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In vivo evidence for the essential function of 2,4-dienoyl-CoA reductase in the β-oxidation of unsaturated fatty acids

You Yi, Seh-Yoon, Ph.D.

City University of New York, 1989

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IN VIVO EVIDENCE FOR THE ESSENTIAL FUNCTION OF 2,4-DIENOYL-COA REDUCTASE IN THE β-OXIDATION OF UNSATURATED FATTY ACIDS

BY

SEH-YOON YOU YI

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirments for the degree of Doctor of Philosophy, The City University of New York

1989

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

March 16, 1989
Date

Chair of Examining Committee

March 16, 1989
Date

Executive Officer

Supervisory Committee

The City University of New York

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My sincere thanks to my parents and my husband for their constant support, patience, and love throughout the years.

Abstract

IN VIVO EVIDENCE FOR THE ESSENTIAL FUNCTION OF 2,4-DIENOYL-COA REDUCTASE IN THE β–OXIDATION OF UNSATURATED FATTY ACIDS.

BY

SEH-YOON YOU YI

Adviser: Professor Horst Schulz

E. coli mutants able to grow on oleate (9-cis-octadecenoic acid) but unable to utilize petroselinic acid (6-cis-octadecenoic acid) were isolated for the purpose of demonstrating in vivo the essential function of 2,4-dienoyl-CoA reductase (EC 1.3.1.34) in the β-oxidation of unsaturated fatty acids. One mutant (fadH) exhibited 12% of the 2,4-dienoyl-CoA reductase activity present in the parental strain with other β-oxidation enzymes being essentially unaffected.

Anti-reductase antibodies were used to prove the presence of a fadH gene product at a level similar to that observed in the parental strain. Thus, the mutation seems to have resulted in the synthesis of a fadH gene product with lower specific activity.

The mutation was mapped in the 71 to 75 min region of the E. coli chromosome where no other gene for β-oxidation enzymes has so far been located. A transconjugant of the mutant complemented with F'141 which carries the 67 to 75.5 min region of the E. coli genome, exhibits 80% of the 2,4-dienoyl-CoA reductase activity of parental Pet^+ strain. Measurements of respiration rates of the mutant, the transconjugant and parental Pet^+ strain on petroselinic acid as the sole carbon source

demonstrated a linear relationship between rates of respiration and levels of 2,4-dienoyl-CoA reductase activity.

Although the mutant was unable to grow on petroselinic acid, it grew on oleate at a rate indistinguishable from that of its parental strain. 2,4-Dienoyl-CoA reductase, like other enzymes of β-oxidation, was induced when E. coli was grown on oleate as the sole carbon source. Glucose repressed its synthesis while cAMP partially relieved the repression. It is concluded that 2,4-dienoyl-CoA reductase is required in vivo for the β-oxidation of unsaturated fatty acids with double bonds extending from even-numbered carbon atoms.

Additionally, the gene for 2,4-dienoyl-CoA reductase was isolated by complementation of the mutant with a genomic library of E. coli for the purpose of sequencing the gene and purifying 2,4-dienoyl-CoA reductase.

Table of Contents

TITLE PAGE.	i
COPYRIGHT PAGE	ii
APPROVAL PAGE	iii
ACKNOWLEDGEMENTS	iv
ABSTRACT	v
TABLE OF CONTENTS	vii
LIST OF TABLES	ix
LIST OF FIGURES	x
ABBREVIATIONS	xi
INTRODUCTION	1
MATERIALS	15
METHODS	16
1) Bacterial strains	16
2) Growth conditions	16
3) Preparation of substrates	18
4) Determination of acyl-CoA concentration	18
5) Preparation of bacterial extracts and enzyme assays.	19
6) Isolation of reductase mutants	21
7) Genetic complementation of mutant SY14 with F'kit.	22
8) Immunological studies	23
Immunoprecipitation with Staphylococcous aureus cell suspensions containing protein A.	23
Ouchterlony double immunodiffusion	23

Western blotting 24

SDS-polyacrylamide gel electrophoresis. 24

Transfer-blotting 25

9) Respiration measurements 26

10) Preliminary cloning of the gene for 2,4-dienoyl-CoA reductase 27

Transformation of E. coli strain SY14 with plasmid pSE103. 27

Complementation of E. coli strain SY141 with bacteriophage vector λSE_6 library 28

Isolation of phasmid DNA 28

Amplification of bacteriophage phSY142 29

Purification of lysates of phage produced on a large-scale 30

RESULTS 32

1) Isolation of E. coli mutants with a defective 2,4-dienoyl-CoA reductase 32

2) Characterization of mutant SY14 33

3) Immunological studies. 43

Immunoprecipitation with Staphylococcous aureus cell suspension containing proteinA 43

Ouchterlony immunodiffusion 46

Western blotting 46

4) Genetic complementation of reductase mutant SY14 49

5) Respiration measurements 57

6) Induction of 2,4-dienoyl-CoA reductase 57

7) Effect of cyclic AMP on the expression of reductase level 62

8) Preliminary cloning of the gene for 2,4-dienoyl-CoA reductase 65

DISCUSSION 67

REFERENCES 73

List of Tables

Table		Page
I.	The bacterial strains, phasmids or phages	17
II.	Activities of 2,4-dienoyl-CoA reductase and 3-hydroxyacyl-CoA dehydrogenase in E. coli mutants able to grow on oleic acid but not on petroselinic acid	34
III.	Specific activities of β-oxidation enzymes in parental strain RS3010 and mutant SY14	42
IV.	Specific activities of 2,4-dienoyl-CoA reductase and 3-hydroxyacyl-CoA dehydrogenase in E. coli strain RS3084 and RS3010.	55
V.	Specific activities of some β-oxidation enzymes in different E. coli strains.	56
VI.	Respiration rate of E. coli strains in a medium containing oleic acid	58
VII.	Respiration rate of E. coli strains in a medium containing petroselinic acid and specific activities of 2,4-dienoyl-CoA reductase present in these strains	59
VIII	Induction of 2,4-dienoyl-CoA reductase and 3-hydroxyacyl-CoA dehydrogenase in wild-type K-12 Ymel as a function of the carbon source in the growth medium.	63
IX	Effect of cyclic AMP on the levels of 2,4-dienoyl-CoA reductase and 3-hydroxyacyl-CoA dehydrogenase in wild-type K-12 Ymel grown on different carbon sources.	64

List of Figures

Figure		Page
1.	Pathway of fatty acid degradation and the organization of the β-oxidation enzymes in E. coli.	2
2.	Pathway of degradation of linoleic acid (A) Old-pathway proposed by Stoffel and Caesar, (B): Revised pathway proposed by Kunau and Dommes.	4
3.	Genetic map of fad regulon on the E. coli chromosome.	12
4.	Relative specific activities of 2,4-dienoyl-CoA reductase and 3-hydroxyacyl-CoA dehydrogenase in the muants which did not grow on petroselinic acid	35
5.	Growth behavior of parental strain RS3010 and mutant SY14 in acetate as the sole carbon source.	37
6.	Growth behavior of parental strain RS3010 and mutant SY14 on oleic acid or petroselinic acid as the sole carbon source	39
7.	Immunotitration of 2,4-dienoyl-CoA reductase present in a soluble extract of E. coli strain RS3010.	44
8.	Ouchterlony immunodiffusion analysis of 2,4-dienoyl-CoA reductase in cellular extracts of (A) RS3010 and (B) SY14	47
9.	Western blotting of 2,4-dienoyl-CoA reductase in cellular extracts of RS3010 and SY14.	51
10.	Genetic map of E. coli chromosome which coodinates of the points of origin of some Hfr strains and F-prime factors	53
11.	Relative respiration rates of cells in a medium containing petroselinic acid as a function of their reductase levels.	60

ABBREVIATIONS

AMP	adenosine 5'-monophosphate
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
BCIP	5-*bromo*-4-*chloro*-3-*indolyl* phosphate
BSA	bovine serum albumin
cAMP	adenosine 3',5'-cyclic monophosphate
CAP	catabolite activator protein
CoA	coenzyme A
CoASH	reduced coenzyme A
DCPIP	2,6-dichlorophenolindophenol
DEAE	diethylamino ethyl
DMF	N,N-dimethyl formamide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTNB	5,5'-dithio-*bis*-(2-nitrobenzoic acid)
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
EMS	ethyl methane sulfonate
FAD	flavin adenine dinucleotide
FADH	reduced flavin adenine dinucleotide
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
Hfr	high frequency recombinant
IG	immunoglobin
met	methionine
NBT	nitro blue tetrazolium *chloride*
NAD^+	nicotinamide adenine dinucleotide

NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NFDM	non fat dry milk
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
pFU	plaque forming unit
PMSF	phenyl methyl sulfonyl floride
pro	proline
S. aureus	*Staphylococcous aureus*
SDS	sodium dodecyl sulfate
TEMED	N,N,N',N',-tetramethyl-ethylenediamine
TRIS	tris (hydroxymethyl) amino methane
UV	ultraviolet

INTRODUCTION

The degradation of saturated fatty acids (e.g. palmitic acid and stearic acid) to acetyl-CoA occurs by a sequence of reactions referred to as β-oxidation (Fig. 1). Acyl-CoA synthetase (EC.6.2.1.3.) activates free fatty acids to fatty acyl-CoA's, which are converted to 2-trans-enoyl-CoA by acyl-CoA dehydrogenase (EC.1.3.99.3.). The second step of the β-oxidation cycle is the hydration of 2-trans-enoyl-CoA to L-3-hydroxyacyl-CoA by enoyl-CoA hydratase (EC.4.2.1.17.) The third reaction is the oxidation of the hydroxyl group substituted carbon atom to the keto group by L-3-hydroxyacyl-CoA dehydrogenase (EC.1.1.1.35.). The final step is the cleavage reaction by thiolase (EC.2.3.1.9.) to acetyl-CoA and an acyl-CoA that is two carbon atoms shorter than the starting fatty acyl-CoA.

The degradation of unsaturated and polyunsaturated fatty acids which usually contain cis double bonds also occurs by β-oxidation. All double bonds found in polysaturated fatty acids can be classified either as double bonds extending from odd-numbered carbon atoms or as double bonds extending from even-numbered carbon atoms. For example, oleic acid contains a 9-cis double bond which extends from an odd-numbered carbon atom, however, linoleic acid possesses both types of double bonds, 9-cis and 12-cis double bonds, extending from odd-numbered and even-numbered carbon atoms, respectively.

In 1965, Stoffel and Caesar[1] proposed a pathway for the β-oxidation of linoleic acid which requires two auxiliary enzymes in addition to the enzymes needed for the β-oxidation of saturated fatty acid (Fig. 2a). According to their proposal, linoleic acid after conversion to its CoA thioester (I) by acyl-CoA synthetase is degraded to 3-cis, 6-cis-dodecadienoyl-CoA (II) by three cycles of β-oxidation . The

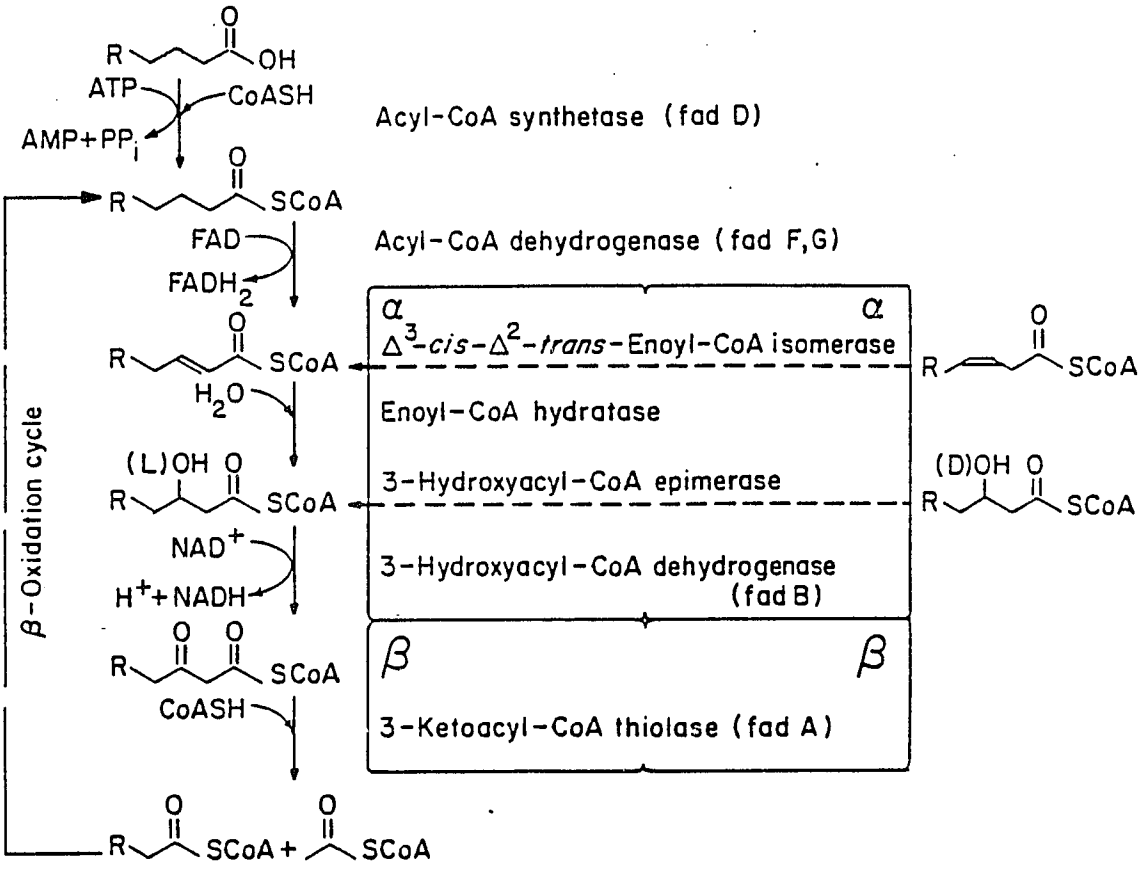


Fig. 1.

Pathway of the fatty acid degradation and the organization of the β-oxidation enzymes in *E. coli*.

The five component enzymes of the fatty acid oxidation complex associate with the large subunit (α) and the small subunit (β).

first auxiliary enzyme 3-cis, 2-trans-enoyl-CoA isomerase (EC.5.3.3.8.) catalyzes the isomerization of 3-cis, 6-cis-dodecadienoyl-CoA to 2-trans, 6-cis-dodecadienoyl-CoA (III), which is β-oxidized to 4-cis-decenoyl-CoA and converted to 2-trans, 4-cis-decadienoyl-CoA by medium-chain acyl-CoA dehydrogenase (V). This compound enters another β-oxidation cycle to be converted to 2-cis-octenoyl-CoA (VI). Hydration of 2-cis-octenoyl-CoA by enoyl-CoA hydratase yields D-3-hydroxyoctanoyl-CoA (VII). The second auxiliary enzyme, 3-hydroxyacyl-CoA epimerase (EC.5.1.2.3.), converts the D-form of 3-hydroxyoctanoyl-CoA to the L-isomer which can be completely degraded to acetyl-CoA by passing three times through the β-oxidation cycle. This old-pathway was supported by indirect evidence but key intermediates were never identified.

