# STUDIES ON

# PHYSIOLOGICAL FUNCTIONS OF THIOLASE ISOZYMES

# OF *N*-ALKANE-ASSIMILATING YEAST

## NAOKI KANAYAMA

## 1998

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# PREFACE

This is a thesis submitted by the author to Kyoto University for the degree of Doctor of Engineering. The studies collected here have been carried out under the direction of Professor Atsuo Tanaka in the Laboratory of Industrial Biochemistry, Department of Industrial Chemistry and in the Laboratory of Applied Biological Chemistry, Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, during 1991–1998.

The author expresses sincere gratitude to Professor Atsuo Tanaka for his continuous guidance and encouragement throughout this study.

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# CONTENTS

# INTRODUCTION

*Candida tropicalis* is an asporogenic diploid yeast, which can utilize *n*-alkanes and fatty acids as a carbon source. The most striking feature of this yeast is the profound proliferation of a subcellular organelle, peroxisome, by specific carbon sources such as *n*-alkanes and fatty acids (1, 2). In *Candida tropicalis* peroxisomes, the fatty acid β-oxidation system is present, suggesting the indispensable role of peroxisomes in the alkane assimilation (3, 4). Since these characteristics of the yeast peroxisomes are similar to those of mammalian peroxisomes, this yeast is invaluable as a model system for the investigation of the functions of mammalian peroxisomes. On the other hand, since this yeast has been used for the production of valuable compounds such as dicarboxylic acids from *n*-alkanes (5, 6), it is industrially important to elucidate *n*-alkane metabolism in this yeast. The metabolic system of *n*-alkanes is composed of a number of enzymes, which are compartmentalized to the specific intracellular locations related to the physiological functions (3, 4). Some of these enzymes occur as isozymes and are localized in the same and/or different subcellular compartment. Therefore, it is also of great interest to clarify the physiological roles of isozymes.

In the present study, the author has investigated the biochemical and genetical fundamentals of peroxisome functions in *Candida tropicalis*, particularly the expression and physiological functions of thiolase isozymes, which catalyze the final step of the fatty acid β-oxidation system.

## *n*-Alkane assimilation in the yeast, *Candida tropicalis*

When *Candida tropicalis* is cultivated on *n*-alkanes whose carbon-length is between 10 and 13, conspicuous numbers of peroxisomes appears (Fig. 1) (1, 2). Biochemical investigations revealed that there were various metabolic systems in the *n*-alkane-grown cells for *n*-alkane assimilation (Fig. 2) (3, 4). *n*-Alkanes incorporated by the cells are first oxidized to alcohols in microsomes. This hydroxylation system is presumed to be catalyzed by

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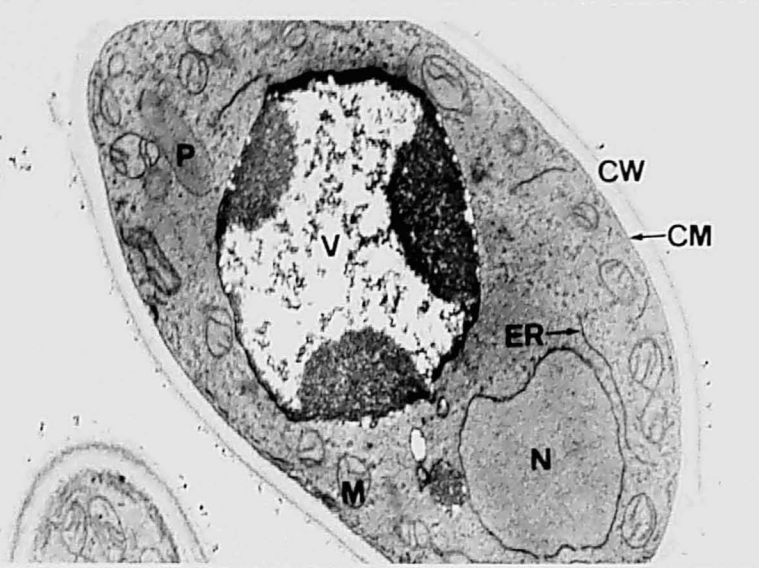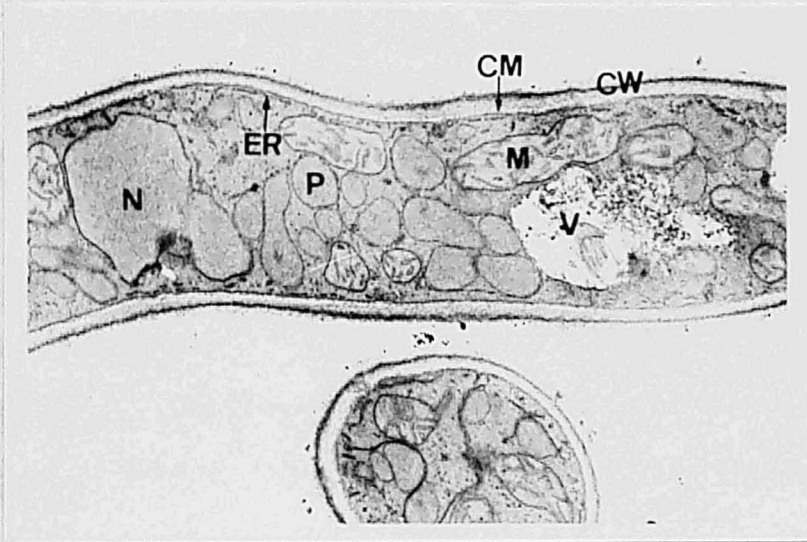
Fig. 1.    Electron micrographs of the glucose-grown (A) and n-alkane-grown cells (B) of

Candida tropicalis pK233.

CM, cell membrane; CW, cell wall; ER, endoplasmic reticulum; M, mitochondrion; N,

nucleus; P, peroxisome; V, vacuole.
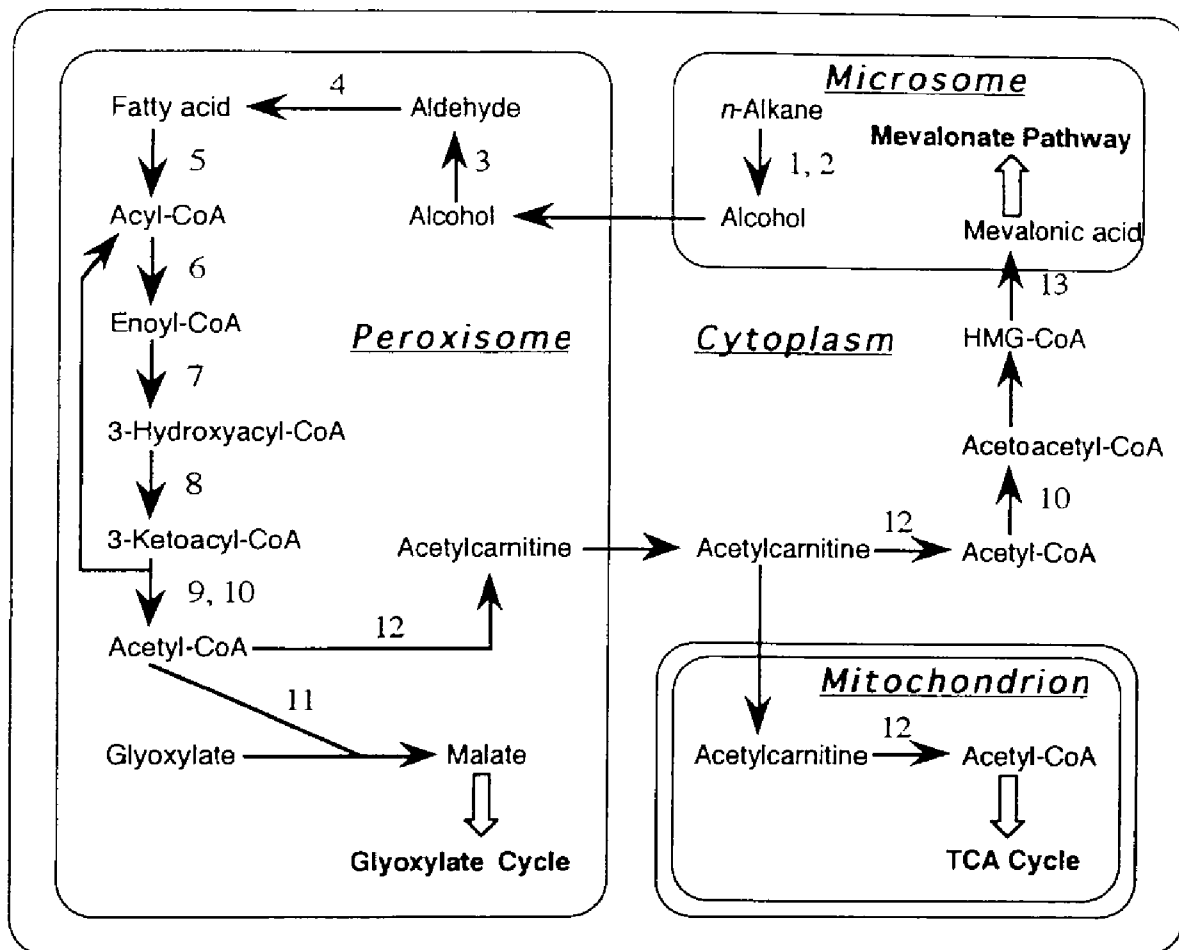
Fig. 2.  Presumptive roles of peroxisome, mitochondrion, microsome, and cytoplasm in
*n*-alkane-assimilating *Candida tropicalis*.

The β-oxidation system is composed of enzymes represented by 6, 7, 8, 9, and 10. Of the glyoxylate cycle enzymes, two key enzymes (isocitrate lyase and malate synthase) are localized in peroxisomes , while the others in mitochondria. Enzymes: 1, cytochrome P-450; 2, NADPH-cytochrom *c* (P-450) reductase; 3, long-chain alcohol dehydrogenase; 4, long-chain aldehyde dehydrogenase; 5, acyl-CoA synthetase; 6, acyl-CoA oxidase; 7, enoyl-CoA hydratase; 8, 3-hydroxyacyl-CoA dehydrogenase; 9, 3-ketoacyl-CoA thiolase; 10, acetoacetyl-CoA thiolase; 11, malate synthase; 12, carnitine acetyltransferase; 13, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase. Abbreviation: TCA cycle, tricarboxylic acid cycle.

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cytochrome P-450 and NADPH-cytochrome c (cytochrome P-450) reductase in microsomes (7). The alcohols are transported into peroxisomes and oxidized via aldehydes to fatty acids by NAD-linked alcohol dehydrogenase and aldehyde dehydrogenase (8). In peroxisomes, the fatty acids are activated to acyl-CoAs by acyl-CoA synthetase (8) and then degraded to acetyl-CoA via the fatty acid β-oxidation system (3, 9). The acetyl-CoA is metabolized through two main pathways. In one pathway, it is utilized to produce malate by malate synthase and further metabolized in the glyoxylate cycle, of which two key enzymes are localized in peroxisomes (4). Metabolites in this cycle are utilized for the biosynthesis of cellular components including carbohydrates and amino acids. In the other pathway, acetyl-CoA is transported to cytoplasm and mitochondria via the acetylcarnitine shuttle system (10). In cytoplasm, acetyl-CoA is utilized in the early steps of the mevalonate pathway, which synthesizes the precursor of physiologically important isoprenoid groups (11–13). In mitochondria, acetyl-CoA is further metabolized in the tricarboxylic acid (TCA) cycle, which produces the reducing power leading to ATP via the respiratory chain. In order to obtain the detailed insight of metabolism of n-alkane, it is important to disclose the peroxisomal functions from the view of molecular biological and genetical aspects as well as from that of biochemical aspects.

**Peroxisomes**

Peroxisomes are the ubiquitous subcellular organelles which are present in most eukaryotic species. Peroxisomes were first discovered in rat kidney as a single membrane bounded vesicle with a fine granular matrix (14). These subcellular organelles were shown to contain several kinds of oxidases and catalase which degrades hydrogen peroxide produced by oxidases (15). Based on this metabolic feature, these organelles were named as peroxisomes. As for the physiological role of these organelles, the fatty acid β-oxidation system was first demonstrated by Lazarow and de Duve, which had been believed to be present only in mitochondria (16). Yeast peroxisomes were first found in Saccharomyces cerevisiae grown on glucose (17). Fukui and coworkers discovered that in the n-alkane-grown Candida tropicalis, conspicuous numbers of peroxisomes appeared (1, 2) and the β-oxidation system was present

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in these organelles (3). Recent biochemical and genetical investigations revealed that peroxisomes contained various metabolic functions. For example, mammalian peroxisomes contain various metabolisms such as glyoxylate metabolism, cholesterol metabolism, and ether lipid synthesis in addition to the β-oxidation (18).

In response to changes in the extracellular environment, peroxisomes in the cells increase significantly both in number and volume. Cells can adjust the biogenesis of peroxisomes in accordance with the demand of particular functions that are provided by peroxisomes. Such peroxisome proliferation is induced in liver cells of rats fed a diet containing peroxisome proliferators, such as hypolipidemic drugs and phthalate ester plasticizers (19, 20). In *Candida tropicalis* cells grown on *n*-alkanes and fatty acids, peroxisomes are proliferated in number and volume much higher than in cells grown on glucose (Fig. 1) (1, 2, 21). The morphological response of peroxisomes is in harmony with the inductive synthesis of peroxisomal proteins. The elucidation of the regulated expression of the individual peroxisomal protein-encoding genes would be a important clue to understand the peroxisome proliferation.

Protein import is one of important processes in the peroxisomal biogenesis. Peroxisomal proteins are synthesized on free ribosomes in cytoplasm and are posttranslationally imported into the organelle (22). Peroxisomal targeting signals (PTSs) in peroxisomal proteins are recognized in the protein import process. Genetic and biochemical evidence has underscored the conservation of PTSs from yeast to humans and implicated at least three pathways for the transport of proteins to the peroxisome matrix (23–25). It is noted that components in these pathways were identified as the indispensable factors, peroxins (PEXs), for the peroxisomal biogenesis (26). On the other hand, recent evidence suggests that the peroxisomal targeting and assembly of proteins do not follow the same rules as those for targeting and import into other organelles, such as mitochondria and endoplasmic reticulum (25). Analysis of the structures of several different peroxisomal proteins would be a clue to gain information of peroxisomal targeting and importing systems. Furthermore, the manipulation of peroxisomal targeting signal would be a means to elucidate the physiological functions of peroxisomal proteins.

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## Fatty acid β-oxidation system

Although the fatty acid β-oxidation system is recognaized as a process to degrade fatty acids, and has been investigated for a century, overall image for the metabolic functions of this system has not been completed yet. The β-oxidation system consists of four steps of reactions, and is classified into two types by the fate of reductive hydrogen produced in the first step (Fig. 3) (27). One system is peroxisomal and the other is mitochondrial. The β-oxidation of fatty acids in both systems is preceded by their conversion to coenzyme A (CoA) thioesters. This reaction is catalyzed by acyl-CoA synthetase (9, 28). In the peroxisomal system, the first step is catalyzed by acyl-CoA oxidase which oxidizes acyl-CoA to enoyl-CoA accompanied by the production of hydrogen peroxide (29–31). Hydrogen peroxide is decomposed by catalase (32). In the mitochondrial system, acyl-CoA is converted to enoyl-CoA by acyl-CoA dehydrogenase which is coupled with the electron transfer system (33). The rest of reaction steps are catalyzed by enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase in both β-oxidation systems.

Compared with other metabolic systems, the β-oxidation system possesses two main characteristics. One is that the β-oxidation system consists of kinds of isozymes (34, 35), which are different mostly in substrate specificity. The other is that multiple steps of β-oxidation are catalyzed by a single-peptide protein, which can be defined as a multifunctional protein. Furthermore, it has been proposed that the enzymes of the mitochondrial β-oxidation system are organized as a multi-protein complex to preform the channelling of metabolic intermediates between enzymes (35–37). In rat peroxisomes, there are three acyl-CoA oxidases (34, 38), two multifunctional proteins (formerly bifunctional enzymes) (39, 40), and two 3-ketoacyl-CoA thiolases (41, 42) (Fig.4). Multifunctional proteins catalyze the second and third steps of the β-oxidation system (enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase). In the case of *Candida tropicalis*, peroxisomes possesses two acyl-CoA oxidases (43, 44), one bifunctional enzyme (45, 46), and two thiolases (47, 48). Since the organization of enzymes is suggested to be related to the channelling of metabolites and the regulation of metabolism, it is important to elucidate how these β-oxidation enzymes are organized in peroxisomes.

$$R-CH_2CH_2-\overset{\overset{O}{\|}}{C}-OH \qquad HS-CoA$$

**Mitochondrial**  *ACS*  **Peroxisomal**

*ETF*

FAD $\qquad$ $R-CH_2CH_2-\overset{\overset{O}{\|}}{C}-S-CoA$ $\qquad$ FAD $\qquad$ *KAT* $\qquad$ $H_2O_2 \longrightarrow H_2O$

*ACD* $\qquad$ *ACO*

FADH$_2$ $\qquad$ $R-CH=CH-\overset{\overset{O}{\|}}{C}-S-CoA$ $\qquad$ FADH$_2$ $\qquad$ O$_2$

**Eelectron Transport Chain**

*ECH*

$$R-\overset{\overset{OH}{|}}{C}H-CH_2-\overset{\overset{O}{\|}}{C}-S-CoA$$

*HCD*

$$R-\overset{\overset{O}{\|}}{C}-CH_2-\overset{\overset{O}{\|}}{C}-S-CoA$$

*KCT*

$$R-\overset{\overset{O}{\|}}{C}-S-CoA \qquad CH_3-\overset{\overset{O}{\|}}{C}-S-CoA$$
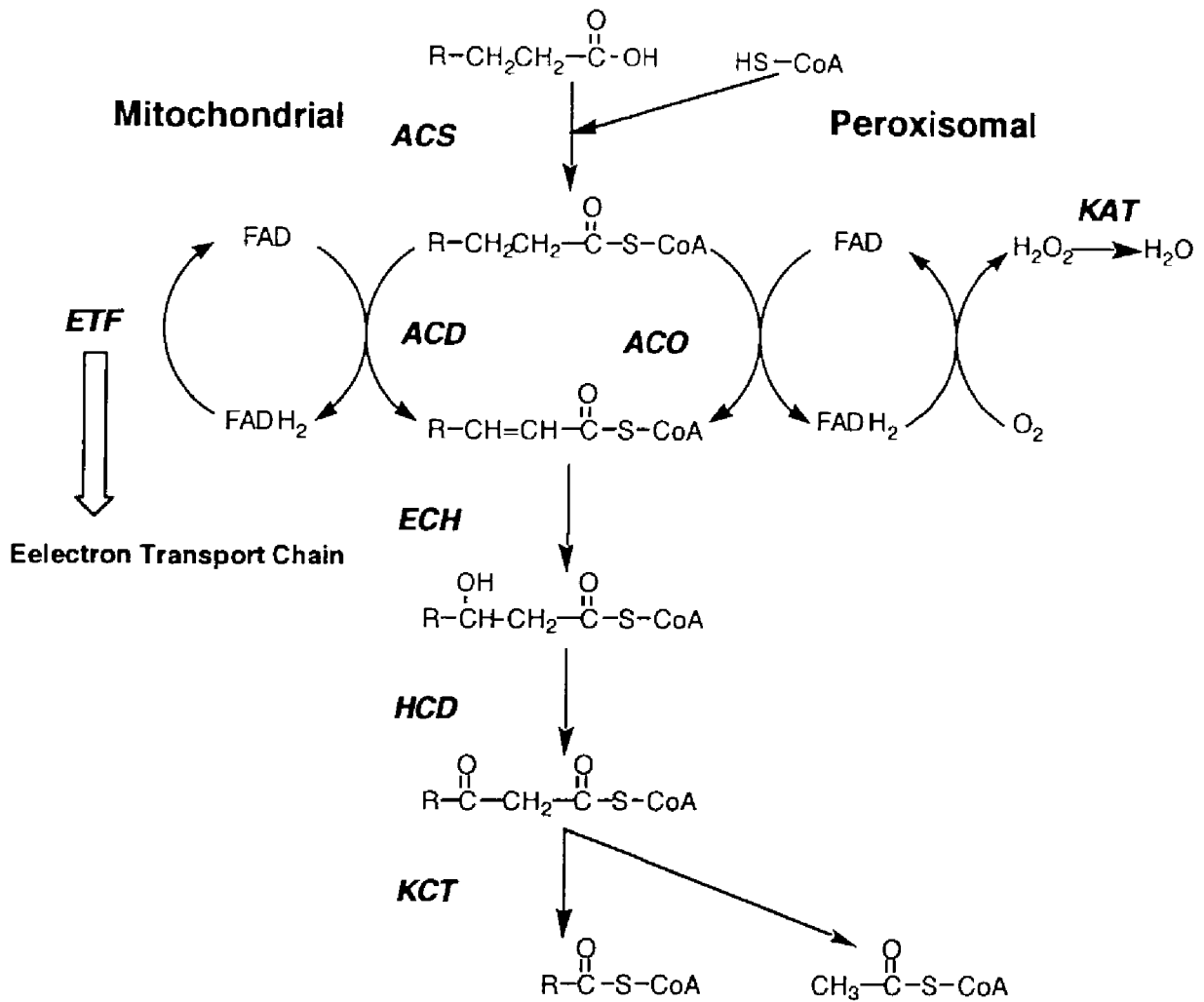
Fig. 3.  Peroxisomal and mitochondrial fatty acid β-oxidation systems.

ACD, acyl-CoA dehydrogenase; ACO, acyl-CoA oxidase; ACS, acyl-CoA shyntetase; ECH, enoyl-CoA hydratase; ETF, electon transfer flavoprotein; HCD, 3-hydroxyacyl-CoA dehydrogenase; KAT, catalase; KCT, 3-ketoacyl-CoA thiolase.

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**Mammalian**  **Candida tropicalis**

**ACO**

| Palmitoyl-CoA oxidase |
| Pristanoyl-CoA oxidase |
| Trihydroxycoprostanoyl-CoA oxidase |

| PXP4 |
| PXP5 |

**ECH**

| MFP-1 |
| MFP-2 |

| BFE |

**HCD**

**KCT**

| Peroxisomal 3-ketoacyl-CoA thiolase |
| SCP-2/thiolase |

| Ps-Thiolase I |
| Thiolase III |
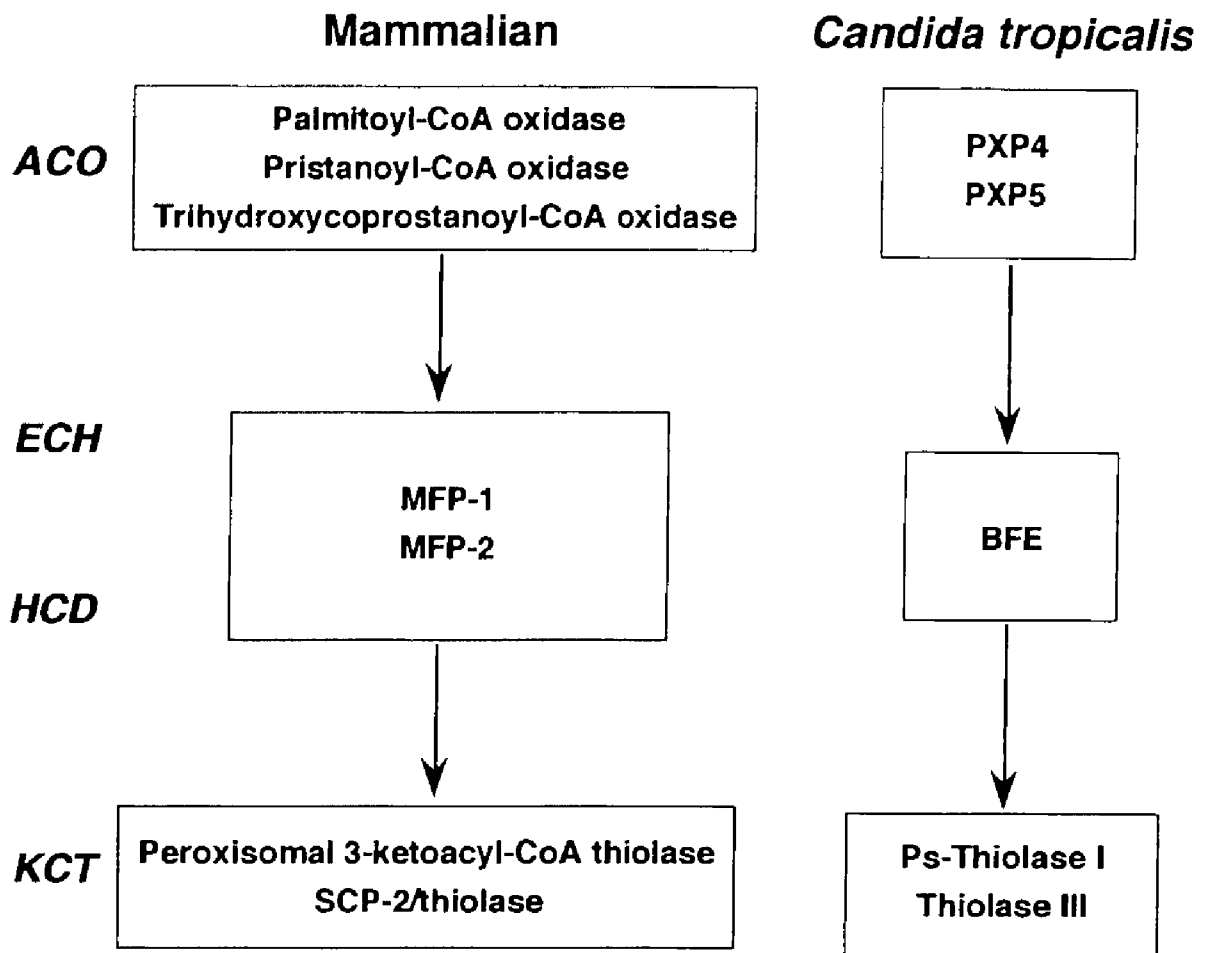
Fig. 4.    Enzymes of the peroxisomal β-oxidation system in mammalian cells and *Candida tropicalis*.

ACO, acyl-CoA oxidase; BFE, bifunctional enzyme; ECH, enoyl-CoA hydratase; HCD, 3-hydroxyacyl-CoA dehydrogenase; KCT, 3-ketoacyl-CoA thiolase; MFP, multifunctional protein; Ps-Thiolase I, *Candida tropicalis* peroxisomal acetoacetyl-CoA thiolase; PXP, peroxisomal protein; SCP-2, sterol carrier protein 2; Thiolase III, *Candida tropicalis* 3-ketoacyl-CoA thiolase.

## Thiolase

Thiolase is an ubiquitous enzyme which catalyzes the thiolytic cleavage of 3-ketoacyl-CoA to acyl-CoA and acetyl-CoA. This enzyme is classified into two types by substrate specificity. One type is acetoacetyl-CoA thiolase, which catalyzes the cleavage of acetoacetyl-CoA and the reverse condensation of acetyl-CoA. The other is 3-ketoacyl-CoA thiolase, which has broad substrate specificity for 3-ketoacyl-CoAs in carbon length ($\geq C_4$). In eukaryotic cells, thiolases exhibit diversity in intracellular localization related to their metabolic functions. For example, in mammalian cells, it is suggested biochemically that mitochondrial trifunctional protein, mitochondrial 3-ketoacyl-CoA thiolase, peroxisomal 3-ketoacyl-CoA thiolase, and peroxisomal SCP-2/thiolase participate in fatty acid degradation (41, 42, 49-51), while mitochondrial acetoacetyl-CoA thiolase participates in the ketone body metabolism (50) and cytosolic acetoacetyl-CoA thiolase participates in the mevalonate pathway (50).

In *Candida tropicalis* pK233, there are at least three thiolase isozymes, cytosolic acetoacetyl-CoA thiolase (Cs-Thiolase I), peroxisomal acetoacetyl-CoA thiolase (Ps-Thiolase I), and peroxisomal 3-ketoacyl-CoA thiolase (Thiolase III) (13, 47, 48). Ps-Thiolase I and Thiolase III were purified and characterized (24), and gene analysis of Ps-Thiolase I was carried out hitherto (52). However, Cs-Thiolase I have not been purified, and the genes encoding Cs-Thiolase I and Thiolase III have not been identified yet. To make a complete map of thiolases in this yeast, the enzymatic properties and gene structures of thiolase isozymes must be studied in detail. On the other hand, since both acetoacetyl-CoA thiolase (Ps-Thiolase I) and 3-ketoacyl-CoA thiolase (Thiolase III) are found in *C. tropicalis* peroxisomes, the comparison of primary structures of thiolases can be a means to assume the evolution of thiolase. The information of physiological functions of thiolase isozymes also will be a clue to understand the evolution of the β-oxidation system and the organization of the peroxisomal β-oxidation system.

From this point of view, the author has analyzed the structure and expression of genes encoding peroxisomal thiolase isozymes. In comparison of properties of Cs-Thiolase I and recombinant Thiolase I expressed from Ps-Thiolase I gene, the author has demonstrated the dual compartmentalization of the same gene product. Furthermore, the author has first examined

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the genetic studies for thiolases in *Candida tropicalis*, in order to reveal directly the physiological functions of thiolase isozymes, particularly the contribution of peroxisomal thiolase isozymes to the β-oxidation system, and consequently has gave new insight into the regulation and the organization of the peroxisomal β-oxidation system.

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# SYNOPSIS

**Part I.** **Gene structure and expression of thiolase isozymes of *n*-alkane-assimilating yeast, *Candida tropicalis***

Thiolase catalyzes the final step of fatty acid β-oxidation, the thiolytic cleavage of 3-ketoacyl-CoA to produce acyl-CoA and acetyl-CoA. Based on substrate specificity, thiolase can be categorized to acetoacetyl-CoA thiolase, which is specific to acetoacetyl-CoA, and 3-ketoacyl-CoA thiolase, which acts on various chain-length substrates. *Candida tropicalis* possesses at least three thiolase isozymes: cytosolic acetoacetyl-CoA thiolase (Cs-Thiolase I), peroxisomal acetoacetyl-CoA thiolase (Ps-Thiolase I), and peroxisomal 3-ketoacyl-CoA thiolase (Thiolase III). In part I, the author disclosed the molecular information of the structures and examined the expression of thiolase isozyme genes of *Candida tropicalis*.

