-conjugated substrate kit and hydroxylapatite were purchased from Bio-Rad Laboratories. Antisera to thioesterase II and thioesterase III from *E. coli* were raised in rabbits by Pocono Rabbit Farm and Laboratory, Inc. (Canadensis, PA). Amicon\* Ultrafree Centrifugal Filter Unit (NMWL of 10,000) and 0.22- $\mu$ m syringe driven filter membrane were from Millipore. Standard cellulose dialysis tubing was from Spectrum Medical Industries, Inc. Methyl- $\beta$ -cyclodextrin (cyclodextrin), diethylaminoethyl (DEAE)-cellulose, octyl Sepharose CL-4B, oleic acid, chloramphenicol, kanamycin, acyl-CoA oxidase from *Arthrobacter* species and most of the standard biochemicals were

obtained from Sigma-Aldrich. QIAprep® spin miniprep kit, QIAquick PCR purification kit, QIAGEN genomic tips, pQE-81L expression vector and nitrilotriacetic acid-Ni<sup>2+</sup>-agarose (NTA-Ni<sup>2+</sup>-agarose) were bought from QIAGEN Inc. (Valencia, CA). Restriction endonucleases, T<sub>4</sub> DNA ligase, Taq DNA polymerase, and 2-long DNA ladder were obtained from New England Biolabs Inc. Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. XL10-Gold® ultracompetent cells were purchased from Stratagene. *E. coli* strain HC74 (*tesA::kan<sup>r</sup>* & *tesB::cml<sup>r</sup>*, *fadE*), and strain LE392 (*hasR*, *galK*, *trpR*, *metB*, *lacY*) were kindly provided by Dr. Stuart Smith (Children's Hospital Oakland Research Institute) and from the *E. coli* Genetic Stock Center (Yale University), respectively.

*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 (24). 3,5-*cis*-Dodecadienoyl-CoA and 3,5-*cis*-tetradecadienoyl-CoA were prepared from the corresponding 5-*cis*-enoyl-CoA thioesters by dehydrogenation with acyl-CoA oxidase as described by Ren and Schulz (13). 2-*trans*,5-*cis*-Tetradecadienoyl-CoA (13), 2-*trans*-tetradecenoyl-CoA (14), L-3-hydroxytetradecanoyl-CoA (14), 3-*trans*-tetradecenoyl-CoA (25), and 3-ketohexadecanoyl-CoA (26) were synthesized by published procedures. All acyl-CoA thioesters were analyzed and purified by reverse-phase HPLC on a Waters  $\mu$ Bondapak C<sub>18</sub> column (30cm x 3.9mm), 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.

*Isolation of fadE Revertants from E. coli Strain HC74* - A *fadE* mutation had been introduced into *E. coli* strain LE392 by use of the transposable element Tn10 (23). Subsequently, the  $\Delta tesA::kan^r$  (23) mutation followed by the  $\Delta tesB::cml^r$  (27) mutation had been introduced into this strain to create strain HC74 (27). Strain HC74 is unable to grow on fatty acids as the sole carbon source because  $\beta$ -oxidation is inactive due to the absence of functional electron transferring flavoprotein that is encoded by the *fadE* gene. For the purpose of creating an *E. coli* strain that retained the *tesA* and *tesB* mutations but was able to grow on fatty acids as the sole carbon source, spontaneous revertants of *fadE* were selected by plating strain HC74 on a medium that contained kanamycin and chloramphenicol and palmitic acid as the sole carbon source. Colonies detected after seven days of incubation were found to grow on long-chain fatty acids as the sole carbon source in the presence of both antibiotics. This strain was named YR1. It was always cultured in the presence of kanamycin (20  $\mu$ g/ml) and chloramphenicol (20 $\mu$ g/ml) that did not affect its growth behavior.

*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<sub>4</sub>, 10  $\mu$ M CaCl<sub>2</sub>, 1  $\mu$ M FeCl<sub>3</sub>, 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 growth medium without tryptone, which contained either glucose (0.1%, w/v), oleic acid (0.1%, v/v), palmitic acid (0.1%, w/v), or conjugated linoleic acid (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 absorbance change at 600 nm. For the preparation of cell extracts, cultures were harvested at an absorbance of 1 at 600 nm by centrifugation at 2,300 x 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 sec x 12) at 0 °C and centrifuged at 33,000 x g for 1 hr at 4 °C. The resultant supernatants were collected for enzyme and protein assays.

*Separation of E. coli Thioesterases on DEAE-cellulose* – *E. coli* cell pellets (1.5 g) grown on either glucose or oleate were suspended in 0.1 M  $KP_i$  (pH 7.0) containing 10% glycerol, sonicated for a total of 2 min (10 sec x 12) at 0 °C and centrifuged at 33,000 x g for 1 hr 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 x 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, and those with high activities were pooled, concentrated, and stored at -80 °C.

*Purification of E. coli Thioesterase III* – Thirty three grams of cell paste of *E. coli* strain YR1 grown on oleate were suspended in 70 ml of 0.1 M  $\text{KPi}$  (pH 7.0) containing 10% glycerol, sonicated for a total of 2 min (10 sec x 12) at 0 °C and centrifuged at 33,000 x g for 1 hr at 4 °C. The resultant supernatants were dialyzed overnight against 0.02 M Tris-HCl (pH 7.8) containing 10% glycerol (buffer A), and applied to a DEAE-cellulose column (41 x 2.5 cm) previously equilibrated with buffer A. The column was washed with 300 ml of buffer A containing 50 mM NaCl, and then developed with a linear gradient made up of 500 ml each of buffer A containing either 50 mM NaCl or 250 mM NaCl. Fractions eluted from the DEAE-cellulose column were collected and assayed for thioesterase activity with 20  $\mu\text{M}$  myristoyl-CoA as substrate, and those with high activity were combined, concentrated in an Amicon\* Ultrafree Centrifugal Filter Unit (NMWL of 10,000), and dialyzed against 0.02 M  $\text{KPi}$  (pH 6.8) containing 10% glycerol (buffer B) for 2 hours. After dialysis, the sample was applied to a hydroxylapatite column (11 x 2.5 cm), which had been equilibrated with buffer B. After washing the column with 250 ml of buffer B containing 0.1 M KCl, the column was developed with a gradient made up of 250 ml of buffer B containing 0.1 M KCl and 250 ml of 0.07 M  $\text{KPi}$  (pH 6.8) containing 10% glycerol and 0.1 M KCl. Active fractions were combined, concentrated, and dialyzed against 1 mM  $\text{KPi}$  (pH 7.0) containing 0.5 M  $(\text{NH}_4)_2\text{SO}_4$  (buffer C). The dialyzed sample was applied to an octyl Sepharose CL-4B column (3.0 x 1.0 cm) previously equilibrated with buffer C. The column was washed with a linear gradient made up of 15 ml of buffer C and 15 ml of 1 mM  $\text{KPi}$  (pH 7.0), washed with another 9 ml of 1 mM  $\text{KPi}$  (pH 7.0), and then developed with an ethanol gradient made up of 16 ml of 1 mM  $\text{KPi}$  (pH 7.0) and 16 ml of 1 mM  $\text{KPi}$  (pH 7.0)

containing 25% ethanol. Fractions with high thioesterase activity were combined, concentrated and, after dialysis against 0.02 M  $\text{KPi}$  (pH 7.0) containing 30% glycerol, stored at  $-80\text{ }^{\circ}\text{C}$ .

*Enzyme and Protein Assays* – Thioesterase activity was assayed by measuring the release of CoASH from acyl-CoAs with Ellman's reagent (28). A standard assay mixture contained 0.175 M  $\text{KPi}$  (pH 8.0), 0.2 mM 5,5'-dithiobis (2-nitrobenzoic acid) (Ellman's reagent), and 20  $\mu\text{M}$  acyl-CoA. The progress of the reaction was determined spectrophotometrically at 412 nm and rates were calculated using an extinction coefficient of  $13,600\text{ M}^{-1}\text{cm}^{-1}$ . Kinetic analyses were performed with recombinant 6xHis-tagged thioesterase III with either lauroyl-CoA, 3,5-*cis*-dodecadienoyl-CoA, myristoyl-CoA, 3,5-*cis*-tetradecadienoyl-CoA, 3-hydroxytetradecanoyl-CoA and palmitoyl-CoA as substrates. Kinetic constants ( $K_m$ ,  $V_{max}$ ) were obtained by nonlinear curve fitting using the SigmaPlot 2000 program. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the conversion of 1  $\mu\text{mol}$  of substrate to product in 1 min. Protein concentrations were determined by the dye-binding assay as described by Bradford (29) with bovine serum albumin as standard.

*Identification of Fatty Acid Metabolites in the Bacterial Growth Medium* – *E. coli* cells were grown to an absorbance of 1 at 600 nm in M9 medium containing oleic acid or 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  $2,300 \times g$  for 30 min at  $4\text{ }^{\circ}\text{C}$ . The supernatant was acidified to pH 1-2 with 2 N  $\text{H}_2\text{SO}_4$  and then extracted

three times with 100 ml ether each. The organic phase was extracted with aqueous sodium bicarbonate. After acidifying the aqueous phase with 2 N H<sub>2</sub>SO<sub>4</sub>, it was extracted three times with 8 ml 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<sub>2</sub>, was methylated by reacting it with 2 ml of BCl<sub>3</sub>-methanol (12%, w/w) for 10 min at 60 °C. After allowing the reaction mixture to cool down, 1 ml of H<sub>2</sub>O 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, a sample containing 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 hr. The reaction mixture was acidified to pH 1-2 with 2 N H<sub>2</sub>SO<sub>4</sub> 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) interfaced 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 °C 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.

*Sodium Dodecylsulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting* – Proteins were subjected to SDS-PAGE on ready gels at 80 V for 2.0 hours (30), and then either stained with Coomassie brilliant blue R or subjected to immunoblotting. For immunoblotting, the proteins were transferred from ready gels to nitrocellulose membranes by use of TransBlot® SD semi-dry electrophoretic transfer cell system with a current of 1.0 mA /cm<sup>2</sup> of gel for 1.5 hours. In order to block nonspecific binding sites, the transferred membrane was soaked in 5% non-fat milk in Tris buffered saline (TBS) for 1 hour at 37°C or overnight at 4°C. The membrane was washed three times with 0.2% non-fat milk in TBS, and then incubated with rabbit antiserum raised against *E. coli* thioesterase III or rabbit antiserum against *E. coli* thioesterase II (diluted 500-fold) for 1.5 hours under gentle shaking at room temperature. The membrane was washed three times with Tris buffered saline with Tween-20 (TBST), then incubated with the second antibody (3,000 times diluted AP-conjugated goat anti-rabbit antibody) for 1 hour. After that, the membrane was washed three times with TBST buffer, and then incubated with AP-conjugated substrate until bands appeared on the membrane.

*Peptide Mass Mapping* – The partially purified *E. coli* thioesterase III was fractionated by SDS-PAGE into six major components, three of which were subjected to matrix-assisted laser desorption ionization (MALDI) mass spectrometry at the Howard Hughes Medical Institute/ Columbia University. Proteins were reduced and alkylated, digested with trypsin, and the resulting peptide mixture was analyzed by MALDI-MS

without separation. The peptide masses obtained by MS were entered into a search program that scans the database, in most cases NCBI or Genpept, to find a match (31-34).

*Cloning, Expression, and Purification of Recombinant E. coli Thioesterase III* – *E. coli* thioesterase III was cloned, expressed, and purified to homogeneity. Purified genomic DNA, which was isolated from *E. coli* strain YR1 by QIAGEN genomic tips, was used for the amplification of the *ybaW* gene by polymerase chain reaction (PCR). The primers used were 5'-TTGGGATCCGCAAACACAAATCAAAG-3' and 5'-GGGCTGCAGTAATTATTCGGGTGTC-3'. The PCR product was purified by use of the QIAquick PCR purification kit, digested with *Bam*HI and *Pst*I, purified by agarose gel electrophoresis, and ligated into plasmid pQE-81L that had been cut with the same restriction enzymes to form a new expression plasmid. The 6xHis-tag was placed on the N-terminus of the mature protein. The resultant vector containing the coding sequence for the 6xHis-tagged form of putative thioesterase III was amplified in XL10-Gold® ultracompetent *E. coli* cells, purified, and used to transform *E. coli* BL21 cells according to the method of Chung et al. (35). Transformants were grown on LB medium to an absorbance of 0.7 at 600 nm and the expression of putative thioesterase III was induced by the addition of 1 mM IPTG to the cell suspension for 4 hours at 37 °C under shaking at 200 rpm. Cells were harvested by centrifugation, disrupted by sonication and centrifuged to obtain an extract of soluble proteins, which was expected to contain the *ybaW* gene product. The soluble extract was purified by chromatography on an NTA-Ni<sup>2+</sup> column, and in some cases was further purified by column chromatographies on DEAE-cellulose and octyl Sepharose CL-4B. Fractions were assayed for thioesterase

activity with myristoyl-CoA as substrate and those with high thioesterase activity were combined, concentrated and, stored at -80 °C after dialysis against 0.05 M KPi (pH 7.4) containing 30% glycerol.