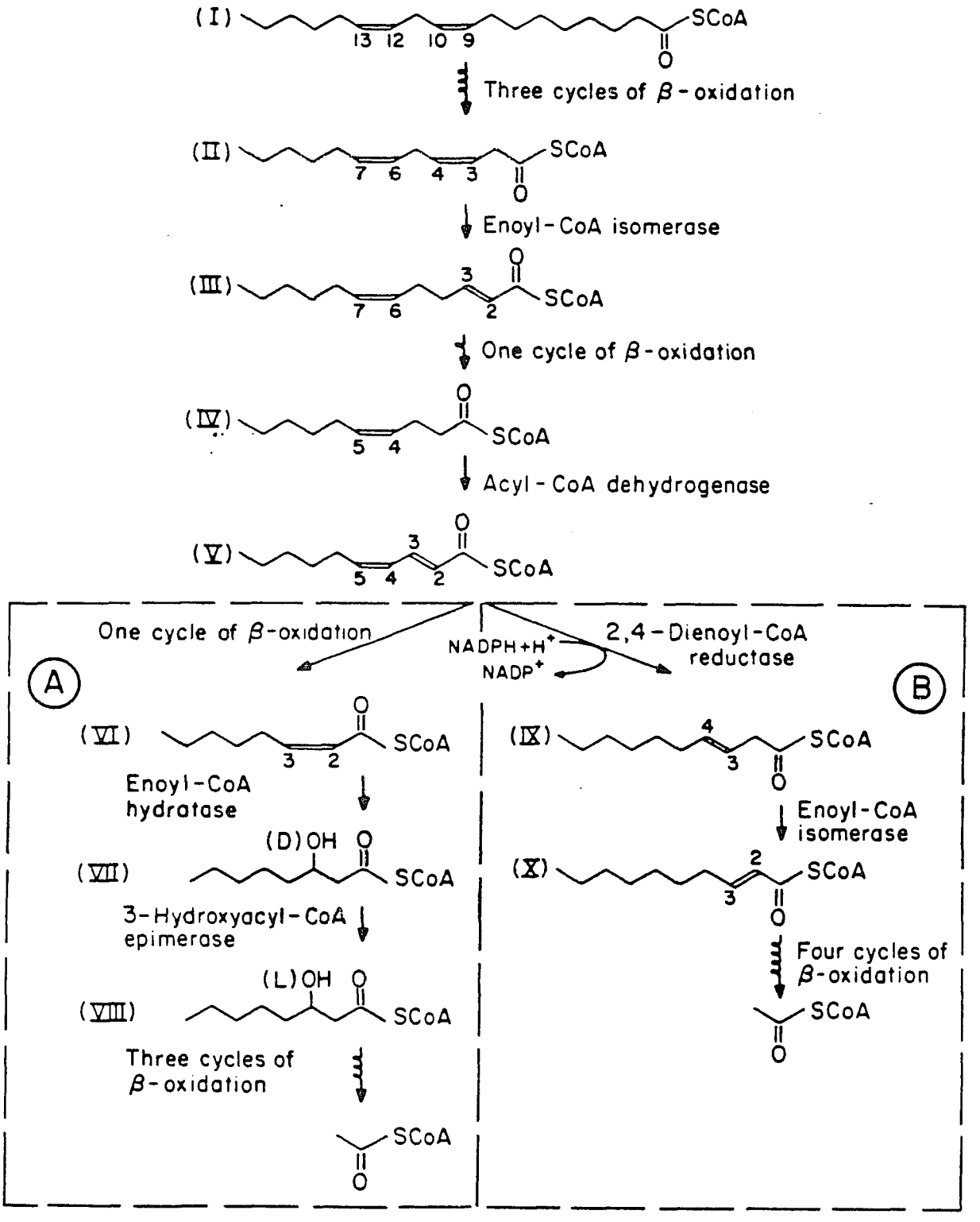
In 1978 Kunau and Dommes[2] proposed a revised pathway according to which a newly discovered NADPH-dependent 2,4-dienoyl-CoA reductase (EC.1.3.1.34.) functions as an auxiliary enzyme in the β-oxidation of polyunsaturated fatty acids. They reported that 2-trans, 4-cis-decadienoyl-CoA, a putative β-oxidation metabolite of linoleic acid, could be reduced by NADPH-dependent 2,4-dienoyl-CoA reductase to 3-trans-decenoyl-CoA (IX). 3-trans-Decenoyl-CoA can be isomerized to 2-trans-decenoyl-CoA (X) by 3-cis, 2-trans-enoyl-CoA isomerase and can be completely degraded to acetyl-CoA by four cycles of β-oxidation. The most convincing evidence for polyunsaturated fatty acid being degraded via the revised pathway was provided by Cuebas and Schulz[3] who demonstrated that 2-trans, 4-cis-decadienoyl- CoA, a metabolite of linoleic acid, was not degraded by a reconstituted β-oxidation system composed of purified crotonase, L-3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase plus NAD^+ and CoASH. However, 2-trans, 4-cis-decadienoyl-CoA was reduced by NADPH in the presence of rat heart mitochodria. This finding

Figure 2.

Linoleic Acid Degradation.

(A) : Old-Pathway : Stoffel and Caesar proposed a pathway for the β-oxidation of linoleic acid which requires 3-hydroxyacyl-CoA epimerase as an auxiliary enzyme for the degradation of unsaturated fatty acid..

(B): Revised pathway : Kunau and Dommes proposed a revised pathway for unsaturated fatty acid oxidation which requires NADPH-dependent 2,4-dienoyl-CoA reductase as an auxiliary enzyme.


(I)
SCoA
13 12 10 9
Three cycles of β-oxidation
(II)
SCoA
7 6 4 3
Enoyl-CoA isomerase
(III)
SCoA
7 6 3 2
One cycle of β-oxidation
(IV)
SCoA
5 4
Acyl-CoA dehydrogenase
(V)
SCoA
5 4 3 2
One cycle of β-oxidation
NADPH+H+
NADP+
2,4-Dienoyl-CoA reductase
A
B
(VI)
SCoA
3 2
Enoyl-CoA hydratase
(D)OH
(VII)
SCoA
3-Hydroxyacyl-CoA epimerase
(L)OH
(VIII)
SCoA
Three cycles of β-oxidation
SCoA
(IX)
SCoA
4 3
Enoyl-CoA isomerase
(X)
SCoA
2 3
Four cycles of β-oxidation
SCoA

suggested that 2-trans, 4-cis-decadienoyl-CoA is not metabolized via the pathway proposed by Stoffel and Caesar but could be degraded via the revised pathway.

When 3--hydroxyacyl-CoA epimerase, the key enzyme in the original pathway, was assayed in extracts of rat heart mitochondria its activity was found to be very low and insufficient to account for the rate of linoleic acid degradation(4). A careful evaluation of the subcellular location of 3-hydroxyacyl-CoA epimerase in rat liver revealed that the epimerase was associated with peroxisomes but not with mitochondria(5). Based on a kinetic study, Yang et al.(6) reported that 2-trans, 4-cis-decadienoyl-CoA is not directly β-oxidized in mitochondria, but instead is reduced by NADPH-dependent 2,4-dienoyl-CoA reductase. However, 2-trans,4-cis-decadienoyl-CoA can be slowly oxidized by the bifunctional β-oxidation enzyme from rat liver peroxisomes, as well as by the fatty acid oxidation complex from E. coli. The observed β-oxidation of 2-trans, 4-cis-decadienoyl-CoA in peroxisomes and E. coli is most likely a consequence of the direct transfer of L-3-hydroxy-4-cis-decenoyl-CoA from the active site of enoyl-CoA hydratase to that of 3-hydroxyacyl-CoA dehydrogenase on the same protein molecule. The estimated percentage of 2-trans, 4-cis-decadienoyl-CoA metabolized via the original pathway is 0.02% in mitochondria, 2% in peroxisome and 2.8% in E. coli(7). The above results lead to the conclusion that the major pathway for the degradation of 2-trans,4-cis-decadienoyl-CoA involves the NADPH-dependent 2,4-dienoyl-CoA reductase as an auxiliary enzyme, whereas 3-hydroxyacyl-CoA epimerase is involved in the minor pathway(8).

Since the NADPH-dependent 2,4-dienoyl-CoA reductase has been detected in crude extracts of Escherichia coli(9,10), liver mitochondria(2,11,12) and peroxisome(13-15) as well as in Candida lipolytica(16,17), it seems that this reductase is widely distributed in living organisms. The reductase has been purified from several sources and the properties of these enzymes have been studied. The

reductase is separated by phosphocellulose chromatography from the E. coli multi-enzyme complex of fatty acid oxidation which exhibits enoyl-CoA hydratase (crotonase), 3-hydroxyacyl-CoA epimerase, 3-cis, 2-trans-enoyl-CoA isomerase, 3-hydroxyacyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase activities. The E. coli 2,4-dienoyl-CoA reductase was completely purified after ion-exchange chromatography on DEAE-cellulose followed by affinity chromatography on 2',5'-ADP-Sepharose 4B[18].

The bovine liver and E. coli 2,4-dienoyl-CoA reductases have similar substrate specificities but different molecular properties. Both enzymes are specific for NADPH and 2,4-dienoyl-CoA as substrate, and at higher concentrations are inhibited by substrate or coenzyme. However, structurally the two reductase are different; the bovine liver enzyme is a tetramer of four apparently identical subunits with estimated molecular weights of 32,000, whereas the bacterial enzyme is a monomer with a molecular weight of 73,000. The latter enzyme is a flavo-protein containing 1 mol of FAD/mole of enzyme, whereas the animal enzyme does not contain any flavin[19]. The bovine liver enzyme reduces 2,4-decadienoyl-CoA, a metabolite of linoleic acid, to 3-trans-decenoyl-CoA which can be isomerized to 2-trans-decenoyl-CoA by 3-cis, 2-trans-enoyl-CoA isomerase[10]. In contrast, the E. coli enzyme reduces 2-trans, 4-cis-decadienoyl-CoA to 2-trans-decenoyl-CoA[10,19]. The activity of 2,4-dienoyl-CoA reductase is dependent on the supply of NADPH which is controlled by the energy state of mitochondria[20]. Intramitochondrial NADPH required by the reductase can be formed either by the energy-requiring $NAD(P)^+$ transhydrogenase (EC.1.6.1.1.) or by $NADP^+$-dependent isocitrate dehydrogenase (EC.1.1.1.42)[21] or by glutamate dehydrogenase[22].

In addition to 2,4-dienoyl-CoA reductase, E. coli contains two additional NADPH-dependent enoyl-CoA reductases. One is NADPH-dependent 2-cis-enoyl-

CoA reductase[23,24], and the other is NADPH-dependent 2-trans-enoyl-CoA reductase[25]. These two enzymes differ from one another and from 2,4-dienoyl-CoA reductase, and they are also distinct from enoyl acyl carrier protein (ACP) reductases, which are involved in the de novo synthesis of fatty acids[24]. 2-cis-Enoyl-CoA reductase may participate in the β-oxidation of unsaturated fatty acids containing cis double bonds on even-numbered carbon atoms and 2-trans-enoyl-CoA reductase may participate in the acetyl-CoA-dependent chain elongation of fatty acid in E. coli[25-28]. However, no direct evidence has so far been obtained for these proposed functions of 2-enoyl-CoA reductases. 2,4-Dienoyl-CoA reductase is also distinct from the two 2-enoyl-CoA reductase in E. coli because of the stereochemistry of their catalytic reactions. The two 2-enoyl-CoA reductases catalyze the direct hydride transfer from NADPH to the substrates and they do not contain flavin nucleotides[29,30], whereas the hydride of NADPH is first transferred to the flavin adenine nucleotide of 2,4-dienoyl-CoA reductase and followed by its transfer to the substrate[31]. The 2-enoyl-CoA reductases can be separated from the 2,4-dienoyl-CoA reductase[32]. For example, the 2-enoyl-CoA reductase from bovine liver mitochondria is separated from 2,4-dienoyl-CoA reductase by ion-exchage chromatography on DEAE cellulose followed by affinity chromatography on Blue Dextrin Sepharose and 2',5'-ADP-Sepharose 4B. The purified 2-enoyl-CoA reductase reduces the double bond of 2-trans-enoyl-CoA esters, but does not exhibit significant activity toward the 2-trans-double bond of 2,4-dienoyl-CoA esters. Thus 2,4-dienoyl-CoA reductase is clearly distinct from 2-enoyl-CoA reductases.

β-Oxidation of fatty acids in E. coli has been carefully studied by Overath and co-workers[33] who demonstrated that the enzymes of fatty acid oxidation in E. coli are highly induced when cells are grown on long-chain fatty acids (having 12 or more carbon atoms) as the sole carbon source. Growth on short-chain and medium-chain

fatty acids does not result in the induction of β-oxidation enzymes, however, medium-chain fatty acids can be degraded by fatty acid β-oxidation enzymes. The synthesis of at least three β-oxidation enzymes, namely 3-ketoacyl-CoA thiolase, enoyl-CoA hydratase, and 3-hydroxyacyl-CoA dehydrogenase, is induced at identical rates when cells are grown on various media containing either oleate, acetate, succinate, or tryptone and combinations thereof. However, the induction of acyl-CoA synthetase is slower than the induction of the above enzymes[34]. These results are easily understood if the structural genes for 3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase and enoyl-CoA hydratase form an operon, which is not linked to the gene of acyl-CoA synthetase. However, the expression of all fad genes is controlled by the same regulatory gene. This fatty acid regulon encodes the enzymes responsible for the transport, activation and β-oxidation of medium-chain and long-chain fatty acids.

The synthesis of β-oxidation enzymes in E. coli is strongly repressed in the presence of glucose. Addition of long-chain fatty acids, like oleate, can not relieve the repression; however, addition of cyclic AMP can partially restore the synthesis of enzymes[35]. The synthesis of the fad enzymes is most likely regulated by a mechanism similar to that of lac operon postulated by Jacob and Monod[36]. When the concentration of cAMP is low, the catabolite activator protein (CAP) does not bind to the fad promotors, thereby preventing the transcription of the fad regulon. Thus the expression of the fad regulon seems to be subject to catabolite repression involving CAP protein and cAMP.

The availability of E. coli mutants, which are unable to grow on fatty acids as the sole carbon source, facilitates genetic studies of the β-oxidation system and provides structural information of the fad regulon. On the basis of genetic studies with several fad mutants, the genes for the fatty acid oxidation enzymes have been mapped on the

E. coli chromosome at four different locations (see Fig. 3)[37]. The gene for a regulatory protein (repressor) is mapped at a fifth location. The structural gene encoding the fadAB locus harbors the genes for 3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA epimerase and 3-cis,2-trans-enoyl-CoA isomerase[38]. Overath and co-workers[33] observed the coodinate induction for 3-ketoacyl-CoA thiolase, enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase, while acyl-CoA synthetase and acyl-CoA dehydrogenase showed different patterns of induction. Mutant fad5, which is devoid of fadAB enzyme activities, has inducible wild-type levels of acyl-CoA synthetase and acyl-CoA dehydrogenase. The above results support the conclusion that expression of the genes 3-ketoacyl-CoA thiolase, enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase is coordinate as expected of "an operon", but different from the genes of acyl-CoA synthetase and acyl-CoA dehydrogenase[39,40]. The fadAB genes codes for a multi-enzyme complex which has an $\alpha_2\beta_2$ subunit structure and a native molecular weight of 260,000[41]. The fadA gene codes for the 42,000 Da β-subunit which harbors thiolase activity while the fadB gene carries the genetic information for the 78,000-Da α subunit which contains the remaining four enzyme activities. The fadAB genes are closely linked and are located between the loci metE and rha in the 86 min region of the E. coli chromosome[42,43]. The fadD gene codes for acyl-CoA synthetase and is located at 40 min of the E. coli chromosome. This acyl-CoA synthetase has been purified[44,45] and the molecular weights of the native enzyme and its subunit were determined to be 130,000 and 47,000, respectively. Acyl-CoA synthetase activates free fatty acids to acyl-CoA thioesters in the presence of ATP, CoASH and Mg^{2+}. This enzyme acts on medium-chain as well as long-chain fatty acids[45]. The fadFG genes which are poorly characterized code for short-chain and long-chain acyl-CoA dehydrogenase. Mutants of gene F & G, lacking acyl-CoA dehydrogenase activity, are unable to oxidize fatty acids. This mutation has been