Chapter 1 deals with the gene analysis of Thiolase III of *Candida tropicalis*. Two transcribed genes encoding Thiolase III were isolated. Both genes contained open reading frame of 1224 bp corresponding to 408 amino acid residues. The deduced amino acid sequences of Thiolase III was characteristic in its amino-terminal region which is considered to be a peroxisomal targeting signal. The primary structure of peroxisomal thiolase isozymes (Ps-Thiolase I and Thiolase III) showed 35% identity. The regulation of biosynthesis of these thiolases was compared by Northern and Western blot analyses. The results revealed that the biosynthesis of Thiolase III was strongly induced in cells grown on butyrate or alkanes, while Ps-Thiolase I was less strongly expressed. Thus, the regulations of biosynthesis of Ps-Thiolase I and Thiolase III were found to be different, although the enzymes are thought to participate in the final step of the peroxisomal fatty acid β-oxidation system.

Chapter 2 deals with the molecular evolution of thiolases. The coexistence of two thiolase isozymes (Ps-Thiolase I and Thiolase III) in peroxisomes of the *n*-alkane-utilizing yeast *Candida tropicalis* is unique in eukaryotic cells. As one of the methods of analysis of molecular information from these isozymes, the calculation of the evolutional distance among

thiolases from various organisms was applied. The result suggests that yeast peroxisomal thiolase isozymes are important enzymes to examine the molecular evolution of the fatty acid metabolic pathway and the biogenesis of peroxisomes.

Chapter 3 deals with the characterization of Cs-Thiolase I and recombinant proteins individually expressed from two genes encoding Ps-Thiolase I. In order to compare the expressed products of Thiolase I isozyme-encoding genes in *Candida tropicalis*, Cs-Thiolase I was first purified from the glucose-grown *Candida tropicalis* in which the proliferation of peroxisomes and the expression of Ps-Thiolase I were repressed. Cs-Thiolase I was virtually identical to Ps-Thiolase I in molecular mass, kinetic and immunochemical properties, and primary structure at the N-terminus. Amino acid sequence analysis revealed that Cs-Thiolase I was the mixture of products of two genes, as was the case with the peroxisomal enzyme. Two genes encoding Ps-Thiolase I were expressed independently in the yeast *Saccharomyces cerevisiae* and the recombinant proteins were purified. Recombinant thiolases exhibited practically identical enzymatic properties to Cs-Thiolase I and Ps-Thiolase I from *Candida tropicalis*. These results revealed that Cs-Thiolase I and Ps-Thiolase I were encoded not by different genes, but by the same genes and are present as a mixture of products expressed from both genes, although their subcellular localization is different.

## Part II. Physiological functions of thiolase isozymes in *n*-alkane-assimilating yeast, *Candida tropicalis*

By the genetic approach, it is possible to examine the functions of cellular components directly. In Part II, the author developed the system to recycle the *URA3* marker in *Candida tropicalis* and applied it to the disruption of multiple thiolase isozyme genes and the introduction of a site-directed mutaion onto these genes, in order to elucidate the physiological functions of thiolase isozymes

Chapter 1 deals with genetic evaluation of physiological functions of thiolase isozymes in *Candida tropicalis*. As described in Part I, *Candida tropicalis* possesses three isozymes encoded by two pairs of alleles: Thiolase III, Cs-Thiolase I, and Ps-Thiolase I. The physiological

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functions of these thiolases have been examined by gene disruption. The homozygous Thiolase I gene null mutation abolished the activity of acetoacetyl-CoA thiolase and resulted in mevalonate auxotrophy. The homozygous Thiolase III gene null mutation abolished the activity of 3-ketoacyl-CoA thiolase and resulted in growth deficiency on $n$-alkanes ($C_{10}$-$C_{13}$). To further clarify the functions of peroxisomal thiolase isozymes, the site-directed mutation leading to Thiolase I without a putative C-terminal peroxisomal targeting signal was introduced on the locus of Thiolase I gene. The truncated Thiolase I was solely present in cytoplasm, and the absence of Thiolase I in peroxisomes had no effect on growth on all carbon sources employed. Growth on butyrate was not affected by a lack of Ps-Thiolase I, while growth was retarded by a lack of Thiolase III. A defect of both peroxisomal isozymes completely inhibited the growth on butyrate. These results demonstrated that Cs-Thiolase I was indispensable for the mevalonate pathway, and that both Ps-Thiolase I and Thiolase III could participate in the peroxisomal β-oxidation system. In addition to the essential contribution to the β-oxidation of longer-chain fatty acids, Thiolase III greatly contributed even to the β-oxidation of a $C_4$ substrate butyrate.

Chapter 2 deals with the clarification of the contribution of Ps-Thiolase I and Thiolase III to the peroxisomal fatty acid β-oxidation system. In the yeast *Candida tropicalis*, two thiolase isozymes, Ps-Thiolase I and Thiolase III, participate in the peroxisomal fatty acid β-oxidation system. As shown in Part I, Chapter 1, the disruption of Thiolase III genes resulted in the growth retardation on butyrate, suggesting that Thiolase III contributes to the β-oxidation system more greatly than Ps-Thiolase I. In this chapter, the contribution of two thiolase isozymes to the peroxisomal fatty acid β-oxidation system was investigated in cells grown on butyrate, on which *Candida tropicalis* can grow with either the absence of Thiolase III or that of Ps-Thiolase I. A lack of Thiolase III protein resulted in the induced expression of Thiolase I and other peroxisomal proteins, whereas a lack of Ps-Thiolase I did not. Overexpression of Ps-Thiolase I could not suppress the induction and growth retardation in cells without Thiolase III, although much amount of overexpressed Ps-Thiolase I was detected in most peroxisomes of butyrate-grown cells. These results gave evidence of the greater contribution of Thiolase III to the peroxisomal fatty acid β-oxidation system than Ps-Thiolase

I and also implies that components of the peroxisomal fatty acid $\beta$-oxidation system are organized in peroxisome matrix of *Candida tropicalis* and that the $\beta$-oxidation system is regulated in a feedback manner.

**Part I**     Gene sturcture and expression of thiolase isozymes of *n*-alkane-

assimilating yeast, *Candida tropicalis*

# Chapter 1 Comparison of molecular structures and regulation of biosynthesis of unique thiolase isozymes localized only in peroxisomes of *Candida tropicalis*

## INTRODUCTION

Fatty acid degradation systems of mammalian cells are localized in peroxisomes, which play a role in the degradation of long-chain fatty acids to middle-chain ones, and in mitochondria, which degrade middle-chain fatty acids to short-chain ones (1). Such discrimination of subcellular fatty acid $\beta$-oxidation systems is dependent on the substrate specificities of the enzymes constituting the system, especially on those of thiolases in peroxisomes and mitochondria. Furthermore, considering the evolution of the fatty acid degradation system, the yeast system is unique because only the peroxisomal system for fatty acid degradation is present and plays a role in degrading fatty acids of various chain lengths ranging from long- to short-chain ones (2, 3). This feature has been supported by the finding of the peroxisomal localization of thiolase isozymes such as peroxisomal 3-ketoacyl-CoA thiolase (Thiolase III) which has broad substrate specificity and peroxisomal acetoacetyl-CoA thiolase (Ps-Thiolase I) which is specific to acetoacetyl-CoA in the *n* -alkane-assimilating yeast, *Candida tropicalis* (4, 5). In addition to these two thiolase isozymes, *Candida tropicalis* possesses cytosolic acetoacetyl-CoA thiolase (Cs-Thiolase I), which can not be discriminated immunochemically from Ps-Thiolase I, and which is suggested to have a role in the sterol biosynthesis (6). The gene encoding Ps-Thiolase I has been isolated and analyzed (7).

In this chapter, the author reports the nucleotide sequence and the deduced amino acid sequence of another thiolase isozyme, Thiolase III, of *Candida tropicalis* and compare the molecular structures and the regulation of gene expression of Ps-Thiolase I and Thiolase III in the yeast. Discussion is also made on the difference of these thiolase isozymes to reveal

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the uniqueness of yeast peroxisomes in fatty acid metabolism.

# MATERIALS AND METHODS

## Cultivaion of *Candida tropicalis*

*Candida tropicalis* pK233 (ATCC 20336) was cultivated aerobically at 30°C in a medium containing glucose (16.5 g/l), sodium acetate $3H_2O$ (13.6 g/l), sodium butyrate (11.0 g/l) or *n*-alkane mixture (10 ml/l) as the sole source of carbon and energy (8, 9). *n*-Alkane mixture was composed of $C_{10}$ 16.4, $C_{11}$ 50.4, $C_{12}$ 32.5, and $C_{13}$ 0.7% by weight. The basic composition of the medium was as follows: 5.0 g of $NH_4H_2PO_4$, 2.5 g of $KH_2 PO_4$, 1.0 g of $MgSO_4 \cdot 7H_2O$, 0.02 g of $FeCl_3 \cdot 6H_2O$, and 1.0 ml of corn steep liquor per liter of tap water (8).

## Strain and vector used for DNA manipulation

*Escherichia coli* DH5α [$F$, *endA1*, *hsdR17*($r_k^-$, $m_k^-$), *supE44*, *thi-1*, λ, *recA1*, *gyrA96*, Δ*lacU169* (φ*80lacZΔM15*)] was used as the host cell for cloning and pUC19 as a plasmid for recombination.

## Screening of clones

A λgt11 cDNA expression library constructed with poly(A)-rich RNA from the *n*-alkane-grown *Candida tropicalis* pK 233 was screened with rabbit antiserum raised against peroxisomal Thiolase III (5). A λEMBL3 genomic DNA recombinant library prepared with genomic DNA isolated from *Candida tropicalis* cells was further screened using the Thiolase III cDNA probe obtained (7).

## Preparation of probes

A cDNA fragment encoding a part of Thiolase III was labeled with biotin-14-dATP (Bethesda Research Laboratories Life Technologies (BRL), Gaithersburg, MD, USA) by nick translation and used as a probe. Detection was performed either by a color formation reaction with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate or by a luminescence reaction with a Photo Gene Nucleic Acid Detection System kit (BRL).

## Restriction mapping and DNA sequencing

Restriction endonucleases were purchased from Toyobo (Osaka, Japan). Reactions

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were carried out under the conditions recommended by the manufacturer. The DNA fragments were prepared using endonucleases. The respective DNA fragments were subcloned into pUC19. Sequence analysis was carried out by the dideoxy-chain-termination method (11) using the universal 17-primer, the reverse 17-primer (Pharmacia Fine Chemical, Uppsala, Sweden), and oligonucleotide primers synthesized by a 381 A DNA synthesizer (Applied Biosystem, Foster City, CA, USA).

## Southern and Northern blot analyses

Southern blot analysis of total genomic DNA was carried out as described by Kurihara *et al.* (7). Northern blot analysis was carried out as described by Hikida *et al.* (12). The biotin-labeled cDNAs were used as probes.

## Amino acid sequence analysis

Thiolase III was purified from the peroxisome-containing particulate fraction of the *n*-alkane-grown *Candida tropicalis* cells as described by Kurihara *et al.* (4) and digested with *Achromobacter* protease I. Peptide fragments obtained were separated by HPLC and analyzed with the Edman degradation procedures.

## Preparation of cell-free extracts

Cells were harvested and disintegrated by sonication (20 kHz, 2.5 min, 0 °C) in the PGD buffer [50 mM potassium phosphate buffer (pH 7.2), 10% glycerol, and 1 mM dithiothreitol], containing protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 0.05 g/l of pepstatin A, 0.05 g/l of leupeptin, 0.05 g/l of antipain, and 0.05 g/l of chymostatin). Cell-free extracts were obtained by centrifugation of the sonicated cells at 10,000 x g for 20 min at 0 °C.

## Western blot analysis

Sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a slab gel (acrylamide, 12.5%) (13). After electrophoresis, Western blot analysis was performed using antisera against Ps-Thiolase I, Thiolase III, acyl-CoA oxidase, catalase, isocitrate lyase, and malate synthase with the procedure reported by Ueda *et al.* (13).

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# RESULTS AND DISCUSSION

**Isolation and analysis of peroxisomal 3-ketoacyl-CoA thiolase (Thiolase III) gene**

By immunoscreening of an 80,000 pfu (plaque-forming units) cDNA library with anti-(Thiolase III) antiserum, 12 positive clones were obtained. Since the clones were grouped into those having two kinds (presence or absence of the *PstI* site) of partial Thiolase III cDNAs (the longest fragments were 1060 bp and 870 bp, respectively, as shown in Fig. 1A), these DNA fragments were subcloned into pUC19. Their restriction maps were very similar and only one restriction enzyme site (*PstI*) was different. They were named TIII-cDNA-A and TIII-cDNA-B, respectively.

A λEMBL 3 genomic DNA library was then screened with the Thiolase III cDNA probe (TIII-cDNA-A). Two independent clones (λCT-KCT-A and λCT-KCT-B), which could be distinguished by Southern blot analysis, were isolated (Fig. 1B). When these clones were digested with *Eco*RI and *Bam*HI, a 7.5-kbp band was detected for the λCT-KCT-A clone and a 5-kbp band for the λCT-KCT-B clone. These bands corresponded to those detected in total Southern blotting (Fig. 2). The *Eco*RI-*Sal*I fragments of λCT-KCT-A and λCT-KCT-B were subcloned into pUC19 and the subcloned plasmids were named pCTKCTA and pCTKCTB, respectively. Figure 1B shows the restriction sites conserved between these two fragments. It was confirmed that the *PstI* site was different in the coding regions, as was the case with the analysis of cDNAs. The isolation of the two genomic genes for Thiolase III confirmed the presence of two positive bands by the total Southern blot analysis with the Thiolase III cDNA probe (Fig. 2). When digested with *Bam*HI, *Eco*RI, *Bam*HI+*Eco*RI, and *Xho*I, two bands appeared.

The nucleotide sequence analyzed is shown in Fig 3. Open reading frames consisting of 1224 bp corresponding to 408 amino acid residues were found in both pCTKCTA and pCTKCTB. The amino acid sequences deduced from the nucleotide sequences shown by underlines in Fig. 3 were found to be identical to those analyzed by Edman degradation of the peptide fragments obtained after protease digestion. The N-terminal amino acid residue was not detected because it seemed to be blocked. Therefore, the peptides encoded in these

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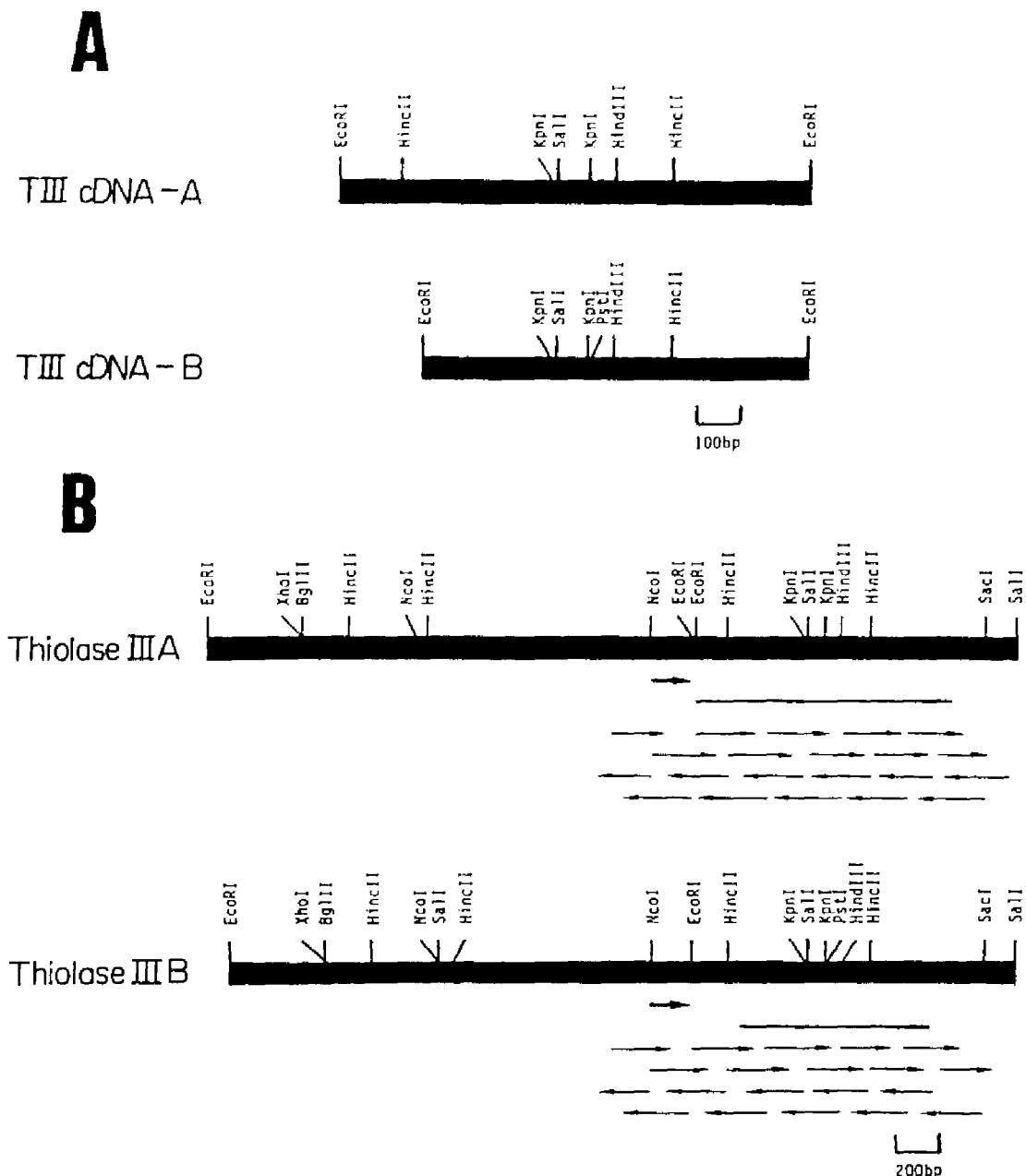
Fig. 1.    Restriction maps of cDNAs (A) and genomic DNAs (B) containing Thiolase III genes.

(A) Two kinds of partial Thiolase III cDNAs from λgt11 expression library are illustrated. They are referred to as TIII-cDNA-A (top) and TIII-cDNA-B (bottom).

(B) λCT-KCT-A (top) and λCT-KCT-B (bottom) contain Thiolase IIIA and Thiolase IIIB, respectively. The thick arrows indicate the direction of transcription and thin arrows show the sequence strategy. Thin bars correspond to TIII-cDNA-A (top) and TIII-cDNA-B (bottom), respectively.

Fig. 2.  Total Southern blot analysis of *Candida tropicalis* DNA.

cDNA encoding 3-ketoacyl-CoA thiolase (TIII-cDNA-A) was used as a probe. DNA (40 μg) was digested with *Eco*RI (lane 1), *Bam*HI (lane 2), *Eco*RI+*Bam*HI (lane 3), *Xho*I (lane 4) and *Xba*I (lane 5).

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Thiolase IIIA :-163   TCACATTCTCCCCACACATTCCCACCCTCAAGATTTCACAGGCAA

Thiolase IIIB :          --G-----------------------------------------


-120  AAACTGCAAATATATATAAATCTACAACCAATTCTTTCCCCAGATGGAAAATCTAATTTT

      ------------------------------------------------------------


-60   TGTTCACCCTTTTTCTTTCTCCTGCTATCACTGCTACTGCACATATTCAACACCACAACC

      -------------------CA----------------------------T-------


  1   ATGGATAGATTAAACCAATTAAGCGGCCAATTAAAGCCAAACGCCAAACAATCCATCTTG
  1   MetAspArgLeuAsnGlnLeuSerGlyGlnLeuLysProAsnAlaLysGlnSerIleLeu
      -------------------------------------------T--------------
      +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +


 61   CAAAAAAACCCAGACGACGTCGTTATCGTTGCTGCATACAGAACCGCCATCGGTAAAGGT
 21   GlnLysAsnProAspAspValValIleValAlaAlaTyrArgThrAlaIleGlyLysGly
      --------------------------------------------------------G---
      +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +


121   TTCAAAGGTTCCTTCAGAAGCGTCCGCTCTGAATTCATCTTGACTGAGTTCTTGAAAGAA
 41   PheLysGlySerPheArgSerValArgSerGluPheIleLeuThrGluPheLeuLysGlu
      -------------------A-----A---------------------------------
      +  +  +  +  +  + Asn + His +  +  +  +  +  +  +  +  +  +  +


181   TTCATTAAAAAGACCAACATCGACCCATCTTTGATTGAAGATGTCGCTATCGGTAACGTC
 61   PheIleLysLysThrAsnIleAspProSerLeuIleGluAspValAlaIleGlyAsnVal
      --T--C----------------------------------------------------
      +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +


241   TTGAACCAGGCCGCCGGTGCCACCGAACACAGAGGTGCTTGTTTGGCTGCCGGTATCCCA
 81   LeuAsnGlnAlaAlaGlyAlaThrGluHisArgGlyAlaCysLeuAlaAlaGlyIlePro
      --------------A-------------------------------------------
      +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +
                                         *

301   TACACCGCCGCTTTCATCGCCGTCAACAGATTCTGCTCATCCGGTTTGATGGCCATCTCC
101   TyrThrAlaAlaPheIleAlaValAsnArgPheCysSerSerGlyLeuMetAlaIleSer
      -----------C---------T--------------T--C-----------------
      +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +


361   GACATTGCCAACAAGATCAAGACTGGTGAAATCGAGTGTGGTTTGGCTGGTGGTGCCGAA
121   AspIleAlaAsnLysIleLysThrGlyGluIleGluCysGlyLeuAlaGlyGlyAlaGlu
      ----------------------------------------------------------
      +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +


421   TCCATGTCCACCAACTACCGTGATCCTAGAGTTGCCCCAAGAATCGACCCACACTTGGCT
141   SerMetSerThrAsnTyrArgAspProArgValAlaProArgIleAspProHisLeuAla
      -------------------C--A---------------------------T------
      +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +
```

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 481   GACGACGCCCAAATGGAAAAGTGTTTGATTCCTATGGGTATCACCAACGAAAACGTTGCT
 161   AspAspAlaGlnMetGlnLysCysLeuIleProMetGlyIleThrAsnGluAsnValAla
       ------------------------------------------------------------
       +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +


 541   AACCAATTCAACATCTCCAGAGAAAGACAAGACGAGTTCGCCGCCAAGTCCTACAACAAG
 181   AsnGlnPheAsnIleSerArgGluArgGlnAspGluPheAlaAlaLysSerTyrAsnLys
       ---------------------------------T--------------------------
       +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +


 601   GCTGCCAAGGCTGTTGCCGCTGGTGCTTTCAAGAGCGAAATCTTGCCAATCAGATCCATC
 201   AlaAlaLysAlaValAlaAlaGlyAlaPheLysSerGluIleLeuProIleArgSerIle
       -----------G------T-----------------T-----------------------
       +   +   +   +   +   + Ser +   +   +   +   +   +   +   +   +   +   +   +


 661   ATCAGAAACTCTGACGGTACCGAAAAGGAAATCATTGTCGACACTGACGAAGGTCCAAGA
 221   IleArgAsnSerAspGlyThrGluLysGluIleIleValAspThrAspGluGlyProArg
       ------------------------------------------------------------
       +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +


 721   GAAGGTGTCACCGCTGAATCCTTGGGCAAGTTGAGACCAGCTTTCGACGGTACCACCACT
 241   GluGlyValThrAlaGluSerLeuGlyLysLeuArgProAlaPheAspGlyThrThrThr
       ----------------------T-------------------------------------
       +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +


 781   GCCGGTAACGCTTCCCAAGTCTCTGACGGTGCTGCCGCCGTCTTGTTGATGAAGAGAAGC
 261   AlaGlyAsnAlaSerGlnValSerAspGlyAlaAlaAlaValLeuLeuMetLysArgSer
       --A------------T-----------------C--------------------------
       +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +


 841   TTGGCTGAAGCCAAGGGATACCCAATCATTGGTAAGTACGTCCTTTGTTCCACCGCCGGT
 281   LeuAlaGluAlaLysGlyTyrProIleIleGlyLysTyrValLeuCysSerThrAlaGly
       ------------------------------------------------------------
       +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +


 901   GTTCCTCCAGAAATTATGGGTGTTGGTCCAGCCTACGCTATCCCAGAAGTCTTGAAGAGA
 301   ValProProGluIleMetGlyValGlyProAlaTyrAlaIleProGluValLeuLysArg
       -----A----------C--------------------T----------------------
       +   +   +   +   +   +   +   +   +   +   + Phe +   +   +   +   +   +   +   +


 961   ACTGGTTTGACTGTTGACGACATTGATGTTTTCGAAATCAACGAAGCCTTTGCTGCTCAA
 321   ThrGlyLeuThrValAspAspIleAspValPheGluIleAsnGluAlaPheAlaAlaGln
       -----C----------------------------------------------C------
       +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +


1021   TGTCTCTACTCTGCTGAACAAGTCAATGTGCCTGAAGAGAAGTTGAACATCAACGGTGGT
 341   CysLeuTyrSerAlaGlnGlnValAsnValProGluGluLysLeuAsnIleAsnGlyGly
       -----T---------------------------------A--------------------
       +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +


1081   GCCATTGCCTTGGGCCACCCATTGGGTGAAACCGGTGCTCGTCAATACGCCACCATCATC
 361   AlaIleAlaLeuGlyHisProLeuGlyGluThrGlyAlaArgGlnTyrAlaThrIleIle
       ------------------T-----------------------------------------
       +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +   +
```

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```
1141   CCATTGTTAAAACCAGGTCAAATTGGATTGACTTCAATGTGTATTGGTTCTGGTATGGGT
 381   ProLeuLeuLysProGlyGlnIleGlyLeuThrSerMetCysIleGlySerGlyMetGly
       ----------G-------------------------------------------------
       +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +  +


1201   TCTGCTTCTATTTTGGTTAGAGAATAGAGAGGAAGCGG          AAGTGTTTTATAGT
 401   SerAlaSerIleLeuValArgGlu***
       ------------------------------------------TAGGTCAA------------
       +  +  +  +  +  +  +  +