*Construction of E. coli Strains with ybaW or/and tesB Deletion(s)* – Strains containing a deletion of the *ybaW* gene ( $\Delta ybaW$ ) or/and *tesB* gene ( $\Delta tesB$ ) were constructed by the method of Datsenko and Wanner (36). 50-bp Primers were synthesized, which were homologous to regions adjacent to the *ybaW* gene encoding thioesterase III or *tesB* gene and to regions of the template plasmid pKD3 that carried the chloramphenicol resistance gene. These primers were used to amplify by PCR the chloramphenicol resistance gene of plasmid pKD3 (36) 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-inducible  $\lambda$ Red helper plasmid (36). Transformants were selected on LB medium containing 20  $\mu$ g/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 sec x 12) at 0 °C and centrifuged at 33,000 x g for 1 hr 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 x 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  $\mu$ l of protein A Sepharose<sup>TM</sup> CL-4B agarose, 50  $\mu$ l of primary antiserum (rabbit anti-*E. coli* thioesterase II or anti-*E. coli* thioesterase III antiserum), and 300  $\mu$ l 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 hour at 4 °C under gentle shaking. The sample was centrifuged at 10,000 x g for 1 min, and after careful removal of the supernatant, the beads were washed with 1 ml washing buffer for at least three times. One ml of *E. coli* cell extract after partial purification on DEAE-cellulose was added to the tube and this mixture was incubated for 1.5 hours at 4 °C. The immunoprecipitated complex was collected by centrifugation at 10,000 x g for 1 min and washed five times with 1 ml washing buffer. After the last wash, the pellet was suspended in 50  $\mu$ l of SDS-PAGE sample buffer to a final concentration of 1x sample buffer, and then vortexed and heated at 95 °C for 10 min. After centrifugation for 1 min at 10,000 x g at room temperature, the solubilized immunoprecipitate was collected and kept frozen for SDS-PAGE and

immunoblotting using rabbit anti-*E. coli* thioesterase II or anti-*E. coli* thioesterase III antiserum as described above.

## RESULTS

### **Part I: Identification and Characterization of *E. coli* Thioesterase III That Functions in Fatty Acid $\beta$ -Oxidation**

*Partial Purification of a Novel Thioesterase* – In an attempt to determine if thioesterase II is essential for growth of *E. coli* on oleic acid, strain YR1, which is devoid of thioesterases I and II due to deletion mutations in *tesA* and *tesB*, was cultured on oleic acid as the sole carbon source. This thioesterase mutant grew on oleate and, most importantly, produced 3,5-tetradecadienoic acid that was detected in the growth medium. Acidic compounds were extracted from the growth medium, converted to methyl esters, and analyzed by GC/MS. As shown in Fig. 6A, many acidic compounds were present in the growth medium and the methyl esters of several of them were detected in the 18-20 min region where methyl 3,5-tetradecadienoate would be eluted (Fig. 6B). Mass spectra of the materials corresponding to peaks 1-4 were almost identical and all showed a molecular ion with mass-to-charge ratios ( $m/z$ ) of 238 as expected of methyl 3,5-tetradecadienoate. Shown in Fig. 6C is the mass spectrum of the material corresponding to peak 3, which is identical with the spectrum of synthetic methyl 3,5-tetradecadienoate (14). The formation of 3,5-tetradecadienoate by strain YR1, which is devoid of thioesterases I and II, is suggestive of the presence of one or more additional thioesterases in *E. coli*. Direct evidence in support of this idea was obtained by assaying a soluble extract of mutant YR1 cells grown on oleate and detecting a low but significant myristoyl-CoA thioesterase activity of 4.6 milliunits/mg of protein (see Table 2). Hence this *E. coli* strain must contain one or more additional enzymes with myristoyl-CoA

thioesterase activity. The extract was subjected to column chromatography on DEAE-cellulose from which slightly more than half of the myristoyl-CoA thioesterase activity was eluted by a NaCl gradient as a symmetrical peak (see Fig. 7). The myristoyl-CoA thioesterase activity corresponding to this peak was absent when the same *E. coli* strain was grown on glucose (Fig. 8A) or tryptone (data not shown) as carbon source. This myristoyl-CoA thioesterase, henceforth referred to as novel thioesterase, was further purified by column chromatography on hydroxylapatite (see Fig. 9) followed by chromatography on an octyl-Sepharose CL-4B column. The novel thioesterase was eluted from octyl-Sepharose CL-4B with a 0 to 25% ethanol gradient (see Fig. 10). This purification step resulted in a 26-fold increase of its specific thioesterase activity and led to the removal of most, but not all proteins that were unrelated to this enzyme. As summarized in Table 2, the novel thioesterase was purified almost 900-fold and was obtained in 10% yield based on the total myristoyl-CoA thioesterase activity present in the cell extract. SDS-PAGE analysis of this thioesterase preparation revealed the presence of close to ten protein bands with six bands considered to be major ones (see Fig. 11).

*Identification of the Gene Encoding the Novel Thioesterase* – The proteins corresponding to bands marked 1-3 (see Fig. 11) were considered the more likely ones to have thioesterase activity because they were the most prominent bands. Proteins associated with bands 1-3 were partially digested with trypsin and the resultant peptides were analyzed by MALDI mass spectrometry. Searches of databases for matching sequences revealed the presence of two proteins in each of bands 1 and 3 and of three proteins in band 2 (see Table 3). Each of the three bands contained the same protein of

unknown function while the other proteins were well known housekeeping enzymes like isocitrate dehydrogenase, tryptophanase, aldehyde dehydrogenase, and phenylalanine tRNA synthase that are not known to have thioesterase activity. The protein of unknown function, which was only a minor component of the band 2 material, but a major component of the proteins associated with bands 1 and 3, is encoded by the *ybaW* gene. Shown in Fig. 12 is the predicted sequence of the *ybaW* gene product that is comprised of 132 amino acid residues and has a molecular mass of close to 15 kDa. Also shown are the peptides that were derived from the partially purified thioesterase and identified by MALDI mass spectrometry. All peptides marked in Fig. 12 were identified in the tryptic digest of material corresponding to band 1 while the proteins of bands 2 and 3 yielded some of the peptides from the N-terminal and C-terminal regions of the *ybaW* protein.

Upstream of the *ybaW* coding region, extending from nucleotide -49 to -33, is a 17 base pair sequence with 53% identity to the consensus sequence for fatty acid degradation repressor (*fadR*)-binding sites (see Fig. 13). The identity of well characterized *fadR*-binding sites with the consensus-binding sequence is 70% to 85% (24). The existence of a *fadR*-binding site upstream of the *ybaW* coding region explains why the novel thioesterase is expressed when *E. coli* is grown on oleic acid as the sole carbon source but not when grown on glucose (see Fig. 8A) or tryptone (data not shown).

#### *Molecular Cloning, Expression, and Partial Characterization of Thioesterase III*

– It remained to be demonstrated that the novel thioesterase isolated from *E. coli* strain YR1 and the *ybaW* gene product are one and the same protein. For this purpose, the *ybaW* gene was cloned and the encoded protein was expressed in *E. coli* as described under Experimental Procedures. After purification on an NTA-Ni<sup>2+</sup> column, the 6xHis-

tagged *ybaW* protein had a specific myristoyl-CoA thioesterase activity of 20 units/mg compared to 4.1 units/mg of the partially purified novel thioesterase preparation. These two thioesterase preparations were compared by SDS-PAGE and immunoblotting. The purified 6xHis-tagged *ybaW* protein gave rise to three bands on SDS-PAGE with the fastest moving material corresponding to the 16 kDa-subunit of the protein while the two slower moving bands corresponded to proteins with estimated molecular masses of 43 kDa and 53 kDa (see Fig. 14, lanes 2). The same pattern of bands was observed when the *ybaW* protein was subjected to immunoblotting (Fig. 14, lane 4). The immunoblot result is not surprising because the 6xHis-tagged *ybaW* protein was used to raise the antiserum. The immunoblot of the partially purified novel thioesterase resembled the blot obtained with the *ybaW* protein except that the proteins of the former preparation moved slightly faster than the recombinant proteins (compare lanes 4 and 5 of Fig. 14) because they lacked the 6xHis tags. The partially purified novel thioesterase gave rise to more than six bands on SDS-PAGE (see lane 3 of Fig. 14); three of these bands were also detected on the immunoblot, (compare lanes 3 and 5 of Fig. 14). Because the materials corresponding to three separate bands of the partially purified novel thioesterase had yielded peptides that matched the sequence of the *ybaW* protein, all seem to be related to the *ybaW* protein. However the identity of the materials with molecular masses larger than 15 kDa needed to be established more rigorously. For this purpose, the materials corresponding to the two slower-moving bands of the *ybaW* protein were subjected to limited proteolysis and analyzed by MALDI-MS. Only peptides matching the sequence of the *ybaW* protein were detected. Hence the two slower moving materials of the *ybaW* protein and of the novel thioesterase must be multimeric forms of the protein. Activity

measurements together with the SDS-PAGE and immunoblot data strongly support the conclusion that the novel thioesterase and the *ybaW* gene product are the same protein that functions as a long-chain acyl-CoA thioesterase. Because this thioesterase is the third *E. coli* enzyme known to hydrolyze long-chain acyl-CoAs, we propose naming it thioesterase III.

The activity of thioesterase III was measured with different acyl-CoAs and the kinetic parameters of this enzyme were determined with the best substrates in an attempt to gain an understanding of its metabolic function. The substrate profile of thioesterase III (see Fig. 4B) shows that the activity of this enzyme increases with increasing length of the substrate's acyl chain. The enzyme is virtually inactive with substrates having acyl chains with less than twelve carbon atoms, but it exhibits increasing activity as the acyl chain increases from 12 carbon atoms of lauryl-CoA to 18 carbon atoms of stearoyl-CoA. The *9-cis* double bond in oleoyl-CoA makes it a better substrate than stearoyl-CoA. Most noteworthy is the fact that the best substrate is 3,5-tetradecadienoyl-CoA, a metabolite of oleic acid, that is hydrolyzed during oleate  $\beta$ -oxidation in *E. coli* (14). The next best of the substrates is 3,5-dodecadienoyl-CoA, a metabolite of *9-cis,11-trans*-octadecadienoyl-CoA (conjugated linoleic acid). Most other long-chain metabolites of  $\beta$ -oxidation were found to be poor or very poor substrates of thioesterase III except for 3-hydroxytetradecanoyl-CoA that is a substrate as good as palmitoyl-CoA and a much better substrate than myristoyl-CoA (tetradecanoyl-CoA). The kinetic parameters for the hydrolysis of the best substrates were determined and are shown in Table 4. The  $K_m$  values for the listed substrates, which are in the low micromolar range, vary only moderately (4-fold). Differences between activities with various substrates are mainly

due to different  $k_{\text{cat}}$  values. For example, the faster hydrolysis of 3,5-*cis*-dodecadienoyl-CoA compared to that of dodecanoyl-CoA (lauroyl-CoA) is due to a 13.5-fold higher  $k_{\text{cat}}$  value with the former substrate while the  $K_m$  values for the two substrates are nearly the same. The substrate specificity of thioesterase III prompts the conclusion that this enzyme is well suited to hydrolyze acyl-CoAs with long-chain acyl residues that either are saturated, carry a 3-hydroxy group, or have a 3,5 diene structural element.

## **Part II: A Novel Paradigm of Fatty Acid $\beta$ -Oxidation Exemplified by the Thioesterase-Catalyzed Partial Degradation of Conjugated Linoleic Acid That Fully Supports Growth of *E. coli***

*Growth of E. coli on Fatty Acids as the Sole Carbon Source* – Growth of *E. coli* is fully supported by the  $\beta$ -oxidation of oleic acid even though a fraction of this fatty acid is only partially degraded (14). 3,5-Tetradecadienoyl-CoA, a metabolite of oleic acid that is resistant to further  $\beta$ -oxidation, is hydrolyzed, presumably by a long-chain acyl-CoA thioesterase (thioesterase), and the resultant acid is released into the growth medium (14). In an attempt to evaluate the function of thioesterases in the  $\beta$ -oxidation of fatty acids in *E. coli*, the growth behaviors of wild-type *E. coli* (strain LE392) and *E. coli* strain YR1 that is devoid of thioesterases I and II were compared. As shown in Fig. 5A, 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 it grew slower than wild-type *E. coli* on oleate (Fig. 5B). The observation that *E. coli* can grow on oleic acid

in the absence of both known long-chain acyl-CoA thioesterases suggests that one or more thioesterases other than thioesterases I and II are operative in this organism.