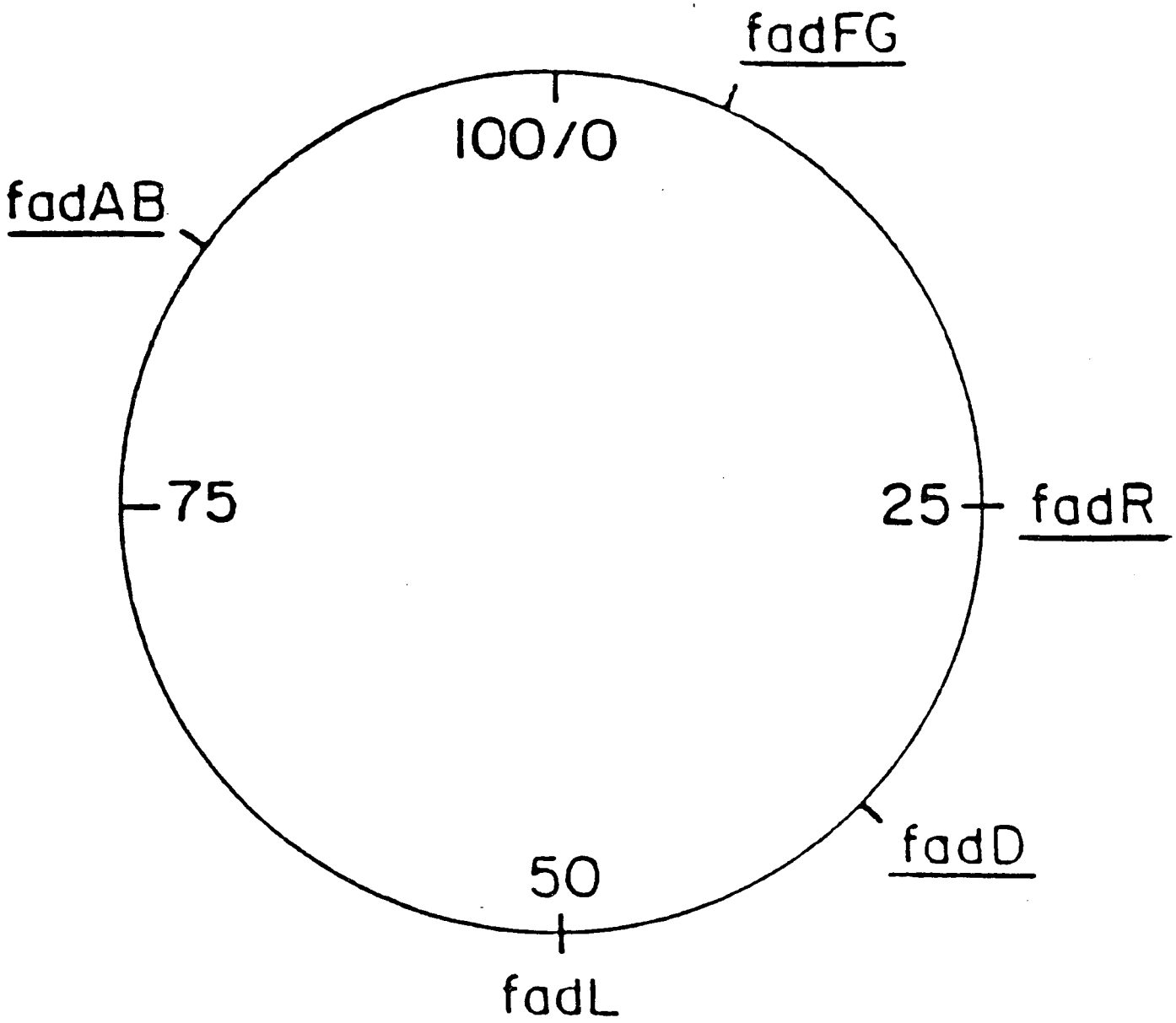
mapped in the 5 min region between the genes proA and proB on the E. coli chromosome[46]. The fadL gene, which is located at 50 min on the E. coli chromosome, codes for a 43,000-Da membrane protein[47,49]. The fadL gene product is an essential protein for long-chain fatty acids transport, but is not required for medium-chain fatty acid uptake[50]. A model for the transport of fatty acids through the E. coli membranes was proposed by Dr. W. Nunn[51]. According to his proposal, long-chain fatty acids initially bind to the fadL gene product (FLP), which is a component of the outer membrane. Once the long-chain fatty acids have passed the outer membrane, they are transferred across the cytoplasmic membrane to the membrane bound acyl-CoA synthetase, where they are activated and released into the cell[52]. The fadR gene, a multifunctional regulatory gene, was mapped at 25.5 min on the E. coli chromosome. The fadR gene product exerts negative control over fatty acid degradation(fad) and acetate metabolism (ace). It has also been suggested that fadR plays a role in the regulation of unsaturated fatty acid biosynthesis (fab)[53,54]. Medium-chain fatty acids can serve as substrates of the fatty acid oxidation system, but cannot themselves induce the fad system. However, fadR mutants that are able to utilize medium-chain fatty acids as the sole carbon source have been isolated by plating wild-type cells onto minimal medium containing decanoate (C_{10}) as the sole carbon source[55]. These fadR mutants are constitutive for the synthesis of all β-oxidation enzymes and oxidize both medium-chain and long-chain fatty acids. The repressor produced by the fadR gene was identified as a 29,000-dalton protein which regulates the expression of the fad regulon at the level of transcription.

Genetic and molecular biological methods including chemical mutagenesis provide powerful approaches for the identification of isolation of genes from E. coli. More than 1,000 E. coli genes have been identified by mutations. Especially complementation of E. coli mutants provides a powerful way of identifying E. coli

Figure 3.

Genetic map of the fad regulon on the E. coli chromosome.

fadAB , gene for the multienzyme complex located at 86 min; fadFG , gene for short and long-chain acyl-CoA dehydrogenases located at 5 min; fadR , gene for the repressor located at 25 min; fadD , gene for the acyl-CoA synthetase located at 40 min; fadL , gene for the fatty acid uptake protein which is membrane-bound located in the 50 min region of the E. coli chromosome.


fadFG
100/0
fadAB
75
25
fadR
fadD
50
fadL

genes. Elledge and Walker[56] constructed the bacteriophage cloning vector λSE_6 which has been designed for the purpose of isolating and cloning genes that could be expressed in E. coli by complementation of E. coli mutants. The cloning vector λSE_6 was constructed by attaching a very-low-copy number replication system and kanamycin resistance gene to the left arm of λ1059. When λSE_6 is introduced into a cell containing the lambda repressor cI gene, its lambda functions can be repressed and the phasmid would replicate by the low-copy-number replication system and can be selected for its kanamycin resistance. In a strain lacking λcI gene, the phasmid would grow as a lytic phage and form plaques. Many libraries based on bacteriophage lambda have problems such as altered phenotype expression or incompleteness due to the lethality or instability of genes when genes are present at high copy number. However, λSE_6 which contains a low-copy-number replication system and an antibiotic selection marker is optimal for the isolation and identification of genes of interest[56,57].

Since all evidence supporting the operation of the reductase-dependent pathway was obtained by in vitro experiments utilizing isolated mitochondria, cell extracts or isolated enzymes, a need existed to demonstrate in vivo the essential function of 2,4-dienoyl-CoA reductase in the β-oxidation of unsaturated and polyunsaturated fatty acids. Toward this end I have isolated an E. coli mutant with a defective 2,4-dienoyl-CoA reductase and studied its growth on and oxidation of unsaturated fatty acids. This mutation will provide genetic and structural information of the gene of 2,4-dienoyl-CoA reductase by mapping and isolating the gene of 2,4-dienoyl-CoA reductase from E. coli.

MATERIALS

Diketene, 2-trans,4-trans -decadienal, crotonic anhydride and 2-trans -decenoic acid were obtained from Aldrich Chemical Co. Ethyl methane sulfonate (EMS), phenyl methyl sulfonyl floride (PMSF), petroselinic acid (99%), nitro blue tetrazolium *chloride* (NBT), 5-*bromo*-4-*chloro*-3-*indolyl* phosphate, *p-toluidine* salt (BCIP), a suspension of Staphylococcous aureus cells, pig heart 3-hydroxyacyl-CoA dehydrogenase and all other standard biochemicals were purchased from Sigma. Ouchterlony immunodiffusion plates were obtained from Miles Laboratory. Antibodies against 2,4-dienoyl-CoA reductase from E. coli were generously provided by Dr. W-H Kunau, Ruhr-Universitat Bochum, West Germany. Prestained standard proteins, alkaline phosphatase-anti Ig conjugate, nitrocellulose membranes and Whatman 3MM filter paper were obtained from Bio-Rad. The cloning vector λSE_6 library and pSE103 were obtained from American Type Culture Collection (ATCC), Rockville Md. Strain K-12 Ymel and the F' kit were obtained from the Coli Genetic Stock Center (CGSC), Yale University. E. coli strains RS3010 and RS3084 were provided by Dr. W. D. Nunn, University of California at Irvine. Enoyl-CoA hydratase or crotonase[58] and 3-ketoacyl-CoA thiolase[59] were isolated from bovine and pig heart, respectively, and purified by published procedures. 3-Octyn-1-ol, obtained from ICN Pharmaceuticals, was converted to 3-cis-octenoic acid as described by Stoffel and Ecker [60]. 2,4-trans-Decadienoic acid was prepared from 2-trans, 4-trans-decadienal by oxidation with Ag_2O according to a general procedure for the oxidation of aldehydes to acids that are sensitive to strong oxidizing agent[61]. 3-Hydroxy-octenoic acid was prepared by alkaline hydrolysis of methyl-3-hydroxy octanoate which was synthesized from hexanal and methyl bromoacetate by the procedure of Ruppert and White[62].

METHODS

1) Bacterial Strains.

All bacterial strains, plasmids or phages in this study are listed in Table I.

2) Growth Conditions

E. coli K-12 Ymel was grown in LB medium from single colonies. The starter culture was diluted 10-fold into M9 mineral salts medium containing as carbon source either oleic acid (0.05%, w/v), petroselinic acid (0.05%, w/v), tryptone (0.5%, w/v), acetate (0.5%, w/v), succinate (0.5%, w/v), glucose (0.5%, w/v) or combinations thereof[63]. Cultures were grown to stationary phase overnight. The overnight culture was diluted 20-fold into M9 mineral medium containing a fatty acid dispersed with Triton X-100 (0.4%, w/v) and was grown again to stationary phase. Finally, the cell suspensions were diluted 10-fold with the same medium into a total volume of 2 liters. Cells were grown to cell density of 80 Klett or an absorbance of 1.5 at 420 nm and harvested at 6,000Xg at 4°C for 20 min. Cells were washed twice with M9 mineral buffer and stored at -70°C

For experiments testing the effects of cyclic AMP, the cultures growing on glucose or glucose plus oleate were divided into two parts at an OD420 of 0.35. To one part cyclic AMP was added to a final concentration of 50mM. All cultures were then grown for another two generations before cells were harvested by centrifugation[35].

RS3010, SY14 and its transconjugant were routinely grown in M9 mineral medium with oleate (0.05%, W/V) plus acetate (0.5%, W/V) as carbon source to a cell density of Klett 80.

Table I.

The bacterial strain, plasmid and phages

Strain,plasmid or phage	Genotype of phenotype	Source
Strains		
K-12 Ymel	prototroph	CGSC[a]
RS3010	fadR	W.D.Nunn(55)
RS3084	fadR::Tn10 *rspL* atoC fad85 ::Tn5-14	W.D. Nunn
SY14	fadR fadH	This lab
SY141	fadH fadR (pSE103)	This lab
SY142	SY141 (phSY142)	This lab
JC7623	*recB recC sbcB*	A.J. Clark (64)
Plasmids or phage		
λSE6	E. coli genomic library	ATCC[b]
pSE103	cI857 kan^r	ATCC[b]
phSY142	pet^+ (from λSE6)	This lab
F'kit	CGSC[a]	

[a] CGSC, E. coli Genetic Stock Center, Yale University.
[b] ATCC, American Type Culture Collection, Rockville, Md.

All agar plates contained 1.5% bacto-agar or 0.7% when soft agar plates were used. Davis minimal agar plates supplemented with appropriate amino acids at 50μg/ml were used in conjugation experiments. To make LB-kanamycin plates, stock solution of kanamycin sulfate in sterile H_2O (25mg/ml) was added to autoclaved LB media (at 50°C) to a final concentration of 50μg/ml.

3) Preparation of Substrates

Crotonyl-CoA[65] and acetoacyl-CoA[66] were synthesized as previously described. The CoA derivatives of cis-3-octenoic acid, 2-trans-decadienoic acid, DL-3-hydroxydodecanoic acid and 2,4-trans-decadienoic acid were prepared by the mixed anhydride method as detailed by Goldman and Vagelos[67]. The concentration of 2,4-trans-decadienyl-CoA was determined by measuring the absorbance at 298 nm due to the dienoyl thioester chromophore and calculating the concentration by use of a extinction coefficient of 27,800 $M^{-1}cm^{-1}$.

4) Determination of Acyl-CoA concentration.

An aliquot of the acyl-CoA solution (5 μl) was mixed with an equal volume of 2M hydroxyamine (pH 7.0) and allowed to react at room temperature for 5 to 15 min. To this solution was added 10 μl of Ellman's reagent[68], 300 μl of 0.1 M potassium phosphate buffer (pH 8.0), and distilled water to give a total volume of 1 ml. The absorbance at 412 nm was measured in a Gilford spectrophotometer. The value obtained was corrected for free CoASH in the solution by performing the same determination without the addition of hydroxylamine. The resultant free CoASH will react with 5,5'-dithio-*bis*-(2-nitrobenzoic acid) to produce 5-thio-2-nitrobenzoate which absorbs light at 412 nm with an extinction coefficient of 13,600 $cm^{-1} M^{-1}$.

5) Preparation of Bacterial Extracts and Enzyme Assays.

Cells grown to late logarithemic phase on M9 mineral salts medium containing various carbon sources were harvested and washed twice with M9 mineral salts medium. One g of cell paste suspended in 2 ml of 10 mM KPi (pH 7.0) containing 10 mM 2-mercaptoethanol, 2 mM PMSF and 25% glycerol was sonicated with an ultrasonic sonicator (W-385 from Heat System Ultrasonics. Inc.) for 2 min (12x10s) at 0°C and centrifuged for 30 min at 180,000xg at 4°C. The resultant supernatant was assayed for enzymes of β-oxidation with the exception of acyl-CoA synthetase which was assayed in the sonicate before centrifugation.

Protein concentrations were determined by the method of Lowry using bovine serum albumin as standard(69).

Acyl-CoA synthetase (EC 6.2.1.3.) was assayed essentially as described by Konberg and Pricer(70) and as modified by Overath et.al.(34). Two ml of a mixture containing 4 mmol oleic acid, 40 mmol NaF, 0.5 mg of Triton X-100 were combined with 1 ml of buffer containing 250 mmol Tris, 1 mmol hydroxylamine 10 mmol ATP, 0.6 mg of CoA , 20 mmol $MgCl_2$, 40 mmol 2-mercaptoethanol adjusted to pH 8.5 with ammonia. A mixture without ATP and CoA served as a control. After addition of soluble E. coli extract (3 mg of protein) the reaction mixture was incubated in a stoppered flask at 37°C for 1 hour. To stop the reaction, bovine serum albumin (2.5 mg) and 0.2 ml of 70% HCl04 were added. The formed hydroxamic acid was extracted from the precipitate with 1 ml of methanolic Hill's reagent and its absorbance was measured at 520 nm. A molar extinction coefficient of $1.1X10^3$ M^{-1} cm^{-1} was used for calculating the amount of hydroxamic acid formed.

Enzymes of β-oxidation were assayed spectrophotometrically on a Gilford recording spectrophotometer at 25°C. 2,4-Dienoyl-CoA reductase (EC 1.3.1.34.) was assayed by measuring the decrease in absorbance at 340 nm due to the oxidation of NADPH. The reaction mixture contained 0.2 M of potassium phosphate (pH 7.0), 60 μM of NADPH, 30 μM of 2.4-decadienoyl-CoA and enzyme. The assay was started by the addition of 2,4-decadienoyl-CoA. An extinction coefficient of 6,220 M^{-1} cm^{-1} was used to calculate rates.

Enzymes of the multienzyme complex were assayed as described by Binstock and Schulz[71]. Enoyl-CoA hydratase (EC 4.2.1.17) was assayed by recording the decrease in absorbance at 263 nm due to the hydration of the 2-trans double bond of the substrate. An assay mixture contained 0.2 M potassium phosphate (pH 8.0), bovine serium albumin (0.2 mg/ml) and 30 μM crotonyl-CoA or 20 μM 2-decenoyl-CoA. The reaction was started by the addition of enzyme. An extinction coefficient of 6,700 $M^{-1}cm^{-1}$ was used to calculate rates.

L-3-Hydroxyacyl-CoA dehydrogenase (EC1.1.1.35.) was assayed by measuring the decrease in absorbance at 340 nm due to the dehydrogenation of NADH. An assay mixture contained 0.1 M potassium phosphate (pH 7.0), bovine serum albumin (0.2 mg/ml), 0.1 mM NADH, and 30 μM acetoacetyl-CoA. The assay was started by the addition of enzyme. An extinction coefficient of 6,220 $M^{-1}cm^{-1}$ was used to calculate rates. 3-Ketoacyl-CoA thiolase (EC 2.3.1.9.) was assayed by measuring the decrease in absorbance at 303 nm due to the disappearence of the Mg^{2+}-enolate complex of the substrate. An assay mixture contained 0.1M HEPES(pH 8.1), 25 mM $MgCl_2$, bovine serum albumin (0.2 mg/ml), 2 mM mercaptoethanol, 5% (v/v) glycerol, 0.1 mM CoASH, 30 μM acetoacetyl-CoA. The reaction was started by the

addition of enzyme. An extinction coefficient of 12,000 $M^{-1}cm^{-1}$ was used for calculating rates.