1261   CATTGAATGAATATATGTTTATAAAAGAAAGCAAGATGAGTCTACCACACGAGATGATGT

       --------------------------------A---------G-C--G--G-A----A----


1321   ATAGTCTACTTTTATTTATAATTATATGTACTAATTAAAGTATAACAAGTATCTAAACAT

       ----------C------C------------------------------------------


1381   GACTTGCTAAACAAAGTATAAAACATTAAAAAAGCAGCTAAACCAAAAAAAAAAAAAAAA

       --T-----------------------------------C---------------CC-----


1441   TAACCCAGAAT          GAATTAAATACCTTACTTGAAATAAACATTAG

       A----------AAATAAGAAT---A-----------------------------
```

Fig. 3.    Nucleotide sequence of the two genes for Thiolase III and their flanking regions. The amino acid sequence was deduced from the nucleotide sequence. Identical nucleotides between two genes are indicated with (–) and identical amino acid residues with (+). Gaps have been inserted to achieve the maximum similarity. Position 1 corresponds to the first nucleotide of the ATG initiation codon. The TATA box sequence is boxed on the Thiolase IIIA gene, and a candidate of the poly(A) addition signal is double-underlined. The underlined amino acid residues were identical to the sequences analyzed by Edman degradation of the purified enzyme.

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genes were referred to as Thiolases IIIA and IIIB, respectively. An extremely high similarity was observed between these two genes (97% of nucleotides in the coding region and 99% of amino acids deduced). The predicted molecular masses were 43,262 Da for Thiolase IIIA and 43,270 Da for Thiolase IIIB, which were in good agreement with the subunit mass of the purified enzyme (43 kDa). The occurrence of two types of cDNAs revealed that the two genomic DNAs for Thiolase III are both transcribed, although the functional difference between the two genes remains unknown, as is the case with Ps-Thiolase I and malate synthase (7, 12). Two cysteine residues which were supposed to be active sites (marked by stars in Fig. 4) were conserved, similar to thiolases from different sources (7). A putative peroxisomal targeting signal was found at the N-terminal region of Thiolase III as demonstrated for peroxisomal 3-ketoacyl-CoA thiolases of mammalian cells (7, 14, 15). Comparison of the deduced amino acid sequences of Ps-Thiolase I and Thiolase III revealed that the degree of identity was relatively low (35%) despite the same peroxisomal localization and the similarity in catalytic function of these enzymes (Fig. 4).

**Regulation of biosynthesis of Thiolase I and Thiolase III**

Northern blot analysis using RNAs isolated from the cells grown on different carbon sources indicated a large difference in the level mRNA between Thiolase I and Thiolase III (Fig. 5). With respect to the level of protein synthesis, Thiolase III was synthesized in the cells grown on acetate and strongly induced on butyrate or alkanes, whereas Thiolase I was synthesized in the cells grown on all the carbon sources employed with a slight induction on butyrate and alkanes (Fig. 6). It is not clear whether the probe prepared from Ps-Thiolase I cDNA can discriminate between mRNA of Ps-Thiolase I and that of Cs-Thiolase I, and anti-Ps-Thiolase I antiserum can not distinguish these Thiolase Is (6). However, Kurihara *et al.* demonstrated by subcellular fractionation that Cs-Thiolase I was constitutively synthesized, and Ps-Thiolase I was inductively synthesized in the cells grown on butyrate or alkanes although the induction was weaker than that of Thiolase III (6). Therefore, the slightly induced parts of the mRNA and protein levels of Thiolase I may correspond to the levels of Ps-Thiolase I, and Ps-Thiolase I could be classified as one of the less strongly inductive peroxisomal enzymes, such as malate synthase and isocitrate lyase, which are house-keeping

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```
Thiolase IA    MA-------------------LPPVYIVSTARTPIG-SFQGSLSSLTYSDLGAHAVKA 38
Thiolase IIIA  MDRLNQLSGQLKPHAKQSILQKNPDDVVIVAAYRTAIGKGFKGSFRS-VRSEFILTEFLK 59
               *                 * **    ** **   * **            *


                                                          ★
Thiolase IA    ALAKVPQIKPQDVDEIVFGGVLQAHVGQAPARQVALKAGLPDSIVASTINKVCASGMKAV 98
Thiolase IIIA  EFIKKTNIDPSLIEDVAIGHVLNQAAGATEHRGACLAAGIPYTAAFIAVNRFCSSGLMAI 119
                *   * *        * **    *    *  * ** *        *   * **   *


Thiolase IA    IIGAQHIICGTSDIVVVGGAESMSHTPYYLPSARSGARYGDAIMVDGVQKDGLLDVYEEK 158
Thiolase IIIA  SDIANKIKTGEIECGLAGGAESMSTN---YRDPRVAPRIDPHLADDAQMEKCLIP----- 171
                *  *  *    ******            *    *          *       *


Thiolase IA    LMGVAAEKCAKDHGFSREDQDNFAIHSYKKAGKALSEGKFKSEIAP--VTIKGFRGKPDT 216
Thiolase IIIA  -MGITNENVANQFNISRERQDEFAAKSYNKAAKAVAAGAFKSEILPIRSIIRNSDGTEKE 230
                **   *  *   *** **  **  **  ** **   ** ***** *    *    *


Thiolase IA    VIENDEEIGK--FNEERLKSARTVFQKENGTVTAPNASKLHDGGAALVLVSEAKLKQLGL 274
Thiolase IIIA  IIVDTDEGPREGVTAESLGKLRPAF---DGTTTAGNASQVSDGAAAVLLMKRSLAEAKGY 287
                *     *       *   *     ** ** ***   ** **  *  *        *


Thiolase IA    KPLAKISGWGEAARTPFDFTIAPALAVPKAVKHAGLTVDRVDFFELNEAFSVVGLANAEL 334
Thiolase IIIA  PIIGKYVLCSTAGVPPEIMGVGPAYAIPEVLKRTGLTVDDIDVFEINEAFAAQCLYSAEQ 347
                  *     *     ** * *     *  ****  * ** ****    *   **


                                                        ★
Thiolase IA    VNIPLEKLNVYGGAVAMGHPLGCSGARIIVTLLSVLTQEGGRFGVAGVCNGGGGASAVVI 394
Thiolase IIIA  VNVPEEKLNINGGAIALGHPLGETGARQYATIIPLLK--PGQIGLTSMCIGSGMGSASIL 405
                * * **** *** * ***** **    *     *     *   * *   ** *


Thiolase IA    EKIDADAKL 403
Thiolase IIIA  ------VRE 408
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Fig. 4.    Alignment of the amino acid sequences of Ps-Thiolases I and III.

Amino acid sequences are shown by the one-letter amino acid notation. The amino acid sequences of Thiolases IA and IIIA are aligned as representatives. Gaps have been inserted to achieve the maximum homology according to the ALIGN program of ODEN package supplied from DNA Databank Japan (DDBJ). Two stars mark the putative active-site cysteine residues as described by Kurihara et al. (7). The amino acids of each enzyme are numbered at the right side. Identical residues among these thiolases are indicated with (*).
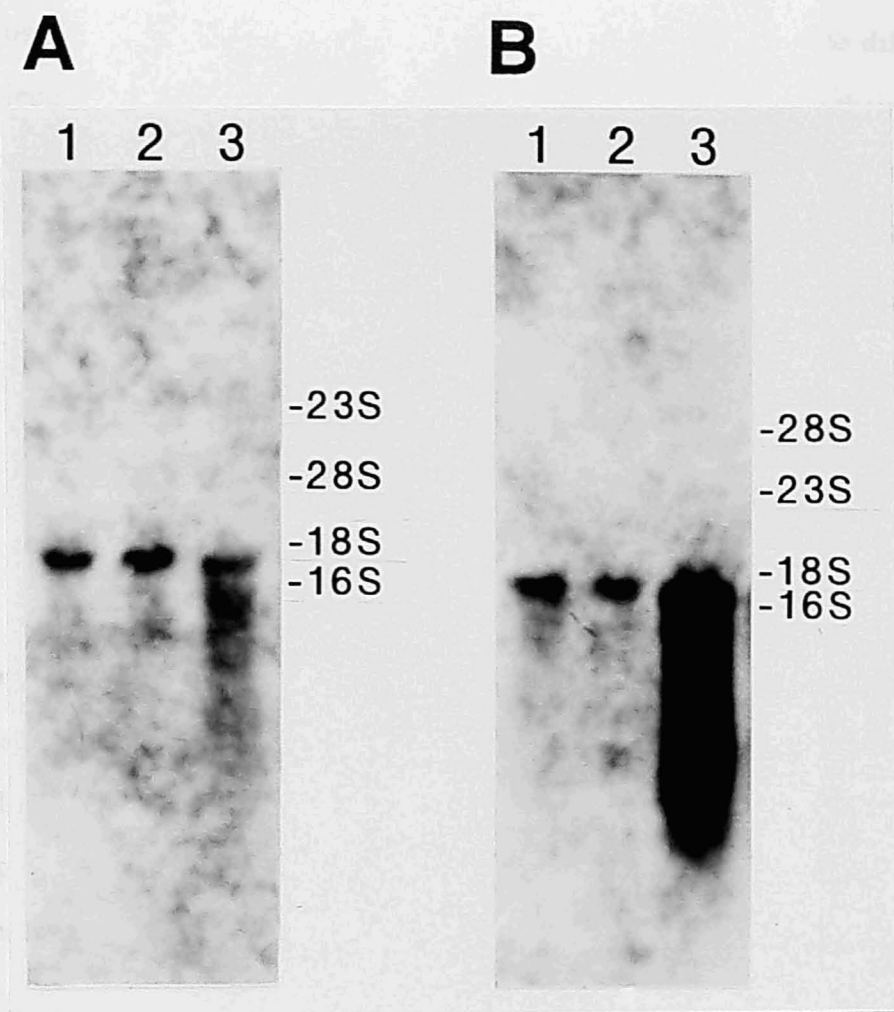
Fig. 5.    Northern blot analysis of Thiolase I (A) and Thiolase III (B) mRNAs. Total RNAs (50 µg) were isolated from *Candida tropicalis* cultured on acetate (lane 1), glucose (lane 2), and *n*-alkanes (lane 3) at the early exponential growth phase. Blots were hybridized with the probes prepared from Ps-Thiolase I (A) and Thiolase III cDNAs (B). To increase the intensity of the faint positive bands on lanes 1 and 2, the exposure time was adjusted. Calf rRNAs (28S and 18S) and *Escherichia coli* rRNAs (23S and 16S) were used as the size markers.

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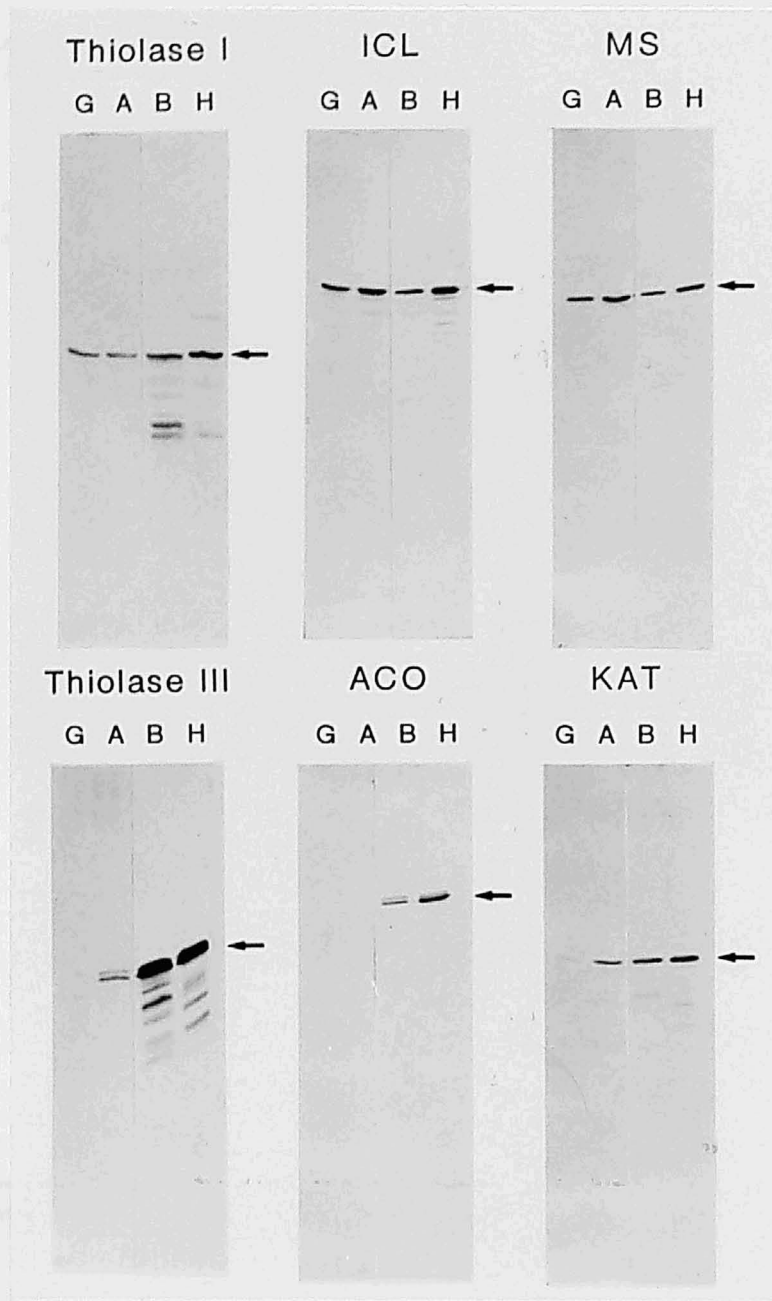
Fig. 6.    Western blot analysis of peroxisomal proteins from the cells grown on various carbon sources.

*Candida tropicalis* cells were harvested from glucose (G), acetate (A), butyrate (B), and *n*-alkane (H) media at the exponential growth phase. Aliquots of cell-free extracts (40 μg of protein for Thiolase I and 10 μg for others) were run on SDS-PAGE. Arrows represent the respective sbunit bands (Thiolase I, 41 kDa; isocitrate lyase (ICL), 65 kDa; malate synthase (MS), 61 kDa; Thiolase III, 43 kDa; acyl-CoA oxidase (ACO), 74 kDa; catalase (KAT), 54kDa). The degradated products were observed below the bands indicated by arrows.

proteins and are synthesized constitutively even in the cells grown in glucose or acetate medium (12, 13), whereas Thiolase III could be classified as one of the more strongly inducible peroxisomal enzymes like acyl-CoA oxidase and catalase.

In conclusion, Ps-Thiolase I and Thiolase III were demonstrated to be different in terms of the molecular structures and the regulation of biosynthesis, although they are both localized in peroxisomes and are similar in their reaction and cooperative function in the fatty acid $\beta$-oxidation system. Further investigation of the transcriptional regulation and subcellular localization mechanism of Ps-Thiolase 1 and Thiolase III would be useful to analyze the evolution of the fatty acid $\beta$-oxidation system and that of peroxisomes from yeasts to mammalian cells.

## SUMMARY

Two transcribed genes encoding peroxisomal 3-ketoacyl-CoA thiolase (Thiolase III), one of the peroxisomal thiolase isozymes, of the n-alkane-assimilating yeast, Candida tropicalis , were isolated. Restriction maps of these two genes were very similar. Both genes had only one open reading frame consisting of 1224 bp corresponding to 408 amino acid residues. The amino acid sequences of Thiolase III contains a putative peroxisomal targeting signal and showed 35% identity to Ps-Thiolase I. The regulation of biosynthesis of these peroxisomal thiolases in Candida tropicalis was compared by Northern and Western blot analyses. The results revealed that the biosynthesis of Thiolase III was strongly induced in a medium containing butyrate or alkanes as a carbon source, while Ps-Thiolase I was expressed even in a medium containing glucose or acetate with the slight induction in a medium containing butyrate or alkanes. In conclusion, the regulation of biosynthesis of Ps-Thiolase I and Thiolase III was found to be different, althoufh the enzymes can participate in the final step of the peroxisomal fatty acid $\beta$-oxidation system.

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# Chapter 2  Molecular evolution of yeast thiolase isozymes

## INTRODUCTION

Thiolases catalyze the final step of fatty acid β-oxidation in prokaryotic (1) and eukaryotic cells (2-6) and are classified into two types, acetoacetyl-CoA thiolase, which is specific to acetoacetyl-CoA, and 3-ketoacyl-CoA thiolase, which has broad substrate specificity. For the complete and efficient degradation of fatty acids having various chain lengths, the coexistence of these two types of thiolases is important. In eukaryotes, thiolases exhibit diverse subcellular localization. In rat liver cells, there are at least two distinct 3-ketoacyl-CoA thiolases, one localized in peroxisomes responsible for long-chain 3-ketoacyl-CoAs (4) and another in mitochondria for middle-chain 3-ketoacyl-CoAs (2), whereas acetoacetyl-CoA thiolases are localized in cytosol and in mitochondria (2).

The presence of both acetoacetyl-CoA thiolase (Ps-Thiolase I) and 3-ketoacyl-CoA thiolase (Thiolase III) in peroxisomes was first found in the *n*-alkane-assimilating yeast *Candida tropicalis* (5-7). In this organism, neither of them was present in its mitochondria. The peroxisomal localization of the two thiolases is unique and provides a basis for examining the evolution of the fatty acid β-oxidation system. Since the fatty acid β-oxidation system was also exclusively detected in peroxisomes in the yeast cells (8), fatty acids could be completely degraded with cooperation of these Ps-Thiolase I and Thiolase III in peroxisomes (6, 7). These facts are quite different from the case of mammalian cells, which possess two β-oxidation pathways: a peroxisomal one involved in long-chain fatty acid degradation and a mitochondrial one responsible for medium- or short-chain fatty acid degradation (9-11).

The genes encoding Ps-Thiolase I and Thiolase III of *Candida tropicalis* were cloned and their primary protein structures were determined as described by Kurihara *et al.* (12) and in Part I, Chapter 1. By calculating the evolutional distance among thiolases from various sources (13, 14), the author has constructed a phylogenetic tree of thiolases and have discussed the molecular evolution of thiolase isozymes. The symmetric divergent branch points of this

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tree strongly suggested that the ancestors of peroxisomal thiolase isozymes, Ps-Thiolase I and Thiolase III, had occurred at the same time in the yeast *Candida tropicalis*, as predicted by the endosymbiotic theory of peroxisomes (15-17).

## MATERIALS AND METHODS

### Alignment of amino acid sequences

The multiple alignment of protein sequences and the number of similarity among sequences were obtained with the program ALIGN contained within the ODEN program provided by DNA Databank Japan (DDBJ). Nucleotide sequences of thiolases have been deposited in DDBJ with accession numbers.

### Construction of phylogenetic tree

The number of amino acid substitutions was estimated with the DISTA program (18). The distance matrix was made with the DMATA program (18). The phylogenetic tree was constructed by the neighbor-joining (N-J) method with the TREENJ program (19). Bootstrap resampling was performed with the BSTRAP program (20). These programs were contained within the ODEN program provided by DDBJ.

## RESULTS AND DISCUSSION

The author compared the primary amino acid sequences of thiolases from various organisms reported so far (Fig. 1), and constructed a phylogenetic tree of thiolases based on the estimation of amino acid substitutions (18) and the neighbor-joining method (19) (Fig. 2). The phylogenetic tree clearly indicates that three types of thiolases, acetoacetyl-CoA thiolase, 3-ketoacyl-CoA thiolase, and mitochondrial 3-ketoacyl-CoA thiolase, diverged simultaneously from one original branch point. It can be found that the divergence of one original thiolase to thiolases with different substrate specificities —that is, to 3-ketoacyl-CoA thiolase and acetoacetyl-CoA thiolase— occurred before the divergence of prokaryotic and eukaryotic thiolases. The high similarity of eukaryotic acetoacetyl-CoA thiolases beyond species and

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Thiolase III    MD-RLNQLSGQLK---PNA--------KQSIL-QKNPD--------DVVIV-AAYRTAI   37
SCPKCT          MSQRLQSIKDHLV---ESAMGKGESKRKNSLL-EKRPE--------DVVIV-AANRSAI   46
RPKCTB          MH-RLQVVLGHLAGRSESSSALQAAPCSAGFP-QASAS--------DVVVV-HGQRTPI   48
ECKCT           M--------------------------------E--------QVVIV-DAIRTPM      14
PFKCT           M--------------------------------SLNPR-----DVVIV-DFGRTPM     18
RMKCT           M---------------------------------A------LLRGVFIV-AAKRTPF   17
Ps-Thiolase I   M---------------------------------A----LP---PVYIV-STARTPI    16
SUCACT          M---------------------------------S-----Q---NVYIV-STARTPI    15
RMACT           MA-ALAVLHGVVRRPLLRGLLQEVRCLGRSYASKPTLN--------DVVIV-SATRTPI   49
AEACT           M---------------------------------T--------DVVIV-SAARTAV    14
ZRACT           M---------------------------------S--------TPSIVIASARTAV    15
                                                          *           *      *


Thiolase III    G-KGFKGSFRSVRSEFILTEFLKEFIKK----T-NIDP-SLIEDVAIGNVL-NQAA-GAT   88
SCPKCT          G-KGFKGAFKDVNTDYLLYNFLNEFIGRFPEPL-RADL-NLIEEVACGNVL-NVGA-GAT   101
RPKCTB          G-RAGRGGFKDTTPDELLSAVLTAVLQD----V-KPKP-ECLGDISVGNVL-QPGA-GAA   99
ECKCT           GRSKG-GAFRNVRAEDLSAHLMRSLLAR----NPALEA-AALDDIYWGCVQ-QTLEQGFN   67
PFKCT           GRSKG-GMHRNTRAEDMSAHLISKVLER----NSKVDP-GEVEDVIWGCVN-QTLEQGWN   71
RMKCT           G-AYG-GLLKDFTATDLTEFAARAALSA----GKV-PP-ETIDSVIVGNVMQSSSD-AAY   68
Ps-Thiolase I   G-SFQ-GSLSSLTYSDLGAHAVKAALAK----VPQIKP-QDVDEIVFGGVL-QANV-GQA   67
SUCACT          G-SFQ-GSLSSKTAVELGAAALKGALAK----VPELDASKDFDEIIFGNVL-SANL-GQA   67
RMACT           G-SFL-GSLASQPATKLGTIAIQGAIEK----A-GIPK-EEVKEVYMGNVI-QGGE-GQA   99
AEACT           G-KFG-GSLAKIPAPELGAVVIKAALER----AGV-KP-EQVSEVIMGQVL-TAGS-GQN   64
ZRACT           G-SFN-GAFAHTPAHELGATVISAVLER----AGV-AA-GEVHEVILGQVL-PAGE-GQN   65
                *       *                                        *  *


                                    ★
Thiolase III    EHRGACLAAGIPYTAAFIAVNRFCSSGLMAISDIANKIKTGEIECGLAGGAESMS---TN   145
SCPKCT          EHRAACLASGIPYSTPFVALNRQCSSGLTAVNDIANKIKVGQIDIGLALGVESMT---NN   158
RPKCTB          MARIAQFLSGIPETVPLSAVNRQCSSGLQAVANIAGGIRNGSYDIGMACGVESMT---LS   156
ECKCT           IARNAALLAEVPHSVPAVTVNRLCGSSMQALHDAARMIMTGDAQACLVGGVEHMGHVPMS   127
PFKCT           IARMASLMTQIPHTSAAQTVSRLCGSSMSALHTAAQAIMTGNGDVFVVGGVEHMGHVSMM   131
RMKCT           LARHVGLRVGVPTETGALTLNRLCGSGFQSIVSGCQEICSKDAEVVLCGGTESMSQSPYS   128
Ps-Thiolase I   PARQVALKAGLPDSIVASTINKVCASGMKAVIIGAQNIICGTSDIVVVGGAESMSNTPYY   127
SUCACT          PARQVALTAGLGNHIVATTVNKVCASAMKAIILGAQSIKCGNADVVVAGGCESMTNAPYY   127
RMACT           PTRQATLGAGLPIATPCTTVNKVCASGMKAIMMASQSLMCGHQDVMVAGGMESMSNVPYV   159
AEACT           PARQAAIKAGLPAMVPAMTINKVCGSGLKAVMLAANAIMAGDAEIVVAGGQENMSAAPHV   124
ZRACT           PARQAAMKAGVPQEATAWGMNQLCGSGLRAVALGMQQIATGDASIIVAGGMESMSMAPHC   125
                *                   * *                          *  *  *


Thiolase III    Y--RD--PRVAPRIDP-HLADDAQMEKC-LIPMGITNENVANQFNISRERQDEFAAKSYN   199
SCPKCT          Y--KN--VNPLGMISSEELQKNREAKKC-LIPMGITNENVAANFKISRKDQDEFAANSYQ   213
RPKCTB          E--RGNPGNISSRLLENEKARDCL------IPMGITSENVAERFGISRQKQDAFALASQQ   208
ECKCT           H-----GVDFHPGLSRNVAKAAGM--------MGLTAEMLARMHGISREMQDAFAARSHA   174
PFKCT           H-----GVDPNPHMSLYAAKASGM--------MGLTAEMLGKMHGISREQQDAFAVRSHQ   178
RMKCT           VRNVRFGTKFGLDLKLEDTLWAGLTDQHVKLPMGMTAENLAAKYNISREDCDRYALQSQQ   188
Ps-Thiolase I   LPSARSGARYGDAIMVDGVQKDGLLDVYEEKLMGVAAEKCAKDHGFSREDQDNFAINSYK   187
SUCACT          MPAARGGAKFGQTVLIDGVERDGLNDAYDGLAMGVHAEKCARDWDITRDQQDSFAIESYQ   187
RMACT           M--SRGATPYGGVKLEDLIVKDGLTDVYNKIHMGNCAENTAKKLSISREEQDKYAIGSYT   217
AEACT           LPGSRDGFRMGDAKLVDTMIVDGLWDVYNQYHMGITAENVAKEYGITREAQDEFAVGSQN   184
ZRACT           --AHLAGVKMGDFKMIDTMIKDGLTDAFYGYHMGTTAENVAKQWQLSRDEQDAFAVASQN   183
                                          **    *         *    *   *  *
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Thiolase III   KAAKAVAAGAFKSEILPIRSIIRHSDGTEKEI-I-VDTDEGPREGV-TAESLGKLRPAF-   255
SCPKCT         KAYKAKNEGLFEDFILPIKL----PDGS-----I-CQSDEGPRPNV-TAESLSSIRPAFI   262
RPKCTB         KAASAQSKGCFRAEIVPVTTVLDDKGDRKTI-T-VSQDEGVRPST-TMEGLAKLRPAF-   264
ECKCT          RAWAATQSAAFKNEIIPTGGHDAD-----GVL-KQFNYDEVIRPET-TVEALATLRPAFD   227
PFKCT          LAHKATVEGKFKDEIIPMQGYDEN-----GFL-KIFDYDETIRPDT-TLESLAALKPAFN   231
RMKCT          RWKAANEAGYFNEEMAPIEVKTKK-----GKQ-T-MQVDEHARPQT-TLEQLQNLPPVFK   240
Ps-Thiolase I  KAGKALSEGKFKSEIAPVTIKGFR-----GKP-D-TVIENDEEIGKFNEERLKSARTVFQ   240
SUCACT         KSQQSQKEGKFDNEIVPVTIKGFR-----GKP-D-TQVTNDEEPARLHVERLKSARTVFQ   240
RMACT          RSKEAWDAGKFANEITPITISVKG-----KPD-V-VVKEDEEYKRV-DFSKVPKLKTVFQ   269
AEACT          KAEAAQKAGKFDEEIVPVLIPQRK-----GDPVA-FKTDEFVRQGA-TLDSMSGLKPAFD   237
ZRACT          KAEAAQKDGRFKDEIVPFIVKGRK-----GDI-T-VDADEYIRHGA-TLDSMAKLRPAFD   235
                         *   *   *                                        *

Thiolase III   --DGTTTAGNASQVSDGAAAVLLMKRSLAEAKGYPIIGKYVLCSTAGVPPEIMGVGPAYA   313
SCPKCT         KDRGTTTAGNASQVSDGVAGVLLARRSVANQLNLPVLGRYIDFQTVGVPPEIMGVGPAYA   322
RPKCTB         KDGGSTTAGNSSQVSDGAAAVLLARRSKAEELGLPILGVLRSYAVVGVPPDIMGIGPAYA   324
ECKCT          PVNGMVTAGTSSALSDGAAAMLVMSESRAHELGLKPRARVRSMAVVGCDPSIMGYGPVPA   287
PFKCT          PKGGTVTAGTSSQITDGASCMIVMSAQRAKDLGLEPLAVIRSMAVAGVDPAIMGYGPVPA   291
RMKCT          KE-GTVTAGNASGMSDGAGVVIIASEDAVKKHNFTPLARVVGYFVSGCDPAIMGIGPVPA   299
Ps-Thiolase I  KENGTVTAPNASKLNDGGAALVLVSEAKLKQLGLKPLAKISGWGEAARTPFDFTIAPALA   300
SUCACT         RENGTVTAANASPINDGAAAIILVSERVLKERNLKPLAIVKGWGEAAHLPADFTWAPSLA   300
RMACT          KENGTVTAANASTLNDGAAAVVLMTAEAAQRLKVKPLARIAAFADAAVDPIDFPLAPAYA   329
AEACT          KA-GTVTAANASGLNDGAAAVVVMSAAKAKELGLTPLATIKSYANAGVDPKVMGMGPVPA   296
ZRACT          KE-GTVTAGNASGLNDGAAAALLMSEAEASRRGIQPLGRIVSWATVGVDPKVMGTGPIPA   294