*β-Oxidation of 9-Cis,11-Trans-Octadecadienoic Acid (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 conjugated linoleic acid (CLA). As shown in Fig. 5A, 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 density. According to the hypothetical pathway for the  $\beta$ -oxidation of CLA, shown in Scheme 4, this fatty acid, after conversion to its CoA thioester, passes three times through the  $\beta$ -oxidation cycle to yield 3-*cis*,5-*trans*-dodecadienoyl-CoA (3,5-dodecadienoyl-CoA) that is assumed to be resistant to further  $\beta$ -oxidation because of the absence of dienoyl-CoA isomerase from *E. coli* (14). Disposal of 3,5-dodecadienoyl-CoA might occur by its hydrolysis to 3,5-dodecadienoic acid followed by the release of the latter metabolite into the growth medium. If such alternative pathway, also referred to as thioesterase-dependent pathway (see Scheme 4), is operative in *E. coli*, only 3 moles of acetyl-CoA would be generated per mole of CLA compared to 7 and 8 moles 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 Fig. 5A), agrees with a reduced yield of acetyl-CoA due to the partial  $\beta$ -oxidation of CLA. Proof for the operation of the pathway shown in Scheme 4 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 gas chromatography (GC) and mass spectrometry (MS). The most prominent peak in the GC spectrum was observed at an elution time of close to 14 minutes (see Fig. 15A) in a region where methyl 3,5-dodecadienoate is expected to be eluted. An expanded spectrum of the region between 13 and 18 minutes is shown in Fig. 15B with the most prominent peak (Peak 1) centered at 13.7 minutes. The mass spectrum of the material corresponding to Peak 1 is shown in Fig. 15C. A mass-to-charge ratio of 210 of the presumed molecular ion is that of methyl 3,5-dodecadienoate. Furthermore, the mass spectrum shown in Fig. 15C is virtually identical with the spectrum of authentic methyl 3,5-dodecadienoate (Fig. 15D) that was prepared by hydrolysis of 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 Fig. 15D. It is likely that these materials are the methyl esters of stereoisomers of 3-*cis*,5-*trans*-dodecadienoic acid, the main metabolite of 9-*cis*,11-*trans*-octadecadienoic acid. The same products were identified when *E. coli* strain YR1, the thioesterase I and II double mutant, was grown on CLA, whereas growth of *E. coli* on either oleic acid or palmitic acids did not produce 3,5-dodecadienoic acid (data not shown). The identification of 3,5-dodecadienoic acid as the major acidic component in the growth medium proves that the  $\beta$ -oxidation of CLA proceeds by the thioesterase-dependent pathway shown in Scheme 4.

*Long-chain Acyl-CoA Thioesterases in E. coli* – The conclusion that CLA is degraded via the thioesterase-dependent pathway of  $\beta$ -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 thioesterases present in *E. coli* (23), only thioesterase II could be involved because thioesterase I is a periplasmic protein (23). The substrate specificity of thioesterase II (see Fig. 4A) agrees with its possible function in the thioesterase-dependent  $\beta$ -oxidation of CLA. It has a preference for substrates with long-chain acyl chains, but is most active with 3,5-tetradecadienoyl-CoA and highly active with 3,5-dodecadienoyl-CoA, which are metabolites of oleic acid and CLA, respectively. The disposal of these two intermediates of  $\beta$ -oxidation requires their thioesterase-catalyzed hydrolyses. When an extract of *E. coli* mutant YR1 that does not contain thioesterases I and II was assayed for thioesterase with myristoyl-CoA as substrate, a low level of activity was detected, especially in cells grown on oleate as the sole carbon source (see Table 5). Although the myristoyl-CoA thioesterase activity of the YR1 strain was much lower than the activity of the wild-type cells, a measurable level of activity was present that apparently was sufficient to support growth of the mutant on oleate, albeit at a slower rate than growth of wild-type cells (see Fig. 5B). Separation of soluble proteins extracted from the mutant YR1 by chromatography on a DEAE-cellulose column revealed the presence of two peaks of myristoyl-CoA thioesterase activity in cells grown on glucose and three peaks of activity in cells grown on oleate (see Fig. 8). The thioesterase corresponding to Peak 3 in Fig. 8B, which was eluted ahead of where thioesterase II would have emerged, was identified as a novel thioesterase that is encoded by the *ybaW* gene. This thioesterase, named thioesterase III, was identified, molecular cloned and purified as described in Part I. The substrate specificity of thioesterase III, shown in Fig. 4B, proves 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.

*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 in cooperation with thioesterase II functions in the thioesterase-catalyzed  $\beta$ -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 & III mutant) were created. The successful deletion of the two thioesterase genes was confirmed by immunoblotting. Shown in Fig. 16 are immunoblots that were obtained with preparations of thioesterase II or III, which had been partially purified by column chromatography of cell extracts on DEAE-cellulose followed by immunoprecipitation of the two thioesterases with antisera raised against them. The absence of thioesterases II, III or II & III from mutants TE I & II, TE III, and TE II & III, respectively, 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 & III mutant, and TE I & 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 Fig. 17A 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 & II mutant was most likely due to the absence of thioesterase I that is encoded by the *tesA* gene because the absence of both

thioesterase II and thioesterase III did not show 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 thioesterase II and III were eliminated (see Fig. 17B). 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 Fig. 17B). This conclusion is based on the growth inhibition that was observed with the thioesterase I and II double mutant, but not with the thioesterase II mutant when palmitate was the sole carbon source. Hence a thioesterase I mutation seems to negatively affect the growth on fatty acids in a manner that is unrelated to the function of thioesterases in the  $\beta$ -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 & 2 in Fig. 8, also function in the thioesterase-dependent pathway of  $\beta$ -oxidation in *E. coli*.

## DISCUSSION

$\beta$ -Oxidation of fatty acids in *E. coli* is carried out by a simple system of enzymes, several of which are organized as a multienzyme complex (5). Expression of the  $\beta$ -oxidation enzymes is induced by long-chain fatty acids that provide all energy and carbon precursors when *E. coli* is grown on fatty acids as the sole carbon source. The simplicity of this  $\beta$ -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  $\beta$ -oxidation.

It has been reported that the  $\beta$ -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 (14). 3,5-Tetradecadienoyl-CoA, a metabolite of the alternative pathway cannot be further degraded by  $\beta$ -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 result raised the question as to which long-chain acyl-CoA thioesterase catalyzes the hydrolysis of 3,5-tetradecadienoyl-CoA? Two of such enzymes, thioesterases I and II, are known to be expressed in *E. coli*, but only thioesterase II is located in the cytosol where  $\beta$ -oxidation takes place, whereas thioesterase I has a periplasmic location. In an attempt to identify the enzyme(s) that functions in oleate  $\beta$ -oxidation, the growth of *E. coli* strain YR1 with deletion mutations in *tesA* and *tesB* that encode thioesterase I and thioesterase II,

respectively, was investigated. Because *E. coli* YR1 cells grew on oleate as the sole carbon source and 3,5-tetradecadienoic acid was detected in the growth medium, a long-chain acyl-CoA thioesterase other than thioesterases I and II must be present and operative in this *E. coli* strain. The presence of such thioesterase was demonstrated by assaying a soluble extract of strain YR1 with myristoyl-CoA as substrate. This thioesterase, which was expressed when cells were grown on oleate, but not when grown on glucose or LB medium, was partially purified by a three-step purification procedure. Most effective was the purification by hydrophobic chromatography on octyl Sepharose CL-4B from which the enzyme was eluted with an ethanol gradient. This result is suggestive of an enzyme that is relatively hydrophobic for a soluble protein. Mass spectrometric analysis of peptides derived from several component proteins of the partially purified thioesterase provided evidence for this protein being encoded by the *ybaW* gene. This tentative conclusion was verified by cloning and expressing the *ybaW* gene, testing the enzymatic activity of the recombinant protein and demonstrating its immunological identity with the partially purified thioesterase. The 15-kDa monomer of this thioesterase, referred to as thioesterase III, was detected by SDS-PAGE, but so were two slower-moving forms that may reflect the presence of multimers. As part of a structural genomics project, the *ybaW* protein has been crystallized and its crystal structure has been solved by X-ray diffraction. That data were deposited in the Protein Data Bank (Kim et al., PDB file 1NJK), but have not been published. According to its crystal structure, the *ybaW* protein is a homotetramer with a hot dog-fold. The two slower moving forms of thioesterase III that were detected by SDS-PAGE under standard reducing conditions might be the dimer and tetramer of the protein. It is possible that the

hydrophobic property of thioesterase III inferred from its behavior during hydrophobic chromatography is related to or even is the cause of its incomplete dissociation in a boiling SDS solution. Such resistance of proteins to dissociation by SDS has previously been observed and attributed to a very stable structure (37).

The search for thioesterase III was prompted by the recognition that one or more additional long-chain acyl-CoA thioesterases must be present in an *E. coli* mutant that is devoid of thioesterases I and II, which were the only long-chain acyl-CoA thioesterases known to exist in this organism up to now. The identification of thioesterase III raises the question whether this enzyme participates in the  $\beta$ -oxidation of oleic acid? The substrate spectrum proves it to be a long-chain acyl-CoA thioesterase and because it hydrolyzes 3,5-tetradecadienoyl-CoA, which in fact is the best of all tested substrates, thioesterase III most likely functions in oleate  $\beta$ -oxidation. Another clue to the function of this enzyme is its induction when *E. coli* cells are grown on oleic acid as the sole carbon source. The underlying mechanism seems to be the fadR-dependent regulation of *ybaW* expression. The basis for this hypothesis is the identification of a potential consensus sequence for fadR binding upstream of the *ybaW* coding region. As with the regulation of other *fad* genes, fadR is thought to bind to this 17 bp sequence in the absence of fatty acids and block transcription. However, when fatty acids are present in the growth medium and especially when they are the only carbon source to supply energy and carbon precursors, long-chain fatty acyl-CoA will bind to fadR and displace it from the promoter thereby allowing transcription to proceed.

Given that 90% of oleic acid is completely degraded by  $\beta$ -oxidation and only 10% is converted to 3,5-tetradecadienoyl-CoA, it seems justified to ask how important the thioesterase-catalyzed hydrolysis of this metabolite is for  $\beta$ -oxidation to continue unimpeded? Although a definite answer is outstanding, it is reasonable to assume that in the absence of thioesterases 3,5-tetradecadienoyl-CoA would accumulate and thereby tie up free CoA that is required for  $\beta$ -oxidation. When finally no free CoA is left,  $\beta$ -oxidation and the energy production supported by this process would come to a halt. It is also possible that 3,5-tetradecadienoyl-CoA inhibits one or more enzymes of  $\beta$ -oxidation and thereby would inhibit  $\beta$ -oxidation before free CoA is depleted. In either situation, the hydrolysis of 3,5-tetradecadienoyl-CoA would be essential to maintain an optimal rate of  $\beta$ -oxidation and energy production.

In conclusion, a novel long-chain acyl-CoA thioesterase, named thioesterase III, is described that effectively hydrolyzes 3,5-tetradecadienoyl-CoA, a minor metabolite of oleic acid in *E. coli*, that would inhibit  $\beta$ -oxidation if allowed to accumulate. Thioesterase III is induced when *E. coli* is grown on long-chain fatty acids and seems to function in the thioesterase-dependent pathway of  $\beta$ -oxidation.

It has been reported that the  $\beta$ -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 (14). 3,5-Tetradecadienoyl-CoA, a metabolite of the alternative pathway cannot be further

degraded by  $\beta$ -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 also raised the question if a fatty acid that is only partially degraded via the thioesterase-dependent pathway can support the growth of *E. coli*? Is the thioesterase activity in *E. coli* high enough to permit a rapid flux through such pathway without being so high that  $\beta$ -oxidation is inhibited by the hydrolysis of all  $\beta$ -oxidation intermediates? To address these questions, CLA (9-*cis*,11-*trans*-octadecadienoic acid) was tested as a carbon source for growth of *E. coli*. CLA is predicted to be chain shortened by three cycles of  $\beta$ -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 be hydrolyzed to 3,5-dodecadienoic acid that would be released into the growth medium. The data shows 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  $\beta$ -oxidation in *E. coli*, was identified in the growth medium, thus confirming the predicted pathway of  $\beta$ -oxidation for CLA. The slower growth of *E. coli* on CLA as compared to its growth on oleate could be due to the reduced production of acetyl-CoA from the former fatty acid, but could also be the consequence of CLA being degraded at a slower rate than is oleic acid. It is possible that the hydrolysis of 3,5-dodecadienoyl-CoA limits the rate of CLA  $\beta$ -oxidation, but it is also conceivable 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  $\beta$ -oxidation. 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 CLA as the carbon source.

The least known aspect of the novel thioesterase-dependent pathway of  $\beta$ -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  $\beta$ -oxidation. At least three of 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 (23,27). It is unlikely that thioesterase I functions in the thioesterase-dependent pathway of  $\beta$ -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 one or more additional thioesterases participate in this process.

Altogether, this work demonstrates the operation of a novel pathway of  $\beta$ -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 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  $\beta$ -oxidation for this cofactor and to yield a partially degraded fatty acid that is released into the growth medium.