3-cis-2-trans-Enoyl-CoA isomerase (EC 5.3.3.8.) was assayed by measuring the increase in absorbance at 340 nm due to the formation of NADH. The assay mixture contained 0.15 M potassium phosphate (pH 8.0), 0.5 mM NAD^+, 0.1 mM CoASH, 30 μM 3-cis-octenoyl-CoA, beef liver crotonase (7.5 ug/ml), pig heart L-3-hydroxyacyl-CoA dehydrogenase (0.3 U/ml), and pig heart 3-ketoacyl-CoA thiolase (33 mU/ml). The reaction was started by the addition of enzyme. 3-Hydroxyacyl-CoA epimerase (EC 5.1.2.3.) was measured by a coupled assay in which the formation of NADH at 340 nm was recorded. The assay mixture contained 0.15 M potassium phosphate (pH 8.0), 0.33 mM NAD^+, 0.1 mM CoASH, 60 μM DL-3-hydroxydecanoyl-CoA, pig heart 3-hydroxyacyl-CoA dehydrogenase (0.3 U/ml), and pig heart 3-ketoacyl-CoA thiolase (33 mU/ml). When assaying 3-hydroxyacyl-CoA epimerase, the reaction was initiated by the addition of enzyme after the oxidation of the L-3-hydroxydecanoyl-CoA was complete.

One unit of enzyme activity is defined as the amount that catalyzes the conversion of 1 μmol of substrate to product per min.

6) Isolation of Reductase Mutants.

E. coil cells (strain RS3010) were grown overnight in LB medium[63] and diluted 100-fold into M9 mineral salts[63] medium containing glucose (0.5%, w/v). After growth at 37°C to a density of $3x10^8$ cells/ml, cells were harvested by centrifugation, washed with M9 mineral salts medium and suspended in one half the original volume of M9 mineral salts medium containning 0.2 Tris-HCl (pH 7.5). Ethyl methane sulfonate (30 μl) was added to each 2 ml of cell suspension was

aerobically incubated for 2 hours at 37°C under shaking. The cell suspension which was then diluted 10-fold into LB medium and grown at 37°C overnight. The overnight culture was harvested, washed twice with M9 mineral salts medium and suspended in the same medium. One tenth of 1 ml of this suspension was transferred to 10 ml of M9 mineral salts medium containing petroselinic acid (0.05%, w/v) and grown at 37°C to a cell density of Klett 20. Ampicillin was then added to the cell culture to a final concentration of 40 μg/ml and shaking was continued until the cell density had dropped to Klett 10. The ampicillin selection was repeated after washing the cells with M9 mineral salts medium. The cell suspension was finally diluted 10^5-fold with M9 mineral salts medium and plated on agar plates containing M9 mineral salts and acetate (0.5%, w/v). After growth overnight these plates served as master plates of which replica plates were made which contained either petroselinic acid (0.05%, w/v) or oleic acid (0.05%, w/v) as carbon source. Colonies which could be detected on the master plate as well as on the oleate plate but were not present on the plate containing petroselinic acid were picked and grown on LB plates to be re-checked for their inability to grow on petroselinic acid and growth oleate.

7) Genetic Complementation of Mutant SY14 with F'kit.

Strains of an F' kit were cross-streaked against SY14 on agar plates containing M9 mineral salts medium plus petroselinic acid (0.05%, w/v) as the sole carbon source. Transconjugants were purified and assayed for 2,4-dienoyl-CoA reductase as well as for other enzymes of β-oxidation.

F'kits were maintained on Davis minimal medium supplemented with amino acids. Specially, F'102 (CGCC strain #4247), F'140 (CGCC strain #4289) and

F'141 (CGCC strain #4248) were maintained on Davis minimal medium containing histidine, leucine and methionine(63).

8) Immunological Studies.

a. Immunoprecipitation with Staphylococcous aureus cell suspensions containing protein A.

A soluble extract (20 ul) of E. coli cells RS3010 containing 0.4 mg of protein was incubated for 30 min at 25°C with varying amounts (0 to 2.4 mg of protein in 40 µl) of antiserum raised against 2,4-dienoyl-CoA reductase from E. coli. To this incubation mixture was added 0.2 M KPi (pH 7.0) to give a total volume of 120 µl. After the addition of 0.5 ml of a 10% cell suspension of S. aureus, the mixture was kept for 2 min at 25°C, then combined with 0.5 ml of 0.2 M KPi (pH 7.0) and centrifuged in a Beckman Microfuge B for 2 min at top speed. Part (0.9 ml) of the resultant supernatant was assayed for 2,4-dienoyl-CoA reductase activity by adding first NADPH followed by 2,4-decadienoyl-CoA.

b. Immunodiffusion.

Double immunodiffusion experiments were performed according to Ouchterlony(72). Soluble cell extrates (20 µl) of E. coli strains RS3010 and SY14 containing between 40 µg and 1.2 mg of protein were placed in the outer wells of immunodiffusion plates. After placing antiserum to E. coli 2,4-dienoyl-CoA reductase (10 µl containg 0.6 mg of protein) in the center well, the plates were kept for 3 days at 4°C and 100% humidity. The plates were washed with 1 mM KPi (pH 7.2) containing 0.9% NaCl for 2 days at 4°C by replacing the buffer twice, thereafter they were stained for protein with 0.5% solution of Amido black and destained with a 1:1 mixture of water and methanol containing 1% acetic acid.

c. Western Blotting.

Crude cell extracts were solubilized with SDS and subjected to PAGE. Proteins were electrophoretically transferred to a nitrocellulose membrane and then probed with the rabbit antibody to E. coli 2,4-dienoyl-CoA reductase. The primary antibody was detected by reaction with goat-anti rabbit IgG-alkaline phosphatase conjugate and visualized by incubating the nitrocellulose membrane in the presence of the alkaline phosphatase substrate(73).

I. SDS-polyacrylamide gel electrophoresis. : The SDS-PAGE of crude extracts of E. coli strains RS3010 and SY14 was performed on separating gels prepared with 8% acrylamide. The separating gel solution was prepared by combining 4 ml of 30% acrylamide/0.8% bisacrylamide with 3.75 ml of 1.5 M Tris·Cl containing 0.4% SDS adjusted to pH 8.8 and 7.25 ml of water into flask(73). The flask was attached to a water aspirator for 15 min. Then 50 µl of 10% (w/v) ammonium persulfate and 10 µl of TEMED (N,N,N',N',-tetramethyl-ethylenediamine) were added to the solution. The gel solution was immediately transferred into the minigel apparatus (8 cm X 7 cm gels) which was presealed with 1% agarose. The height of the solution in the gel apparatus was approximately 6 cm leaving 1.5 cm for the stacking gel. The gel was allowed to polymerize for 30 min. The stacking gel solution was prepared by combining 0.65 ml of 30% acrylamide/0.8% bisacrylamide with 1.25 ml of 0.5 M Tris·Cl containing 0.4% SDS adjusted to pH 6.8 and 3.05 ml of water. After degassing under vacuum for 15 min, 25 µl of 10% ammonium persulfate and 5 µl of TEMED were added. The solution was poured on top of separating gel in the gel apparatus. The stacking gel solution polymerized within 30 min. To prepare protein samples, 10 µl of 0.12 M Tris.Cl containing 20% glycerol(v/v), 2% SDS, 2% 2-mercaptoethanol (v/v) and 1% Bromophenol blue (w/v) adjusted to pH 6.8 was

mixed with 20 µl of sample solution and then boiled for 5 min at 100°C. The samples were previously prepared by diluting crude extrates of E. coli strains of RS3010 and SY14 (19 mg/ml) 10-, 50-, 250-,1,250-, and 6,250-fold. After application of the samples to the gels together with prestained proteins as molecular weight markers, the gels were subjected to electrophoresis for 2.5 hours at 30 mA constant currency.

II. Transfer-Blotting. ; The SDS-PAGE gel was prewetted in transfer buffer (0.25 M Tris (pH.8.0), 0.2 M glycine, 20% methanol). Two pieces of Whatman 3 MM filter paper, which were cut to the size of the gel, were prewetted in anode buffer (25 mM Tris (pH.10.4), 20% methanol) and cathode buffer (25 mM Tris (pH.9.4), 40 mM 6-aminohexanoic acid, 20% methanol), respectively. The nitrocellulose membrane was soaked in transfer buffer for 20 min. A transfer unit was prepared in the following manner ; one layer of filter paper soaked in anode buffer was placed first on the unit. A prewetted nitrocellulose membrane was placed on top of the filter paper soaked in anode buffer. The prewetted gel was placed on top of the membrane. The air bubbles between nitrocellulose membrane and gel were removed by use of a piece of glass rod. The filter paper soaked in cathode buffer was placed on top of the gel. The transfer-blotting was performed for one hour at 65 mA of constant currency. The nitrocellulose membrane from the transfer-unit was placed in 5% NFDM (non fat dry milk, w/v) containing 0.1% Tween 80 in TBS (20 mM Tris.Cl (pH 7.4), 0.9% NaCl) at 4°C overnight. The membrane was transferred to a miniblotter and incubated with 500-fold diluted E. coli-2,4-dienoyl-CoA reductase rabbit antibody for 2 hours at room temperature with gentle agitation. The membrane was washed with gentle agitation. The membrane was washed with a solution containing 0.2% NFDM, 0.05% Tween 20 in TBS three times for 3 min. Goat anti

rabbit IgG-alkaline phosphatase conjugate which was diluted 500-fold was added to the blotter and incubated for 2 hours[74-76]. After washing the membrane, the blot spots were developed by incubating the nitrocellulose membrane with freshly prepared McGadey reagent [77]. This solution was prepared by combining 0.2 ml of a 50 mg/ml stock solution of NBT (nitro blue tetrazolium chloride) in 50% DMF (N, N-dimethyl formamide) and 0.1 ml of a 50 mg/ml stock solution of BCIP (5-bromo-4-chloro-3-indoyl phosphate, *p* -toluidine salt) in 50% aqueous DMF and 30 ml Tris-saline (0.1 M Tris-Cl, 0.1 M NaCl, 50 mM $MgCl_2$ adjusted to pH.9.5). After completion of the reaction, the nitrocellulose membrane was rinsed in distilled water and dried at room temperature.

9) Respiration Measurements.

Cells of E. coli strains K-12 Ymel, F'141, RS3010, SY14 and its transconjugant were grown to late logarithmic phase (absorbance of 1.6 at 420 nm), harvested, washed twice with M9 mineral salts medium, and resuspended in the same medium to obtain suspensions of 5-times higher cell density. These cell suspensions were starved by incubating them at 37°C for 2 hours under shaking. Aliquots of 2 ml were then placed in the main compartment of 25 ml respiration flasks which contained in their central wells pieces of filter paper impregnated with 10% KOH. The filter paper absorbed carbon dioxide produced by the cells. Placed into the side arms of the flasks were 0.2 ml of 50 mM potassium oleate or petroselinate solubilized with 10% Triton X-100. The flasks were attached to a Gilson differential respirometer and oxygen consumption was recorded every 5 min for 40 min. Rates thus obtained represent endogenous oxygen consumption which was between 0.3 and 0.6 $\mu l \cdot O_2$/min per mg of cellular protein. The exogenous fatty acids were then added to cell suspensions by tilting the reaction flasks. After allowing the system to re-equilibrate for 15 min,

oxygen consumption was recorded every 5 min for 40 min. The rates so obtained were not corrected for respiration supported by endogenous substrates because the latter rates could not be determined separately in the presence of exogenous fatty acids.

10) Preliminary Cloning of Gene for 2,4-Dienoyl-CoA reductase.

The gene of 2.4-dienoyl-CoA reductase was isolated by the following protocol.

a) Transformation of E. coli strain SY14 with pSE103 (Kan^R, λcI857).

b) Infection of the transformant SY141 with a phage λSE_6 library.

c) Isolation of phasmid DNA (phSY142).

d) Amplification of bacteriophage phSY142.

e) Isolation of phasmid DNA from lysate of phage produced on a large-scale.

a. Transformation of *E. coli* strain SY14 with pSE103.

Aliquots of SY14 cells grown on LB were inoculated into 5 ml of LB at 1:20 dilution and grown at 37°C to a cell density of Klett 60. The cell suspension was chilled in ice/water for 10 min, centrifuged at 5,000xg and 4°C for 5 min and suspended in an equal volume of 50 mM $CaCl_2$. After rechilling the cells for 30 min, centrifuging and resuspending them into 1/10 of the volume of 50 mM $CaCl_2$, they were placed in ice/water for 20 min. Prechilled pSE103 (10 µl) and 40 µl of TE (10 mM Tris (pH 8.0), 1 mM EDTA) were added to 100 µl of the cells. Cell were heat shocked at 42°C for 2 min in the water bath and then the cell mixture was moved into ice water. LB medium (600 µl) was added to the cells and they were aerobically incubated at 37°C for one hour for phenotype expression(78). Cells were plated onto LB-kanamycin and incubated at 37°C overnight. The transformants (SY141) were isolated and re-checked for their inability to grow on petroselinic acid.

b. Complementation of SY141 with an *E. coli* genomic library in bacteriophage λSE6 .

E. coli strain SY141, which carried plasmid pSE103 containing the gene of the λ phage repressor protein grown at 30 C overnight in 5 ml of TB-maltose (0.5% maltose)(79). After centrifugation at 6,000xg and 4°C for 20 min, the cells were suspended in the same volume of 10-fold diluted MC buffer (10 mM MgSO4, 10 mM Tris (pH 7.5)). This operation was repeated twice. In the meantime, a λSE6 library which previously was amplified to obtain highter titers, was diluted with 10-fold diluted MC buffer (10 mM Tris (pH 7.5), 10 mM MgS04) 10-, 10^2-, 10^4-, or 10^6-fold. Two hundred microliters of cells suspension and the serially diluted λSE6 library were mixed and incubated at 30°C for 20 min without shaking. Aliquots of each mixture were plated onto M9 containing petroselinic acid and then incubated at 30°C. Under these conditions, λSE6 complementing the mutation behaved as a "phasmid", a single-copy plasmid. The Pet^+ transductants (SY142) were isolated after 2 to 5 days. The strain SY142 was checked for its ability to grow on LB-kanamycin plate.

C. Isolation of phasmid DNA.

The phasmid DNA from pet^+ transductant (SY142) was purified and isolated by the following procedure. A single colony of SY142 was grown on 5 ml of LB-kanamycin at 30°C overnight. After centrifugation at 6,000Xg at 4°C for 20 min, cells were suspended in 100 µl of 25% sucrose in H_2O with vigorous vortexing. To this cell suspension 300 µl of M-STET (5% Triton X-100, 50 mM EDTA, 50 mM Tris (pH 8.0), 5% sucrose) were added and mixed well. The mixture was transferred to a 1.5 ml Eppendorf tube and 25 µl of lysozyme (10mg/ml) in H_20 was added. The tube was heated in boiling water for one minute, and chilled in ice/water for 10 min.

After spining down the mixture in a table top centrifuge for 15 min, 200 µl of the supernatant was transferred into a sterile Eppendorf tube. The phasmid DNA was precipitated by adding an equal volume of ice-cold isopropanol, chilled to -80°C for 30 min and centrifuged for 15 min at room temterature. The pellet was dried and resuspended in 100 µl of H_2O (80).

d. Amplification of bacteriophage phSY142.

The phasmid DNA (phSY142) was amplified by transfection into JC7623. The overnight culture of JC7623 in LB was inoculated into 30 ml of LB to obtain a 1:20 dilution and was grown at 37°C to a cell density of Klett 60. The transfection followed the transformation protocol which was described above. After incubation in cold 50 mM $CaCl_2$, the mixture which contained 100 µl of cells and phSY142 was heated at 42°C for 2 min (heat shock). The mixture was added to 2.5 ml of soft TB agar at 47°C and poured onto a TB plate. The plate was incubated right-side up at 37°C (79). A single plaque was picked with a sterile glass capillary, added to 0.5 ml of TB medium and kept at room temperature for one hour. Most of phage was absorbed into TB. The bacteriophage was added to 0.2 ml a JC7623 cell suspension in 10-fold diluted MC buffer which had been grown in TB-maltose medium overnight. The mixture was incubated at 37°C for 30 min to allow cells to preadsorb phage. The mixture was then added to 2.5 ml of soft TB agar at 47°C. The soft agar mixture was poured onto a TB plate and incubated at 37°C overnight. After confluent lysis, the overlay was scraped and transferred into a tube. After scrapping, residual plaques on the plate were suspended with 3 ml of TB medium and collected in a tube which was kept at room temperature for one hour. After centrifuging the tube to remove agar debris, 0.5 ml of $CHCl_3$ was added to the supernatant which was incubated at room temperature for 30 min. The phage in the supernatant was diluted

10^2-, 10^4-, 10^6-, or 10^8-fold with 10-fold diluted MC buffer. Transduction of bacteriophage into JC7623 was performed as mentioned above. The titer of the phage solution was determined after visualizing plaques at 37°C.

e. Purification of the lysates of phage produced on a large-scale.