                  *   **   *   **                               *     *   *

Thiolase III   IPEVLKRTGLTV-DDIDVFEINEAFAAQCLYSAE---QVNVPEEKLNINGGAIALGHPLG   369
SCPKCT         IPKVLEATGLQV-QDIDIFEINEAFAAQALYCIH---KLGIDLNKVNPRGGAIALGHPLG   378
RPKCTB         IPAALQKAGLTV-NDIDIFEINEAFASQALYCVE---KLGIPAEKVNPLGGAIALGHPLG   380
ECKCT          SKLALKKAGLSA-SDIGVFEMNEAFAAQILPCIKDLGLIEQIDEKINLNGGAIALGHPLG   346
PFKCT          TQKALKRAGLNM-ADIDFIELNEAFAAQALPVLKDLKVLDKMNEKVNLHGGAIALGEPFG   350
RMKCT          ITGALKKAGLSL-KDMDLIDVNEAFAPQFLAVQK---SLDLDPSKTNVSGGAIALGHPLG   355
Ps-Thiolase I  VPKAVKHAGLTV-DRVDFFELNEAFSVVGLANAE---LVNIPLEKLNVYGGAVAMGHPLG   356
SUCACT         VPKALKHAGIEDINSVDYFEFNEAFSVVGLVNTK---ILKLDPSKVNVYGGAVALGHPLG   357
RMACT          VPKVLKYAGLKK-EDIAMWEVNEAFSVVVLANIK---MLEIDPQKVNVHGGAVSLGHPIG .385
AEACT          SKRALSRAEWTP-QDLDLMEINEAFAAQALAVHQ---QMGWDTSKVNVNGGAIAIGHPIG   352
ZRACT          SRKALERAGWKI-GDLDLVEANEAFAAQACAVNK---DLGWDPSIVHVNGGAIAIGHPIG   350
                  ****                             *   ***   *** *

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Thiolase III   ETGARQYATIIPLLKP--GQ-IGLTSMC-IGSGMGSASILV-R--------E   408
SCPKCT         CTGARQVATILRELKK--DQ-IGVVSMC-IGTGMGAAAIFI-K--------E   417
RPKCTB         CTGARQVVTLLNELKRRGRRAYGVVSMC-IGTGMGAAAVFE-Y------PGN   424
ECKCT          CSGARISTTLLNLMERKDVQ-FGLADGCVSGLGQGIATVFE-R------V--   388
PFKCT          CSGARISGTLLNVMKQNGGT-FGLSTMC-IGLGQGIATVFE-R------V--   391
RMKCT          GSGSRITAHLVHELRRRGGK-YAVGSAC-IGGGQGISLIIQNT------A--   397
Ps-Thiolase I  CSGARIIVTLLSVLTQEGGR-FGVAGVC-NGGGGASAVVIE-KIDADAKL--   403
SUCACT         CSGARVVVTLLSILQQEGGK-IGVAAIC-NGGGGASSVVIE-K------L--   398
RMACT          MSGARIVVHLAHALRQ--GE-FGLASIC-NGGGGASAVLIE-K------L--   424
AEACT          ASGCRILVTLLHEMKRRDAK-KGLASLC-IGGGMGVALAVERK--------   393
ZRACT          ASGARILNTLLFEMKRRGAR-KGLATLC-IGGGMGVAMCIESL--------   391
                  *  *                      *   *  *
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Fig. 1. Alignment of amino acid sequences of thiolases from various sources.

All amino acid sequences are shown by the one-letter amino acid notation. The stars mark the active site cysteines. Identical residues among these thiolases are indicated with (*).

Abbreviations: Thiolase III, *Candida tropicalis* peroxisomal 3-ketoacyl-CoA thiolase; SCPKCT, *Saccharomyces cerevisiae* peroxisomal 3-ketoacyl-CoA thiolase; RPKCTB, rat peroxisomal 3-ketoacyl-CoA thiolase B; ECKCT, *Escherichia coli* 3-ketoacyl-CoA thiolase; PFKCT, *Pseudomonas fragi* 3-ketoacyl-CoA thiolase; RMKCT, rat mitochondrial 3-ketoacyl-CoA thiolase; Ps-Thiolase I, *Candida tropicalis* peroxisomal acetoacetyl-CoA thiolase; SUCACT, *Saccharomyces uvarum* cytosolic acetoacetyl-CoA thiolase; RMACT, rat mitochondrial acetoacetyl-CoA thiolase; AEACT, *Alcaligenes eutrophus* acetoacetyl-CoA thiolase; ZRACT, *Zoogloea ramigera* acetoacetyl-CoA thiolase.

Accession numbers in DDBJ: Thiolase III, D17320; SCPKCT, X53946; RPKCTB, J02749; ECKCT, J05498; PFKCT, D90447; RMKCT, X05341; Ps-Thiolase I, D13470; SUCACT, X07976; RMACT, D00511; AEACT, J04987; ZRACT, J02631.
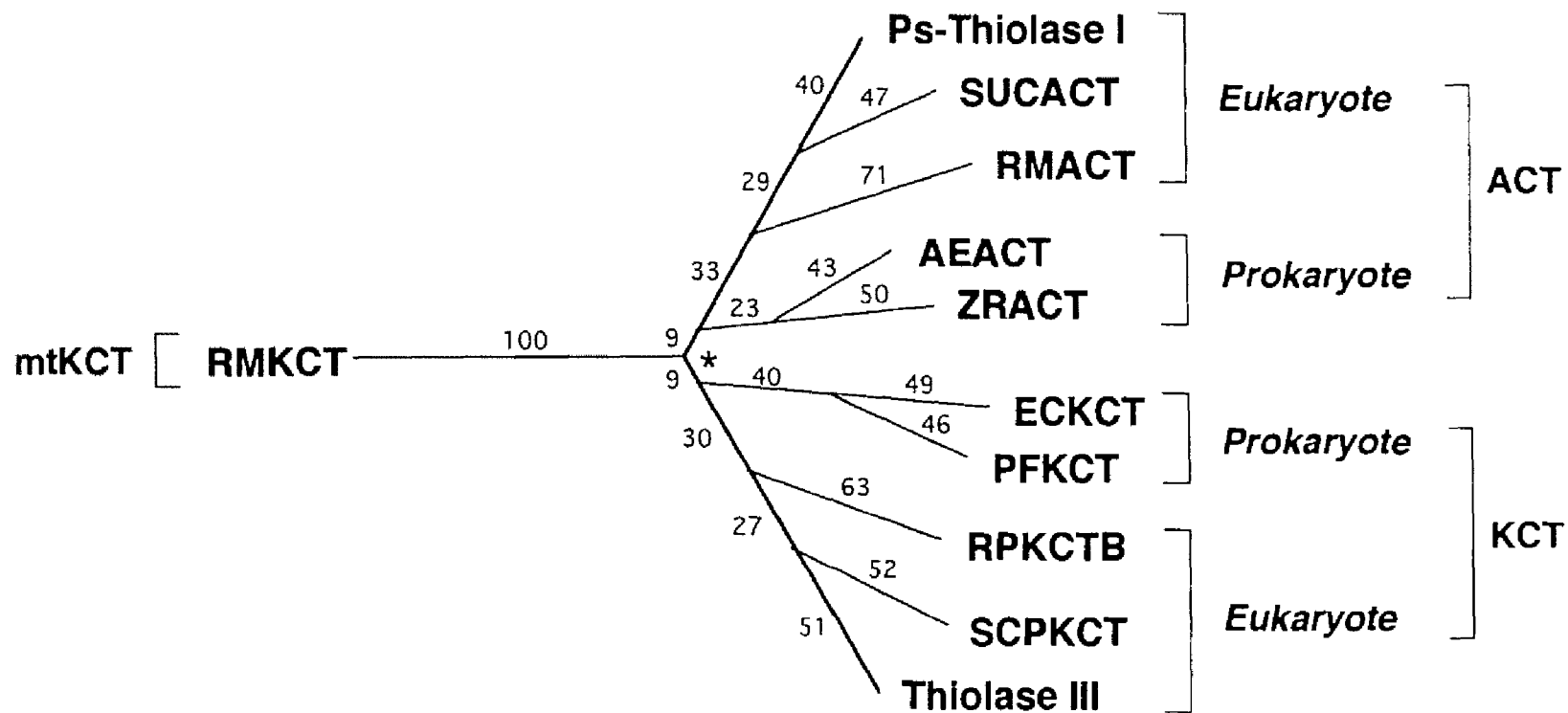
Fig. 2. Phylogenetic relationship of thiolases from various sources.

The phylogenetic tree of thiolases with all aligned sequences (Fig. 1) containing amino-terminal and carboxyl-terminal extensions, which are considered to be important in examining the evolution, was constructed. Therefore, the main replicates in 100 bootstrap replicates are low (40%). The number shows the relative branch length to RMKCT. The mark (*) demonstrates the origin of the divergence. Abbreviations are used as Fig. 1., ACT, KCT, and mtKCT represent acetoactyl-CoA thiolase, 3-ketoacyl-CoA thiolase, and mitochondrial 3-ketoacyl-CoA thiolase, respectively.

subcellular localization seen in Ps-Thiolase I [111, branch length from the origin of the divergence shown in Fig. 2], *Saccharomyces uvarum* cytosolic acetoacetyl-CoA thiolase [118], and rat mitochondrial acetoacetyl-CoA thiolase [113] is probably due to the very strict substrate specificity of acetoacetyl-CoA thiolase (14). Mitochondrial 3-ketoacyl-CoA thiolase may have followed a completely different evolutionary pathway after the diverging point. This indicates that mitochondria have their own fatty acid β-oxidation system. Surprisingly, the branching point and the branch length of prokaryotic acetoacetyl-CoA thiolase and eukaryotic peroxisomal acetoacetyl-CoA thiolase of *Candida tropicalis* (Ps-Thiolase I) closely resemble those of prokaryotic 3-ketoacyl-CoA thiolase and eukaryotic peroxisomal 3-ketoacyl-CoA thiolase of *Candida tropicalis* (Thiolase III). A slightly shorter branch length of acetoacetyl-CoA thiolase than that of 3-ketoacyl-CoA thiolase is probably due to the very strict substrate specificity of acetoacetyl-CoA thiolase, which slowed its evolution. The symmetrical divergence of Ps-Thiolase I and Thiolase III isozymes suggests that ancestral thiolases corresponding to Ps-Thiolase I and Thiolase III appeared at the same time and independently in an original microorganism of *Candida tropicalis*, which had a function of fatty acid assimilation. This simultaneous appearance of two different enzymes (isozymes) in the evolution, having similar functions in the fatty acid metabolism, in a single cell may be explained by endosymbiosis.

Furthermore, the notion of gene duplication (21, 22) could help to explain the molecular evolution of thiolase isozymes. Rat peroxisomal 3-ketoacyl-CoA thiolase (A and B), Ps-Thiolase I (A and B) and Thiolase III (A and B) have two very similar genes, respectively (12, 23). Each pair of genes may be the products of a recent gene duplication, and they may be in the transition period of diversification.

There are differences in the localization of the fatty acid β-oxidation system among eukaryotes. For example, *Candida tropicalis* has only the peroxisomal β-oxidation system, while rat has the peroxisomal and mitochondrial β-oxidation systems. Coexistence of acetoacetyl-CoA thiolase and 3-ketoacyl-CoA thiolase is essential for the complete degradation of fatty acids. According to analysis of the molecular evolution of thiolase isozymes, the final step enzyme of the β-oxidation system, it is presumed as follows. The β-oxidation systems

had existed both in peroxisomes and in mitochondria immediately after the endosymbiosis of a specific microorganism which evolved to peroxisomes in eukaryotes. However, the mitochondrial system was degenerated in *Candida tropicalis* because the peroxisomal system had a broader substrate specificity than the mitochondrial one (24, 25). On the other hand, in rat liver cells, the mitochondrial β-oxidation system remained to participate in the degradation of short- and middle-chain substrates by mitochondrial 3-ketoacyl-CoA thiolase (RMKCT), probably due to the occurrence of mitochondrial actoacetyl-CoA thiolase (RMACT) and the deletion of peroxisomal acetoacetyl-CoA thiolase.

DNA has not been detected in peroxisomes and these organelles have only a single-membrane structure (26). These characteristics differ from those of mitochondria and chloroplasts, about 90% of whose DNAs have been transferred to the nucleus by so-called horizontal gene transfer (27, 28). Endosymbiosis of mitochondria and chloroplasts was suggested judging from the fact that they contain characteristic DNAs (16). Peroxisomes, however, differ in these respects. Several groups discussed the possibility of endosymbiosis of peroxisomes based on the mechanism of posttranslational protein transport and the primitive or bacterial character of the metabolic functions (14-17). The phylogenetic tree of thiolases and peroxisomal localization of these Ps-Thiolase I and Thiolase III isozymes in *Candida tropicalis* support the concept presented by de Duve (17), Borst (15), and Igual *et al.* (14) regarding of molecular evolution. The construction of the following model of the evolution of peroxisomes in *Candida tropicalis* is also possible from the phylogenetic trees. First, a eukaryote and a specific microorganism, having a peroxisomal function, diverged from one original organism (an ancestor). Second, endosymbiosis of the specific microorganism into eukaryotes and development to prokaryotes occurred independently. The specific microorganism evolved to peroxisomes in eukaryotes as a result of endosymbiosis and, on the other hand, the microorganism developed to prokaryotes which can assimilate fatty acids. This hypothesis could be tested through analysis of many as yet unknown amino acid sequences of other peroxisomal enzymes and the comparison of their phylogenetic trees.

# SAMMARY

The coexistence of two thiolase isozymes (acetoacetyl-CoA thiolase and 3-ketoacyl-CoA thiolase), essential for the complete degradation of fatty acids, in peroxisomes of an *n*-alkane-assimilating yeast *Candida tropicalis* is unique in eukaryotic cells. As one of the methods of analysis of molecular information from these isozymes, the evolutional distance among thiolases from various organisms was estimated. The results suggests that yeast peroxisomal thiolase isozymes are important enzymes in examining the molecular evolution of the fatty acid metabolic pathway and the biogenesis of peroxisomes.

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# Chapter 3 Expression of acetoacetyl-CoA thiolase isozymes derived from the same genes in two intracellular compartments of *Candida tropicalis*

## INTRODUCTION

There have been many reports of isozymes localized in distinct compartments of eukaryotic cells. In many cases, two counterparts are encoded by different genes with the information needed for their respective localization. In some cases, two isozymes are encoded by the same gene as reviewed by Danpure (1), for example, carnitine acetyltransferase of yeast *Candida tropicalis* (2). One mechanism that has been elucidated is an alternative transcription or translation, which leads to distinct protein products destined for their proper intracellular compartments.

Thiolase is a ubiquitous enzyme which catalyzes the thiolytic cleavage of 3-ketoacyl-CoA to acetyl-CoA and acyl-CoA in the final step of the fatty acid $\beta$-oxidation system. It is classified into two types according to substrate specificity. One type is acetoacetyl-CoA thiolase, which catalyzes the cleavage of acetoacetyl-CoA and the reverse condensation of acetyl-CoA . The other is 3-ketoacyl-CoA thiolase, which has broad substrate specificity for 3-ketoacyl-CoAs in carbon chain length ($\geq C_4$). It has been reported that thiolases participate in a variety of metabolic pathways as well as the $\beta$-oxidation system (3-7), and that in eukaryotic cells, especially in mammalian cells, thiolases also exhibit diversity in their intracellular localization (6-9) and are encoded by respective genes (10-14).

In the *n*-alkane-assimilating yeast *Candida tropicalis* pK233, Kurihara *et al.* reported that there are two thiolase isozymes in peroxisomes (Ps-Thiolase I and Thiolase III), which differ in their substrate specificities (15, 16). Ps-Thiolase I corresponds to peroxisomal acetoacetyl-CoA thiolase, and Thiolase III to peroxisomal 3-ketoacyl-CoA thiolase. Biochemical evidence suggests that Ps-Thiolase I and Thiolase III participate in the peroxisomal

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β-oxidation system because of the exclusive localization of the β-oxidation system in peroxisomes (17, 18), as well as the inductive expression of these peroxisomal isozymes in *n*-alkane-grown cells. This yeast also has cytosolic acetoacetyl-CoA thiolase (Cs-Thiolase I), which is indistinguishable from Ps-Thiolase I immunochemically and shows a similar subunit molecular mass to Ps-Thiolase I (19-20). However, there is a difference in the manner of expression between Ps-Thiolase I and Cs-Thiolase I. Cs-Thiolase I is expressed constitutively during growth on several carbon sources tested, while Ps-Thiolase I is expressed inductively (19). Therefore, it remains unclear whether they are encoded by the same gene or not.

Ps-Thiolase I is encoded by two genes [Thiolase IA and Thiolase IB genes (*CT-T1A* and *CT-T1B*)], and the A and B genes show an extremely high degree of identity to each other (>96%) (21). Both *CT-T1A* and *CT-T1B* were shown to be translated in *Candida tropicalis*. *CT-T1A* and *CT-T1B* were shown to be located on one pair of chromosomes in this diploid yeast (22), and they are thought to be allelic.

In this chapter, the author has carried out the purification of Cs-Thiolase I from *Candida tropicalis* and recombinant thiolases expressed independently from Thiolase I genes in the yeast *Saccharomyces cerevisiae*, and characterized the enzymatic properties of these proteins. Based on a comparison with Ps-Thiolase I purified from *Candida tropicalis* peroxisomes, the author discuss the mode of expression of Cs-Thiolase I and Ps-Thiolase I isozymes in *Candida tropicalis*.

## MATERIALS AND METHODS

### Strains and media

*Candida tropicalis* pK233 (ATCC20336) was used as a source of Thiolase I. *Escherichia coli* strain DH5α was used for gene manipulation. *Saccharomyces cerevisiae* strain MT8-1 (*MATa ade his3 leu2 trp1 ura3*) (23) was used as the host cell for the expression of each thiolase isozyme gene.

Cultivation of *Candida tropicalis* was carried out as described in Part I, Chapter 1. Media used for *Saccharomyces cerevisiae* were as follows: YPD [10 g of yeast extract

(Difco Laboratories, Detroit, MI, USA), 20 g of peptone (Difco), and 20 g of glucose per liter of deionized water]; a chemically defined medium (SA-W) (0.67 % yeast nitrogen base without amino acids (Difco); 2% anhydrous sodium acetate; 0.002% adenine sulfate dihydrate, 0.002 % uracil, 0.002 % L-histidine monochloride monohydrate, and 0.003 % L-leucine).

## Construction of expression plasmids

Coding regions and their flanking regions of Thiolase I genes were subcloned into pUC19 from clones in λEMBL3 (21). pT16DB containing *CT-T1A* was constructed by insertion of a *Hin*dIII-*Bam*HI fragment of λT16 into pUC19. pT18DX containing *CT-T1B* was constructed by insertion of a *Hin*dIII-*Xba*I fragment of λT18 into pUC19. *Hin*dIII sites of pT16DB and pT18DX were filled with T4 DNA polymerase and ligated with a *Bam*HI linker (named pT16B and pT18B). A multicopy shuttle vector pMW1 (24) carrying *TRP1* as a selection marker was used to introduce and express thiolase isozyme genes in *Saccharomyces cerevisiae*. *Bam*HI fragments containing *CT-T1A* and *CT-T1B* with their promoter and terminator regions derived from pT16B and pT18B were inserted into the *Bam*HI site of pMW1 (named pWT1A and pWT1B, respectively, Fig. 2). These recombinant plasmids were introduced into *Saccharomyces cerevisiae* by electroporation using an EasyJect electroporater EasyJect (Cosmo Bio Co., Tokyo, Japan) (25).

## Preparation of cell-free extracts

*Saccharomyces cerevisiae* carrying plasmids for expression of thiolase genes were cultivated aerobically at 30 °C. After precultivation in 10 ml of YPD medium for 24 h, cells were harvested, and then inoculated in 100 ml of SA-W medium for expression of Thiolase I genes at initial $A_{660}$ of 0.1. Cells were cultivated for 25 h. Cell-free extracts were prepared as described in Part I, Chapter 1.

## Purification of Cs-Thiolase I and recombinant Thiolases

*Candida tropicalis* grown on glucose was harvested at mid-exponential growth phase. Protoplasts prepared from glucose-grown cells were homogenized with a Teflon homogenizer and then fractionated by differential centrifugation (18, 26). The fractions obtained were as follows: $P_2$ fraction (20,000 × *g* pellet), peroxisomes and mitochondria; $S_2$ fraction (20,000 × *g* supernatant), cytosol and microsomes; $S_3$ fraction (139,000 × *g* supernatant), cytosol; $P_3$

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fraction (139,000 x $g$ pellet), microsomes. $S_3$ fraction was used as a crude enzyme preparation of Cs-Thiolase I for further purification.

*Saccharomyces cerevisiae* carrying each thiolase gene was cultivated aerobically in SA-W medium for 25 h (early stationary growth phase, an optimum phase for expression as tested in this study), harvested, suspended in approximately 30 ml of the PGD buffer containing protease inhibitors (described in Part I, Chapter 1), and disintegrated in a Braun cell homogenizer using glass beads (25). Cell-free extracts were prepared by centrifugation at 20,000 x $g$ for 20 min, followed by ultracentrifugation at 139,000 x $g$ for 1 h. These supernatants were used as crude enzyme preparations.

Purification of Cs-Thiolase I and recombinant Thiolase IA and Thiolase IB (rThiolase IA and rThiolase IB) was carried out at 0 to 5 °C as described by Kurihara *et al.* for the purification of Ps-Thiolase I (16), using a DEAE-Sepharose CL-6B column (2.5 x 17.5 cm, Pharmacia Fine Chemicals, Uppsala, Sweden), a Butyl-TOYOPEARL 650M column (2.0 x 15.4 cm, Tosoh, Tokyo, Japan), and a Cellulofine GCL-2000m column (2.0 x 85.5 cm, Seikagaku Kogyo, Tokyo, Japan), sequentially. Ps-Thiolase I was purified as described by Kurihara *et al.* (16).

**Assay of enzymes and protein**

Thiolase activities were determined by measuring the degradation of acetoacetyl-CoA (for acetoacetyl-CoA thiolase activity) or 3-ketooctanoyl-CoA (for 3-ketoacyl-CoA thiolase activity) as described by Kurihara *et al.* (13, 14). The activity for the condensation reaction of two molecules of acetyl-CoA was assayed by measuring the oxidation of NADH, coupled with the reduction of the formed acetoacetyl-CoA by 3-hydroxyacyl-CoA dehydrogenase (27, 28). The reaction mixture (300 µl) was composed of 100 mM Tris-HCl (pH 8.1), 2.4 mM acetyl-CoA, 300 mM NADH, 2.5 U of 3-hydroxyacyl-CoA dehydrogenase from porcine heart and enzyme solution. Protein concentrations were measured with the method of Lowry *et al.* (29).

**Electrophoresis and Western blot analysis**

SDS-PAGE was carried out on a slab-gel (12.5% acrylamide) as described by Ueda *et*

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*al.* (26). Phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa) were used as molecular size markers. After electrophoresis, gels were stained with Coomassie Brilliant Blue or subjected to Western blot analysis using antiserum against Ps-Thiolase I (15, 16, 26).

**Amino acid sequence analysis and determination of native molecular mass**

After electrophoresis, proteins were transferred to a sheet of ProBlott (Applied Biosystems, Foster City, CA, USA). The filter after transfer was set in a protein sequencer 610A (Applied Biosystems) and analyzed as recommended by the vendor. Gel filtration to estimate the molecular masses of the native forms of the enzymes was carried out on a Cellulofine GCL-2000m column, using Calibration protein II for chromatography (Boehringer Mannheim, Mannheim, Germany) as size markers.

**Immunochemical titration**

Immunochemical titration was carried out using anti-Ps-Thiolase I antiserum (30). The reaction mixture (500 μl) containing the enzyme solution (100 μl), the antiserum, and 50 mM potassium phosphate buffer (pH 7.2) (390 μl) was incubated at 4°C for 3 h and the antigen-antibody precipitate was removed by centrifugation at 10,000 x *g* for 15 min at 0°C. Thiolase activity in the supernatant was measured.

**Enzymes and chemicals**

Restriction enzymes and modifying enzymes for gene manipulation were purchased from Toyobo (Osaka, Japan) and Takara Shuzo (Kyoto, Japan). Acetoacetyl-CoA, acetyl-CoA, NADH, and 3-hydroxyacyl-CoA dehydrogenase were purchased from Sigma (St. Louis, MO., USA). CoA was donated by Kojin (Tokyo, Japan). 3-Ketooctanoyl-CoA was prepared enzymatically from octenoyl-CoA synthesized by the mixed anhydride method (31, 32). All other chemicals were obtained from commercial sources.