An interesting question is whether a thioesterase-dependent pathway of  $\beta$ -oxidation exists and is operative 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 (18). Another fraction of 5-*trans*-tetradecenoyl-CoA was converted to its carnitine derivative and both diversions of 5-*trans*-tetradecenoyl-CoA from  $\beta$ -oxidation were the result of an elevated intramitochondrial concentration of 5-*cis*-tetradecenoyl-CoA, which occurred without compromising the rate of mitochondrial respiration. The maximal capacity of the thioesterase-dependent pathway of  $\beta$ -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 degradation to a more water-soluble product that can be excreted as a solute. Future

studies of  $\beta$ -oxidation in mitochondria should take into consideration the operation of a thioesterase-dependent pathway even if such pathway has only a limited capacity.

Table 1

**Kinetic parameters of *E. coli* thioesterase II \***

| Substrate                             | $V_{\max}$ (U/mg) | $K_m$ ( $\mu$ M) | $k_{\text{cat}}$ ( $s^{-1}$ ) | $k_{\text{cat}}/K_m$ ( $\mu\text{M}^{-1}\text{s}^{-1}$ ) |
|---------------------------------------|-------------------|------------------|-------------------------------|--|
| Myristoyl-CoA                         | 60 $\pm$ 4        | 50 $\pm$ 6       | 30.5 $\pm$ 2                  | 0.6  |
| 3,5- <i>cis</i> -Tetradecadienoyl-CoA | 91 $\pm$ 6        | 7 $\pm$ 1        | 46 $\pm$ 3                    | 6.6  |
| Pamitoyl-CoA                          | 166 $\pm$ 17      | 46 $\pm$ 7       | 84 $\pm$ 9                    | 1.8  |

\* Y.Ren and H. Schulz, unpublished observation

Table 2

**Purification of a novel thioesterase from *E. coli***

| Step                         | Total Protein (mg) | Total Activity (mU) | Specific Activity (mU/mg) | Purification (-fold) | Yield (%) |
|------------------------------|--------------------|---------------------|---------------------------|----------------------|-----------|
| Cell pellet                  | 33,000             |                     |                           |                      |           |
| Cell extract(after dialysis) | 1,051              | 4,836               | 4.60                      | 1                    | 100       |
| DEAE-cellulose               | 95                 | 2,148               | 22.61                     | 4.92                 | 44        |
| Hydroxylapatite              | 6.65               | 1,044               | 156.99                    | 34.13                | 22        |
| Octyl – Sepharose CL-4B      | 0.12               | 507                 | 4109                      | 893                  | 10        |

Table 3

**Identification of proteins by peptide analysis via MALDI mass spectrometry**

| Band | Protein Identified                | SwissProt# |
|------|-----------------------------------|------------|
| 1    | aldehyde dehydrogenase            | P25553     |
|      | hypothetical protein ybaW         | P77712     |
| 2    | isocitrate dehydrogenase          | P08200     |
|      | tryptophanase                     | P0A853     |
|      | hypothetical protein ybaW (minor) | P77712     |
| 3    | phenylalanine tRNA synthase       | P08312     |
|      | hypothetical protein ybaW         | P77712     |

Bands were reduced and alkylated, digested with trypsin, and analyzed by MALDI-MS.

Table 4

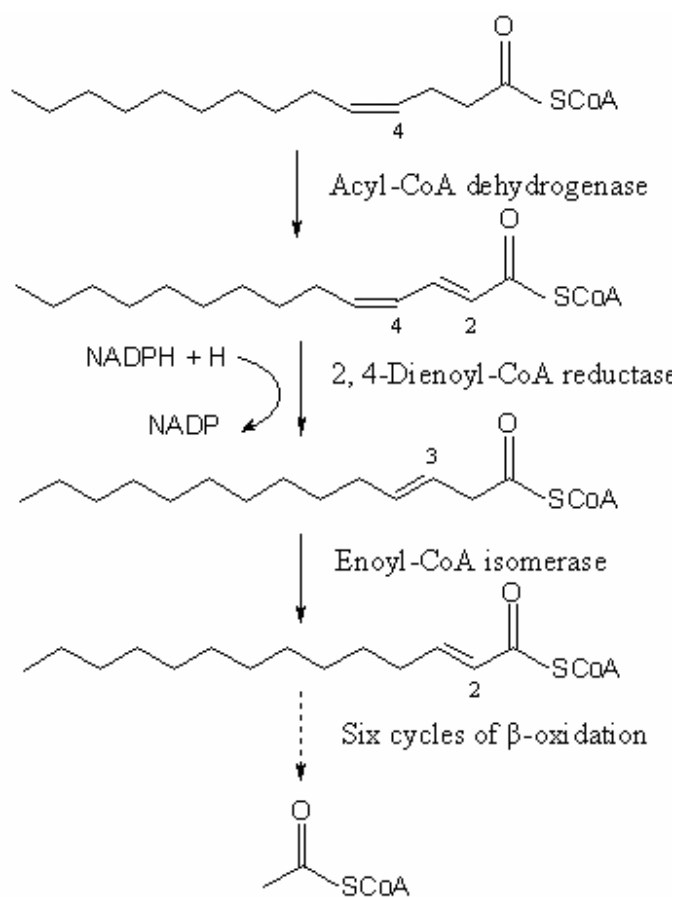
**Kinetic parameters of thioesterase III from *E. coli***

| Substrate                             | $V_{\max}$ (U/mg) | $K_m$ ( $\mu$ M) | $k_{\text{cat}}$ ( $\text{s}^{-1}$ ) | $k_{\text{cat}}/K_m$ ( $\mu\text{M}^{-1}\text{s}^{-1}$ ) |
|---------------------------------------|-------------------|------------------|--------------------------------------|--|
| Lauroyl-CoA                           | 6.9 $\pm$ 0.2     | 13.0 $\pm$ 1.1   | 1.6 $\pm$ 0.04                       | 0.1  |
| 3,5- <i>cis</i> -Dodecadienoyl-CoA    | 92.3 $\pm$ 1.5    | 10.8 $\pm$ 0.5   | 21.6 $\pm$ 0.4                       | 2.0  |
| Myristoyl-CoA                         | 26.4 $\pm$ 0.7    | 6.3 $\pm$ 0.6    | 6.2 $\pm$ 0.2                        | 1.0  |
| 3,5- <i>cis</i> -Tetradecadienoyl-CoA | 101.3 $\pm$ 2.2   | 3.0 $\pm$ 0.3    | 23.7 $\pm$ 0.5                       | 7.9  |
| 3-hydroxytetradecanoyl-CoA            | 45.5 $\pm$ 1.6    | 5.8 $\pm$ 0.7    | 10.7 $\pm$ 0.4                       | 1.8  |
| Pamitoyl-CoA                          | 43.5 $\pm$ 2.2    | 6.0 $\pm$ 0.9    | 10.2 $\pm$ 0.5                       | 1.7  |

Table 5

**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**

| Growth Medium | Thioesterase Activity (mU/mg) |                |
|---------------|-------------------------------|----------------|
|               | Wild-type (LE 392)            | Mutant (YR 1)  |
| LB            | $21.5 \pm 1.6$                | $\geq 0$       |
| Glucose       | $26.2 \pm 1.7$                | $0.5 \pm 0.03$ |
| Oleate        | $32.6 \pm 2.3$                | $1.8 \pm 0.1$  |

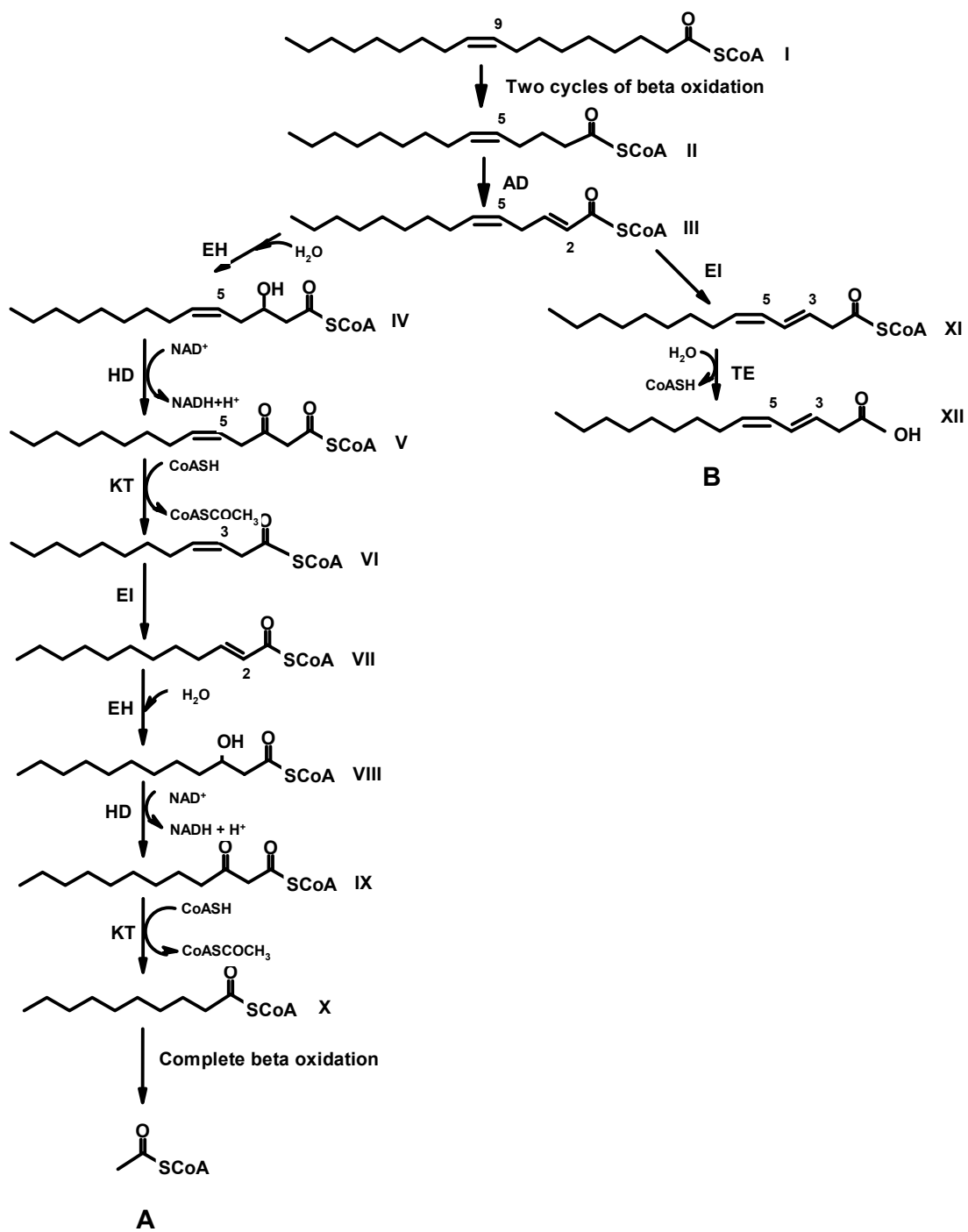


Scheme 1.  $\beta$ -Oxidation of unsaturated fatty acids with even-numbered double bonds

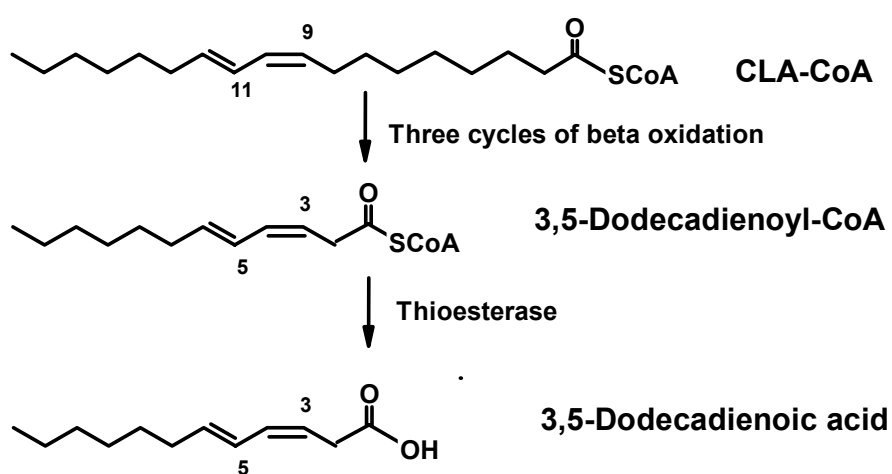
Scheme 2.  **$\beta$ -Oxidation of oleoyl-CoA in rat mitochondria.** *A*, isomerase-dependent pathway; *B*, reductase-dependent pathway. *AD*, acyl-CoA dehydrogenase; *EH*, enoyl-CoA hydratase; *HD*, L-3-hydroxyacyl-CoA dehydrogenase; *KT*, 3-ketoacyl-CoA thiolase; *EI*,  $\Delta^3, \Delta^2$ -enoyl-CoA isomerase; *DI*,  $\Delta^{3,5}, \Delta^{2,4}$ -dienoyl-CoA isomerase; *DR*, 2,4-dienoyl-CoA reductase. (Adapted from Ren, Y., and Schulz, H. (2003) *J. Biol. Chem.* **278**, 111-116)