To prepare phage lysates, a single colony of JC7623 was placed into 100 ml of NZCYM(pH. 7.5) (casein 10g, NaCl 5g, casamino acids 1g, Bacto-yeast extract 5g, $MgSO_4 \cdot 7H_2O$ 2g in one liter.) in a 500 ml flask and incubated at 37°C overnight with vigorous shaking. Aliquotes containing 10^{10} cells were transferred to a tube and centrifuged at 4,000Xg for 10 min at room temperature. The pellet was resuspended in 3 ml of SM buffer(pH.7.5) (NaCl 5.8g, $MgSO_4 \cdot 7H_2O$ 2g, 1 M Tris·Cl and 50 ml 2% gelatin in one liter). The titered bacteriophage with 10^8 pFU/ml was added to the cells and mixed rapidly. The mixture was incubated at 37° for 20 min with intermittent shaking. The infected cells were added to 500 ml of NZCYM and incubated at 37°C with vigorous shaking. The lysis of the bacteria occurred after 9 hours of incubation. Ten mililiters of chloroform were added to the fully lysed culture which was continuously incubated for another 30 min. After centrifugation at 6,000Xg at 4°C for 20 min, the supernatant was saved(78). To an 500 ml liquid lysate of phage, chloroform was added to a final concentration of 2%, in addition to DNase I (1μg/ml), RNase A (1μg/ml) and solid NaCl to a final concentration of 1 M. After incubation at 37°C for 30 min, the solution was centrifuged at 6,000Xg at 4°C for 10 min. Solid polyethylene glycol 8000 (PEG) was added to 10% w/v and the mixture was stored at 4°C for one hour. The intact phage was recovered by centrifugation at 6,000Xg at 4°C for 20 min and resuspended in 3 ml of SM buffer. DNase I and RNase A were added to 5 μg/ml and 100 μg/ml, respectively. After a 30 min incubation at 37°C, the phages were lysed by adding 10% SDS to yield a final concentration of 0.5%, 0.5 M EDTA

(pH 8.0) to 20 mM, and proteinase K to 100 μg/ml. The mixture was heated to 68°C for 30 min. The phage DNA was extracted with an equal volume of phenol, then phenol/chloroform and then chloroform and precipitated by adding 1/2 volumes of 5 M ammonium acetate and two volumes of ethanol. After freezing the mixture at -80°C for 30 min, the precipitate was recovered by centrifugation at 10,000Xg at 4°C for 30 min. To the dry pellets were added 1.6 ml of distilled H_2O, 0.4 ml of 4 M NaCl, 2.0 ml of 13% PEG (w/v)[81]. The resulting precipitate was collected after an hour of incubation on ice followed by a 15 min centrifugation at 10,000Xg for 15 min. The pellet thus obtained was rinsed with 70% ethanol, dried, and resuspended in 500μl of TE buffer (10 mM Tris (pH 8.0), 1 mM EDTA). The purified phasmid DNA was transfected into E. coli strain SY141 which carried plasmid pSE103 and incubated at 30°C for several days in order to re-check for the ability to grow on petroselinic acid as the sole carbon source.

RESULTS

Isolation of E. coli Mutants with a Defective 2,4-Dienoyl-CoA Reductase.

According to the revised pathway of unsaturated fatty acid β-oxidation, the degradation of a monounsaturated fatty acid in E. coli requires only one auxiliary enzyme. If the double bond extends from an odd-numbered carbon atom as in oleic acid, its β-oxidation is dependent on 3-cis,2-trans-enoyl-CoA isomerase while the degradation of a fatty acid with an even-numbered double bond, such as petroselinic acid (6-cis -octadecenoic acid), requires the involvement of 2,4-dienoyl-CoA reductase. Hence, an E. coli mutant lacking 2,4-dienoyl-CoA reductase should be able to grow on oleic acid, but not on petroselinic acid, as the sole carbon source. This reasoning formed the basis for the isolation of E. coli mutants deficient for 2,4-dienoyl-CoA reductase by selecting for growth on oleic acid and no growth on petroselinic acid.

E. coli strain RS3010 which is a fadR mutant of wild-type strain K-12 Ymel was used as the parental strain. Wild-type K-12 Ymel must be grown in the presence of long-chain fatty acid ($> C_{12}$) to induce the synthesis of the five key enzyme involved in the β–oxidation of fatty acids. In contrast, the fadR mutant RS3010 is able to utilize medium-chain as well as long-chain fatty acids as the sole carbon source and it constitutively synthesizes the fad enzymes. Therefore, RS3010 strain can grow on petroselinic acid without a long lag phase. Strain RS3010 was mutagenized with ethyl methane sulfonate, which modifies bases of DNA by alkylation resulting in transition (pyrimidine→ pyrimidine or purine→ purine) or transversion (pyr→ pu or pu →pyr) mutations. The mutants were enriched by

growing mutagenized cells for more than ten hours on a medium containing petroselinic acid and ampicillin. In the previous experiment, it was established that a doubling time of the cells in petroselinic acid containing medium is ten hours. Fourteen mutants were selected which were unable to grow on a medium containing petroselinic acid as the sole carbon source. Cell extracts from these mutants were assayed for 2,4-dienoyl-CoA reductase and 3-hydroxyacyl-CoA dehydrogenase, a component enzyme of the multienzyme complex of fatty acid oxidation (Table II). As indicated by the measured levels of 2,4-dienoyl-CoA reductase activities presented in Fig. 4, none of mutants was completely devoid of 2,4-dienoyl-CoA reductase activity but some exhibited significantly lower reductase activity levels in the presence of normal or near normal dehydrogenase activity. The mutant SY14 which exhibited 12% of the parental reductase activity and a normal level of dehydrogenase activity was chosen for further studies.

Characterization of Mutant SY14.

Growth rates of the parental strain RS3010 and mutant SY14 were determined in three different media containing either acetate, oleate, or petroselinic acid as the sole carbon source. If the inoculum was taken from a saturated culture consisting half-starved, slowly multiplying cells, there was a long lag time before the cells resumed rapid growth. Therefore exponentially growing cells were transferred to the medium to measure cell growth at 555 nm. In media containing either acetate (Fig. 5) or oleate (Fig. 6), RS3010 and SY14 grew at similar rates with doubling time of approximately two hours. However, in a petroselinic acid containing medium (Fig. 6), SY14, as expected, was unable to grow, whereas RS3010 grew slowly with a doubling time of ten hours. The growth rate of RS3010 in oleic acid

Table II.

Activities of 2,4-dienoyl-CoA reductase and 3-hydroxyacyl-CoA dehydrogenase in E. coli mutants able to grow on oleic acid but not on petroselinic acid.[a]

Mutant	2,4-Dienoyl-CoA reductase		3-Hydroxyacyl-CoA dehydrogenase	
	Spec. act.	Rel. act.	Spec. act.	Rel. act
	nmol/min/mg	%	nmol/min/mg	%
RS3010[b]	14.0	100	1000	100
SY01	14.0	100	140	14
SY02	11.1	79	400	40
SY03	9.0	64	450	45
SY04	8.4	60	700	70
SY05	7.4	53	200	20
SY06	5.2	37	600	60
SY07	3.6	26	900	90
SY08	3.5	25	960	96
SY09	3.4	24	1100	110
SY10	2.8	20	700	70
SY11	0.9	14	600	60
SY12	1.8	13	300	30
SY13	1.8	13	180	18
SY14	1.5	11	960	96

[a] All mutants were grown on M9 mineral salts medium with oleate as the carbon source.

[b] Parental strain.

Figure 4.

Relative specific activities of 2,4-dienoyl-CoA reductase (closed box) and 3-hydroxyacyl-CoA dehydrogenase (open box) in the mutants which were not growing on petroselinic acid. The activities of the parental strain RS3010 was set as 100% .

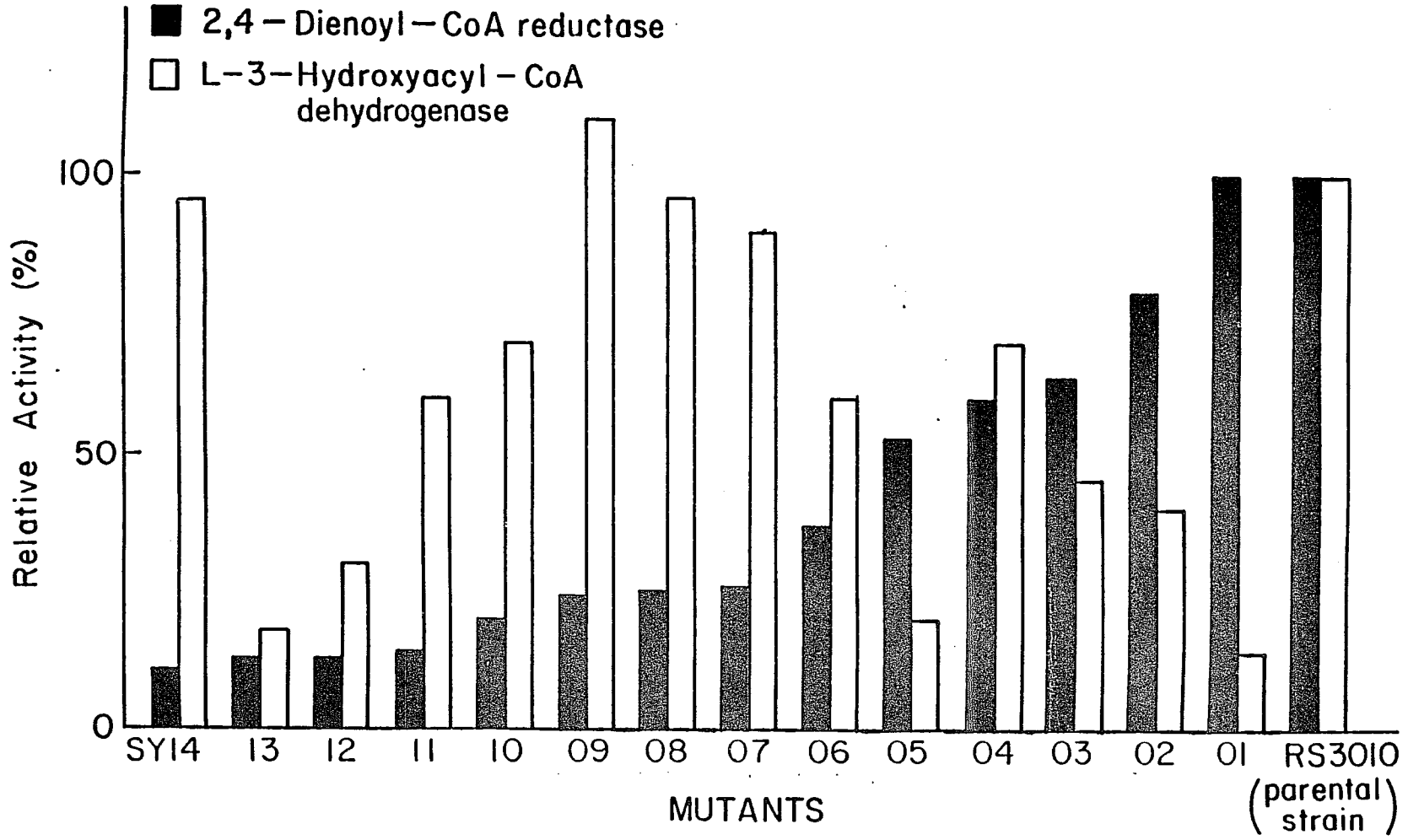

2,4 – Dienoyl – CoA reductase
L–3–Hydroxyacyl – CoA dehydrogenase
Relative Activity (%)
100
50
0
SY14
13
12
11
10
09
08
07
06
05
04
03
02
01
RS3010
(parental strain)
MUTANTS

Figure 5.

Growth behavior of E. coli parental Pet $^{+}$ strain RS3010 (circles) and mutant SY14 (squares) on acetate as the sole carbon source.

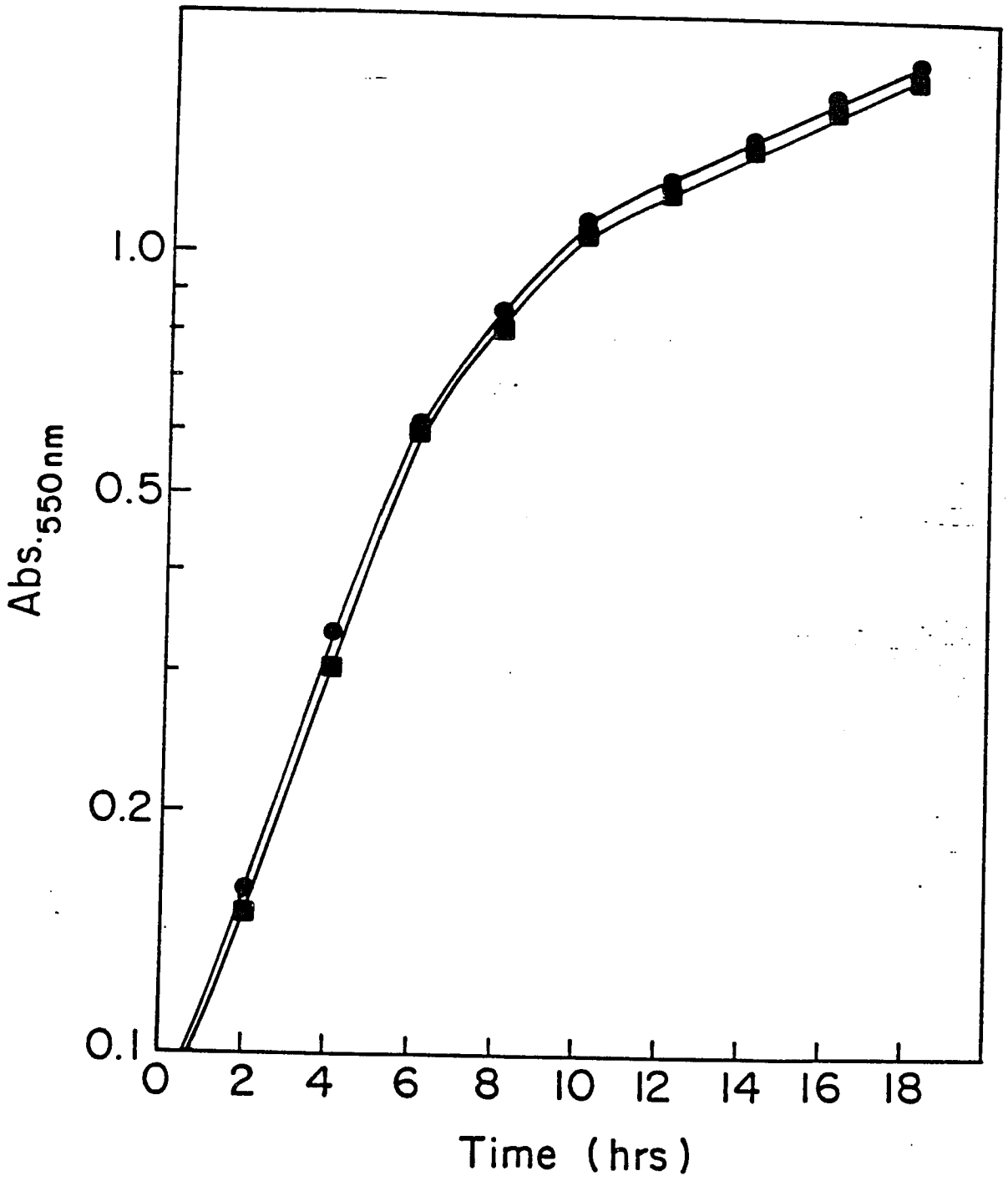
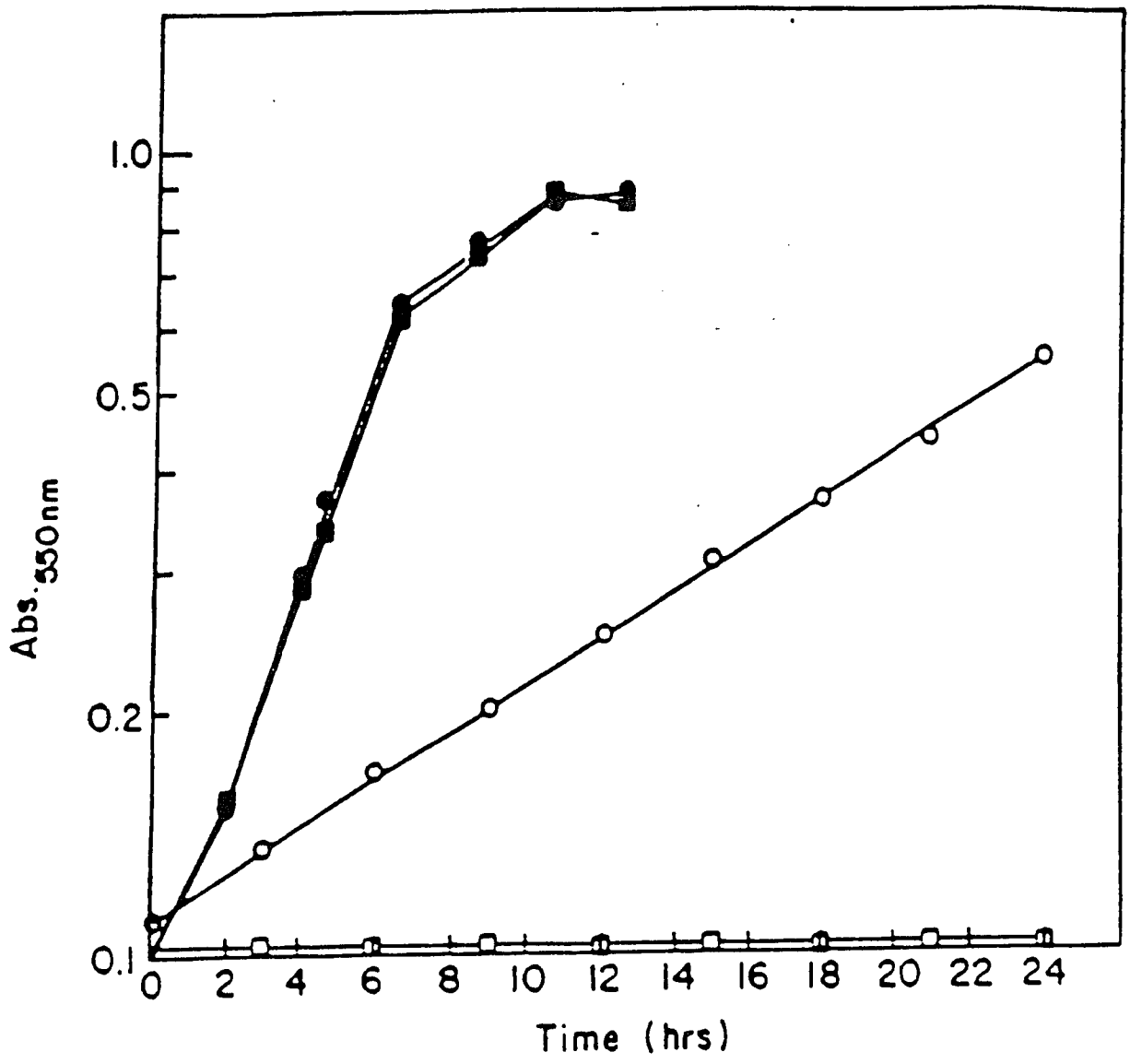