## RESULTS

### Purification of Cs-Thiolase I from *Candida tropicalis*

The enzymatic properties and primary structure of Cs-Thiolase I should provide a

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basis for the comparison of Cs-Thiolase I and Ps-Thiolase I. Cs-Thiolase I was purified from the cytosolic fraction of the glucose-grown *Candida tropicalis*, in which most of the acetoacetyl-CoA thiolase activity exists in this fraction (19). The protein was purified by the same procedure as used for Ps-Thiolase I (Table 1 and Fig. 1) (16). The native molecular mass of Cs-Thiolase I was approximately 250 kDa, essentially identical to that of Ps-Thiolase I (240 kDa). The subunit molecular mass of Cs-Thiolase I (41 kDa) was the same as that of the peroxisomal enzyme (Fig. 1B) (20). This cytosolic protein was cross-reactive to the antiserum against Ps-Thiolase I (Fig. 1B) and the activity was immunochemically titrated completely. The N-terminal amino acid sequence of this protein was $NH_2$-(Ala/Thr)-Leu-Pro-Pro-Val-Tyr-Ile-Val-Ser-Thr-Ala-(Xaa)-Thr-Pro-Ile-Gly-(Xaa)-Phe-, which coincides with that of the peroxisomal enzyme (21). It is noteworthy that both Ala for Thiolase IA and Thr for Thiolase IB were detected as the first amino acid after methionine was processed.

**Individual expression of thiolase isozyme genes in *Saccharomyces cerevisiae* and purification of recombinant thiolases**

Examination of the enzyme characteristics of the proteins from *CT-TIA* and *CT-TIB* is also available as a means to compare Cs-Thiolase I and Ps-Thiolase I. We expressed these genes independently in *Saccharomyces cerevisiae*. pWT1A and pWT1B were constructed for the expression of Thiolase I genes with their own promoters (Fig. 2). Cell-free extracts from cells carrying the respective plasmids showed higher activity of the cleavage reaction of acetoacetyl-CoA than those from cells harboring pMW1 as a control (Table 2). The production of recombinant thiolases under optimum conditions of gene expression was also monitored by protein staining and Western blot analysis (Fig. 3). The subunit molecular mass (Fig. 3) and native molecular mass of recombinant Thiolase IA (rThiolase IA) and recombinant Thiolase IB (rThiolase IB)were shown to be identical to those of both Cs-Thiolase I and Ps-Thiolase I from *Candida tropicalis*. Each recombinant thiolase was purified by the purification procedures as described in MATERIALS AND METHODS (Fig.4). The specific activities of rThiolase IA and rThiolase IB were 23 and 26 $\mu$mol·min$^{-1}$·mg protein$^{-1}$, respectively. The N-terminal amino acid sequence analysis of the purified proteins demonstrated that the N-termini of rThiolase IA and rThiolase IB were the second amino acids after the first

Table 1. Purification of Cs-Thiolase I from glucose-grown *Candida tropicalis*.

| | Total activity (nmol·min$^{-1}$) | Total protein (mg) | Specific activity (nmol·min$^{-1}$·mg protein$^{-1}$) | Recovery (%) | Purification (-fold) |
|---|---|---|---|---|---|
| S$_3$ fraction | 18,400 | 124 | 148 | 100 | 1 |
| DEAE-Sepharose CL-6B | 6,710 | 9.63 | 697 | 36.5 | 4.71 |
| Butyl-TOYOPEARL 650M | 5,300 | 0.732 | 7,230 | 28.8 | 48.9 |
| Cellulofine GCL-2000m | 1,970 | 0.0329 | 59,800 | 10.7 | 404 |

Thiolase activity was monitored using acetoacetyl-CoA as a substrate.
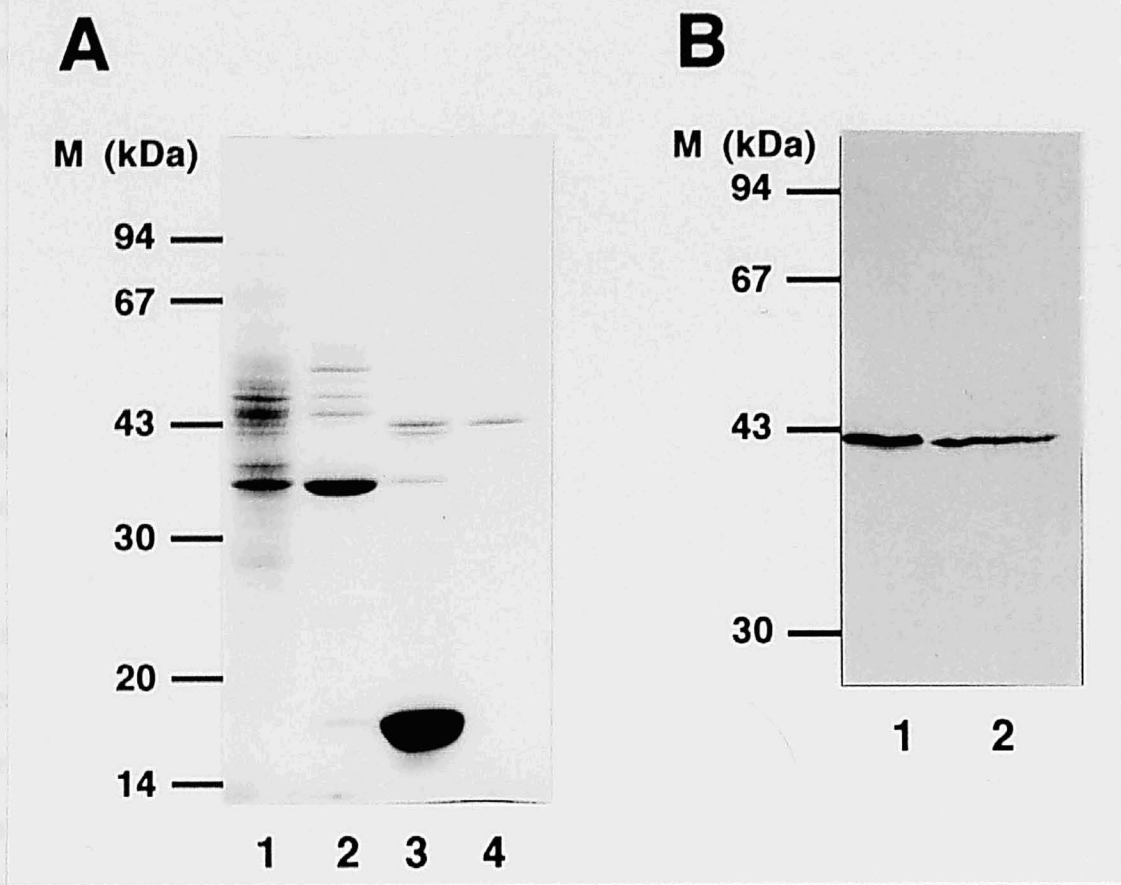
Fig. 1.  SDS-PAGE (A) and Western blot analysis (B) of purified Cs-Thiolase I. (A) Lane 1, $S_3$ fraction (50 μg); lane 2, DEAE-Sepharose CL6-B column eluate (25 μg); lane 3, Butyl-TOYOPEARL 650M column eluate (25 μg); lane 4, Cellulofine GCL-2000m column eluate (5 μg). (B) Lane 1, Cs-Thiolase I (0.1 μg); lane 2, Ps-Thiolase I (0.1 μg). Antiserum against Ps-Thiolase I was used for detection.
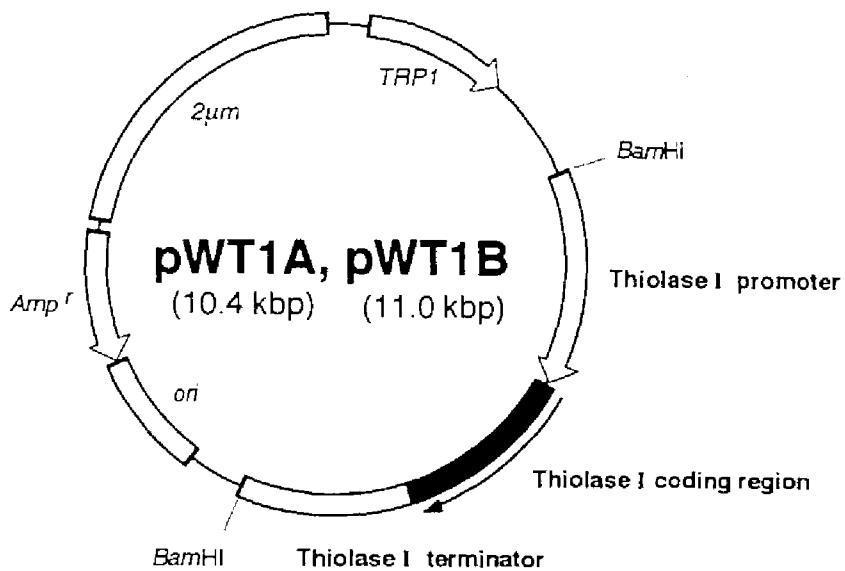
Fig. 2. Construction of expression plasmids (pWT1A and pWT1B) carrying thiolase genes.

Table 2. Specific activity of thiolases in *Saccharomyces cerevisiae*

harboring expression plasmids (pWT1A and pWT1B).

| Plasmid | Specific activity $(nmol \cdot min^{-1} \cdot mg\ protein^{-1})$ |
|---------|---------------------------|
| pMW1 | 43.5 |
| pWT1A | 9,360 |
| pWT1B | 10,800 |

Acetoacetyl-CoA was used as a substrate.

Cells carrying these plasmids were cultivated in SA-W medium.

pMW1 was a control plasmid.
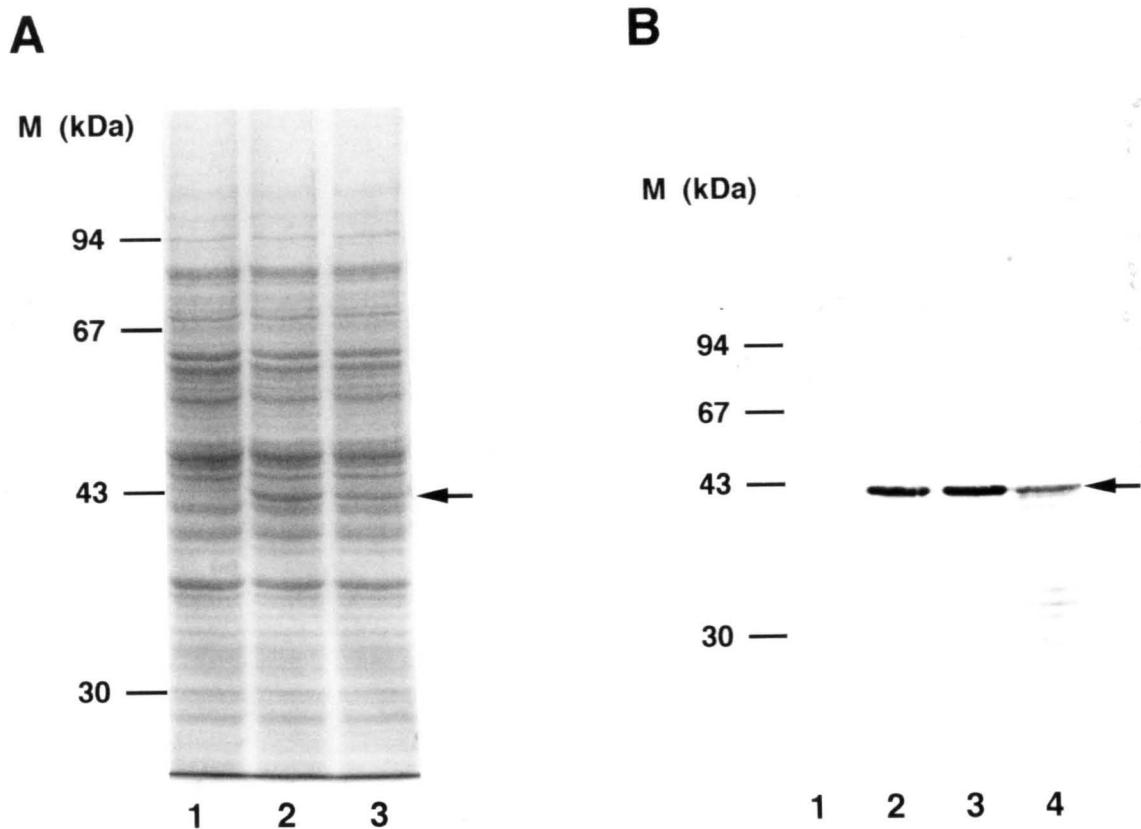
Fig. 3. SDS-polyacrylamide gel electrophoresis (A) and Western blot analysis (B) of cell-free extracts prepared from *S.cerevisiae* harboring pWT1A and pWT1B.

Cell-free extracts were prepared from acetate-grown *Saccharomyces cerevisiae* carrying pWT1A (lane 2), pWT1B (lane 3), and pMW1 as a control (lane 1). Aliquots (40 μg) of cell-free extracts were used for protein staining (A), and those (80 μg) were used for Western blot analysis with antiserum against Ps-Thiolase I (B). Cell-free extract of *n*-alkane-grown *Candida tropicalis* was used as a control of Western blot analysis (B, lane 4). Arrows indicate the protein band corresponding to Thiolase I.

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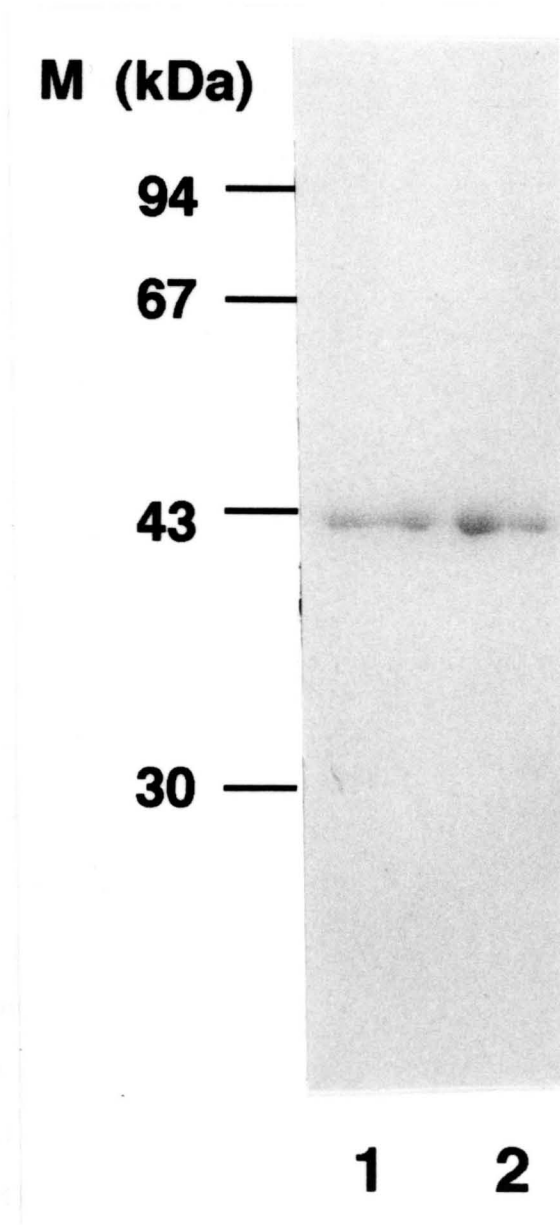
Fig. 4.    SDS-PAGE of purified recombinant thiolases.

Lane 1, rThiolase IA; lane 2, rThiolase IB. Purified recombinant thiolase (3 μg) was run on each lane.

methionine was processed (Ala for rThiolase IA and Thr for rThiolase IB).

## Comparison of kinetic properties of purified thiolase isozymes

Optimum pH and $K_m$ values were determined in both cleavage and condensation reactions to compare the recombinant thiolases and the thiolases purified from *Candida tropicalis* (Table 3). rThiolase IA, rThiolase IB, Cs-Thiolase I, and Ps-Thiolase I could equally catalyze the condensation reaction of acetyl-CoA. rThiolase Is and Cs-Thiolase I did not degrade a longer-chain substrate, 3-ketooctanoyl-CoA, as was the case with Ps-Thiolase I (16). rThiolase Is, Cs-Thiolase I, and Ps-Thiolase I had the same optimum pH values of 8.3 and 8.1 in the cleavage reaction of acetoacetyl-CoA and in the condensation reaction, respectively. The $K_m$ values for each substrate were virtually identical among rThiolase IA, rThiolase IB, Cs-Thiolase I, and Ps-Thiolase I.

## DISCUSSION

As described above, Cs-Thiolase I and Ps-Thiolase I were substantially identical in molecular mass and kinetic and immunochemical properties. Moreover, the primary structure at the N-terminus of Cs-Thiolase I was that of a mixture of Thiolase IA and Thiolase IB, as was the case with Ps-Thiolase I (21). The author also examined the functional expression of two Ps-Thiolase I genes of *Candida tropicalis* independently in *Saccharomyces cerevisiae*. The rThiolase IA and rThiolase IB assembled correctly in *Saccharomyces cerevisiae* cytosol. These recombinant thiolases exhibited essentially identical kinetic properties with, and the same molecular masses as Cs-Thiolase I and Ps-Thiolase I purified from *Candida tropicalis*. These results represent strong evidence that Cs-Thiolase I and Ps-Thiolase I are encoded by the same genes, *CT-T1A* and *CT-T1B*, and both are present as a mixture of Thiolase IA and Thiolase IB in *Candida tropicalis*.

From the results shown in previous reports (15, 16, 19) and in Part I, Chapter 1, it is suggested that cytosolic acetoacetyl-CoA thiolase was identical in glucose-grown and *n*-alkane-grown cells and that Ps-Thiolase I was the sole peroxisomal acetoacetyl-CoA thiolase in the *n*-alkane-grown cells of *Candida tropicalis*. In the chromatography of the cytosolic

Table 3. Summary of kinetic evaluation of thiolases from *Candida tropicalis* (Cs-Thiolase I and Ps-Thiolase I) and recombinant Thiolase I's expressed in *Sacchromyces cerevisiae* (rThiolase IA and rThiolase IB).

| | Cleavage reaction | | | Condensation reaction | |
|---|---|---|---|---|---|
| | Optimum pH | $K_m$ for | | Optimum pH | $K_m$ for |
| | | CoA ($\mu$M) | $C_4$-CoA ($\mu$M) | | $C_2$-CoA (mM) |
| Cytosolic Thiolase I | 8.3 | 37 | 42 | 8.1 | 0.77 |
| Peroxisomal Thiolase I | 8.3 | 30 | 25[a] | 8.1 | 1.05 |
| rThiolase IA | 8.3 | 50 | 23 | 8.1 | 0.69 |
| rThiolase IB | 8.3 | 32 | 57 | 8.1 | 0.74 |

The concentration of CoA was fixed at 50 $\mu$M in the measurement of $K_m$ values for acetoacetyl-CoA.

The concentration of acetoacetyl-CoA was set at 40 $\mu$M in the estimation of $K_m$ values for CoA.

$C_4$-CoA, acetoacetyl-CoA; $C_2$-CoA, acetyl-CoA.

[a]This value was cited from the report of Kurihara *et al.* (16).

fraction of the glucose-grown *Candida tropicalis*, no redundant thiolase activity was detectable other than Cs-Thiolase I. This indicates that the same single thiolase protein, Cs-Thiolase I, is responsible for the acetoacetyl-CoA thiolase activity in the cytosol of both cells. Peroxisomal acetoacetyl-CoA activity can hardly be detected in glucose-grown cells. Therefore, it is suggested that there is no other acetoacetyl-CoA thiolase than Ps-Thiolase I and Cs-Thiolase I in *Candida tropicalis*. Southern blot analysis with a *CT-TIA* gene fragment as a probe also indicated that there were no other genes with high similarity to *CT-TIA* and *CT-TIB* (21).

Ps-Thiolase I and Cs-Thiolase I exhibited essentially identical kinetic properties in both thiolytic cleavage and condensation reaction. Therefore, their physiological functions should be determined mainly by the difference in their subcellular locations. Kurihara *et al.* previously suggested that the Ps-Thiolase I participates in the $\beta$-oxidation system in peroxisomes and that Cs-Thiolase I contributes to sterol synthesis. The present results along with the previous results on Ps-Thiolase I (19) indicate that protein products from *CT-TIA* and *CT-TIB* are both localized in dual intracellular compartments, cytoplasm and peroxisomes, and rule out the possibility that one gene corresponds to the peroxisomal enzyme, and the other to the cytosolic enzyme. The compartmentalization mechanism of a single gene product into several cellular locations has been studied extensively (1),. In many cases, two distinct protein molecules are produced from a single gene, leading to distinct subcellular localization. In the case of Thiolase I, no difference could be detected between the peroxisomal and cytosolic enzymes. There are cases where inefficient targeting and translocation may be the mechanism by which dual compartmentalization to cytoplasm and peroxisomes is achieved as is the case with rat epoxide hydratase (33). However, Thiolase I must be sorted to two locations in a quantitatively regulated manner, as the amount of Ps-Thiolase I is increased in parallel with peroxisome proliferation, whereas that of Cs-Thiolase I is constant (19). Thiolase I has a putative motif of the peroxisome targeting signal type I (20) which has been identified at the C-termini of many peroxisomal proteins (34, 35). This sequence of Thiolase I may represent a clue to elucidate the mechanism of distribution of Thiolase I to two subcellular locations.

# SUMMARY

In the *n*-alkane-assimilating yeast *Candida tropicalis*, there are two isozymes of acetoacetyl-CoA thiolase, peroxisomal acetoacetyl-CoA thiolase (Ps-Thiolase I) and cytosolic acetoacetyl-CoA thiolase (Cs-Thiolase I). Two genes (*CT-T1A* and *CT-T1B*) which encode Thiolase I have been isolated. In order to compare the expressed products of Thiolase I isozyme-encoding genes in *Candida tropicalis*, Cs-Thiolase I was first purified from the glucose-grown *Candida tropicalis* in which the proliferation of peroxisomes and the expression of Ps-Thiolase I were repressed. Cs-Thiolase I was virtually identical to Ps-Thiolase I in molecular mass, kinetic and immunochemical properties, and primary structure at the N-terminus. Amino acid sequence analysis revealed that Cs-Thiolase I was the mixture of products of two genes (*CT-T1A* and *CT-T1B*), as was the case with the peroxisomal enzyme. *CT-T1A* and *CT-T1B* were expressed independently in the yeast *Saccharomyces cerevisiae* and the recombinant proteins were purified. Recombinant Thiolase IA and recombinant Thiolase IB exhibited practically identical enzymatic properties to Cs-Thiolase I and Ps-Thiolase I purified from *Candida tropicalis*. These results revealed that Cs-Thiolase I and Ps-Thiolase I are encoded not by different genes, but by the same genes (*CT-T1A* and *CT-T1B* ) and are present as a mixture of products expressed by both genes, although their subcellular localization is different.

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**Part II**    **Physiological functions of thiolase isozymes in *n*-alkane-**

**assimilating yeast, *Candida tropicalis***

# Chapter 1   Genetic evaluation of physiological functions of thiolase isozymes

## in *Candida tropicalis*

## INTRODUCTION

*Candida tropicalis* is an asporogenic diploid yeast which can utilize *n*-alkanes as a carbon source. The most striking feature of this yeast is profound proliferation of peroxisomes by specific carbon sources such as *n*-alkanes and fatty acids (1). Peroxisomal proteins, including fatty acid β-oxidation enzymes, are induced, in harmony with the proliferation of peroxisomes (2, 3).

Thiolase catalyzes the thiolytic cleavage of 3-ketoacyl-CoA to acetyl-CoA and acyl-CoA, and this enzyme is classified into two types by substrate specificity. One type is acetoacetyl-CoA thiolase, which catalyzes the thiolytic cleavage of acetoacetyl-CoA and the reverse condensation of acetyl-CoA. The other is 3-ketoacyl-CoA thiolase, which has broad substrate specificity for 3-ketoacyl-CoAs in carbon length ($\geq C_4$). In bacterial cells, 3-ketoacyl-CoA thiolase takes part in the fatty acid β-oxidation (4) and acetoacetyl-CoA thiolase takes part in the poly-β-hydroxybutyrate metabolism (5). In eukaryotic cells, especially in mammalian cells, thiolases exhibit diversity in intracellular localization related to their metabolic functions as well as in substrate specificity. For example, they contribute to the fatty acid β-oxidation in peroxisomes and mitochondria (7-10), the ketone body metabolism in mitochondria (7), and the early steps of mevalonate pathway in peroxisomes and cytoplasm (7, 11, 12). In addition to biochemical investigations, analyses of genetic disorders have made clear the basis of their functions (13, 14). Genetic studies have also started to disclose the physiological functions of thiolases in the yeast *Saccharomyces cerevisiae* (15, 16).

In *Candida tropicalis* pK233, there are at least three thiolase isozymes, cytosolic acetoacetyl-CoA thiolase (Cs-Thiolase I), peroxisomal acetoacetyl-CoA thiolase (Ps-Thiolase I), and peroxisomal 3-ketoacyl-CoA thiolase (Thiolase III) (17-19). Ps-Thiolase I and Thiolase

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III are each encoded by two extremely similar genes (*CT-T1A* and *CT-T1B* for Ps-Thiolase I, and *CT-T3A* and *CT-T3B* for Thiolase III) as described by Kurihara *et al.* (20) and in Part I, Chapter 1. The author also demonstrated in Part I, Chapter 3 that Cs-Thiolase I and Ps-Thiolase I are derived from the same genes. As for physiological functions of these isozymes, the exclusive localization of the β-oxidation system in peroxisomes and the inductive expression of peroxisomal isozymes led the author to presume that Ps-Thiolase I and Thiolase III participate in the peroxisomal β-oxidation system, whereas the constitutive localization of Cs-Thiolase I in cytoplasm suggests that Cs-Thiolase I has a role in the mevalonate pathway (2, 19, 21, 22). Information about the physiological roles of thiolase isozymes will be a clue to understand the regulation of the peroxisomal β-oxidation system, additionally, the evolution of the β-oxidation system . The mechanism of sorting of Thiolase I to two intracellular locations is also important with respect to the physiological functions of Thiolase I.

In this chapter, in order to genetically evaluate the physiological functions of thiolase isozymes in *Candida tropicalis*, the author has disrupted their genes and altered the localization of Thiolase I by the deletion of its putative peroxisomal targeting signal sequence. The growth phenotype of strains carrying various combinations of mutations on thiolase genes enabled the author to understand the functions of thiolase isozymes.

## MATERIALS AND METHODS

### Strains and media

*Candida tropicalis* strains used in this study are classified into representative and intermediate strains and are listed in Table 1 (see also Fig.1). *Candida tropicalis* SU-2 (ATCC 20913) (*ura3a/ura3b*) (23), derived from *Candida tropicalis* pK233 (ATCC 20336), was used as a wild type and host strain of transformation. *Escherichia coli* strain DH5α (24) was used for gene manipulation. *Saccharomyces cerevisiae* strain MT8-1 (25) was used as a host strain for the cloning of *Candida tropicalis* URA3. Media for genetic experiments with *Candida tropicalis* were as follows: YPD (its composition is described in Part I, Chapter 3), SD (6.7 g of yeast nitrogen base without amino acid (Difco Laboratories, Detroit, MI, USA)

Table 1. List of *Candida tropicalis* strains used in this study.

| Strain | Genotype | Presence or absence of thiolase genes (CT-T1A/CT-T1B CT-T3A/CT-T3B) |
|---|---|---|
| *Representatives* | | |
| SU-2 | *ura3a/ura3b CT-T1A/CT-T1B CT-T3A/CT-T3B* | +/+ +/+ |
| K6 | *ura3a/ura3b ct-t1aΔ::lacZ/CT-T1B CT-T3A/CT-T3B* | −/+ +/+ |
| K8 | *ura3a/ura3b CT-T1A/ct-t1bΔ::lacZ CT-T3A/CT-T3B* | +/− +/+ |
| K68U | *ura3a/ura3b ct-t1aΔ::URA3/ct-t1bΔ::lacZ CT-T3A/CT-T3B* | −/− +/+ |
| K7 | *ura3a/ura3b CT-T1A/CT-T1B ct-t3aΔ::lacZ/CT-T3B* | +/+ −/+ |
| K0 | *ura3a/ura3b CT-T1A/CT-T1B CT-T3A/ct-t3bΔ::lacZ* | +/+ +/− |
| K70 | *ura3a/ura3b CT-T1A/CT-T1B ct-t3aΔ::lacZ/ct-t3bΔ::lacZ* | +/+ −/− |
| K870 | *ura3a/ura3b CT-T1A/ct-t1bΔ::lacZ ct-t3aΔ::lacZ/ct-t3bΔ::lacZ* | +/− −/− |
| K6870U | *ura3a/ura3b ct-t1aΔ::URA3/ct-t1bΔ::lacZ ct-t3aΔ::lacZ/ct-t3bΔ::lacZ* | −/− −/− |
| K8Δ | *ura3a/ura3b CT-T1AΔC6/ct-t1bΔ::lacZ CT-T3A/CT-T3B* | ΔC/− +/+ |
| K870Δ | *ura3a/ura3b CT-T1AΔC6/ct-t1bΔ::lacZ ct-t3aΔ::lacZ/ct-t3bΔ::lacZ* | ΔC/− −/− |
| *Intermediates* | | |
| K6ZUZ | *ura3a/ura3b ct-t1aΔ::lacZ-URA3-lacZ/CT-T1B CT-T3A/CT-T3B* | |
| K8ZUZ | *ura3a/ura3b CT-T1A/ct-t1bΔ::lacZ-URA3-lacZ CT-T3A/CT-T3B* | |
| K7ZUZ | *ura3a/ura3b CT-T1A/CT-CT-T1B ct-t3aΔ::lacZ-URA3-lacZ/CT-T3B* | |
| K0ZUZ | *ura3a/ura3b CT-T1A/CT-T1B CT-T3A/ct-t3bΔ::lacZ-URA3-lacZ* | |
| K70ZUZ | *ura3a/ura3b CT-T1A/CT-T1B ct-t3aΔ::lacZ/ct-t3bΔ::lacZ-URA3-lacZ* | |
| K870ZUZ | *ura3a/ura3b CT-T1A/ct-t1bΔ::lacZ-URA3-lacZ ct-t3aΔ::lacZ/ct-t3bΔ::lacZ* | |
| K8ΔU | *ura3a/ura3b ct-t1aΔ::(CT-T1AΔC6::URA3)/ct-t1bΔ::lacZ CT-T3A/CT-T3B* | |
| K870ΔU | *ura3a/ura3b ct-t1aΔ::(CT-T1AΔC6::URA3)/ct-t1bΔ::lacZ ct-t3aΔ::lacZ/ct-t3bΔ::lacZ* | |

*CT-T1AΔC6* represents the gene encoding the mutant Thiolase I of which the C-terminal six

amino acid residues were deleted. *ct-t1a*Δ::*(CT-T1AΔC6:: URA3)* indicates that the linearized

plasmid pUT1AΔ containing *CT-T1AΔC6* and *URA3* is integrated on *CT-T1A* locus.

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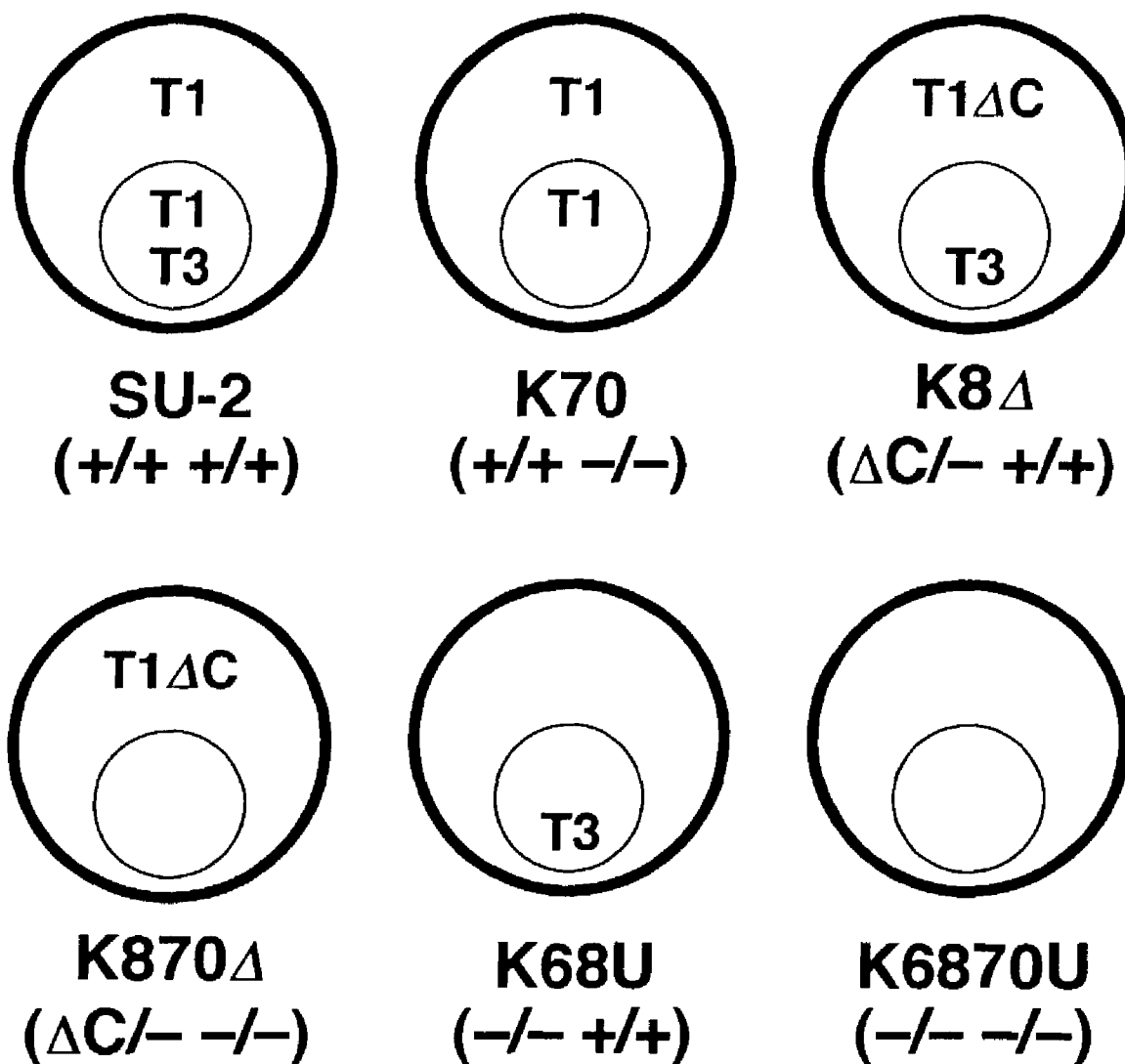
Fig. 1.  Illustration of subcellular distribution of thiolase isozymes in wild type and representative mutant strains prepared in this study.

The presence or absence of each of the four thiolase genes (*T1A/T1B   T3A/T3B*) in each strains is shown by plus or minus signs in parentheses. Outer and inner circles represent the yeast cell and peroxisomes, respectively. Abbreviations: T1, Ps- or Cs-Thiolase 1; T3, Thiolase III; T1ΔC, C-terminal-truncated Thiolase I.