Scheme 3.  **$\beta$ -Oxidation of oleoyl-CoA in *E. coli*.** *A*, classical or isomerase-dependent pathway; *B*, alternative pathway. *AD*, acyl-CoA dehydrogenase; *EH*, enoyl-CoA hydratase; *HD*, L-3-hydroxyacyl-CoA dehydrogenase; *KT*, 3-ketoacyl-CoA thiolase; *EI*,  $\Delta^3, \Delta^2$ -enoyl-CoA isomerase; *TE*, thioesterase. (Adapted from Ren, Y., Aguirre, J., Ntamack, A., Chu, C., and Schulz, H. (2004) *J. Biol. Chem.* **279**, 11042-11050)



Scheme 3



Scheme 4. **Proposed pathway for the  $\beta$ -oxidation of conjugated linoleic acid in *E. coli*.** Abbreviation: CLA-CoA, conjugated linoleoyl-CoA

**Fig. 1. Model of the functional and physical organization of  $\beta$ -oxidation enzymes in mitochondria.** (A)  $\beta$ -Oxidation system active with long-chain (LC) acyl-CoAs; (B)  $\beta$ -oxidation system active with medium-chain (MC) and short-chain (SC) acyl-CoAs. Abbreviations: T, carnitine:acylcarnitine translocase; CPT II, carnitine palmitoyltransferase II; AD, acyl-CoA dehydrogenase; EH, enoyl-CoA hydratase; HD, L-3-hydroxyacyl-CoA dehydrogenase; KT, 3-ketoacyl-CoA thiolase; VLC, very long-chain. (Adapted from Liang, X., Le, W., Zhang, D. and Schulz, H. (2001) *Biochem. Soc. Trans.* **29**, 279-282)

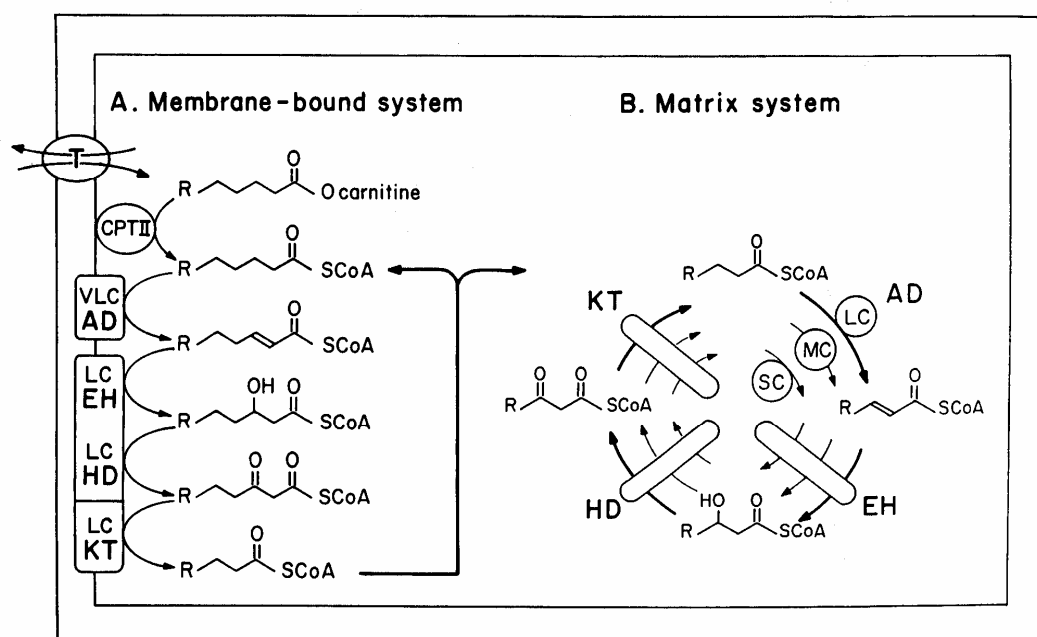


Fig. 1

Fig. 2. **Separation of *E. coli* thioesterases on DEAE-cellulose.** Extracts of soluble proteins from *E. coli* cells grown on either oleate (panel A) or glucose (panel B) as the sole carbon source were subjected to column chromatography on DEAE-cellulose. Fractions were assayed for thioesterase with tetradecanoyl-CoA (myristoyl-CoA) as substrate. (Adapted from Ren, Y., Aguirre, J., Ntamack, A., Chu, C., and Schulz, H. (2004) *J. Biol. Chem.* **279**, 11042-11050)

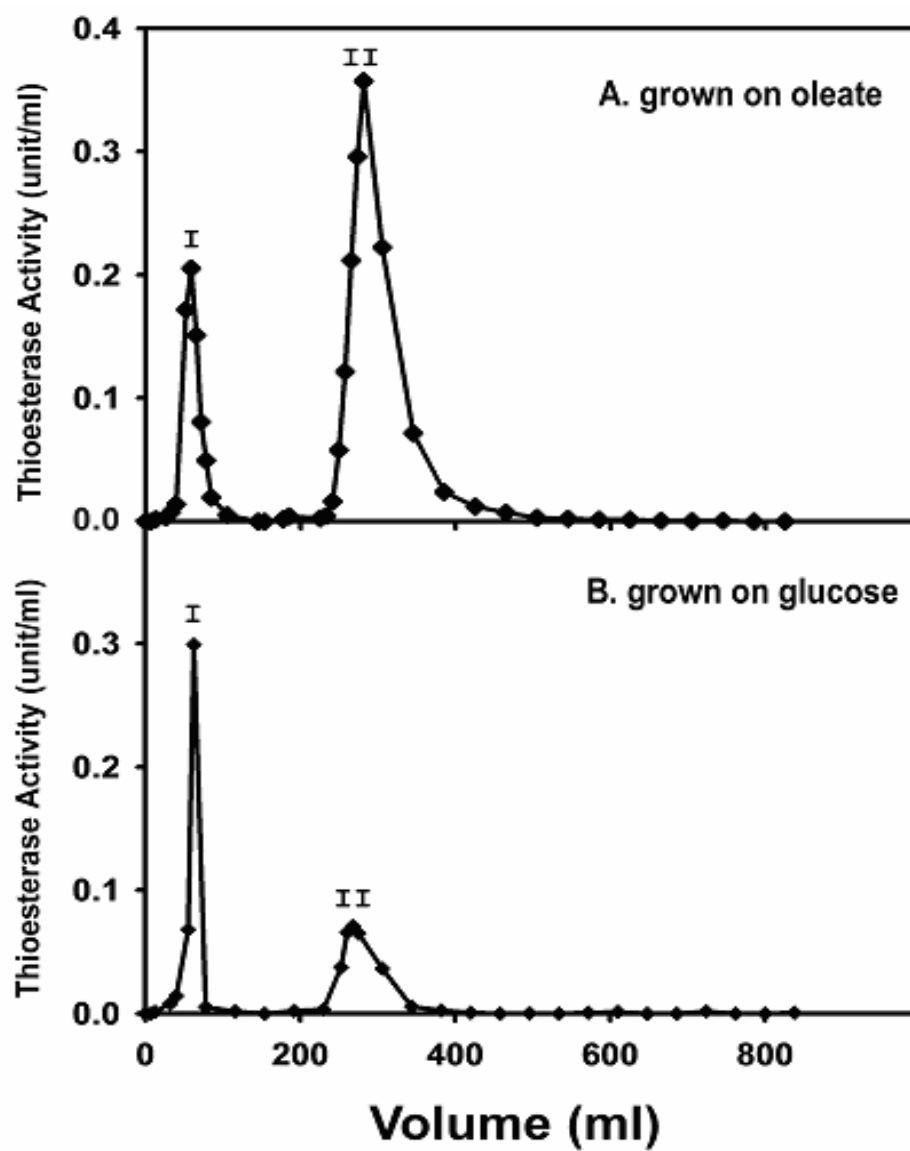


Fig. 2

**Fig. 3. Substrate specificities of partially purified thioesterases I and II from *E. coli*.** Activities of thioesterase fractions I and II were determined at acyl-CoA concentrations of 20  $\mu$ M with: C2:0, acetyl-CoA; C4:0, butyryl-CoA; C8:0, octanoyl-CoA; C14:0, tetradecanoyl-CoA (myristoyl-CoA); 2t-C14:1, 2-*trans*-tetradecenoyl-CoA; 2,5-C14:2, 2-*trans*,5-*cis*-tetradecadienoyl-CoA; 3t-C14:1, 3-*trans*-tetradecenoyl-CoA; 3,5-C14:2, 3,5-*cis*-tetradecadienoyl-CoA; 3OH-C14:0, 3-hydroxytetradecanoyl-CoA; C16:0, hexadecanoyl-CoA (palmitoyl-CoA); 3Keto-C16:0, 3-ketohexadecanoyl-CoA; C18:0, octadecanoyl-CoA (stearoyl-CoA); 9c-C18:1, 9-*cis*-octadecenoyl-CoA (oleoyl-CoA). Values of enzyme activity are means of two measurements that differed by 10% or less. (Adapted from Ren, Y., Aguirre, J., Ntamack, A., Chu, C., and Schulz, H. (2004) *J. Biol. Chem.* **279**, 11042-11050)

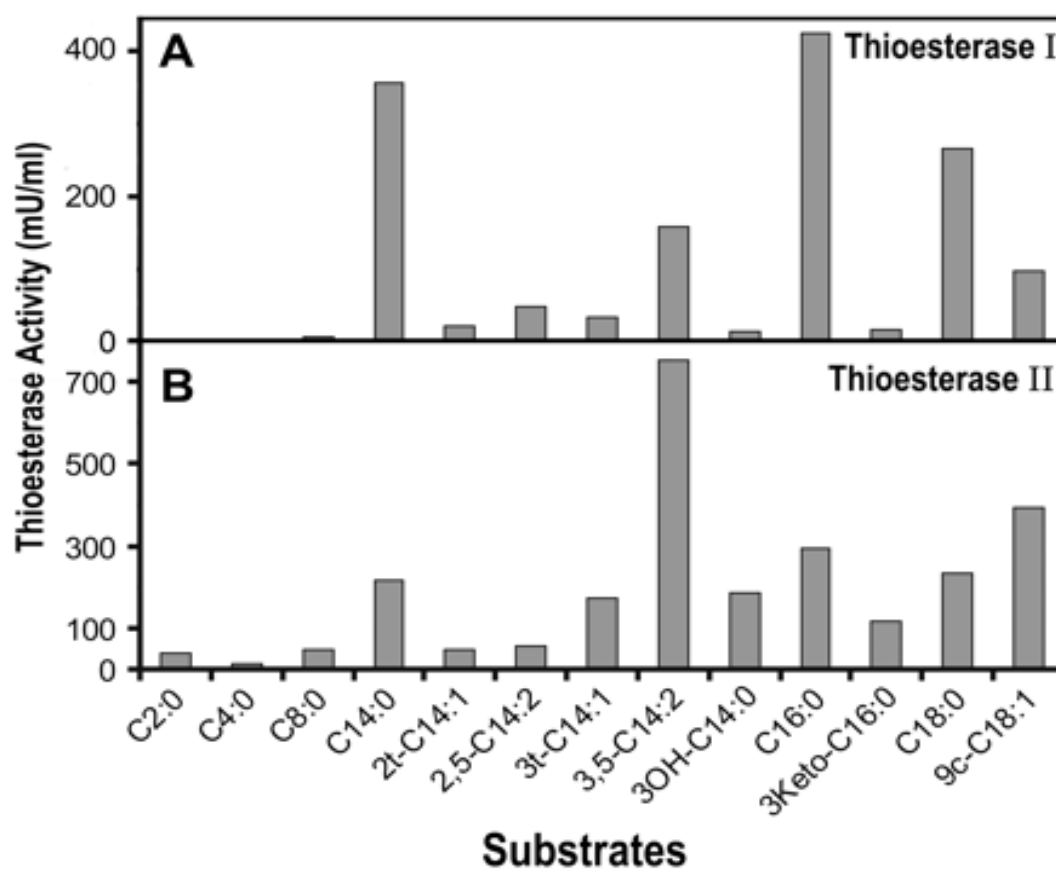


Fig. 3

Fig. 4. **Substrate specificities of *E. coli* thioesterases II and III.** A, purified thioesterase II; B, purified thioesterase III. Thioesterase activities were determined at acyl-CoA concentrations of 20  $\mu$ M with: C2:0, acetyl-CoA; C4:0, butyryl-CoA; C8:0, octanoyl-CoA; C10:0, decanoyl-CoA; C12:0, dodecanoyl-CoA; 3,5-C12:2, 3,5-*cis*-dodecadienoyl-CoA; C14:0, tetradecanoyl-CoA (myristoyl-CoA); 2t-C14:1, 2-*trans*-tetradecenoyl-CoA; 2,5-C14:2, 2-*trans*,5-*cis*-tetradecadienoyl-CoA; 3t-C14:1, 3-*trans*-tetradecenoyl-CoA; 3,5-C14:2, 3,5-*cis*-tetradecadienoyl-CoA; 3OH-C14:0, 3-hydroxytetradecanoyl-CoA; C16:0, hexadecanoyl-CoA (palmitoyl-CoA); 3keto-C16:0, 3-ketohexadecanoyl-CoA; C18:0, octadecanoyl-CoA (stearoyl-CoA); 9c-C18:1, 9-*cis*-octadecenoyl-CoA (oleoyl-CoA). Values of enzyme activity are means of three or four determinations  $\pm$  S. D.