1.0
0.5
0.2
0.1
Abs.550nm
0
2
4
6
8
10
12
14
16
18
Time (hrs)

Figure 6.

Growth behavior of parental Pet^+ strain RS3010 (circles) and mutant SY14 (squares) on oleic acid (solid symbols) or petroselinic acid (open symbols) as the sole carbon source.


1.0
0.5
0.2
0.1
Abs.350nm
0
2
4
6
8
10
12
14
16
18
20
22
24
Time (hrs)

was five times faster than in petroselinic acid. The doubling times were directly estimated from the growth curves.

Strains SY14 and RS3010 were further compared by assaying the cell extracts of both strains for 3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, as well as enoyl-CoA hydratase with crotonyl-CoA (C_4) and 2-decenoyl-CoA (C_{10}) as substrates, and acyl-CoA synthetase with oleic and petroselinic acid as substrates. The data shown in Table III, demonstrate that three enzymes in the multienzyme complex were present at comparable levels in both strains except for 2,4-dienoyl-CoA reductase whose activity in the mutant was only 12% of the activity observed in the parental strain. Especially, acyl-CoA synthetase which is not part of the multienzyme complex was assayed with oleic acid and petroselinic acid as substrates, and activities toward these two fatty acids were not significantly different in parental and mutant strains. This observation supports the suggestion that the uptake of long-chain fatty acids in mutant SY14 is not affected by the mutation. It also seems that the position of the double bonds does not affect the activities of acyl-CoA synthetase. Acyl-CoA dehydrogenases which are also not part of the multienzyme complex, were not assayed because their rates, which are very low, are obliterated by fast nonspecific reactions between dichlorophenol-indophenol (DCPIP), the terminal electron acceptor of the assay, and sulfhydryl containing compounds present in the extract and/or formed during the course of the assay.

Table III.

Specific activities of β-oxidation enzymes in parental strain RS3010 and mutant SY14.

Enzyme	Substrate	Spec. Act. RS3010[a]	Spec. Act. SY14[a]	Rel. Act. in SY14
		nmol/min/mg		%
Acyl-CoA synthetase	Oleic acid	0.18	0.22	122
	Petroselinic acid	0.16	0.16	100
2,4-Dienoyl-CoA reductase	2,4-Decadienoyl-CoA	13.2	1.62	12
3-Hydroxyacyl-CoA dehydrogenase	Acetoacetyl-CoA	1,030	1,420	138
Enoyl-CoA hydratase	Crotonyl-CoA	1,130	1,100	100
	2-Decenoyl-CoA	476	400	84
3-Ketoacyl-CoA thiolase	Acetoacetyl-CoA	62	70	113

[a] Cells were grown in M9-mineral salts medium containing oleate and acetate.

Immunological Studies.

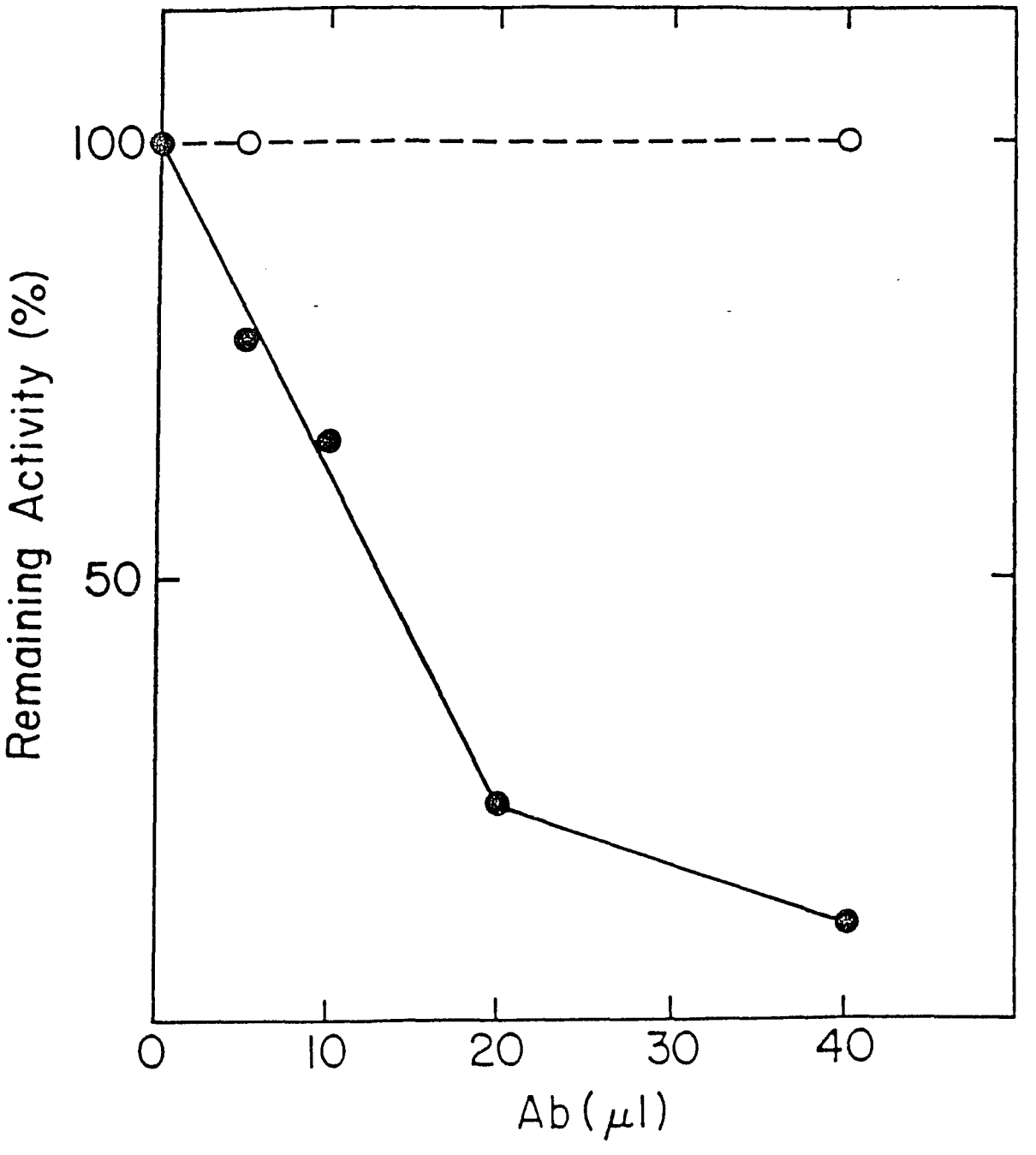
a. Immunoprecipitation with *Staphylococcous aureus* Cell Suspensions Containing Protein A.

In order to determine if the low 2,4-dienoyl-CoA reductase activity of mutant SY14 results from lower amounts of the enzyme or reflects the presence of an enzyme with lower specific activity, an attempt was made to quantitate immunologically the amount of the reductase. Antibodies, against the E. coli. 2,4-dienoyl-CoA reductase were obtained from Dr. Kunau and were used for the following immunological studies.

Generally immunoprecipitation of antigen-antibody complexes from solution can be used to estimate the amount of antigen present in samples. Multivalent antigens may interact with multivalent antibodies to form large insoluble lattices or small soluble complexes. The cell suspension of Staphylococcous aureus containing protein A on the cell surface of cells facilitates the formation of large insoluble precipitates of the antigen-antibody complex. Precipitation of antigen with antibodies and S. aureus cells results in the removal of the reductase from the solution and the 2,4-dienoyl-CoA reductase activity remaining in solution was determined by the standard assay. As shown in Fig. 7, 2,4-dienoyl-CoA reductase activity remaining in solution decreased with increasing amounts of antibodies added to the solution. S. aureus cells by themselves had no effect on the reductase activity (data not shown) nor did they cause its precipitation in the absence of specific anti-reductase antibodies. Also, 2,4-dienoyl-CoA reductase was not precipitated with antibodies in the absence of S. aureus cells (data not shown). Unfortunately, the low reductase activity of mutant strain SY14 prevented quantitation of the amount of reductase by this approach.

Figure 7.

Immunotitration of 2,4-dienoyl-CoA reductase present in a soluble extract of E. coli RS3010. The crude extract of RS3010 (0.4 mg of protein in 20 µl) was titrated with increasing amounts (0 to 2.4 mg of protein in 40 µl) of anti-E. coli-2,4-dienoyl-CoA reductase antibodies in the presence (solid circles) and in the absence (open circles) of Staphylococcous aureus cells (0.5 ml of 10% cell suspension).


100
50
Remaining Activity (%)
0
10
20
30
40
Ab (μl)

b. Ouchterlony Immunodiffusion.

The amounts of 2,4-dienoyl-CoA reductase present in strains RS3010 and SY14 were compared by the double immunodiffusion method of Ouchterlony[71]. Cell extracts of both strains cross-reacted with antibodies raised against E. coli 2,4-dienoyl-CoA reductase and both cell extracts were observed to give rise to single precipitin lines (see Fig. 8). The intensities of the precipitin lines decreased with decreasing amounts of cell extract (see Fig. 8A and 8B). Since the intensities of these bands are similar for corresponding amounts of cellular protein, the quantities of antigen present in both strains appear to be similar. With both extracts the limit of detecting a precipitin line was reached when less than 100 μg of cellular protein were applied to one well. Based on measured activities and published values of the specific activities of the purified E. coli 2,4-dienoyl-CoA reductase[10], the amount of enzyme in the cellular extract of the parental strain can be estimated to be 0.1% of the total protein. Therfore, 100 μg of cellular protein, which are necessary to detect a precipitin line, contain 0.1 μg of 2,4-dienoyl-CoA reductase. Ouchterlony immunodiffusion performed with antibodies raised against the bovine liver 2,4-dienoyl-CoA reductase did not lead to the formation of precipitin lines (data not shown).

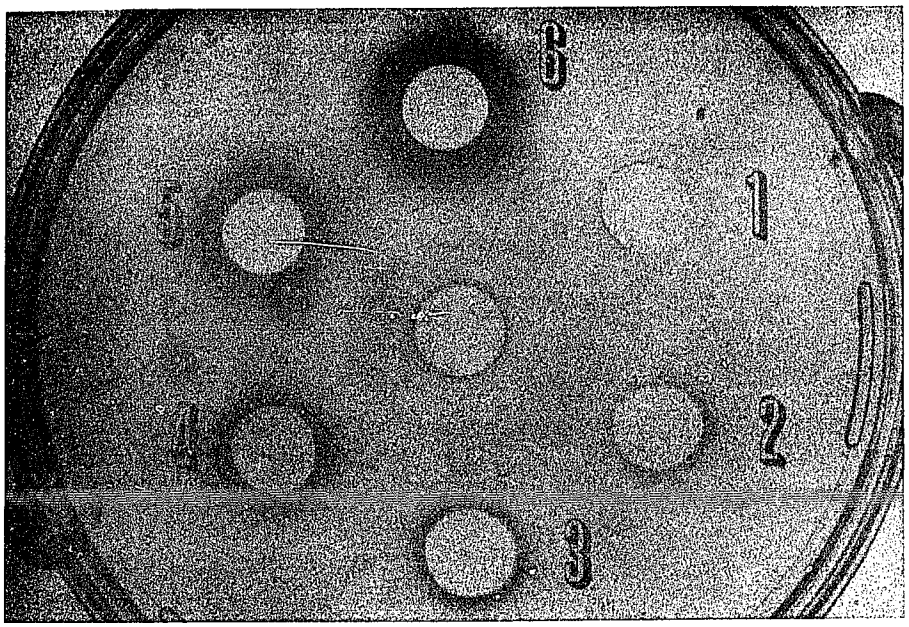
c. Western Blotting.

Western blotting is a rapid and highly sensitive assay for the detection and characterization of proteins ; less than one nanogram of protein may be detected. The cellular extracts of strain RS3010 and SY14 were separated on 8% SDS-PAGE gel. The use of pre-stained SDS-PAGE standards makes it possible to continously monitor the migration of proteins during the electrophoretic run. Moreover, the pre-stained marker proteins permit the visual inspection of the efficiency at which

Figure 8.

Ouchterlony immunodiffusion analysis of 2,4-dienoyl-CoA reductase in extracts of (A) RS3010 and (B) SY14. Anti-E. coli-2,4-dienoyl-CoA reductase antibodies (0.6 mg) were placed in the center wells. Appied to the outer wells were serially diluted cell extracts containing 1) 40 µg, 2) 100 µg,3) 200 µg, 4) 400 µg, 5) 800 µg and 6) 1,200 µg of protein.

A



B

proteins are transferred from gels to nitrocellulose membranes. The intensities of 2,4-dienoyl-CoA reductase bands of serially diluted cellular extracts from the parental and mutant strains were compared (see Fig. 9). The major protein band detected immunologically with antibodies to the E. coli, reductase corresponds to a protein with a molecular weight close to 70,000 which is the reported molecular weight of the E. coli, 2,4-dienoyl-CoA reductase[19]. The amounts of antigens in strains RS3010 and SY14 were also quantitatively evaluated by comparing the intensities of the bands obtained with serially diluted cellular extracts. The intensities of the 70-kDa band with cellular extracts of strains RS3010 and SY14 were not significantly different up to a 1,250-fold dilution of the cellular extracts. Based on the above immunological result, it seems that strains RS3010 and SY14 possess equal or near equal amount of reductase protein. Hence, the mutation resulted in the formation of normal or near normal amounts of reductase with lower specific activity.

Genetic Complementation of Reductase Mutant SY14.