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and 20 g of glucose per liter of deionized water), SD+U (SD, supplemented with 0.1 g of uracil, 0.1 g of uridine, and 0.1 g of uridine 5'-monophosphate per liter), and SD+S (SD containing 1 M sorbitol). L-Mevalonolactone [(R)-(–)-3-hydroxy-3-methyl-5-pentanolide; Wako, Osaka, Japan] (5 g /l) was used to supplement YPD, SD+U, and SD+S, if necessary.

Candida tropicalis was cultivated aerobically at 30°C in a medium containing glucose (16.5 g/l), glycerol (20 g/l), ethanol (20 ml/l), sodium propionate (10 g/l), sodium butyrate (11 g/l) or n-alkane mixture ($C_{10}$ to $C_{13}$) (10 ml/l) as a sole carbon source (21, 26). pH of respective medium was adjusted to 5.2 for glucose-, glycerol-, ethanol-, and n-alkane-media or to 6.0 for propionate- and butyrate-media. Tween 80 (0.5 g/l) was added in the n-alkane medium used for preparation of cell free extracts and for subcellular fractionation. The basic composition of the medium is described in Part I, Chapter 1. Supplemental nutrients were added as described above, if necessary. Cell growth was monitored by measuring light scattering at 570 nm.

## Cloning and sequencing of Candida tropicalis URA3

To make a minimal genomic DNA library of Candida tropicalis (27, 28), genomic DNA of Candida tropicalis was digested with NcoI and fractionated in size by 0.5% agarose gel electrophoresis. A fraction containing 6 to 9-kbp DNA fragments was cloned into the NcoI site of the E. coli–Saccharomyces cerevisiae shuttle vector pMW1 containing the TRP1 selectable marker (29), which was modified to have an NcoI site in multi-cloning sites. This genomic DNA library was introduced into a uracil auxotrophic (Ura⁻) strain, Saccharomyces cerevisiae MT8-1, by the electroporation method (30). Six uracil prototrophs (Ura⁺) were obtained from 4.5 × 10⁴ tryptophan prototrophic (Trp⁺) transformants. Plasmids were recovered from Ura⁺ Trp⁺ candidates. The plasmids contained 7-kbp fragment, in which a 1.7-kbp BglII fragment was enough to complement ura3 of Saccharomyces cerevisiae MT8-1. Sequence analysis of the BglII fragment indicated that this fragment contained an 801-base open reading frame and the deduced amino acid sequence exhibited high similarity to Ura3ps from various organisms. Sequence analysis was carried out with a PRISM DyeDeoxy Terminator Cycle Sequencing Kit and a DNA sequencer (model 373A; Applied Biosystems, Foster City, CA, USA). The 1.7-kbp Candida tropicalis URA3 was subcloned into the BamHI site of

pUC19 and into the *BglII* site of the modified pUC19, where a *BglII* linker was inserted in the *SmaI* site (the subclones were named pUC-URA3 and pUC-URA3Bg, respectively), and was used for the construction of disruption cassettes as described below.

**Construction of disruption cassettes of thiolase isozyme genes**

To disrupt multiple thiolase genes by using the *URA3* selectable marker in *Candida tropicalis* SU-2 (uracil auxotrophy), the Ura-blasting procedure was applied (31). In this procedure, *URA3* was placed between two directly repeated sequences in a disruption cassette (see Fig. 2). The 1.9-kbp part of *lacZ* (*EcoRV-EcoRI* fragment) was used as a repeated sequence. Two *lacZ* fragments were inserted stepwise into pUC-URA3, one in the *SmaI* site and one in the *XbaI* site, and all the ends of the fragments were filled with T4 DNA polymerase. Thus a plasmid, pZUZ, containing the *lacZ-URA3-lacZ* module was constructed.

pT16BE and pT16B contain the coding and flanking regions of *CT-T1A*, and pT18B contains those of *CT-T1B* as described in Part I, Chapter . pT37Bg, carrying the coding and flanking regions of *CT-T3A*, and pT30Bg, carrying those of *CT-T3B*, were constructed by insertion of the *BglII* fragments of λCT-KCT-A and λCT-KCT-B (see Part I, Chapter 1) into the modified pUC19 where a *BglII* linker was inserted into the *HincII* site and where the *EcoRI* site was deleted, respectively. *EcoRI-SalI* fragments (1,400 bp) of pT16B and pT18B were replaced with the *lacZ-URA3-lacZ* fragment (5,500 bp), which was excised from pZUZ by *EcoRI* and *SalI*. The *EcoRI-KpnI* (600 bp) fragments of pT37Bg and pT30Bg were replaced with the *lacZ-URA3-lacZ* fragment after the *KpnI* sites of pT37Bg and pT30Bg had been filled and ligated with a *SalI* linker, respectively. After a *BglII* linker was inserted into the *EcoRV* site of pT16BE, the *EcoRV-BglII* fragment (500 bp) of pT16BE was replaced with the *BglII* fragment (1,700 bp) of *URA3* excised from pUC-URA3Bg. These constructs were named pT16B::ZUZ, pT18B::ZUZ, pT37Bg::ZUZ, pT30Bg::ZUZ, and pT16BE::U, respectively (see Fig. 2). Before these disruption cassettes were used for transformation, pT16BE::U was linearized with *BamHI* and *EcoRI*, pT16B::ZUZ and pT18B::ZUZ were linearized with *BamHI*, and pT37Bg::ZUZ and pT30Bg::ZUZ were linearized with *BglII*.

**Transformation of *Candida tropicalis* by the spheroplast method**

The spheroplast method developed for *Saccharomyces cerevisiae* (32) was applied to

the transformation of *Candida tropicalis* with a slight modification.

*Candida tropicalis* was grown in 10 ml of YPD overnight and inoculated in 100 ml of YPD to $A_{570}$ of 0.01. Cells were cultivated at 30°C to $A_{570}$ of 1 to 2. Cells were harvested, washed by sterilized water and 1 M sorbitol, and then lysed in 20 ml of KPE (1 M sorbitol, 10 mM potassium phosphate buffer (pH 7.2), and 10 mM EDTA) containing 40 µl of mercaptoethanol and 150 µl of Zymolyase solution (10 mg of Zymolyase 20T per ml of KPE) at 30°C for 15 min. Spheroplasts were centrifuged at 250 x g and washed by 1 M sorbitol and STC [1 M sorbitol, 10 mM Tris-HCl (pH 7.5), and 10 mM CaCl₂], and suspended in 1 ml of STC. An aliquot (100 µl) of cell suspension was incubated with 30 µg of a linearized disruption cassette for 10 min at room temperature, and mixed with 1 ml of PEG solution [10 mM Tris-HCl (pH 7.5), 10 mM CaCl₂, and 20% (wt/vol) PEG6000 (Wako, Osaka, Japan)]. After further 10 min incubation at room temperature, cells were centrifuged at 250 x g, suspended in SOS [30% (vol/vol) YPD, 1M sorbitol, 10 mM CaCl₂, and 5 g/l of L-mevalonolactone, if necessary], and incubated at 30°C for 30 min. Transformed spheroplasts were poured on selective media with top agar. Ura⁺ cells formed colonies after 2 to 4 days of incubation at 30°C at a frequency of approximately 10³ colonies/µg of DNA.

In order to pop out *URA3*, Ura⁺ cells in which the disruption cassette containing *lacZ-URA3-lacZ* had been integrated were inoculated on a minimal medium containing 5-fluoroorotic acid (5FOA) (SD+U containing 0.75 g/l of 5FOA) at 30°C for 3 to 4 days. 5FOA-resistant colonies were used as host cells for the next round of transformation. These cells were subjected to Southern blot analysis at each stage of the process.

**Construction of a Thiolase IA mutant with the C-terminus deleted**

A site-directed mutation on *CT-TIA* was generated by PCR (33). PCR conditions were as follows: template, 50 pmol of primers, 0.2 mM deoxynucleoside triphospates, 1x reaction buffer (supplied by vendor), and 5 U of *Pfu* DNA polymerase (Stratagene, La Jalla, CA, USA). Primers used were as follows: PRT1AN1, 5'-AACCATGGACGACGTCGTTATCG-3'; PRT1AD1, 5'-CTTGGCGTCGGTTTAAATCTTTTCA-3'; PRT1C1, 5'-GTGCCGAATCGA-TGTCTAACA-3'; M13 reverse, 5'-CAGGAAACAGCTATGAC-3'.

First, two sets of PCR were carried out with two primers each, one set with PRT1AN

and PRT1AD1, and the other with PRT1CI and M13 reverse. pT16B was used as a template. After the heteroduplex of two PCR products was formed, the second round of PCR was carried out with PRT1AN1 and M13 reverse. The amplified fragment was digested with *BglII* and *SacI* and was cloned into the *BamHI* and *SacI* sites of pUC-URA3, with a *BamHI* linker inserted in the *SmaI* site. The plasmid was named pUT1AΔ. The inserted fragment was sequenced to check whether mutagenesis and PCR had been correctly performed.

The scheme for introducing the mutation on a chromosome by homologous recombination (34) is shown below (See Fig. 6C). pUT1AΔ was linearized with *NcoI* prior to its use in transformation. After pUT1AΔ was integrated into *CT-T1A* on the chromosome, *URA3* and vector parts were popped out by 5FOA selection as described above. Subsequently, the desired strain carrying the mutation on *CT-T1A* was selected from Ura⁻ candidates by Southern blot analysis.

**Preparation of cell-free extracts**

Yeast cells were cultivated in 10 ml of each medium and harvested at mid-exponential phase. Cells were suspended in 500 µl of the PDG buffer (described in Part I, Chapter 1), and disintegrated by vortex with 0.3 g of glass beads (0.4 to 0.45 mm diameter) in a microtube. Cell-free extracts were obtained by centrifugation at 14,000 × $g$ for 20 min at 0°C.

**Other methods**

Thiolase activity was assayed by monitoring acetoacetyl-CoA or 3-ketooctanoyl-CoA degradation as reported by Kurihara et al. (17). Protein concentration was determined by Lowry method (35). Subcellular fractionation (22), Western blot analysis (36), and Southern blot analysis (20) were carried out as described in Part I, Chapter 1 and Chapter 3. General methods for gene manipulation and yeast genetics were used as described in general protocols (24, 37).

**Nucleotide sequence accession number**

The nucleotide sequence of the *Candida tropicalis URA3* has been assigned GenBank/EMBL/DDBJ accession number AB006207.

# RESULTS

## Development of disruptants of thiolase isozymes

In order to genetically evaluate the physiological functions of thiolase isozymes in *Candida tropicalis*, two pairs of genes for thiolase isozymes were individually disrupted. *Candida tropicalis* URA3 was cloned for the construction of disruption cassettes for thiolase isozyme genes and the Ura-blastering procedure was applied to disrupt multiple genes (Fig. 2) (see also MATERIALS AND METHODS) (2).

First, the author disrupted a single gene among the four thiolase genes. Strains, K6ZUZ, K8ZUZ, K7ZUZ, and K0ZUZ were obtained as Ura$^+$ transformants from the wild type strain *Candida tropicalis* SU-2 (23) by using disruption cassettes pT16B::ZUZ, pT18::ZUZ, pT37Bg::ZUZ, and pT30Bg::ZUZ (Fig. 3), respectively. Following selection of Ura$^-$ segregants on the basis of resistance to 5FOA, strains, K6, K8, K7, and K0 were obtained. Southern blot analysis indicated that the desired chromosomal regions were correctly replaced with *lacZ-URA3-lacZ* in the strains, K6ZUZ, K8ZUZ, K7ZUZ, and K0ZUZ and that URA3 was eliminated in the strains, K6, K8, K7, and K0 by the 5FOA treatment. Compared with thiolase genes in the wild type strain SU-2, the increased size of each disrupted thiolase gene on Southern blots also showed that the first round of transformation was successful (Fig. 3). The shift of each band, or the disappearance of a band at the position seen in the SU-2 lane, revealed that each gene was present as a single copy, suggesting that the almost identical A and B genes were allelic in the diploid yeast *Candida tropicalis*. Therefore, the strains, K6 and K8, can be regarded as the hemizygous *CT-T1A/T1B* null mutants, and the strain, K7 and K0, as the hemizygous *CT-T3A/T3B* null mutants.

Second, the homozygous *ct-t1aΔ/t1bΔ* null mutant and the homozygous *ct-t3aΔ/t3bΔ* null mutant were developed from the strains, K8 and K7, respectively. Cs-Thiolase I is expected to have a role in the mevalonate pathway. The *ct-t1aΔ/t1bΔ* mutant, therefore, was supposed to show mevalonate auxotrophy. Thus, in the selection of this mutant, L-mevalonolactone was supplemented in the selective medium. However, the disruption using pT16::ZUZ was not successful despite the use of the medium containing L-mevalonolactone.
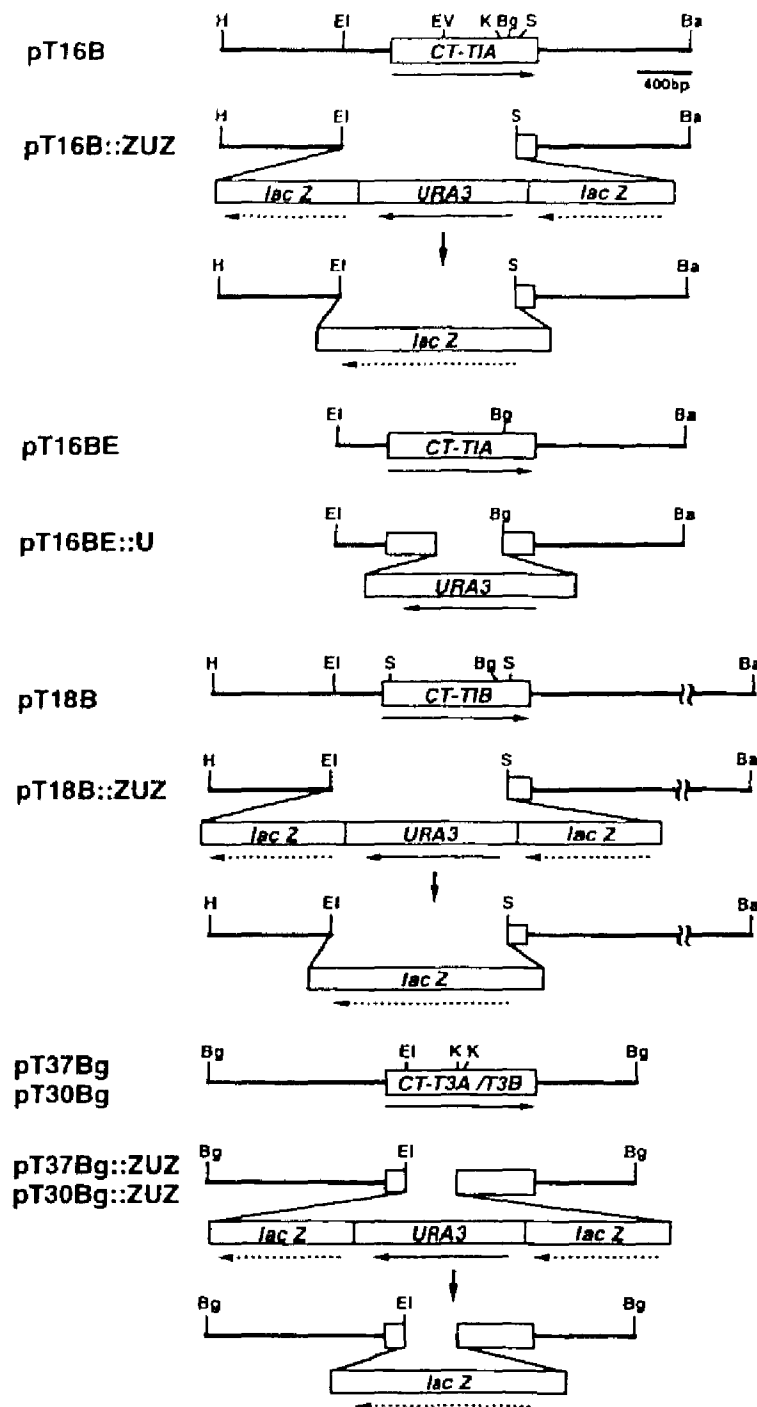
Fig. 2.   Physical maps of thiolase isozyme genes and disruption cassettes, and disruption strategies.

Horizontal arrows indicate the orientation of transcription. Vertical arrows indicate the popping out of the *lacZ-URA3-lacZ* cassette. A boxed region of each thiolase gene shows the open reading frame. Restriction sites: Ba, *Bam*HI; Bg, *Bgl*II; EI, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; S, *Sal*I.

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Fig. 3.    Southern blot analysis of mutant strains derived from *Candida tropicalis* SU-2.
Genomic DNA was digested with *Eco*RI and *Bam*HI (A and B) and with *Eco*RI, *Bam*HI, and
*Ban*III (C). The blots were probed with biotin-labeled cDNA of Ps-Thiolase I (21) (A and C)
or Thiolase III (20) (B). Panels A and B are the same blot in each lane. The presence or
absence of each thiolase gene is indicated as explained in Fig. 1. The genotype corresponding
to each band is given in the key. Asterisk, nonspecific signal. Abbreviations: *T1A, CT-T1A;*
*T1B, CT-T1B; T3A, CT-T3A; T3B, CT-T3B.*

Consequently, an improved disruption cassette, pT16BE::U, for *CT-T1A* was constructed (Fig. 2), in which one of two regions homologous to *CT-T1A* was exchanged with the region eliminated in the *CT-T1B* locus in K8 after the first round of transformation. Using this vector, the author successfully disrupted *CT-T1A* to obtain the *ct-t1aΔ/t1b*Δ mutant K68U as shown in a Southern blot (Fig. 3). The *ct-t3a*Δ/*t3b*Δ mutant K70 was developed using pT30::ZUZ, followed by the elimination of *URA3* (Fig. 3).

Third, to obtain the homozygous *ct-t1a*Δ/*t1b*Δ *ct-t3a*Δ/*t3b*Δ null mutant K6870U, The strain K70 was transformed by the same method that had been applied to develop the strain K68U from the wild type strain SU-2. Stains, K870ZUZ and K870 were obtained as intermediate strains. In K6870U, all bands of the genes encoding thiolase isozymes shifted in size (Fig. 3), revealing the correct disruption of all four genes.

**Expression of thiolase isozymes in mutant strains**

Development of a series of disruptants enabled the author to examine the expression level of each isozyme and its contribution to thiolase activity in the cells. The expression of Thiolase Is and Thiolase III in disruptants was monitored by the thiolase activity and Western blot analysis (Fig. 4 and 5). According to substrate specificity, the activity of acetoacetyl-CoA thiolase was represented mainly by the degradation of acetoacetyl-CoA and the activity of 3-ketoacyl-CoA thiolase exactly by the degradation of 3-ketooctanoyl-CoA (17, 18).

In the hemizygous *CT-T1A/T1B* null mutants, K6 and K8, the thiolase activity for acetoacetyl-CoA was half that in the wild type strain SU-2 on all carbon sources tested (Fig. 4A). Also in the hemizygous *CT-T1A/T1B* null mutants, K7 and K0, the activity for 3-ketooctanoyl-CoA was half in that of the strain SU-2 grown on *n*-alkanes and butyrate (Fig. 4B). The band intensity of Thiolase I and Thiolase III in Western blot analysis paralleled the levels of thiolase activity in the wild type and the disruptant strains (Fig. 5). These results indicated that the expression of the A and B genes of Thiolase I and Thiolase III contributed equally to total intracellular thiolase activity and that their regulation in response to the carbon source was identical. These results confirmed that the A and B genes of both Thiolase I and Thiolase III were allelic. As for the homozygous null mutants, no thiolase activity for acetoacetyl-CoA was detected in the *ct-t1a*Δ/*1b*Δ mutant K68U grown on glucose (Fig. 4A).

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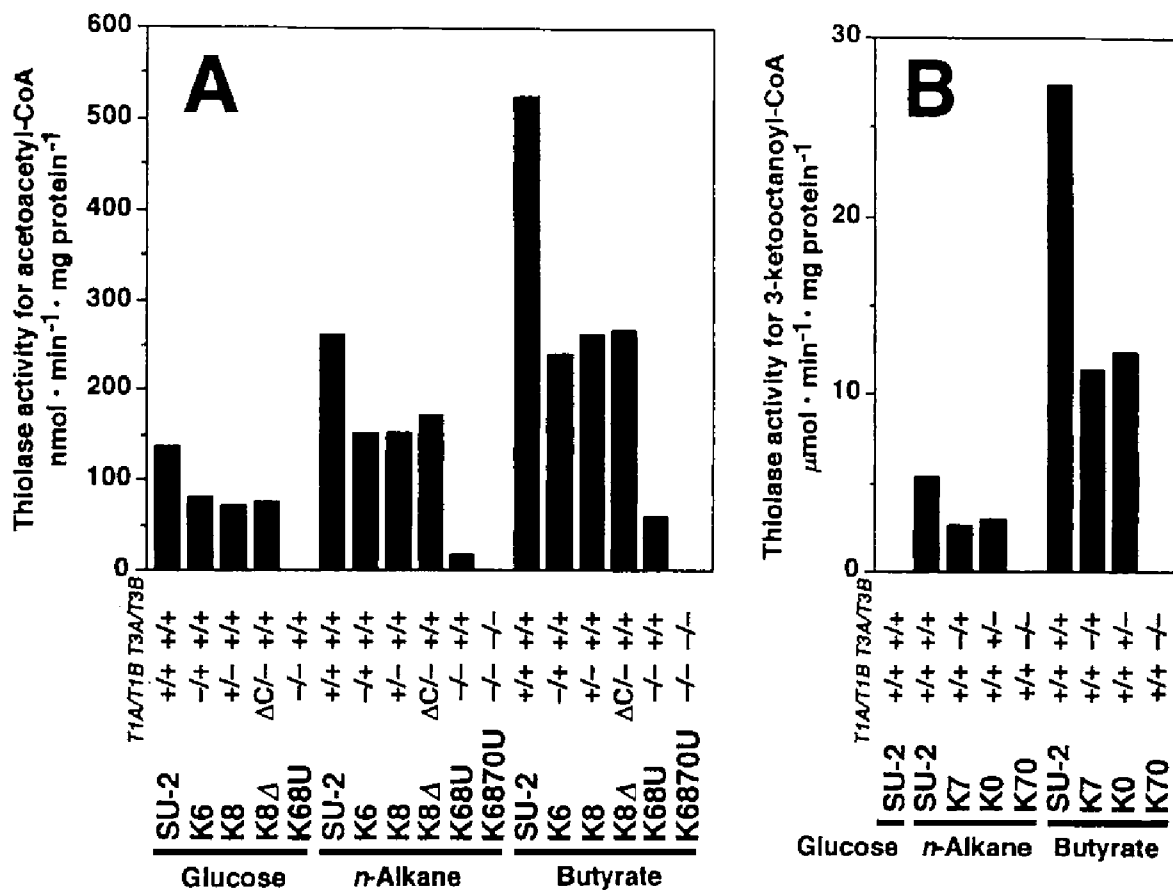
Fig. 4. Thiolase activities of mutants for acetoacetyl-CoA (A) and 3-ketooctanoyl-CoA (B).

The activities for acetoacetyl-CoA and 3-ketooctanoyl-CoA represent the activities of Thiolase I and Thiolase III, respectively. The presence or absence of each thiolase gene is indicated as explained in Fig. 1. Carbon sources for growth are displayed.

A

Thiolase I —
Thiolase IΔC6

T1A/T1B T3A/T3B

| | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ |
| | +/+ | -/+ | +/- | ΔC/- | -/- | +/+ | -/+ | +/- | ΔC/- | -/- | +/+ | -/+ | +/- | ΔC/- | -/- |

SU-2 K6 K8 K8Δ K68U    SU-2 K6 K8 K8Δ K68U    SU-2 K6 K8 K8Δ K68U

Glucose          n-Alkane          Butyrate

B

Thiolase III —

T1A/T1B T3A/T3B

| | +/+ | +/+ | +/+ | +/- | -/- | +/+ | +/+ | +/- | -/- |
| | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ | +/+ |

SU-2    SU-2 K7 K0 K70    SU-2 K7 K0 K70

Glucose    n-Alkane    Butyrate
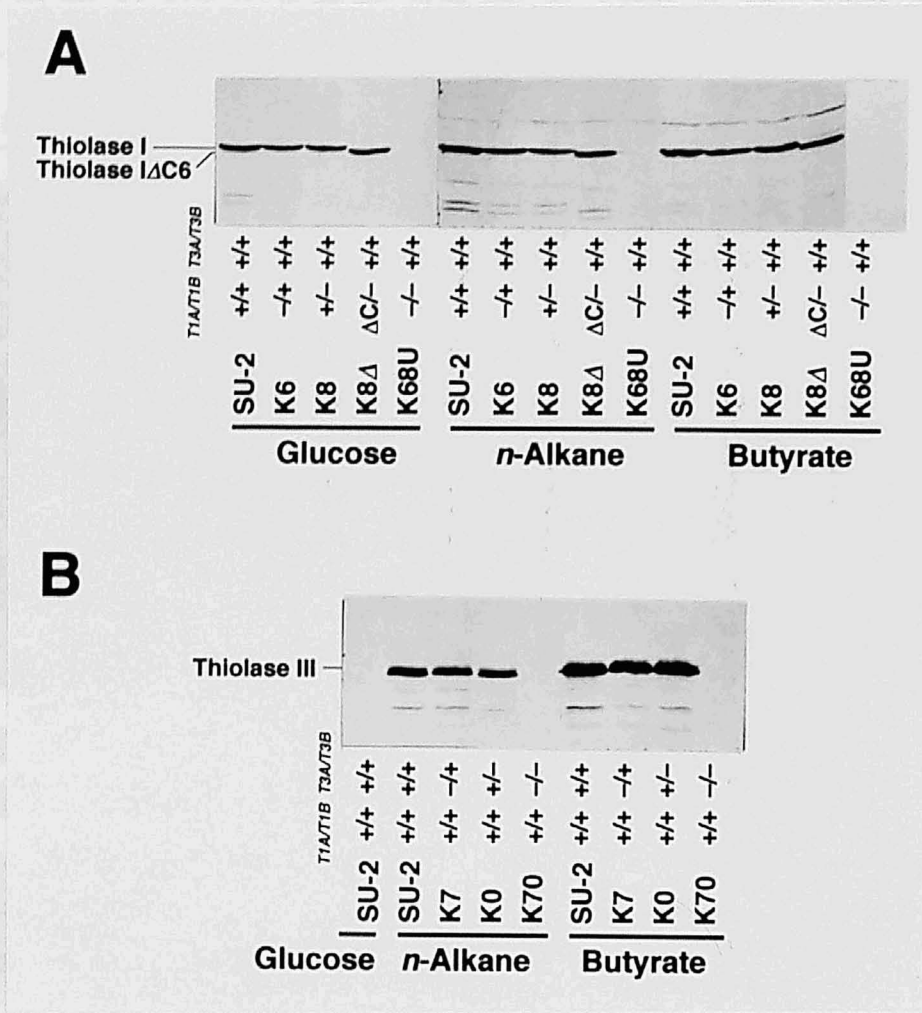
Fig. 5. Western blot analysis of various Thiolase I (A) and Thiolase III (B) mutant strains. Aliquots of 50 μg (glucose) and 25 μg (n-alkane and butyrate) (A) and 20 μg (B) of cell-free extracts were run on gels. Thiolase I and Thiolase III were detected with anti-Ps-Thiolase I and anti-Thiolase III antisera, respectively. The presence or absence of each thiolase gene is indicated as explained in Fig. 1. Carbon sources for growth are displayed. Thiolase IΔC6, C-terminus-truncated mutant of Thiolase I.

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Residual activity for acetoacetyl-CoA was detected in the strain K68U cells grown on *n*-alkanes and butyrate, but it was supposed to be the contribution of Thiolase III, which shows broad chain length specificity. This is strongly supported by the fact that this residual activity was abolished in the *ct-t1a*Δ/*t1b*Δ *ct-t3a*Δ/*t3b*Δ mutant K6870U (Fig. 4A). There was no protein detected by anti-Thiolase I antiserum in the strain K68U grown on any of the carbon sources tested (Fig. 5A). No thiolase activity for 3-ketooctanoyl-CoA was detected in the *ct-t3a*Δ/*t3b*Δ mutant K70 grown on *n*-alkanes and butyrate (Fig. 4B), and no band was detected by anti-Thiolase III antiserum in the strain K70 (Fig. 5B). Furthermore, in the strain K6870U, no thiolase activity was found in the cells (Fig. 4A) and no protein was recognized by either anti-Thiolase I or anti-Thiolase III antiserum (see Fig. 5), indicating that in *Candida tropicalis* there are only three thiolase isozymes encoded by the two pairs of alleles as described in Part I, Chapter 3.

## Development of the strains expressing the C-terminus-truncated protein of Thiolase I

Compared with the amino acid sequences of acetoacetyl-CoA thiolases of other organisms, only Thiolase I of *Candida tropicalis* has an additional six amino acid residues at the C-terminus: DADAKL for Thiolase IA and DSDAKL for Thiolase IB, in which there is a putative motif of peroxisomal targeting signal type I (PTS1) (20, 38) (Fig. 6A). In order to investigate whether this sequence functions as a PTS1 and, furthermore, to distinguish the physiological roles of Cs-Thiolase I and Ps-Thiolase I, the author developed two further strains, K8Δ (*CT-T1A*ΔC6/*ct-t1b*Δ *CT-T3A*/*CT-T3B*) and K870Δ (*CT-T1A*ΔC6/*ct-t1b*Δ *ct-t3a*Δ/*ct-t3b*Δ). These strains express only the C-terminus-truncated Thiolase I with and without Thiolase III, respectively. A nonsense codon was introduced into *CT-T1A* to delete the C-terminal six amino acids of Thiolase I by site-directed mutagenesis (Fig. 6B). A *Dra*I restriction site was also introduced as a marker for this mutation, and then a mutation cassette, pUT1AΔ, was constructed. By using this cassette, these mutations were incorporated on the *CT-T1A* locus in the strains, K8 and K870 (Fig. 6C). Southern blot analysis showed that the *CT-T1A* locus in both strains, K8Δ and K870Δ, could be digested by *Dra*I although *Bam*HI-*Eco*RI fragments were identical to that of the wild type in size, indicating that the mutation was correctly introduced onto the *CT-T1A* locus in these strains (Fig. 3).

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## A