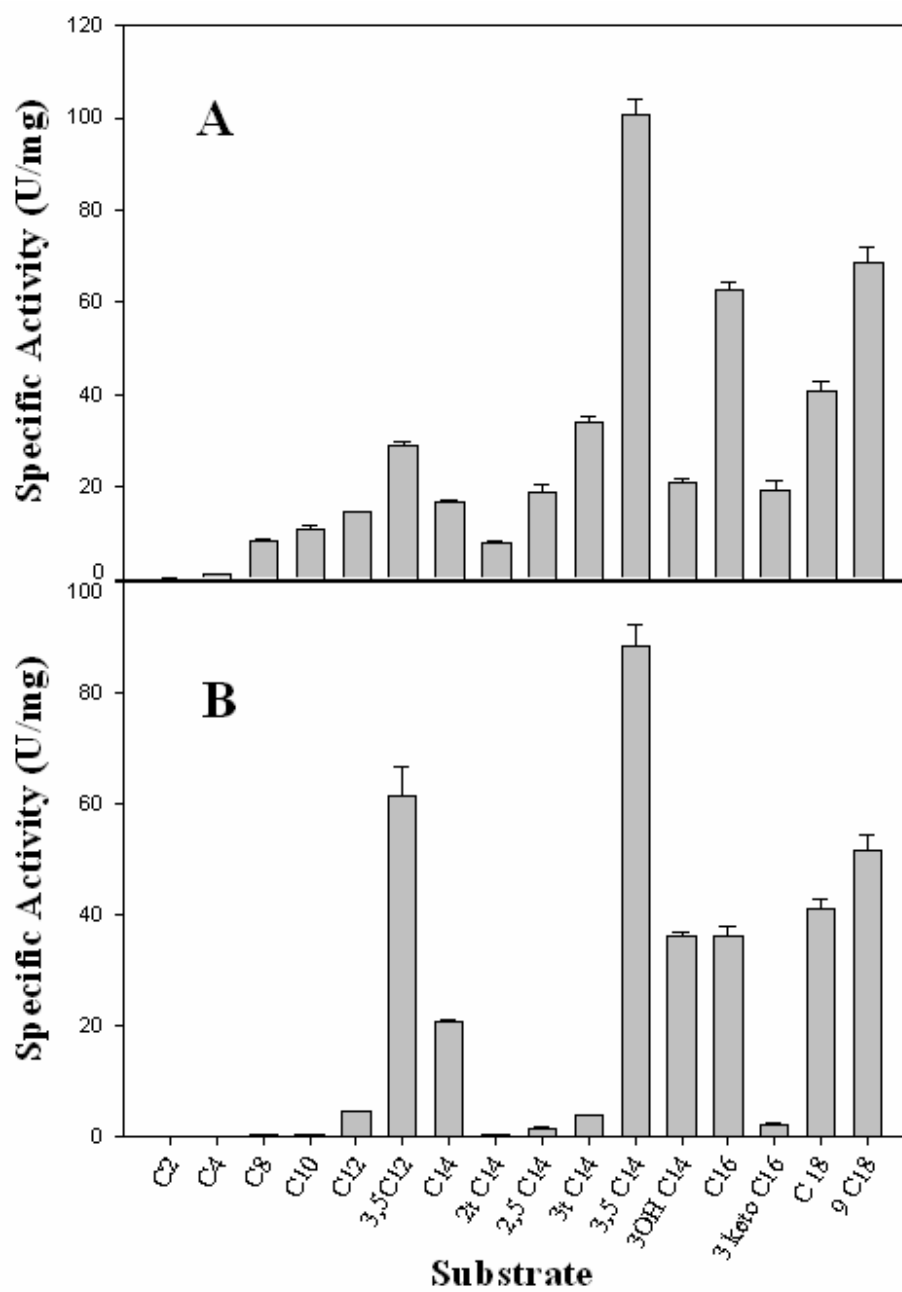


Fig. 4

**Fig. 5. 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; (◆) conjugated linoleic acid. B, wild type *E. coli* grown on (Δ) glucose; (▲) *tesA/tesB* mutant grown on glucose; (○) wild type *E. coli* grown on oleate; (●) *tesA/tesB* mutant grown oleate.

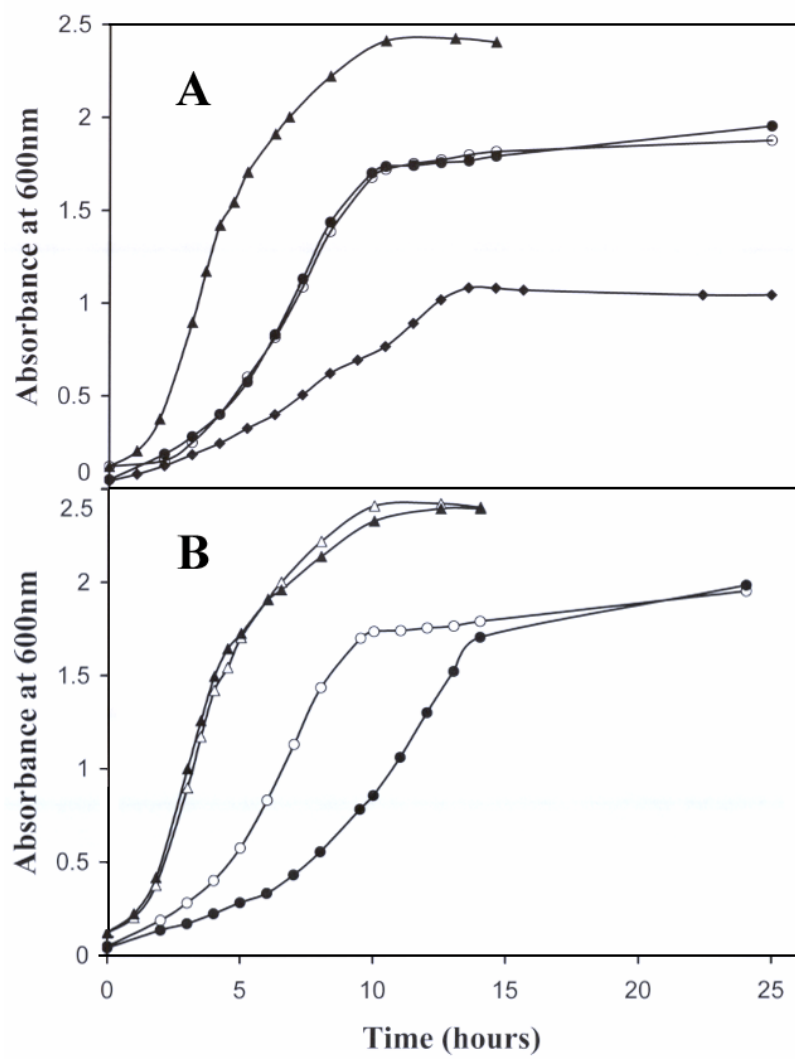


Fig. 5

**Fig. 6. Identification of 3,5-tetradecadienoic acid in the medium after growth of *E. coli* strain YR1 on oleic acid as the sole carbon source.** A, gas chromatogram of the methyl esters of acids extracted from the medium in which *E. coli* strain YR1 was grown to an absorbance of 1 at 600 nm. B, region of the gas chromatogram where methyl 3,5-tetradecadienoate would be eluted. Peaks 1-4 have molecular ions with mass-to-charge ratios ( $m/z$ ) of 238. C, mass spectrum of the material that gave rise to peak 3 of panel B.

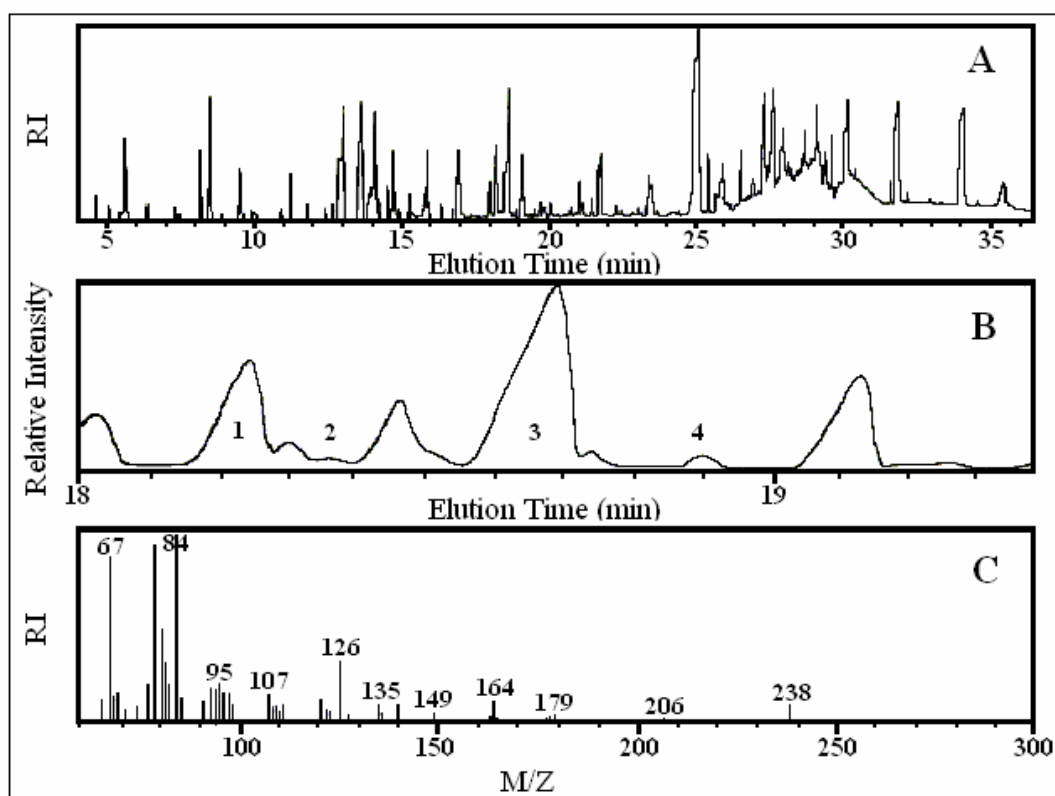


Fig. 6

Fig. 7. **Purification of the novel thioesterase from *E. coli* strain YR1 by column chromatography on DEAE-cellulose.** (▲), thioesterase activity with myristoyl-CoA as substrate; (○) protein concentration; (---) NaCl gradient. For details see “Experimental Procedures.”

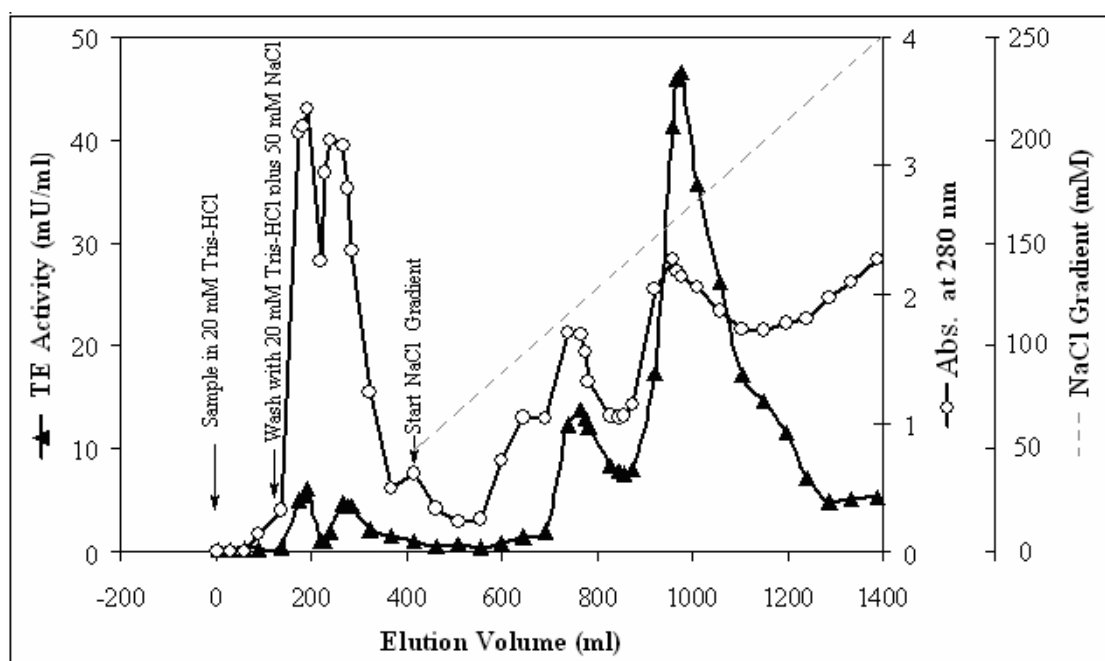


Fig. 7

**Fig. 8. Induction of the novel thioesterase by growth of *E. coli* on oleate.**

Soluble extracts of *tesA/tesB* mutant cells grown on either glucose (Panel A) or oleate (Panel B) as the sole carbon source were subjected to chromatography on DEAE-cellulose. Fractions were assayed for thioesterase with 20  $\mu$ M tetradecanoyl-CoA (myristoyl-CoA) as substrate. The arrow in panel B indicates the position where thioesterase II would have been eluted.