Mapping of the mutation was carried out by use of a number of E. coli strains containing F' s that correspond to different regions of the E. coli chromosome[82, 83]. The segment of the bacterial chromosome carried by the F' is transferred to mutant cells by the same mechanism that brings about the transfer of F factor from F^+ to F^- or from Hfr (high frequency recombinant) to F^-. The F' kit contains all or almost all of the genetic material of the E. coli chromosome. The activity of the F' kit were first confirmed with a several well characterized E. coli mutants. Successful complementation of mutant strain SY14 was achieved with two plasmids, F'141 and F'140, which contain the 67 to 75.5 min region and the 67 to 81 min region of the E. coli chromosome(see Fig. 10). Transconjugants thus

obtained grew on petroselinic acid as the sole carbon source. A small plasmid, F'102, which carries the 67 to 71 min region did not complement the mutation. Based on these data, it was concluded that the gene for 2,4-dienoyl-CoA reductase is located between 71 min and 75 min on the E. coli chromosome. Thus, the reductase gene is not part of any known fad gene including the fadAB operon located at 86 min of the E. coli chromosome which harbors all key β-oxidative enzymes except for acyl-CoA dehydrogenase. This conclusion is also supported by the observation that fadAB mutant RS3084, which is devoid of all activities associated with the fatty acid oxidation complex, exhibits wild-type level of 2,4-dienoyl-CoA reductase activity (Table IV).

To provide further proof that a purified transconjugant derived from the cross of SY14 and the strain carrying F'141 can grow on petroselinic acid because of its higher reductase activity, this construct as well as the mutant, the parental Pet^+ strain and the donor strain carrying F'141 were assayed for 2,4-dienoyl-CoA reductase and several other β-oxidation enzymes. The data presented in Table V demonstrate that introduction of F'141 into the mutant resulted in an increase in the reductase activity from 12% to 80% of the level observed in the parental Pet^+ strain. Interestingly, the donor strain for F'141 has very high reductase activity presumably due to the presence of two reductase genes, one on the plasmid and one on the chromosome. In contrast to the expected change in the reductase activity, the presence or absence of the F' had little effect on the activity levels of 3-hydroxyacyl-CoA dehydrogenase, 3-hydroxyacyl-CoA epimerase and 3-cis,2-trans-enoyl-CoA isomerase all of which are component enzymes of the fatty acid oxidation complex coded for by the fadAB operon.

Figure 9.

Western blotting of 2,4-dienoyl-CoA reductase present in cellular extracts of RS3010 (odd-numbered lanes) and SY14 (even-numbered lanes).

The cellular extracts of both strains (19 mg/ml) were diluted 10-fold (lane 1 &2), 50-fold (lane 3 &4), 250-fold (lane 5 &6), 1,250-fold (lane 7&8) and 6,250-fold (lane 9&10) and equal aliquots were subjected to SDS-PAGE gel electrophoresis. The reductase band was detected by reaction with goat-anti rabbit Ig-alkaline phosphatase conjugate. The prestained low molecular weight protein kit was used as molecular weight markers.

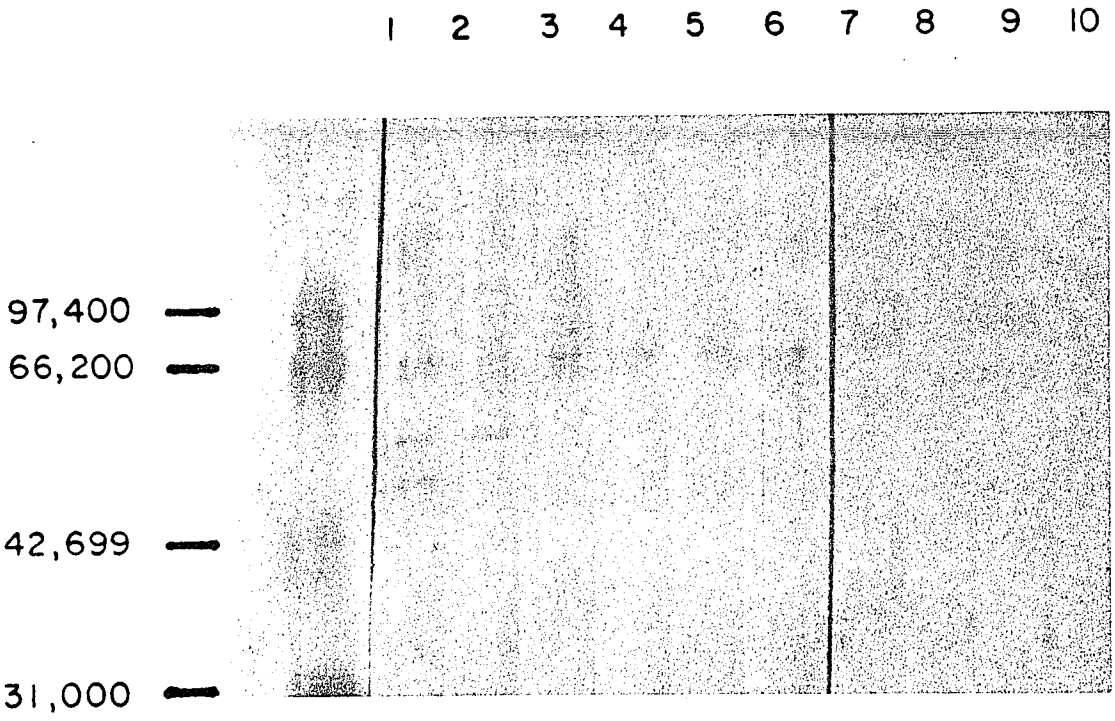
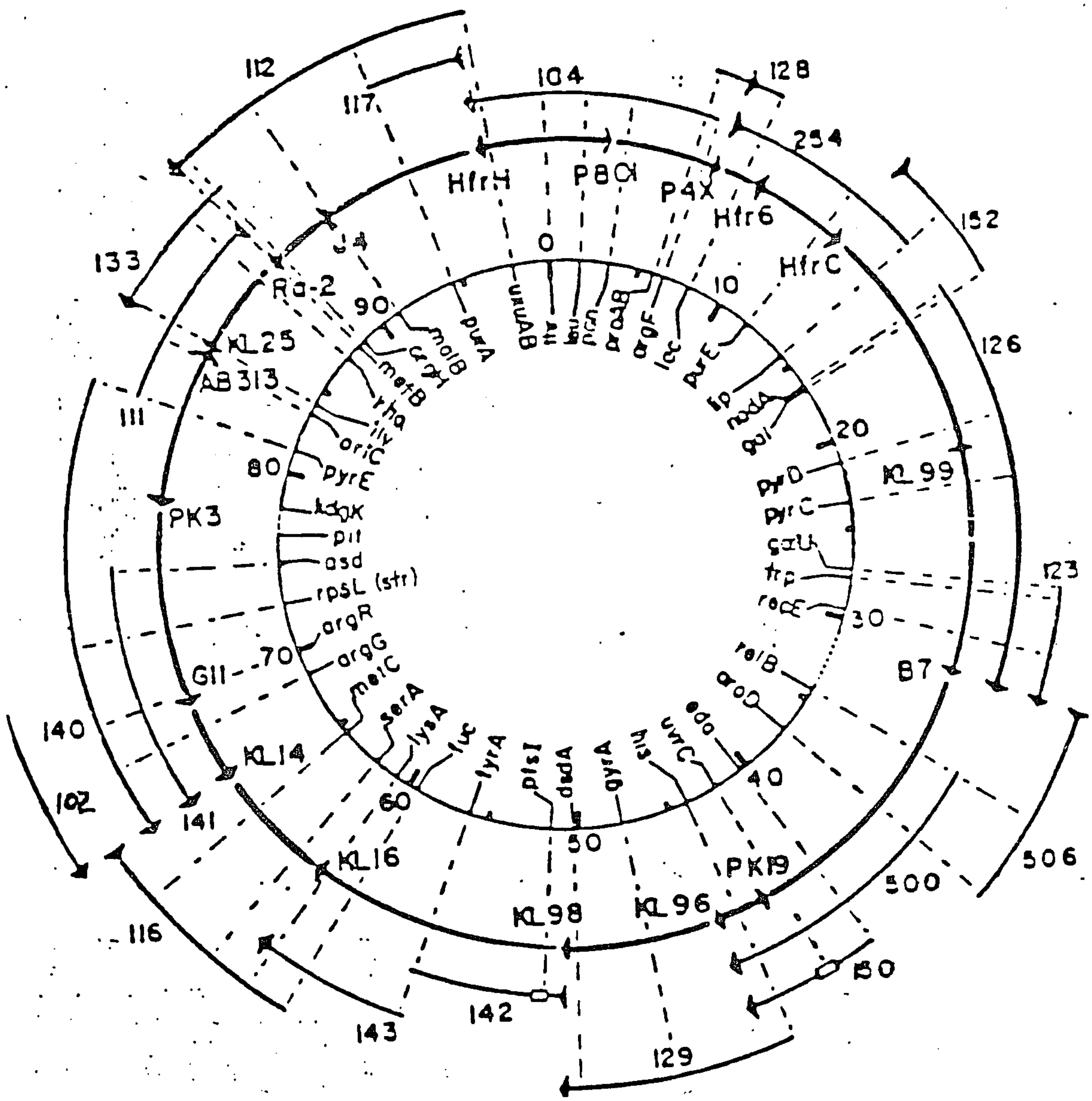

1 2 3 4 5 6 7 8 9 10
97,400
66,200
42,699
31,000

Figure 10.

Genetic map coodinates of the points of origin of some Hfr strains and F-prime factors. The dotted portions of the inner circle indicate gaps in transductional linkage. The point of the arrowhead for any Hfr indicates the point of origin. During conjugational transfer, this is immediately followed by transfer of the base of the arrowhead. Strains of the F'kits were cross-streaked against SY14 on agar plates containing M9 mineral salts medium plus petroselinic acid as the sole carbon source.



104
128
112
117
254
152
133
126
111
123
140
102
141
506
500
116
143
142
129
150
HfrH
P801
P4X
Hfr6
HfrC
J4
Ra-2
KL25
AB313
PK3
G11
KL14
KL16
KL98
KL96
PK19
B7
KL99
0
10
20
30
40
50
60
70
80
90

Table IV.

Specific activities of 2,4-dienoyl-CoA reductase and 3-hydroxyacyl-CoA dehydrogenase in E. coli strain RS3084 and RS3010.

Strain	2,4-Dienoyl-CoA reductase		3-hydroxyacyl-CoA dehydrogenase	
	Spec. act.[a]	Rel. act.	Spec. act.[a]	Rel. act.
	nmol/min/mg	%	nmol/min/mg	%
RS3010	14	100	1,078	100
RS3084	14	100	0.034	0.03

[a] Strains were grown on M9 mineral salts medium with oleate as the sole carbon source.

Table V.

Specific activities of some β-oxidation enzymes in different E. coli strains.

Enzyme	E. coli strains							
	RS3010		SY14		SY14/F'141		F' donor	
	S.A.[a]	R.A.[b]	S.A.[a]	R.A.[b]	S.A.[a]	R.A.[b]	S.A.[a]	R.A.[b]
2,4-Dienoyl-CoA reductase	8.5	100	1.04	12	6.8	80	41.4	487
3-Hydroxyacyl-CoA dehydrogenase	984	100	99	101	762	77	1,444	147
3-Hydroxyacyl-CoA epimerase	62	100	55	89	48	77	54	87
3-cis,2-trans-Enoyl-CoA isomerase	260	100	271	104	252	97	309	119

[a] Specific activity in nmol/min of mg of protein.

[b] Relative activity in percent.

Respiration Measurements.

The capacity of E. coli cells to oxidize either oleic acid or petroselinic acid was evaluated by measuring rates of respiration with one of these fatty acids as the sole carbon source. With oleic acid as a substrate, similar rates were observed for four strains tested except for wild-type K-12 Ymel strain which exhibited a slightly higher activity than other strains (Table VI). In contrast, rates of respiration with petroselinic acid as the substrate were significantly different for strains RS3010, SY14 and the transconjugant all of which have the same genetic background (Table VII). The data presented in Fig. 11 show that rates of respiration supported by petroselinic acid are proportional to the level of 2,4-dienoyl-CoA reductase activity up to a level characteristic of wild-type K-12 Ymel. However, rates of respiration observed with the donor strain F'141 are hardly higher than those of wild-type K-12 Ymel even though the reductase activity is several times greater.

Induction of 2,4-Dienoyl-CoA Reductase.

In order to establish whether or not the expression of 2,4-dienoyl-CoA reductase is subject to the same control as are other enzymes of fatty acid oxidation, E. coli strain K-12 Ymel was grown on different media containing either glucose, acetate, succinate, tryptone, oleate, petroselinic acid or combinations thereof as carbon sources. Soluble extracts of cells thus grown were assayed for 2,4-dienoyl-CoA reductase and 3-hydroxyacyl-CoA dehydrogenase. As is apparent from the data presented in Table VIII, the specific reductase activity is influenced by the growth media. The presence of oleate or petroselinic acid plus tryptone in the growth medium resulted in elevated levels of 2,4-dienoyl-CoA reductase and 3-hydroxyacyl-CoA dehydrogenase activities. In contrast to its effect on 3-hydroxy-

Table VI

Respiration rate of E. coli strains in a medium containing oleic acid as the sole carbon source.

Strain [a]	Phenotype	Rate of oxidation	
		µl·O2/min/mg	%
RS3010	Pet+ Const[b]	7.0	100
SY14/F'141	Pet+ Const[b]	6.8	97
SY14	Pet- Const[b]	6.3	90
K-12 Ymel	Pet+ Indu[c]	9.0	129
F' donor (#4248)	Pet+ Indu[c]	6.5	93

[a] All strains were grown on M9 mineral medium containing acetate and oleate except #4248, for which contained *his*, *leu* and *met*, additionally.

[b] These strains were constitutive for the synthesis of β-oxidation enzymes.

[c] These strains were inducible for the synthesis of β-oxidation enzymes.

Table VII

Respiration rates supported by petroselinic acid and specific activities of 2,4-dienoyl-CoA reductase of several E. coli strains.

Strains	Oxygen consumption [a]	Rel.act.	2,4-Dienoyl-CoA reductase Spec. act.[b]	Rel. act.
	µl·O2/min/mg	%	nmol/min/mg	%
[Exp. 1]				
RS3010	3.0	100	7.5	100
SY14/F'141	2.4	80	6.1	81
SY14	0.4	13	0.8	11
K-12 Ymel	3.5	117	9.6	128
[Exp. 2]				
RS3010	2.7	100	13	100
SY14	0.4	15	1.7	13
F' donor (#4248)	3.3	122	35	269

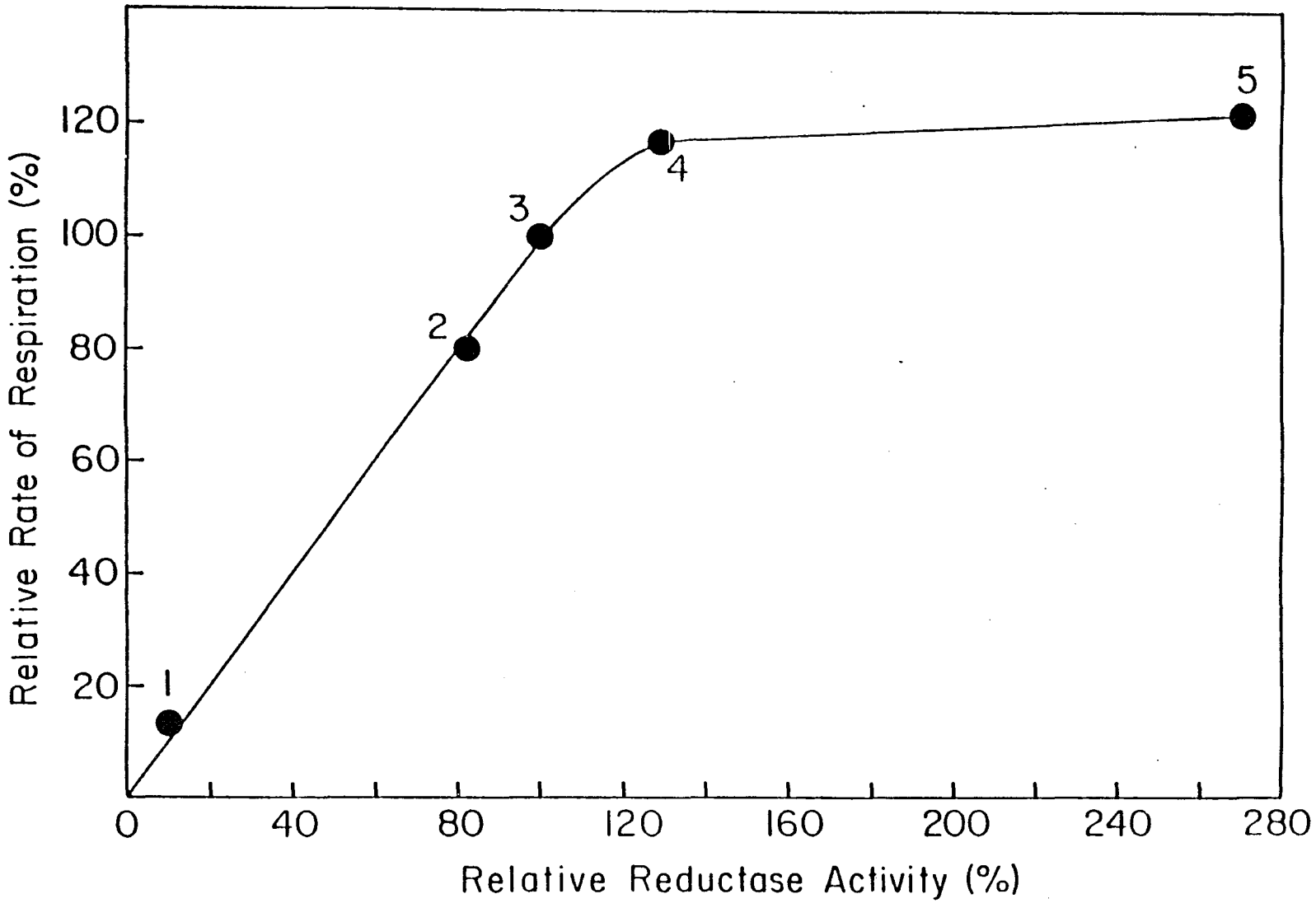
[a] Oxygen consumption were measured in a medium containing petroselinic acid.