```
Thiolase IA :  -FGVAGVCNGGGGASAVVIEKIDADAKL   403
SCCACT      :  -IGVAAICNGGGGASSIVIEKI          398
HCACT       :  -RGVAALCIGGGMGIAMCVQRE          397
RCACT       :  -YGVGGVCNGGGGASALVLEVV          406
RMACT       :  -FGLASICNGGGGASAVLIEKL          424
ZRACT       :  -KGLATLCIGGGMGVAMCIESL          391
```

## B

```
        1180        1190        1200        1210
gctgtcgttattgaaaagattgacgccgacgccaagttgtaaaaa
 A   V   V   I   E   K   I   D   A   D   A   K   L   *
```



```
        1180        1190        1200        1210
gctgtcgttattgaaaaga tttaaa ccgacgccaagttgtaaaaa
 A   V   V   I   E   K   I   *
                       DraI
```

## C



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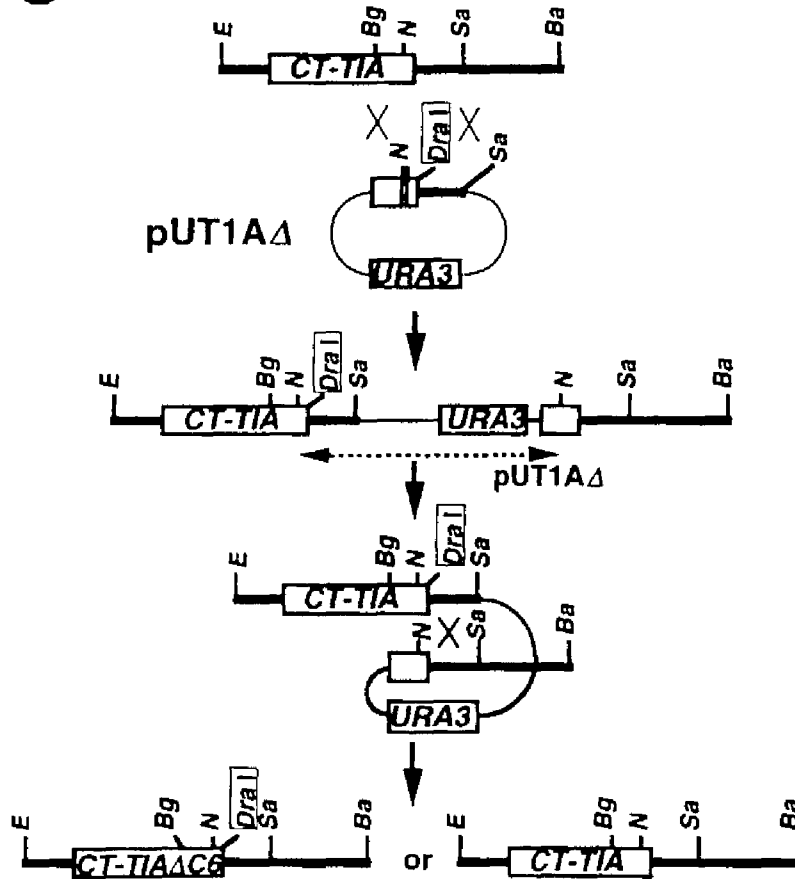
Fig. 6.  Construction of a Thiolase IA mutant with the C-terminus deleted.

(A) Comparison of C-terminal domains of acetoacetyl-CoA thiolases from various sources. The boxed residue is the catalytically important Cys in this domain. Abbreviations: Thiolase IA, *Candida tropicalis* peroxisomal acetoacetyl-CoA thiolase encoded by *CT-TIA*; SCCACT, *Saccharomyces cerevisiae* cytosolic acetoacetyl-CoA thiolase; HCACT, human cytosolic acetoacetyl-CoA thiolase; RCACT, radish cytosolic acetoacetyl-CoA thiolase; RMACT, rat mitochondrial acetoacetyl-CoA thiolase; ZRACT, *Zooglea ramigera* acetoacetyl-CoA thiolase. Amino acid sequences were retrieved from GenBank/EMBL/DDBJ as accession no. D13470 for Thiolase IA, L20428 for SCCACT, S70154 for HCACT, X78116 for RCACT, D00511 for RMACT, and J02631 for ZRACT. (B) Strategy for deletion of a putative peroxisomal targeting signal of Thiolase I by site-directed mutagenesis. Open box, *Dra*I site. (C) Strategy for the introduction of the site-directed mutation on the *CT-TIA* locus. Restriction sites: *Ba*, *Bam*HI; *Bg*, *Bgl*II; *E*, *Eco*RI; *N*, *Nco*I; *Sa*, *Sac*I.

The expression and subcellular localization of the C-terminus-truncated Thiolase I in the strain K8Δ were examined. The truncated Thiolase I was expressed as a slightly smaller protein than the wild type Thiolase I (Fig. 5A). The thiolase activity for acetoacetyl-CoA and the band intensity of Thiolase I in the strain K8Δ were essentially identical to those in the parent strain K8 on all carbon sources tested (Fig. 4A and 5A). These results revealed that the C-terminal six amino acid residues of Thiolase I did not have any function for the enzymatic activity of Thiolase I and that this truncated protein was present in a completely active form. The truncated protein was also expressed in the strain K870Δ. The postnuclear supernatant fractions (S$_1$) of the strains, K8 and K8Δ, grown on $n$-alkanes were separated to cytoplasm/microsome fractions (S$_2$) and organelle fractions (P$_2$) at 20,000 x g (Fig. 7). Thiolase I was present only in the S$_2$ fraction in the strain K8Δ, whereas it was present in both the S$_2$ and P$_2$ fractions in the strain K8. Proper subcellular fractionation was confirmed by the presence of the majority of Thiolase III in the P$_2$ fraction. These results demonstrated that the C-terminal residues of Thiolase I functioned as a PTS1 in *Candida tropicalis* and that the localization of Thiolase I was successfully restricted to the cytoplasm.

**Mevalonate requirement of mutant strains**

The *ct-t1a*Δ/*t1b*Δ mutants, K68U and K6870U, could be obtained in an SD+S medium containing L-mevalonolactone. In the yeast *Saccharomyces cerevisiae*, cytosolic acetoacetyl-CoA thiolase encoded by *ERG10* has been genetically shown to be essential for the mevalonate pathway (15). If a thiolase isozyme in *Candida tropicalis* catalyzes the initial step of this pathway, its deficiency would result in mevalonate auxotrophy. The strains, K68U and K6870U, both of which lacked Thiolase I, could grow on YPD medium only when it was supplemented with L-mevalonolactone (Fig. 8). The strains, K70, which lacks Thiolase III, K8Δ, which lacks Ps-Thiolase I, and K870Δ, which lacks Ps-Thiolase I and Thiolase III, did not require mevalonate, as was the case with the wild type strain SU-2. These results suggest that Cs-Thiolase I has an indispensable role in the mevalonate pathway.

**Cell growth of mutant strains on various carbon sources**

Kurihara *et al.* reported that *Candida tropicalis* could utilize a short chain fatty acid, butyrate, as well as $n$-alkanes and longer-chain fatty acids (21). In butyrate-grown cells,
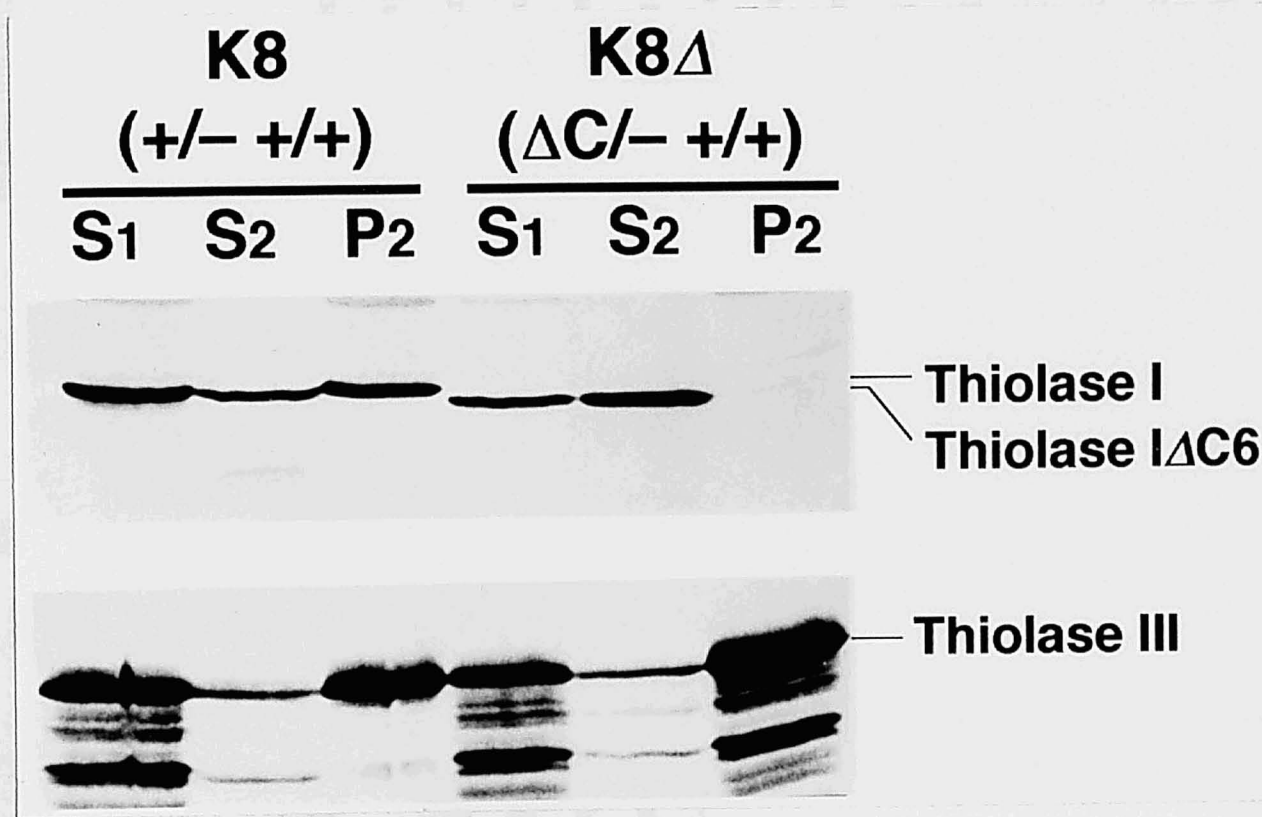
Fig. 7.    Subcellular distribution of the wild type Thiolase I [K8 (+/− +/+)] and C-terminus-deleted Thiolase I [K8Δ (ΔC/− +/+)].

Cells grown on *n*-alkanes were harvested at mid-exponential phase, lysed to protoplast, homogenized, and separated to nuclear and postnuclear fractions (24). $S_1$, $S_2$, and $P_2$ represent postnuclear supernatant, cytoplasm/microsome, and organelle fractions, respectively. Proteins (20 μg) from each fraction were run on gels. Thiolases were detected with anti-Ps-Thiolase I (upper panel) and anti-Thiolase III (lower panel) antisera. Thiolase IΔC6, C-terminus-truncated Thiolase I.
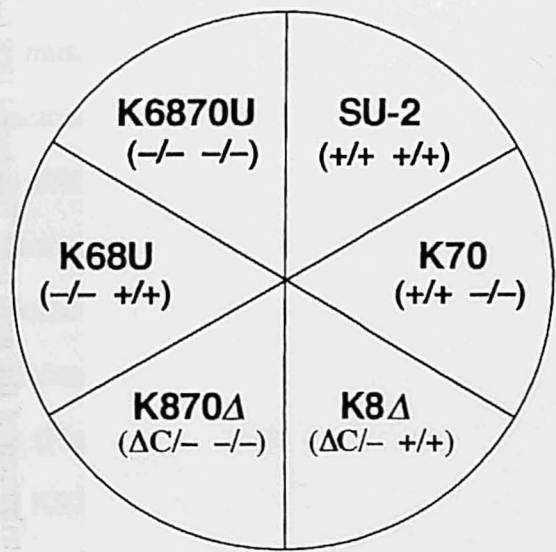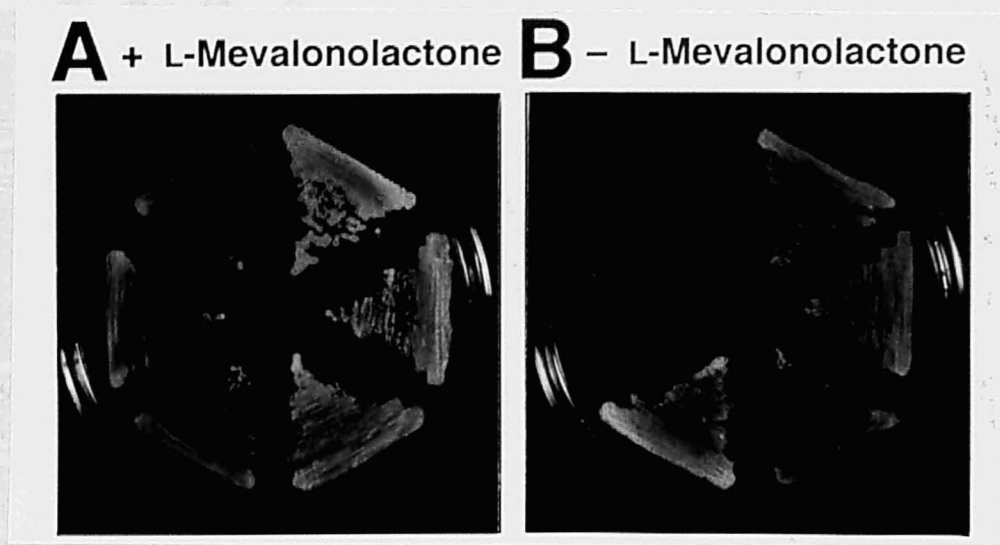
Fig. 8. Requirement of mevalonate for growth of wild type and mutant strains.

Yeast cells were inoculated on YPD medium with L-mevalonolactone (A) and without L-mevalonolactone (B) and were incubated at 30°C for 2 days. The presence or absence of each thiolase gene is indicated as explained in Fig. 1.

peroxisomes and the enzymes of the peroxisomal fatty acid β-oxidation system were induced. Therefore, the contributions of thiolase isozymes to the β-oxidation system and/or to other metabolic processes were examined by observation of the cell growth of thiolase disruptants on various carbon sources (Fig. 9).

Significant differences in growth were not observed among the wild type strain SU-2 and the hemizygous thiolase-gene null mutants, K6, K8, K7, and K0, on glucose, n-alkane, and butyrate, indicating that neither the A nor the B gene has an independent physiological role in cell growth. The strian K70, which lacks Thiolase III, could not grow on n-alkanes $(C_{10}$ to $C_{13})$, whereas the strain K8Δ, which lacks Ps-Thiolase I, exhibited growth on n-alkanes. The strain K70 could not grow on oleic acid either. These results demonstrated that Thiolase III was indispensable for the β-oxidation of long-chain fatty acids. On butyrate, however, the strian K8Δ also showed good growth, while the growth of the strain K70 was retarded, but the growth of both strains reached almost the same level as that of the wild type strain SU-2 in the stationary growth phase. No growth on butyrate was observed for the strain K870Δ, which lacks both Ps-Thiolase I and Thiolase III, suggesting that butyrate was utilized solely through the peroxisomal β-oxidation system. This fact supports the induction of the β-oxidation enzymes in butyrate-utilizing cells (21). There was no significant difference among the strains, SU-2, K70, and K8Δ, in cell growth on another short chain fatty acid, propionate, on glucose, or on the nonfermentable carbon sources glycerol and ethanol (Fig. 9); the results also indicate the indispensable participation of peroxisomal thiolase isozymes in β-oxidation.

## DISCUSSION

In the diploid yeast *Candida tropicalis*, the author disrupted the thiolase isozyme genes and altered the distribution of Thiolase I in order to elucidate the physiological functions of the thiolase isozymes. Intracellular thiolase activity was completely abolished in the double homozygous mutant lacking the Thiolase I and Thiolase III genes, indicating that there is no thiolase in *Candida tropicalis* other than Cs-Thiolase I, Ps-Thiolase I, and Thiolase III, which are encoded by two pairs of alleles. For *Saccharomyces cerevisiae*, it has been reported that

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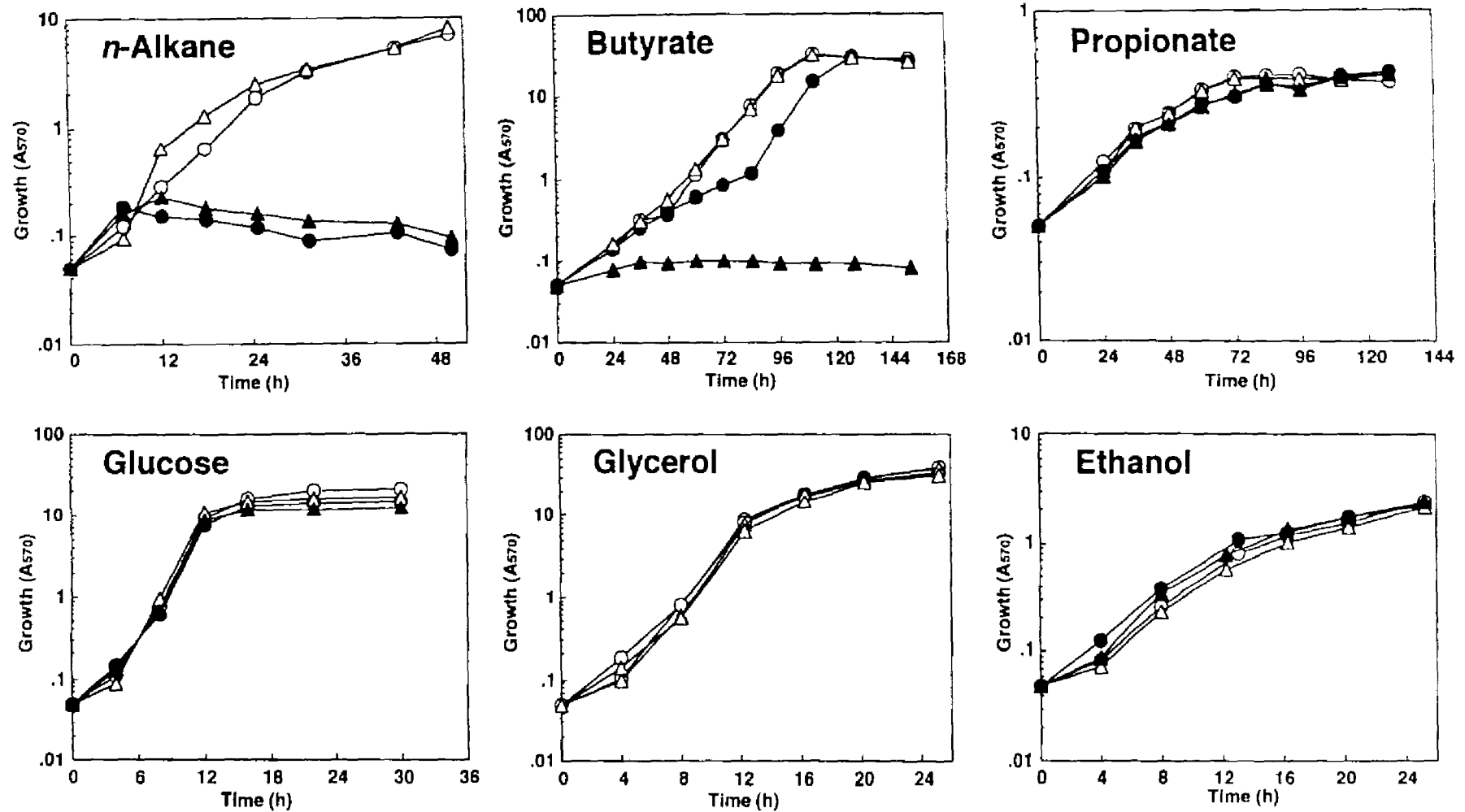
FIG. 9.   Growth kinetics of wild type and mutant strains on various carbon sources.

Open circle, closed circle, open triangle, and closed triangle indicate strains SU-2 (+/+ +/+), K70 (+/+ –/–), K8Δ (ΔC/– +/+),

and K870Δ (ΔC/– –/–), respectively. Carbon sources are indicated.

there are peroxisomal 3-ketoacyl-CoA thiolase (Pot1p/Fox3p), cytosolic acetoacetyl-CoA thiolase (Erg10p), and mitochondrial acetoacetyl-CoA thiolase (15, 16, 39), although the gene encoding the mitochondrial enzyme has not been cloned yet. In mammalian cells, there are at least five thiolase isozymes, and they are encoded by distinct genes. Compared with these systems, *Candida tropicalis* has a simple set of thiolase isozymes encoded by two pairs of allelic genes.

Experiments with deletions of the C-terminal six amino acid residues of Thiolase I, DADAKL, revealed the necessity of the sequence for targeting of the enzyme to peroxisomes in *Candida tropicalis*. The last three residues, AKL, are in good agreement with one motif of PTS1 (38). The transport of peroxisomal proteins of *Candida tropicalis* to peroxisomes has been examined for acyl-CoA oxidase and the multifunctional protein, but these studies were performed in *in vitro* systems or in heterologous *in vivo* systems (40-42). Therefore, the present result marks the first case for *Candida tropicalis* that a peroxisomal targeting signal was identified in a homologous *in vivo* system.

As presented by Kurihara *et al.* (19), in Part I, Chapter 1, and Fig. 5A, the expression of Thiolase I (Ps-Thiolase I and Cs-Thiolase I) genes was totally induced in response to *n*-alkane utilization, but Cs-Thiolase I is present constitutively and contributes indispensably to the mevalonate pathway. Therefore, it is important that Thiolase I is sorted into peroxisomes and cytoplasm in a regulated manner. Many mechanisms for the sorting of a single protein to dual compartments have been proposed (43). Recently, the fourth residue of C-terminal PTS1 of human catalase has been shown to be important in determining the efficiency of transportation of catalase to peroxisomes, which was attributed to the binding affinity of PTS1 for PTS1 receptor (44). Further detailed analysis of the C-terminal six amino acids will be necessary to reveal their relation to the dual sorting mechanism of Thiolase I.

The results in this chapter demonstrated that Cs-Thiolase I was essential for the mevalonate pathway, the early steps of sterol synthesis, in *Candida tropicalis*. This physiological function is consistent with the enzymatic properties of Cs-Thiolase I, because Cs-Thiolase I exhibits the activity for condensation reaction of acetyl-CoA units as shown in Part I, Chapter 3. The author has also showed in Part I, Chapter 3 that Thiolase I which can catalyze the

condensation reaction was present both in cytoplasm and in peroxisomes of *Candida tropicalis*. In mammalian cells also, there is the condensation reaction of thiolase in these two compartments (7, 12, 45), and additionally, 3-hydroxy-3-methylglutaryl-CoA reductase, which is the rate limiting enzyme in the mevalonate pathway, is colocalized with the condensation reaction (46, 47), suggesting two pathways in the early steps of sterol synthesis. However, this reductase has not been detected in peroxisomes of *Candida tropicalis* (19), and a lack of Ps-Thiolase I had no significant effect on growth (Fig. 8 and 9). Therefore, it is suggested that the early steps of sterol synthesis occur only in the cytoplasm of this yeast.

The fatty acid β-oxidation system is present only in peroxisomes in *Candida tropicalis* (2). The present results for thiolase isozymes clarified this observation that the peroxisomal β-oxidation system exclusively contributes to the degradation of fatty acids. On the other hand, unlike a lack of Ps-Thiolase I, lack of Thiolase III resulted in growth retardation on butyrate (Fig. 9), suggesting that Thiolase III degraded acetoacetyl-CoA more efficiently than Ps-Thiolase I did in the peroxisomal β-oxidation system. The results were not consistent with the biochemical observation that Thiolase I has much higher specific activity for a $C_4$ substrate, acetoacetyl-CoA, than Thiolase III (17, 18). Furthermore, most of the thiolase activity for acetoacetyl-CoA was due to Thiolase I (Fig. 4A). Therefore, the author presume that the reasons for the growth retardation brought about by the lack of Thiolase III are as follows. First, the contribution of each isozyme to the β-oxidation system might be determined by its quantity. In *Candida tropicalis* peroxisomes, the amount of Thiolase III is much higher than that of Ps-Thiolase I. The molar ratio of the two isozymes (Thiolase III/Ps-Thiolase I) can be estimated as approximately 16 for the native enzyme and 5.2 for the subunit from specific activities of the peroxisomal fraction and the purified proteins (17, 18). Second, Thiolase III might be one component of a β-oxidation multienzyme complex in *Candida tropicalis*. There is a "metabolon" hypothesis which suggests that enzymes included in a metabolic pathway form a multienzyme complex to bring about an efficient metabolic flux (48, 49). If Thiolase III belongs to a multienzyme complex, a lack of Thiolase III would result in the inhibition of the β-oxidation of fatty acids despite the presence of Ps-Thiolase I.

In *Candida tropicalis* peroxisomes, either Ps-Thiolase I or Thiolase III allows this

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yeast to utilize butyrate through the peroxisomal β-oxidation system. This is the first genetic demonstration that acetoacetyl-CoA thiolase participates in the peroxisomal fatty acid β-oxidation system. This system can be taken advantage of to alter the flux of the peroxisomal β-oxidation system in growing conditions and will give insight into the relation between the control of the flux and the regulation of gene expression in the β-oxidation system.

## SUMMARY

The *n*-alkane-assimilating diploid yeast *Candida tropicalis* possesses three thiolase isozymes encoded by two pairs of alleles; cytosolic and peroxisomal acetoacetyl-CoA thiolases, encoded by *CT-T1A* and *CT-T1B*, and peroxisomal 3-ketoacyl-CoA thiolase, encoded by *CT-T3A* and *CT-T3B*. The physiological functions of these thiolases have been examined by gene disruption. The homozygous *ct-t1aΔ/t1bΔ* null mutation abolished the activity of acetoacetyl-CoA thiolase and resulted in mevalonate auxotrophy. The homozygous *ct-t3aΔ/t3bΔ* null mutation abolished the activity of 3-ketoacyl-CoA thiolase and resulted in growth deficiency on *n*-alkanes ($C_{10}$ to $C_{13}$). All thiolase activities in this yeast disappeared with the *ct-t1aΔ/t1bΔ* and *ct-t3aΔ/t3bΔ* null mutations. To further clarify the function of peroxisomal acetoacetyl-CoA thiolase, site-directed mutation leading to acetoacetyl-CoA thiolase without a putative C-terminal peroxisomal targeting signal was introduced on the *CT-T1A* locus in the *ct-t1bΔ* null mutant. The truncated acetoacetyl-CoA thiolase was solely present in cytoplasm, and the absence of acetoacetyl-CoA thiolase in peroxisomes had no effect on growth on all carbon sources employed. Growth on butyrate was not affected by a lack of peroxisomal acetoacetyl-CoA thiolase, while retardation was observed by a lack of peroxisomal 3-ketoacyl-CoA thiolase. A defect of both peroxisomal isozymes completely inhibited growth on butyrate. These results demonstrated that cytosolic acetoacetyl-CoA thiolase was indispensable for the mevalonate pathway and that both peroxisomal acetoacetyl-CoA thiolase and 3-ketoacyl-CoA thiolase could participate in the peroxisomal β-oxidation system. In addition to its essential contribution to the β-oxidation of longer-chain fatty acids, 3-ketoacyl-CoA thiolase contributed even mainly to the β-oxidation of a $C_4$ substrate, butyrate.

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# Chapter 2  Contribution of acetoacetyl-CoA thiolase and 3-ketoacyl-CoA thiolase to fatty acid β-oxidation in *Candida tropicalis*

## INTRODUCTION

Peroxisomes are subcellular organelles present in most eukaryotic cells. The organelles contain various metabolic functions such as the fatty acid β-oxidation (1). In mammalian cells, peroxisomes are the sites metabolizing very-long-chain fatty acids and fatty acid derivatives such as pristanic acid and bile acid intermediates through the fatty acid β-oxidation system (2). Each step of the peroxisomal β-oxidation system consists of several kinds of isozymes (3). From the spectrum of substrate specificity, these isozymes are thought to participate in the metabolism of substrates corresponding to their specificities. Therefore, the intracellular organization of isozymes is one of the subjects for concentrated study. On the other hand, peroxisomes are proliferated and peroxisomal proteins including the β-oxidation system are induced in response to the change of environment of cells. The transcriptional activation of genes encoding the peroxisomal β-oxidation system is mediated by peroxisome proliferator-activated receptor (PPAR) in mammalian cells (4), and by Oaf1p/Pip2p complex in the yeast *Saccharomyces cerevisiae* (5, 6). However, it remains to be elucidated whether the mechanism of the transcriptional activation is associated with that of the regulation of the β-oxidation flux, although the expression of genes encoding the peroxisomal β-oxidation system was shown to be up-regulated in mice with the disruption of acyl-CoA oxidase genes (7)

*Candida tropicalis* is an asporogenic diploid yeast, which can utilize *n*-alkanes or fatty acids as a carbon source. In cells grown on *n*-alkanes or fatty acids, peroxisomes are profoundly proliferated and peroxisomal proteins are induced in harmony with the peroxisome proliferation (8, 9). In this yeast, the fatty acid β-oxidation system is restricted to peroxisomes (10), and consists of two acyl-CoA oxidases, one multifunctional protein containing the

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activities of enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase, and two thiolases (11–15). *Candida tropicalis* pK233 possesses two acetoacetyl-CoA thiolases (Thiolase I) and one 3-ketoacyl-CoA thiolase: cytosolic acetoacetyl-CoA thiolase (Cs-Thiolase I), peroxisomal acetoacetyl-CoA thiolase (Ps-Thiolase I), and peroxisomal 3-ketoacyl-CoA thiolase (Thiolase III) (14–16). Thiolase III is encoded by a pair of alleles *CT-T3A* and *CT-T3B* as shown in Part I, Chapter 1. In Part I, Chapter 3, the author showed that Cs-Thiolase I and Ps-Thiolase I are encoded by the same pair of alleles *CT-T1A* and *CT-T1B*.