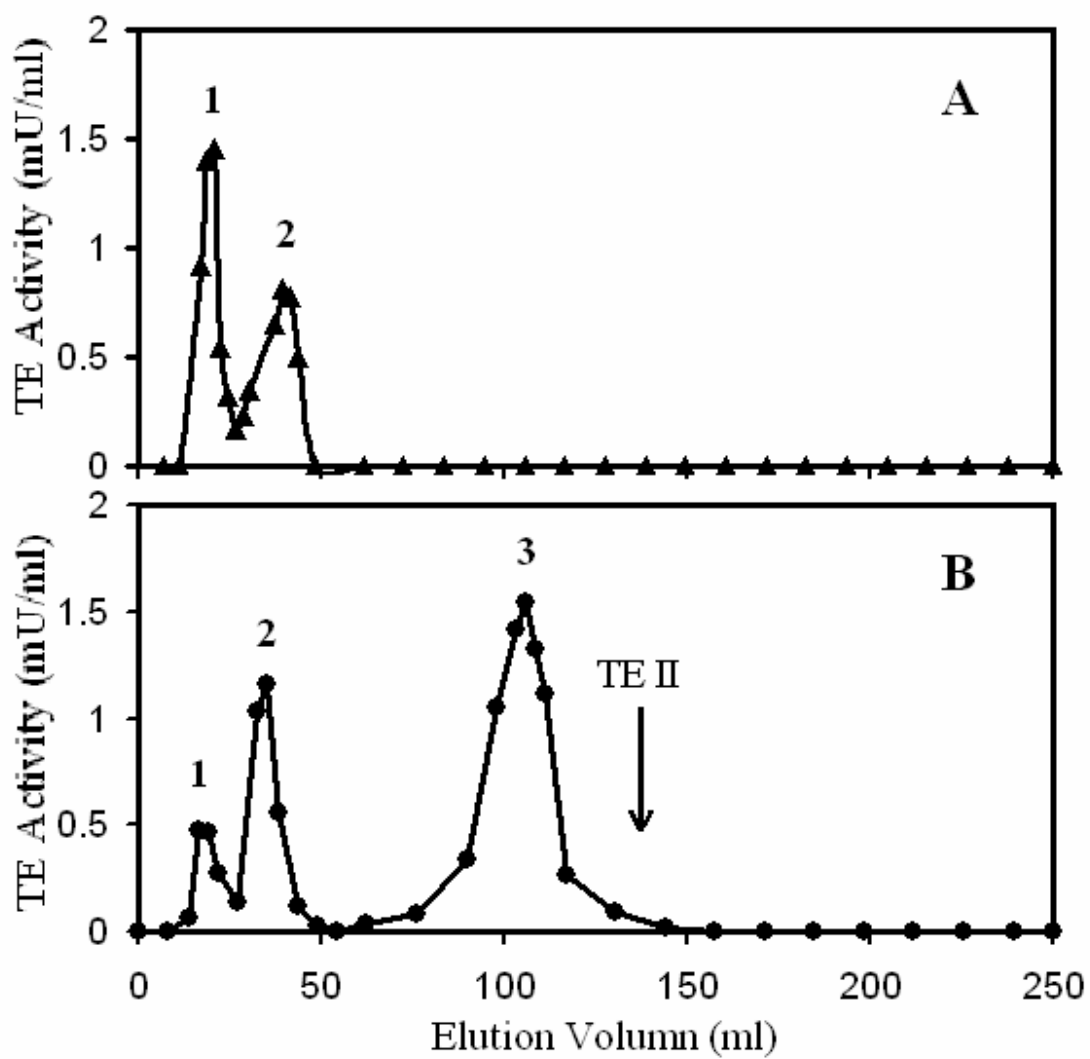


Fig. 8

Fig. 9. **Purification of the novel thioesterase from *E. coli* strain YR1 by column chromatography on hydroxyapatite HTP.** (▲), thioesterase activity with myristoyl-CoA as substrate; (○) protein concentration; (---) phosphate gradient. For details see “Experimental Procedures.”

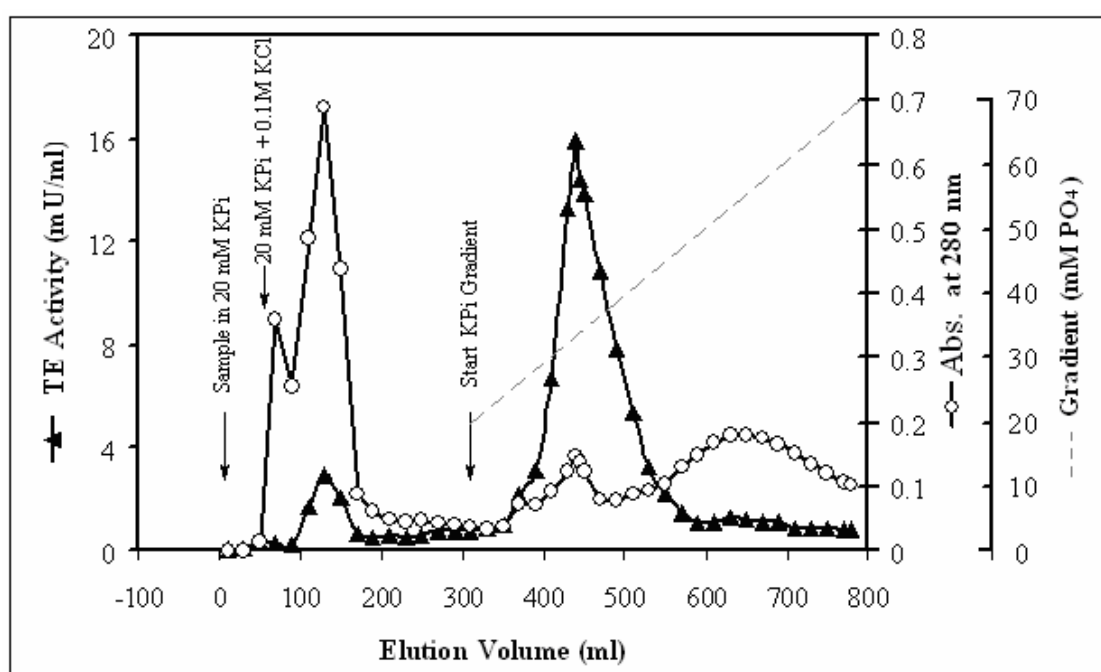


Fig. 9

Fig. 10. **Final step in the purification of the novel thioesterase from *E. coli* strain YR1 by column chromatography on octyl Sepharose CL-4B.** ( $\blacktriangle$ ), Thioesterase activity with myristoyl-CoA as substrate; ( $\circ$ ) protein concentration; (--)  $(\text{NH}_4)_2\text{SO}_4$  gradient; (—) ethanol gradient. For details see Experimental Procedures.

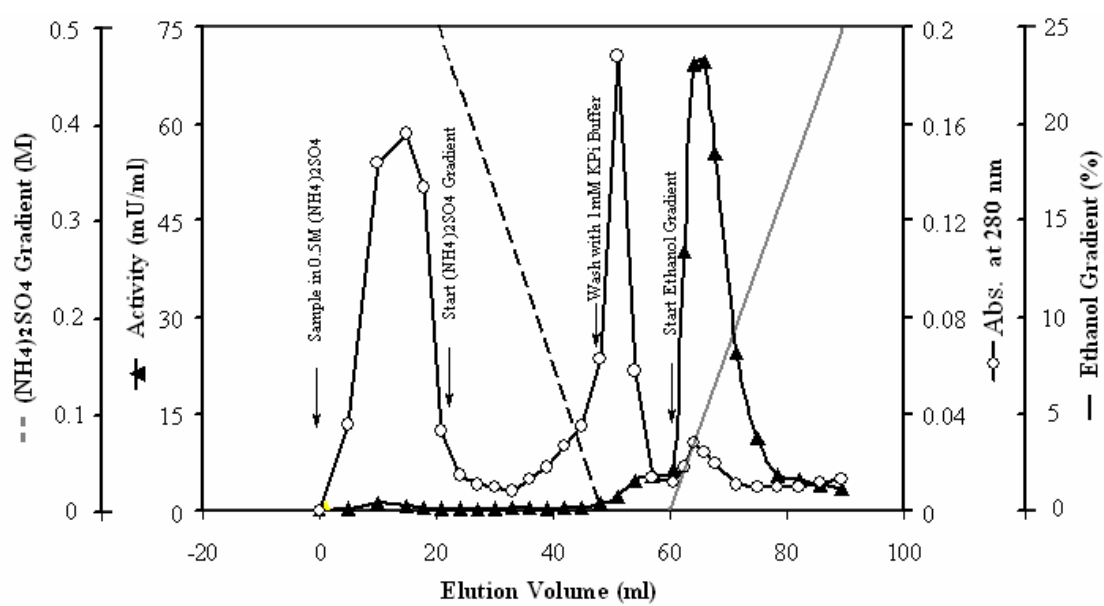


Fig. 10

**Fig. 11. SDS-PAGE of the partially purified novel thioesterase from *E. coli* on a 10% gel after staining with Coomassie brilliant blue R.** Lane A: protein standards to indicate the molecular masses of proteins; lane B, partially purified novel thioesterase.

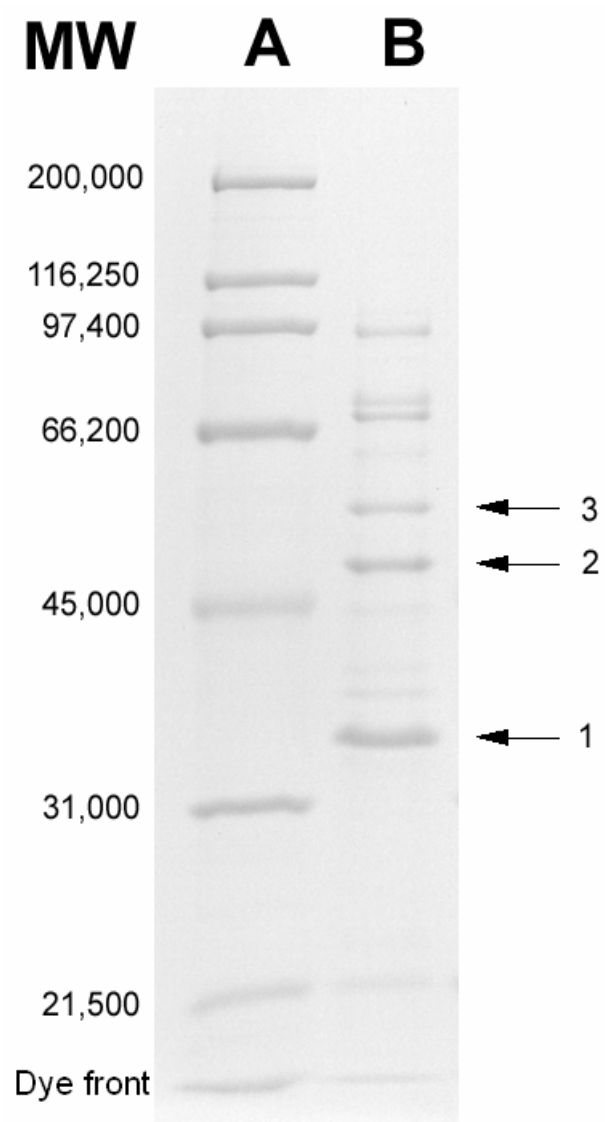


Fig. 11

Fig. 12. **Amino acid sequence of the ybaW protein that was identified as a novel thioesterase from *E. coli*.** Underlined are the tryptic peptides of the novel thioesterase that were identified by MALDI-MS and used to establish the identity of the novel thioesterase with the ybaW protein. Arginine and lysine residues are highlighted to indicate potential tryptic cleavage sites.