[b] All strains were grown on M9-mineral medium with acetate and oleate as the carbon source.

Figure 11.

Relative respiration rates of cells suspended in a medium containing petroselinic acid as the sole carbon source.

1) SY14 (mutant), 2) transconjugant (SY14 complemented with F'141), 3) RS3010 (parental strain) , 4) K-12 Ymel (wild-type), and 5) F'141 (CGSC #4248). The respiration rate of strain RS3010 and its reductase activity were set as 100%.


Relative Rate of Respiration (%)
120
100
80
60
40
20
0
40
80
120
160
200
240
280
Relative Reductase Activity (%)
1
2
3
4
5

acyl-CoA dehydrogenase, long-chain fatty acids caused relatively small increases in the reductase levels when added to media containing either acetate or succinate. However, for both the reductase and dehydrogenase the lowest activity levels were observed when cells were grown on glucose or glucose plus oleate as carbon sources. Thus, it seems that 2,4-dienoyl-CoA reductase, as other β-oxidation enzymes, is induced by long-chain fatty acids. Unfortunately, the induction of both enzyme by petroselinic acid alone could not be determined due to the inability of cells to grow on petroselinic acid as the sole carbon source.

Effect of Cyclic AMP on the Expression of Reductase Level.

Since the low activity levels of 2,4-dienoyl-CoA reductase in cells grown on glucose could be the consequence of catabolite repression, the effect of adding cAMP to the growth medium on the reductase activity levels was studied. Addition of cAMP to the growth medium containing glucose resulted in a 3-fold increase in actvity of the dehydrogenase and the reductase (see Table IX). A further 2- to 3-fold increase was caused when additionally oleate was present. However, maximal reductase as well as dehydrogenase activity levels were obtained with oleate alone. Thus, it appears that the expression of 2,4-dienoyl-CoA reductase, similar to the expression of other fad enzymes, is subject to catabolite repression by glucose which can be partially overcome by the presence of cAMP in the growth medium.

Table VIII.

Induction of 2,4-dienoyl-CoA reductase and 3-hydroxyacyl-CoA dehydrogenase in E. coli wild-type K-12 Ymel as a function of the carbon source in the growth medium.

Medium	2,4-Dienoyl-CoA reductase Rel. act.[b]	3-Hydroxyacyl-CoA dehydrogenase Rel. act.[b]
Oleate [a]	100	100
Oleate & tryptone	94	87
Petroselinate & tryptone	95	100
Tryptone	17	17
Acetate & oleate	44	39
Acetate	15	2.2
Succinate & oleate	43	41
Succinate	21	3.2
Glucose & oleate	3.5	0.5
Glucose	3	0.3

[a] Specific activities of reductase and hydrogenase grown on oleate were 22.8 mU/mg ± 4.6 and 1,078mU/mg ± 352, respectively, based on four separate induction experiments.

[b] Relative activity in percent.

Table IX.

Effect of cAMP on the levels of 2,4-dienoyl-CoA reductase and 3-hydroxyacyl-CoA dehydrogenase in wild-type K-12 Ymel grown on different carbon sources.

Medium	Relative activity	
	2,4-dienoyl-CoA reductase	3-hydroxyacyl-CoA dehydrogenase
Glucose	9	1
Glucose & 50 mM cAMP	27	3
Glucose & oleate	33	1
Glucose & oleate & 50 mM cAMP	56	9
Oleate	100	100

Preliminary Cloning of the Gene for 2,4-Dienoyl-CoA Reductase.

In order to clone and sequence the gene for 2,4-dienoyl-CoA reductase of E. coli, we first isolated the reductase gene by complementation of mutant SY14 with an E. coli genomic library present in λSE6. In a first attempt, five transductants (SY141) of SY14 harboring the plasmid pSE103 with a gene for the λ phage repressor protein (cI) were isolated on LB-kanamycin plates. All of these colonies (Kan^R) were unable to grow on petroselinic acid as the sole carbon source and therefore retained the fadH allele. The λSE6 library containing the E. coli genome in about 400 vectors particles was amplified to give high titers upon growth (10^8 pFU/ml) and then it was stored at -80°C. Transfection efficiency of the λSE6 library was gradually reduced on freezing the cells at -20°C. This E. coli genomic library λSE6 was introduced into SY141 carrying plasmid pSE103. Under these conditions, complementation of SY141 was seen by growth of colonies on petroselinic acid as the sole carbon source. One hundred fifty colonies were counted on the petroselinic acid plate after three days of incubation at 30°C. Fifty transductants were randomly selected and checked for their ability of growth on LB-kanamycin plates. Five of those Pet^+ transductants (SY142) were selected and phasmid DNA was isolated from each. The phasmid DNA was amplified by transfection into JC7623. After transfection of JC7623 with phasmid DNA, the DNA is incoporated into phage particles and subsequent lytic growth, leads to the formation of plaques. Most isolated plaques were picked as soon as they were clearly visible.to prevent possible contamination from nearby plaques. Five clear plaques of different sizes were selected and amplified by transduction. The inoculum of JC7623 was always grown on TB medium in the presence of maltose and the saturated culture was stored in a $MgSO_4$ solution to maximize the transduction efficiency. The bacterial protein which is encoded in the mal operon is

required to absorb the phage and it is induced by maltose. The high-titer plate stocks are those containing plagues that just overlap to give confluent lysis. Too much overlap kills the cell lawn too early giving a very clear plate and low phage yield. If the plaques do not overlap, few cells are infected and resulting in low phage yield. Therefore, the incubation at high concentrations of phage and cells assures good adsorption. The TB agar should not be cooled below 42°C, otherwise it starts to solidify. If the agar is not completely melted, the plates have a grainy appearence after incubation and plaques are poorly defined. All five preparations of phage obtained by the plating method, had relatively high titers of $6X10^7$, $1X10^8$, $5X10^8$, $1X10^8$ and $1X10^9$ pFU/ml, respectively. With the above phage, phage lysates were prepared by incubating the high titer phage preparations and JC7623 in the medium. After 7 hours of shaking at 37°C, a lot of debris was formed in the liquid medium. After two more hours of incubation confluent lysis occured. The exact time for confluent lysis depended on the number of phages and cells as well as on all growth rates. After purification of phage, the phasmid DNA was analyzed by applying it to 0.7% agarose gel electrophoresis. On an agarose gel, a single band was detected, however, its molecular weight could not be estimated since it was too large. The purity of the phasmid DNAs was also determined by measuring the absorbance at 280 nm and 260 nm, the ratio of A 260/280 nm was between 1.8 and 1.83 in each phasmid DNA preparation. The total amount of phasmid DNA from each preparation was approximetely 0.5 mg (1μg/μl). This phasmid DNA will be used for cloning the gene for 2,4-dienoyl-CoA reductase.

DISCUSSION

E. coli mutants lacking 2,4-dienoyl-CoA reductase were isolated, characterized and used to provide in vivo evidence of the essential function of 2,4-dienoyl-CoA reductase in fatty acid oxidation. Mutagenized E. coli cells RS3010 (fadR) were selected by comparing their growth on plates containing as a sole carbon source either oleate or petroselinic acid. Mutants with defective 2,4-dienoyl-CoA reductase were able to grow on either acetate or oleate but not on petroselinic acid (6-cis-octadecenoic acid) because its oxidation requires 2,4-dienoyl-CoA reductase as an auxiliary enzyme to remove the 6-cis double bond after moving to the 4-position due to chain shortening. Other fad mutants were distinguished from reductase mutants by their inability to grow on either oleate or petroselinic acid. Initially, wild-type K-12 Ymel was used as the parental strain. Unfortunately, mutants could not be selected due to their inability to grow on petroselinic acid in the presence of ampicillin. However, the spontaneous repressor mutant RS3010 grew on petroselinic acid in the presence of ampicillin possibly because it possesses constitutive levels of the fad enzymes[84,85]. Although the initial screening of mutagenized cells yielded more than fifty colonies that grew on oleate but not on petroselinic acid, only fourteen exhibited the correct phenotype after purification and none was completely devoid of reductase activity. However, the majority of these mutants showed greatly reduced levels of 2,4-dienoyl-CoA reductase although some had lower than normal activities of 3-hydroxyacyl-CoA dehydrogenase.

Mutant SY14, which had only 12% of the parental reductase activity but exhibited a normal dehydrogenase level, was chosen for further studies. Since all other β-oxidation enzymes assayed for were present in this mutant at near normal levels, it appears that the only defect of this mutant in fatty acid oxidation is its

greatly reduced 2,4-dienoyl-CoA reductase activity. Although 12% of the reductase activity were always detected by the assay based on the oxidation of NADPH, it may be possible that SY14 possesses lower than 12% of the parental reductase activity if all of part of the measured activity was due to a nonspecific reaction of the substrate with other NADPH-dependent reductases. According to a report by Kunau and coworkers[32] 2-trans,4-cis-decadienoyl-CoA is also a poor substrate of 2-enoyl-CoA reductase, which acted on this substrate at approximately 2% of the rate observed with monoene esters of the same chain lengths. The authors suggested that 2,4-dienoyl-CoA was contaminated with 2-trans-enoyl-CoA and that the latter compound was responsible for the reaction observed with 2-enoyl-CoA reductase.

A comparison of the growth behaviors of the strains RS3010 and SY14 proved them to grow at similar rates on oleate or acetate. However, on petroselinic acid RS3010 grew very slowly and SY14 did not grow at all. The doubling time of RS3010 on oleate was only one fifth of the time observed on petroselinic acid. Since growth on acetate or fatty acids as the sole carbon source requires operation of the glyoxylate shunt[86-88], I conclude that the reductase mutant SY14 has a fully functional shunt in addition to a normal capacity of oxidizing oleate and presumably saturated fatty acids.

The immunological characterization of strains RS3010 and SY14, indicated that the reductase antigen is present in the mutant at levels equal or near equal to that of the parental strain. Single precipitin lines were observed when extracts of the parental and mutant strains were tested with monospecific antibodies to the reductase by Ouchterlony double immunodiffusion. The reductase levels in the parental and mutant strains were also tested by Western blotting. The major band after transfer

onto the nitrocellulose membrane corresponded to a protein with a molecular weight of 70,000 which is the reported molecule weight of the purified 2,4-dienoyl-CoA reductase. Interestingly, the intensity of this which band was only sightly reduced when the extracts were diluted 10 to 250-fold. However, the limit of detecting the reductase band with extracts from parental and mutant strains was reached when the extract (19 mg/ml) had been diluted 1,250-fold. On the basis of this immunological results, it is suggested that expression of the reductase gene is not affected in the mutant. It appears that the mutation is in the structural gene producing a reductase of lower specific activity.

The reductase mutant permitted me determine the location of the reductase gene with respect to the known loci of the fad regulon. The mapping of the mutation was attempted by complementation with a F' kit which represented the whole E. coli chromosome. The reductase gene was found to be located between 71 and 75 min on the E. coli chromosome, unliked to any of the other five loci harboring fad genes. The reductase gene is referred to as fadH in accordance with the nomenclature developed for β-oxidation genes (fad) and by making use of the first letter not assigned to any fad genes. The fadH gene seems to have its own operator and is estimated to be 1.7 Kb long. It is especially interesting that the reductase gene is not part of the fadAB operon which carries the genes of all other β-oxidation enzymes with the exception of acyl-CoA dehydrogenases and acyl-CoA synthetase. This conclusion is also supported by the observation that strain RS3084 which is a fadAB mutant devoid of all fadAB activities possess a normal level of reductase activity. All β-oxidation enzymes, except for 2,4-dienoyl-CoA reductase, are required for the degradation of those fatty acids synthesized by E. coli itself. The reductase would only be needed for the oxidation of mono or poly unsaturated fatty

acids with even-numbered double bonds taken up by the organism from the growth medium.

The availability of several E. coli strains with different reductase activity levels provided the tool for evaluating the relationship between the cellular activity of the enzyme and the rates at which cells will oxidize petroselinic acid. The results of this experiment demonstrate that the capacity of E. coli to oxidize petroselinic acid is lower than the capacity of the organism to utilize oleate and is directly proportional to the cellular reductase activity up to the level present in wild-type E. coli . Thus, it seems that the reaction catalyzed by 2,4-dienoyl-CoA reductase is rate-limiting or one of the slow steps in the oxidation of petroselinic acid and possible other unsaturated fatty acids carrying the same type of double bond. However, donor strain #4248 carrying the F'141 episome oxidizes petroselinic acid at a rate only slightly higher than the parental strain RS3010, even though the reductase activity level in strain #4248 is 4-times higher than in strain RS3010. It is possible that the oxidation of petroselinic acid in wild-type E. coli and strains with equal or higher reductase levels is limited by the uptake, activation, or initial cycles of oxidation of petroselinic acid. Or perhaps the availability of NADPH is a limiting factor in the oxidation of this fatty acid.

Based on a kinetic study with purified enzymes, it has been predicted that the epimerase dependent pathway in E. coli. may account for approximately 3% of the degradation of unsaturated fatty acids with double bonds extending from even-numbered carbon atoms. Since the determination of fatty acid oxidation via respiration measurements is not very accurate, more careful measurements should be performed with radioactivelly labeled petroselinic acid to determine whether or not the epimerase-dependent pathway can be detected in the reductase mutant.

Evidence is provided suggesting that the expression of the reductase gene (fadH) like other fad genes is induced by long-chain fatty acids. In addition to oleic acid, petroselinic acid acts as an inducer of the fadH gene as well as the fadAB genes. However, the degree to which the fadH and fadB genes are induced at various growth conditions are similar but not identical as expected for unliked genes. A similar case of unequal expression of fad genes was observed when the inductions of fadB and fadD (gene for acyl-CoA synthetase) were compared[34]. The position of the double bonds in long-chain fatty acids does not seem to affect the induction of the fad regulon. Additionally, it is concluded that the expression of the fadH gene is controlled by the fad repressor (fadR).

The expression of the fadH gene requires the presence of cyclic AMP as has been observed for all other fad genes and thus is subject to catabolite repression by glucose. Another quantitative difference between the expression of the fadH and fadAB genes is the less severe catabolite repression of the former gene by glucose. The expression of the 2,4-dienoyl-CoA reductase gene may be subject to " internal induction " by compounds other than long-chain fatty acids.

Finally, to provide structural information about the fadH gene, the reductase gene from E. coli was isolated by complementation of the mutant with an E. coli genomic library in λSE6 which can replicate either as lytic phage in nonlysogenic hosts or as plasmids in hosts that carry the λcI repressor gene. The phasmid DNA which contained fadH gene was isolated under lysogenic conditions state on petroselinic acid as the sole carbon source and then amplified lytically as phage. Amplication of this phage starts from a single clear plaque to give high titers in order to obtain large amounts of phasmid. The phasmid DNA, which contained a 17 Kb-

base insert of E. coli genomic DNA and 33 kb of phage DNA was purified by a modified protocol which used a biochemical step instead of cesium chloride gradient density centrifugation and dialysis. Polyethylene glycol (PEG) precipitation was used for isolating large molecular weight phasmid DNA from the bacterial DNA and RNA. A 17 kb insert of the E. coli genomic DNA, which contains the fadH gene, will be inserted into a cloning vector after digestion with suitable restriction enzymes for subcloning and sequencing of the reductase gene.

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