MQTQIKVRYGYHLDVYQHVNNARYLEFLEEARWDGLENDSFQWMTAHNIAFVV  
VNININYRRPAVLSDLLTITSQLQQLNGKSGILSQVITLEPEGQVVADALITF  
VCIDLKTQKALALEGELREKLEQMVK

Fig. 12

Fig. 13. **Binding sites for the fatty acid degradation repressor (fadR) in the promoters of several fatty acid degradation (*fad*) genes including *ybaW*.** A base was included in the fadR consensus-binding site when it was present in at least three out of the six compared sequences. The fadR binding sites of the six *fad* genes were experimentally confirmed (18) while the *ybaW* sequence is a proposed fadR binding site.

| Gene            | Position |     |     |     |     |     |     |     |     |     |     |     |    |     |     |     |     |     |
|-----------------|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|----|-----|-----|-----|-----|-----|
|                 | 1        | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  | 13 | 14  | 15  | 16  | 17  |     |
| <i>fadB</i>     | A        | T   | C   | T   | G   | G   | T   | A   | C   | G   | A   | C   | C  | A   | G   | A   | T   | +17 |
| <i>fadL</i> (1) | A        | G   | C   | T   | G   | G   | T   | C   | C   | G   | A   | C   | C  | T   | A   | T   | A   | -9  |
| <i>fadL</i> (2) | C        | A   | C   | T   | G   | G   | T   | C   | T   | G   | A   | T   | T  | T   | C   | T   | A   | +16 |
| <i>fadD</i> (1) | A        | G   | C   | T   | G   | G   | T   | A   | T   | G   | A   | T   | G  | A   | G   | T   | T   | -13 |
| <i>fadD</i> (2) | G        | G   | C   | T   | G   | G   | T   | C   | C   | G   | C   | T   | G  | T   | T   | T   | C   | -99 |
| <i>fabA</i>     | A        | A   | C   | T   | G   | A   | T   | C   | G   | G   | A   | C   | T  | T   | G   | T   | T   | -31 |
| Consensus       | A        | G   | C   | T   | G   | G   | T   | C   | C   | G   | A   | Y   | N  | T   | G   | T   | T   |     |
|                 | 4/6      | 3/6 | 6/6 | 6/6 | 6/6 | 5/6 | 6/6 | 4/6 | 3/6 | 6/6 | 5/6 | 6/6 |    | 4/6 | 3/6 | 5/6 | 3/6 |     |
| <i>YbaW</i>     | A        | C   | C   | A   | G   | T   | T   | A   | T   | G   | A   | C   | C  | T   | C   | T   | G   | -33 |
|                 | -49      |     |     |     |     |     |     |     |     |     |     |     |    |     |     |     |     |     |

Fig. 13

**Fig. 14. SDS-PAGE and immunoblot of the partially purified novel thioesterase and 6xHis-tagged novel thioesterase.** (A) 15% polyacrylamide gel after staining with Coomassie brilliant blue R; (B) Immunoblot after probing with antibodies against *E. coli* novel thioesterase. Lane 1, protein standards to indicate the molecular masses of proteins; lanes 2 and 4, purified 6xHis-tagged novel thioesterase; lanes 3 and 5, partially purified novel thioesterase (native thioesterase III).

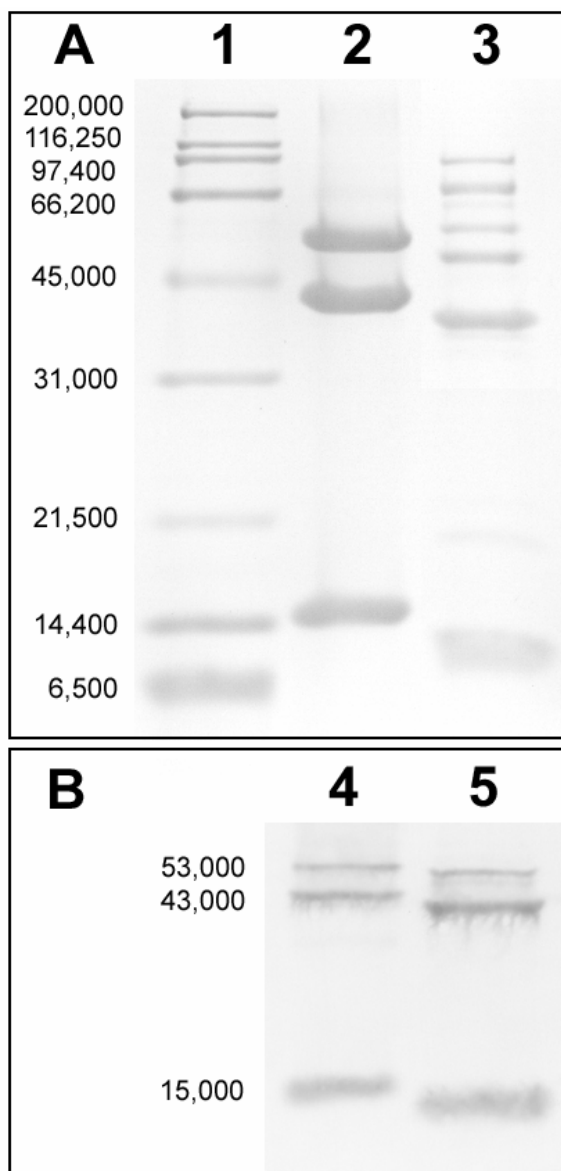


Fig. 14

**Fig. 15. 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 mid-logarithmic 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.

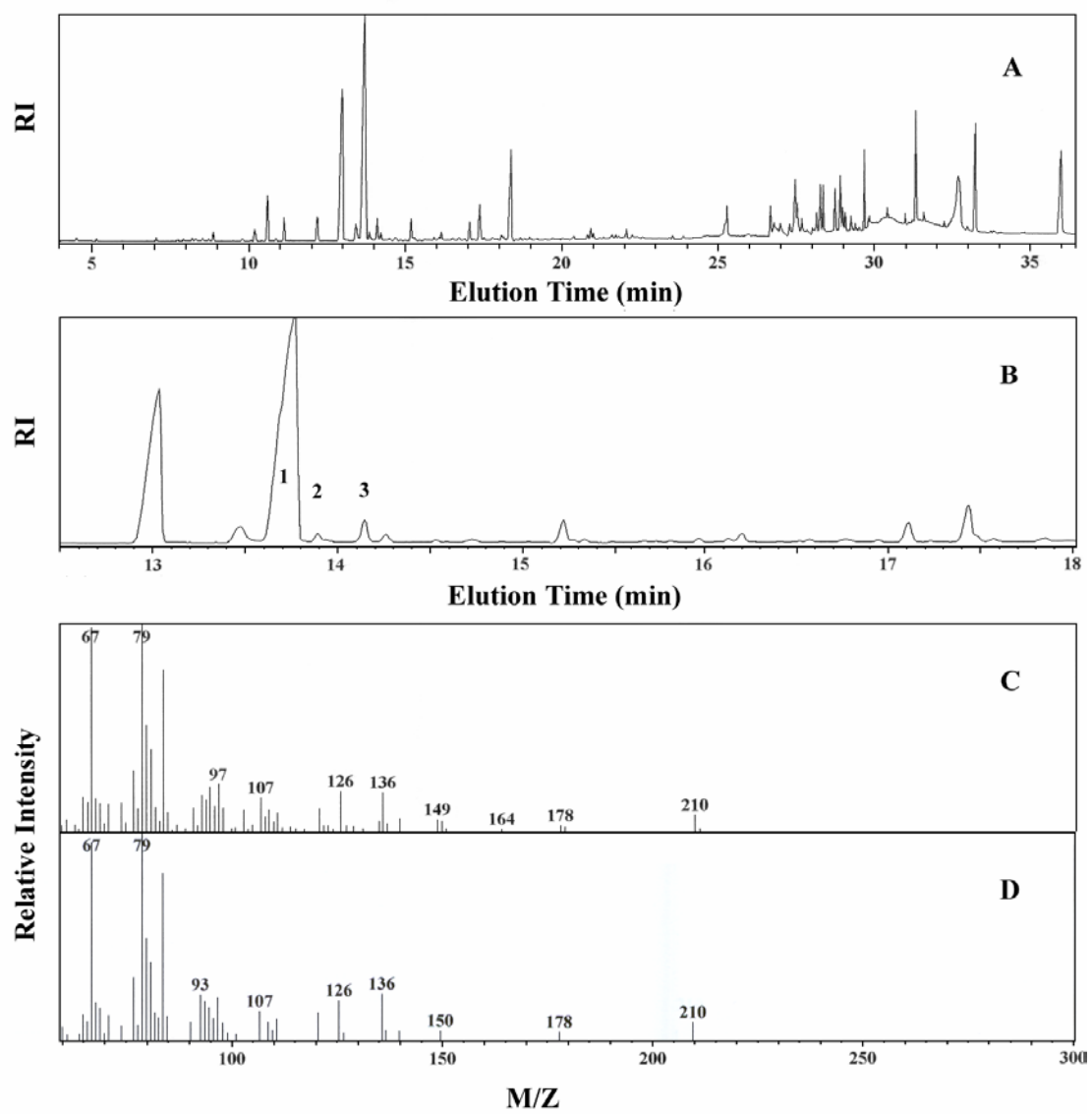


Fig. 15

**Fig. 16. 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. 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.

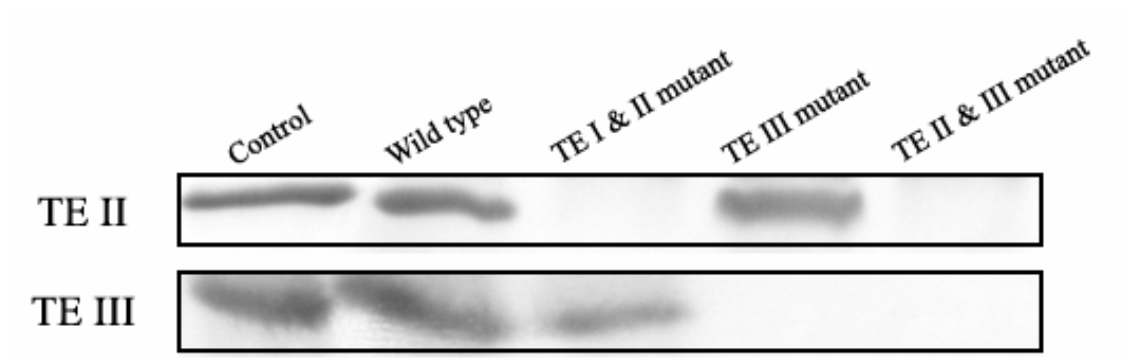


Fig. 16

Fig. 17. **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  $\pm$  S. D. For explanation of mutants, see legend to Fig. 15.

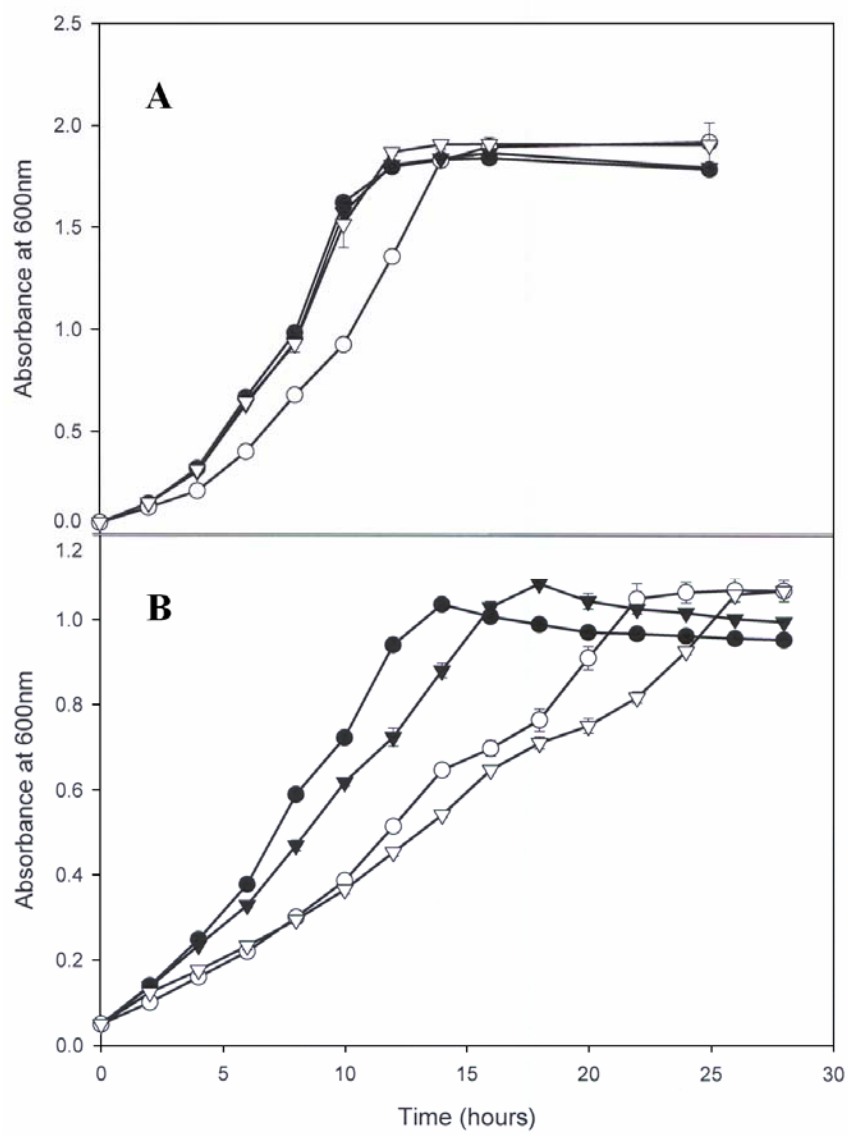


Fig. 17

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