As described in Part II, Chapter 1, from the investigation of these thiolase isozyme functions by gene disruption and mislocalization of an isozyme, it was revealed that Thiolase III is essential to the degradation of long-chain fatty acids and Cs-Thiolase I plays indispensable role in the mevalonate pathway. In addition, either a lack of Ps-Thiolase I or that of Thiolase III allowed cells growing on a short-chain fatty acid butyrate, although a lack of Thiolase III resulted in growth retardation. These characteristics of butyrate-grown cells have prompted the author to investigate the contribution of thiolase isozymes to the peroxisomal fatty acid β-oxidation system and the regulation of the β-oxidation system in cells with the defect of either of peroxisomal thiolase isozymes under the growth on butyrate. From the course of experiments, the author has clarified the contribution of thiolase isozymes to the β-oxidation system and implicated the organization and regulation of the peroxisomal β-oxidation system in *Candida tropicalis*.

## MATERIALS AND METHODS

### Plasmids, strains and growth conditions.

*Candida tropicalis* strains and plasmids used in this study are listed in Table 1. *Candida tropicalis* SU-2 (ATCC 20913) (*ura3a/ura3b*) (17) derived from *Candida tropicalis* pK233 (ATCC 20336) was used as a host strain of transformation. *Escherichia coli* strain DH5α (18) was used for gene manipulation. Media for genetic experiments of *Candida tropicalis* were described in Part II, Chapter 1. *Candida tropicalis* cells were precultured in YPD medium till early stationary growth phase and cultivated at 30°C in a medium containing

Table 1. *Candida tropicalis* strains and plasmids used.

| Strain or plasmid | Description |
|---|---|
| *Candida tropicalis* | |
| SU-2 | *ura3a/ura3b CT-T1A/CT-T1B CT-T3A/CT-T3B* |
| SU-U | *ura3a::( URA3)/ura3b CT-T1A/CT-T1B CT-T3A/CT-T3B* |
| K7ZUZ | *ura3a/ura3b CT-T1A/CT-T1B ct-t3aΔ:: lacZ-URA3-lacZ/CT-T3B* |
| K0ZUZ | *ura3a/ura3b CT-T1A/CT-T1B CT-T3A/ct-t3bΔ::lacZ-URA3-lacZ* |
| K70ZUZ | *ura3a/ura3b CT-T1A/CT-T1B ct-t3aΔ::lacZ/ct-t3bΔ:: lacZ-URA3-lacZ* |
| K70 | *ura3a/ura3b CT-T1A/CT-T1B ct-t3aΔ:: lacZ/ct-t3bΔ::lacZ* |
| K8Δ | *ura3a/ura3b CT-T1AΔC6/ct-t1bΔ:: lacZ CT-T3A/CT-T3B* |
| K70T31 | *ura3a::(T3A:: URA3)/ura3b CT-T1A/CT-T1B ct-t3aΔ:: lacZ/ct-t3bΔ::lacZ* |
| K70T11 | *ura3a/ura3b CT-T1A::( UPR-ICL-T1A:: URA3)/CT-T1B ct-t3aΔ::lacZ/ct-t3bΔ:: lacZ* |
| K8T1A | *ura3a/ura3b CT-T1A::( UPR-ICL-T1AΔC6:: URA3)/CT-T1B CT-T3A/CT-T3B* |
| Plasmid | |
| pWT1A | Source of *CT-T1A* (Thiolase I gene) |
| pUI3 | pUC19 containing *UPR-ICL* |
| pUC-URA3Bg | Source of *Bgl*II fragment of *C. tropicalis URA3* |
| pUIT11 | Thiolase I overexpression under the control of *UPR-ICL* |
| pUC-URA3 | pUC19 containing *Bgl*II fragment of *URA3* |
| pT37Bg | Source of *CT-T3A* (Thiolase III gene) |
| pUT31 | Restration of a sigle copy of *CT-T3A* |

*CT-T1AΔC6* shows the gene encoding the mutant Thiolase I of which C-terminal six amino acid residues were deleted.

*ura3a::( URA3)* and *ura3a::(T3A::URA3)* indicate that the plasmids pUC-URA3Bg and pUT31 have been integrated on the *ura3a* locus.

*T1A::( UPR-ICL-T1A::URA3)* and *T1AΔC6::( UPR-ICL-T1AΔC6::URA3)* display that the plasmids, pUIT11 and pUIT1Δ, have been integrated on the *CT-T1A* and *CT-T1AΔC6* loci, respectively.

butyrate (11 g/liter) as a sole carbon source (19). The compositions of media were described in Part I, Chapter 1. Cell growth was monitored by measuring light scattering at 570 nm.

**Plasmid construction and yeast transformation.**

pWT1A was described in Part I, Chapter 3. pUI3 was described by Kanai *et al.* (20) pUC-URA3, pUC-URA3Bg, and pT37Bg were described in Part II, Chapter 1.

A DNA fragment containing the coding region and the 3'-flanking region of *CT-T1A* was amplified from pWT1A by PCR with the primers 5'-TCCAGGATCCCACACAACTA-TGGCTCTCCC-3' and 5'-CAGGAAACAGCTATGAC-3'. The fragment was digested with *Bam*HI and *Sac*I and inserted between the *Bg*III and *Sac*I sites of pUI3 (20). Subsequently, *URA3* excised from pUC-URA3Bg with *Bg*III was inserted into the *Bam*HI site of the resultant plasmid. The final construct was named pUIT11. pT37Bg containing the coding region and the franking regions of *CT-T3A* was digested with *Bg*III and inserted into the *Sma*I site of pUC-URA3 with *Bg*III linker (named pUT3A).

Transformation of *Candida tropicalis* was carried out by spheroplast method as described in Part II, Chapter 1. Prior to yeast transformation, pUIT11 and pUT31 were linearized with *Bg*III and *Spe*I, respectively. Integration of the linearized pUIT11 and pUT31 on the *CT-T1A* and *ura3a* loci of the strain K70 resulted in strains K70IT11 and K70T31, respectively. Integration of the *Spe*I-linearized pUC-URA3Bg on the *ura3a* locus of SU-2 resulted in the strain SU-U, which was used as a Ura⁺ control-strain. Cells constructed were subjected to Southern blot analysis to check the correct integration into the desired loci.

**Enzyme and protein assays.**

Enzyme activities were determined as described in published papers: acyl-CoA oxidase, enoyl-CoA hydratase (21), acetoacetyl-CoA thiolase (14), catalase, isocitrate lyase, NAD-linked isocitrate dehydrogenase, and glucose-6-phosphate dehydrogenase (22). Protein concentration was assayed by Bradford method using bovine serum albumin as the standard (23).

**RNA methods.**

Isolation of total RNA from yeast cells was carried out as described by Kaiser et al. (24). Total RNA was separated by formaldehyde gel electrophoresis and blotted on positively charged nylon membranes (Boehringer Mannheim, Mannheim, Germany). Blots were

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hybridized with DNA probes labeled with PCR DIG Probe Synthesis Kit (Boehringer Mannheim) and detected by DIG Luminescent Detection Kit (Boehringer Mannheim). Hybridization and detection were carried out under the conditions instructed by the vendor. Primers and templates for the generation of probes are as follows: 5'-TCCAGGATCCC-ACACAACTATGGCTCTCCC-3', 5'-GCCTCGAGTTAAATCTTTTCAATAACGACAGC-AGACG-3', and pWT1A for Thiolase I; 5'-TCCAATGTACTTGGGAAGGTG-3', 5'-GTTCAACATGGCTTCCAAAGC-3', and a cDNA fragment (*POX5*) for acyl-CoA oxidase (11, 25); 5'-GCCACTACTCCAAAGGGTACCAACG-3', 5'-AAACAACTTCATTAGATC-TTTGC-3', and pBFE for multifunctional protein (26, 27); 5'-CAGATTGGTTGCCAT-CAGAG-3', 5'-ACACCAGCACCCATAGCAGC-3', and pUC19-ICL-B1 for isocitrate lyase (28); 5'-AATCCGTCCACCAAGTTATG-3', 5'-ATTT-CCTTCTTGATGAGGTC-3', and pC28 for catalase (29). *Candida tropicalis* actin gene was cloned from genomic DNA by PCR using the primers 5'-TATCGATAACGGTTCTGGTATG-3' and 5'-CGTATTCTTGT-TTTGAGATCCACA-3', inserted into pBluescript II SK+ (named pCTACT1). Actin probe was generated by PCR using the primers for cloning and the plasmid pCTACT1 as a template.

**Quantitative analysis.**

Data obtained by Western blot and Northern blot analyses were captured into Macintosh computer (Apple computer, Cupertino, CA, USA) by ScanJet II scanner (Hewlett Packard, Palo Alto, CA, USA) and analyzed by the public domain NIH Image program (developed at the U. S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

**Immunoelectron microscopy.**

Yeast cells were fixed with a mixture of 0.5% glutaraldehyde (Nisshin EM, Tokyo, Japan) and 3% paraformaldehyde (TA AB laboratories Equipment, Berkshire, England) in PBS buffer [0.1 M potassium phosphate buffer (pH7.6) containing 0.8% NaCl]. Embedding, immunostaining, and microscopic observation were performed as described by Kamasawa *et al*. (30). Anti-Ps-Thiolase I and anti-acyl-CoA oxidase antisera were used as the first antibodies. Goat anti-rabbit IgG conjugated with 10-nm- and 20-nm-diameter gold-particles were used as the second antibodies.

**Antisera.**

Antisera against following peroxisomal proteins were used for Western blot analysis and immunoelectron microscopy: acyl-CoA oxidase, isocitrate lyase, catalase (31), Ps-Thiolase I (30), and Thiolase III (15).
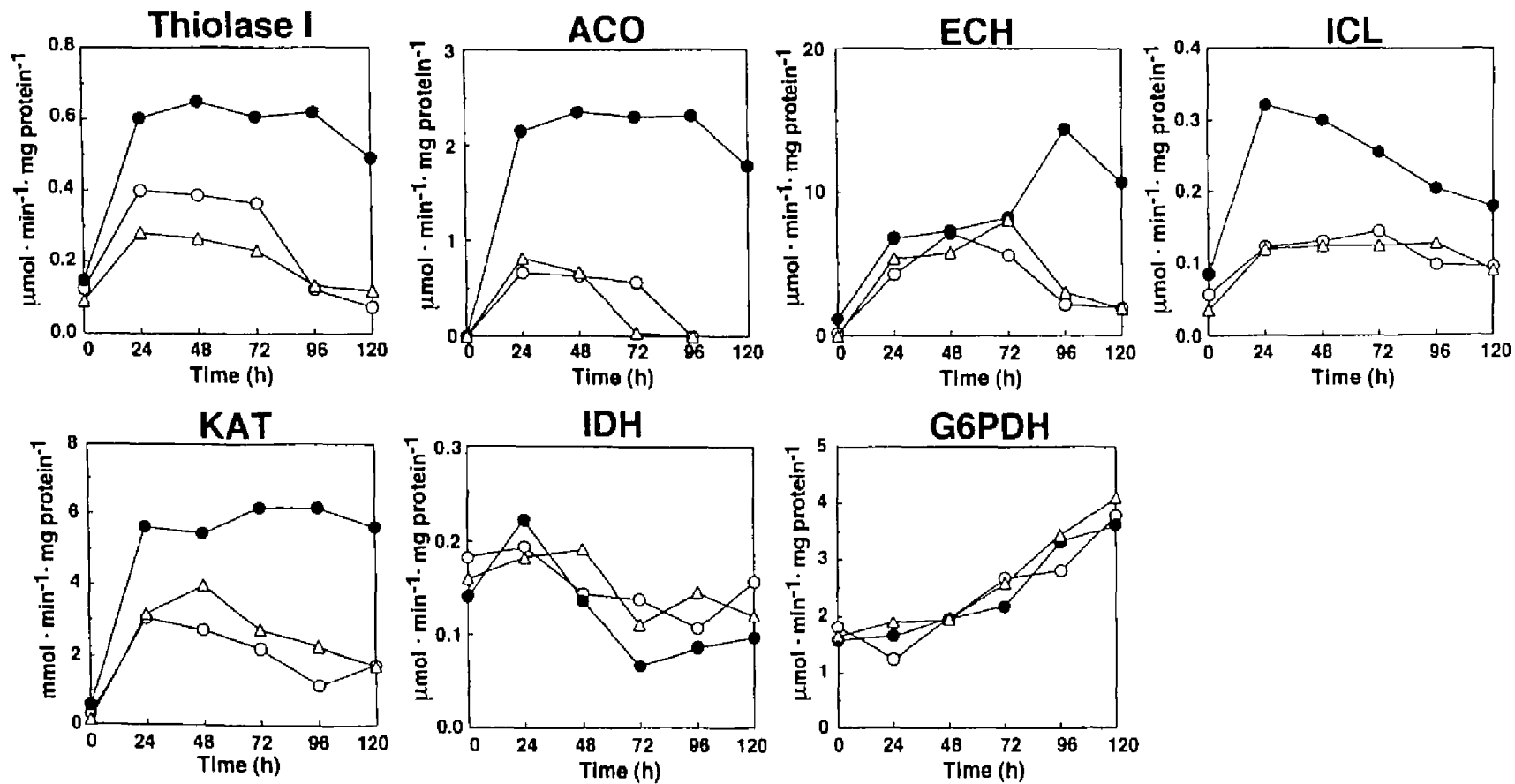
**Other methods.**

Preparation of cell-free extracts was carried out as described in Part I, Chapter 1. Southern blot analysis was performed as described by Kurihara *et al.* (32). Subcellular fractionation and Western blot analysis were conducted as described by Ueda *et al.* (31). Manipulation of nucleic acid and yeast was carried out according to the published methods (18, 24).

# RESULTS

**Effects of the individual defects of peroxisomal thiolase isozymes on the levels of peroxisomal proteins.**

In Part II, Chapter 1, the author showed that a lack of Thiolase III resulted in the retardation of growth on a short-chain fatty acid, butyrate, whereas a lack of Ps-Thiolase I did not. The author has addressed the possibility whether the defect of thiolase isozymes in peroxisomes had effect on the expression levels of peroxisomal protein genes in cells grown on butyrate. Compared with the wild type strain SU-2, the increased activity of Thiolase I was observed in the homozygous *ct-t3aΔ/ct-t3bΔ* null mutant in which Thiolase III had disappeared (the strain K70), whereas, no increase was observed in the mutant in which the localization of Thiolase I was restricted to cytoplasm by deletion of the C-terminal peroxisomal targeting signal of Thiolase I (the strain K8Δ) (Fig. 1). Since the strain K8Δ is a hemizygous Thiolase I gene null mutant, the activity of Thiolase I in the strain K8Δ was a half of that in SU-2 (see Part II, Chapter 1). In the strain K70, the increased levels of activities were also observed for peroxisomal proteins; acyl-CoA oxidase, enoyl-CoA hydratase, isocitrate lyase, and catalase, but those were not observed for glucose-6-phosphate dehydrogenase and NAD-linked isocitrate dehydrogenase, which are only present in cytoplasm and mitochondra in
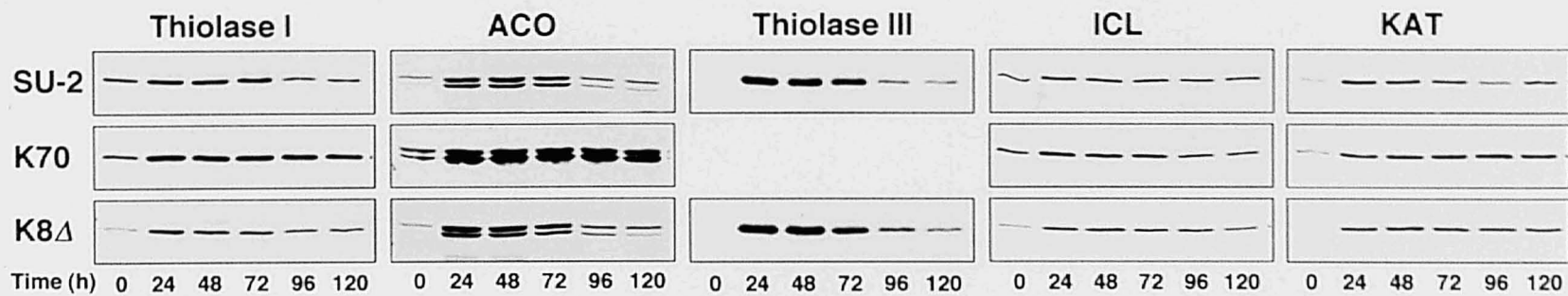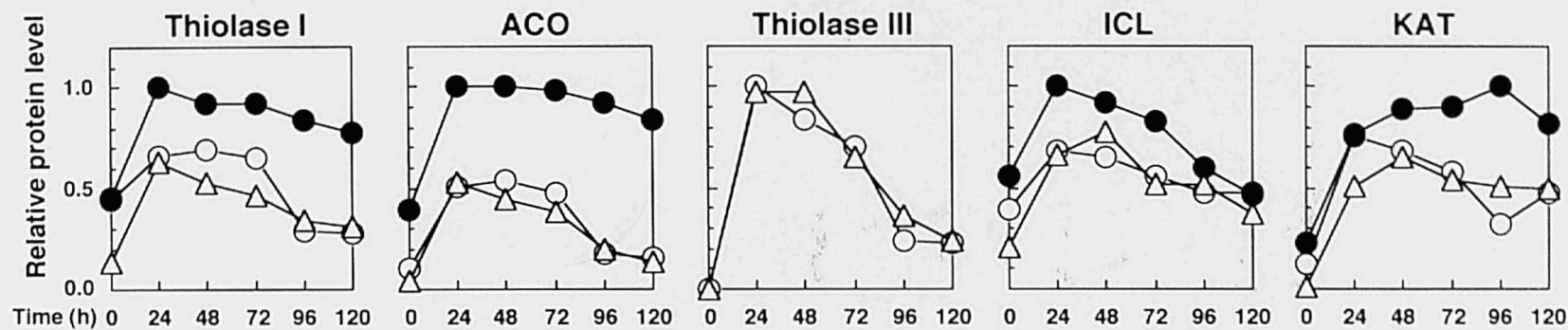
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# A

**B**

| | Thiolase I | ACO | Thiolase III | ICL | KAT |
|---|---|---|---|---|---|
| SU-2 | | | | | |
| K70 | | | | | |
| K8Δ | | | | | |

Time (h)   0   24   48   72   96   120      0   24   48   72   96   120      0   24   48   72   96   120      0   24   48   72   96   120      0   24   48   72   96   120

**C**

Relative protein level

Thiolase I     ACO     Thiolase III     ICL     KAT

1.0

0.5

0.0

Time (h) 0   24   48   72   96   120      0   24   48   72   96   120      0   24   48   72   96   120      0   24   48   72   96   120      0   24   48   72   96   120

Fig. 1.   Enzyme assay and Western blot analysis of Thiolase I and peroxisomal proteins in the *ct-t3aΔ/ct-t3b*Δ mutant grown on butyrate.

Time course of expression levels was followed by measuring specific activities for Thiolase I, acyl-CoA oxidase (ACO), enoyl-CoA

oxidase (ECH), isocitrate lyase (ICL), catalase (KAT), NAD-linked isocitrate dehydrogenase (IDH), and glucose-6-phosphate dehydrogenase

(G6PDH) (A), and by Western blot analysis (B, C). (A) The activity of Thiolase I was assayed using acetoacetyl-CoA as the substrate. (B)

Aliquots (10-μg protein) of cell-free extracts were run on gels and peroxisomal proteins were detected with anti-Ps-Thiolase I, anti-acyl-CoA

oxidase, anti-Thiolase III, anti-isocitrate lyase, and anti-catalase antisera. (C) Band intensity on blots was quantified as described in

MATERIALS AND METHODS. In order to normalize the band intensity between different blots, the same sample was run on each gel.

Strains: ○, SU-2 (*CT-T1A/CT-T1B    CT-T3A/CT-T3B*); ●, K70 (*CT-T1A/CT-T1B    ct-t3aΔ/ct-t3b*Δ) ; △, K8Δ (*CT-T1AΔC6/CT-T1B*

*CT-T3A/CT-T3B*).

*Candida tropicalis* (22, 33), respectively, suggesting that the elevation of enzyme activities occurred on peroxisomal enzymes. The synthesis levels of peroxisomal proteins were reevaluated by Western blot analysis (Fig. 1B and 1C). Two bands were detected for acyl-CoA oxidase and they may be two isozymes in *Candida tropicalis* (11). The change in the quantities of peroxisomal proteins was consistent with that in the enzyme activities.

Northern blot analysis revealed the increased mRNA levels of genes encoding peroxisomal proteins, suggesting that the increase of the enzyme activities and proteins was attributed to that of mRNA levels (Fig. 2). Although mRNA levels of several genes were high at 0 hour, it may be attributed to the condtions of preculture such as medium composition. In addition to the mRNA levels of peroxisomal matrix proteins, the mRNA level of a peroxisomal membrane protein of *Candida tropicalis*, Pmp25p, which was homologous to Pex11p (formerly Pmp27p) (34, 35), was also increased in the strain K70. In *Candida tropicalis*, enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase were involved in a multifunctional protein (MFP) encoded on a single open reading frame (27). The increased mRNA levels of genes encoding acyl-CoA oxidase (Pox5p), MFP, and Thiolase I represented that the disruption of Thiolase III genes lead to the up-regulation of genes of the fatty acid β-oxidation system.

## Up-regulation of peroxisomal proteins led by a lack of Thiolase III protein.

To clarify whether the modifications of the *CT-T3A* and *CT-T3B* loci caused this up-regulation, the author examined the expression of peroxisomal proteins in the hemizygous null mutants K7ZUZ (*ct-t3aΔ/CT-T3A*) and K0ZUZ (*CT-T3A/ct-t3b*Δ), and in the strain K70T31, in which a single copy of *CT-T3A* in the plasmid pUT31 was introduced into the *ura3a* locus in the homozygous *ct-t3aΔ/ct-t3b*Δ null mutant K70. The hemizygous null mutations did not change the expression of peroxisomal proteins (Fig. 3) as well as growth phenotype described in Part II, Chapter 1. In contrast, the expression levels of peroxisomal proteins were restored in the strain K70T31. These results suggest that a lack of Thiolase III protein leads to the induced expression of peroxisomal proteins but the modifications of the *CT-T3A* and *CT-T3B* loci did not.
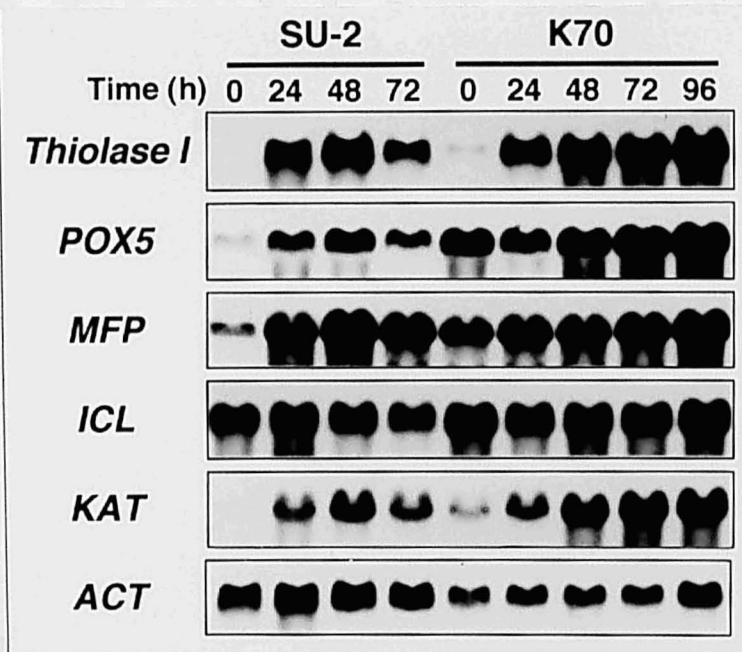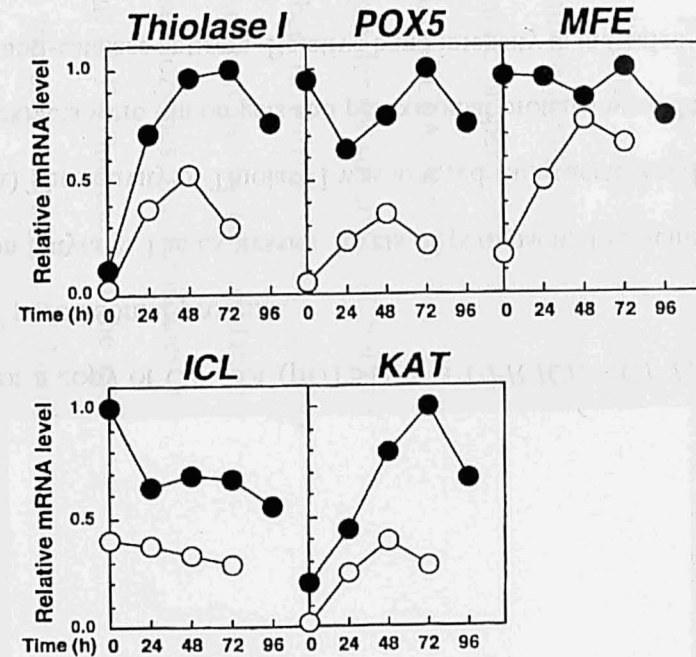
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Fig. 2. Northern blot analysis of genes encoding Thiolase I and peroxisomal proteins in the wild type strain and the *ct-t3a*Δ/*ct-t3b*Δ mutant grown on butyrate.

(A) Total RNAs were run on gels and mRNAs were detected with DIG-labeled DNA probes of genes encoding Thiolase I, acyl-CoA oxidase (POX5), multifunctional protein (MFP), isocitrate lyase (ICL), catalase (KAT), and actin (ACT). (B) The mRNA levels of peroxisomal proteins were densitometrically quantified and standardized toward the actin mRNA level. Strains: ○, SU-2 (*CT-T3A/CT-T3B*); ●, K70 (*ct-t3a*Δ/*ct-t3b*Δ).
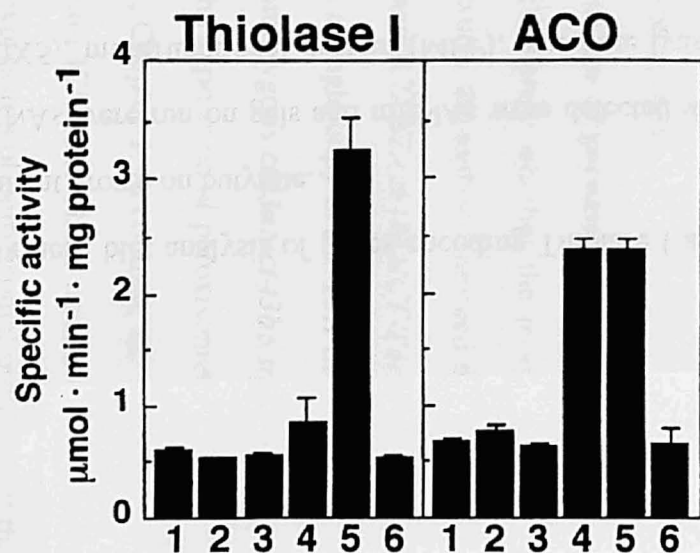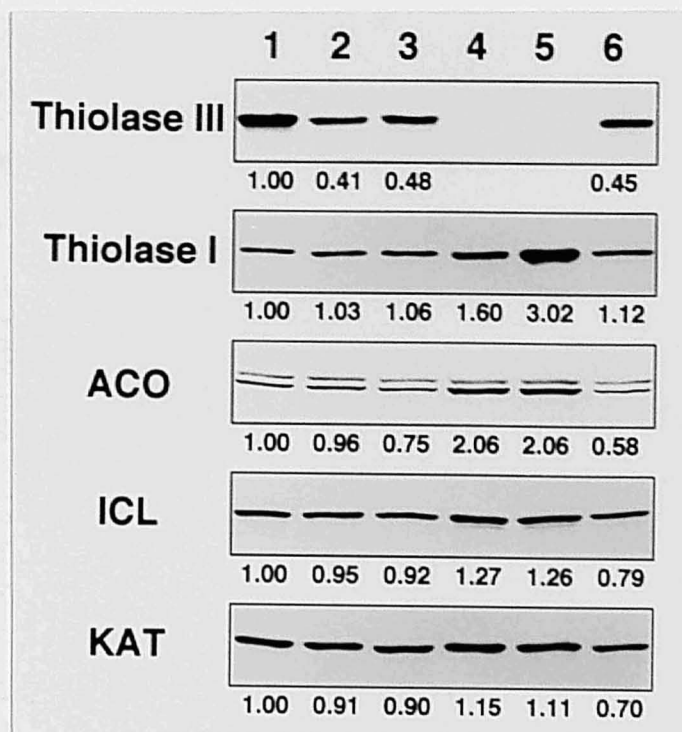
Fig.3.  Effects of the disruption of *CT-T3A* or *CT-T3B*, and of the introduction of a copy of *CT-T3A* (pUT31) or a *UPR-ICL—CT-T1A* fusion gene (pUIT11) into the *ct-t3aΔ/ct-t3bΔ* mutant on the expression of peroxisomal proteins.

Cell-free extracts were prepared from cells harvested at mid-exponential growth phase on butyrate. The expression levels of peroxisomal proteins were determined by measurement of specific activities (A) and by Western blot analysis (B). (A) The activity of Thiolase I was assayed using acetoacetyl-CoA as the substrate. Values are means ±SD (n=2–4) (B) Aliquots (10-μg protein) of cell-free exracts were run on gels and peroxisomal proteins were detected with anti-Ps-Thiolase I, anti-acyl-CoA oxidase, anti-Thiolase III, anti-isocitrate lyase, and anti-catalase antisera. Relative band intensity is indicated below respective bands. Strains: 1, SU-U (*CT-T3A/CT-T3B*); 2, K7ZUZ (*ct-t3aΔ/CT-T3B*); 3, K0ZUZ (*CT-T3A/ct-t3bΔ*); 4, K70ZUZ (*ct-t3aΔ/ct-t3bΔ*); 5, K70IT11 (K70 + pUIT11); 6, K70T31 (K70 + pUT31). All strains are *URA3*$^+$.

## Overexpression of Thiolase I in the *ct-t3aΔ/ct-t3b*Δ mutant.

From the growth phenotype of the strain K70 on butyrate, the author suggested in Part I, Chapter 1 that Thiolase III would contributes to the peroxisomal fatty acid β-oxidation systemmore greatly than Ps-Thiolase I. However, this contribution was not consistent with the kinetic property that acetoacetyl-CoA-specific Ps-Thiolase I has higher activity toward acetoacetyl-CoA than Thiolase III (15). The amount of Thiolase III is approximately five folds greater for subunit than that of Ps-Thiolase I in peroxisomes of *n*-alkane-grown cells as described in Part II, Chapter 1. Therefore, to clarify determinants of the contribution of thiolase isozymes to the β-oxidation system, the author has examined whether the amount of thiolase isozymes in peroxisomes is significant for the contribution. In order to increase the amount of Ps-Thiolase I in the strain K70, the fusion gene, pUIT11, was constructed, in which the promoter region of Thiolase I gene was exchanged with that of isocitrate lyase (*UPR-ICL*) (20), and then introduced the construct pUIT11 into the strain K70 (the resultant strain was named K70IT11), because isocitrate lyase is synthesized much higher than Thiolase I in *Candida tropicalis* and *UPR-ICL* had been used for the high-level expression of heterologous genes in *Saccharomyces cerevisiae* (20). The activity and protein amount of Thiolase I were much greater in the strain K70IT11 grown on butyrate than those in the strain K70ZUZ, which is a *URA3*⁺ strain with the *ct-t3aΔ/ct-t3b*Δ genotype (Fig. 3). However, the activities of all peroxisomal proteins employed were not affected by the overexpression of Thiolase I gene. In addition, whereas the introduction of a copy of *CT-T3A* could restore the growth of the strain K70 on butyrate to that of the wild type strain, the overexpression of Thiolase I gene could not (Fig. 4)

Subcellular franctionation revealed that much amount of overexpressed Thiolase I was present in the organelle fraction (Fig. 5A). Immunoelectron microscopy, furthermore, demonstrated the localization of overexpressed Thiolase I in peroxisomes (Fig. 5B). Signals indicating Thiolase I in the strain K70IT11 were much greater in number than those in the strain K70. Overexpressed Thiolase I was distributed over most peroxisomes in the cells and colocalized with acyl-CoA oxidase (Fig. 5C). These observations revealed that Ps-Thiolase I could not compensate the Thiolase III function in peroxisomes.
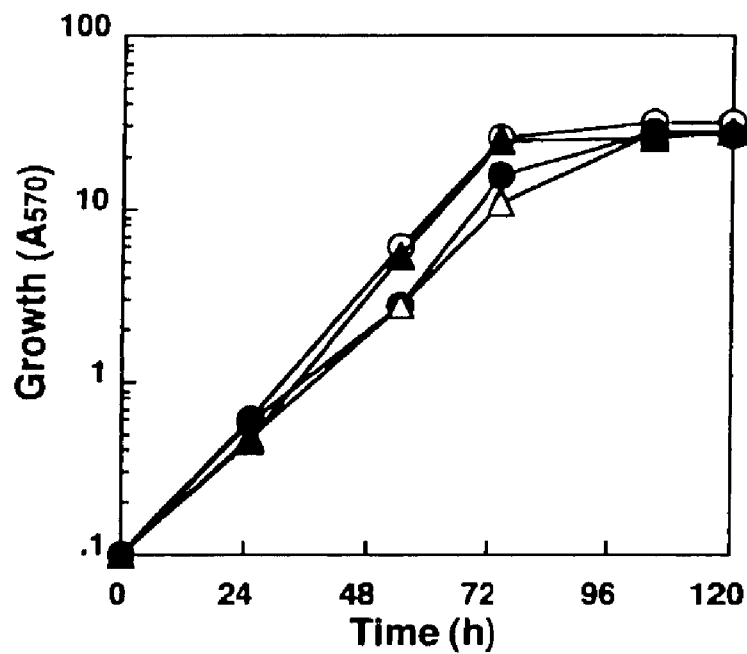
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Fig. 4. Effects of the introduction of a copy of *CT-T3A* (pUT31) or a *UPR-ICL—CT-T1A*

fusion gene (pUIT11) into the *ct-t3aΔ/ct-t3b*Δ mutant on growth kinetics in a

butyrate medium.

Strains: O, SU-U (*CT-T3A/CT-T3B*); ●, K70ZUZ (*ct-t3aΔ/ct-t3b*Δ); Δ, K70IT11 (K70 +
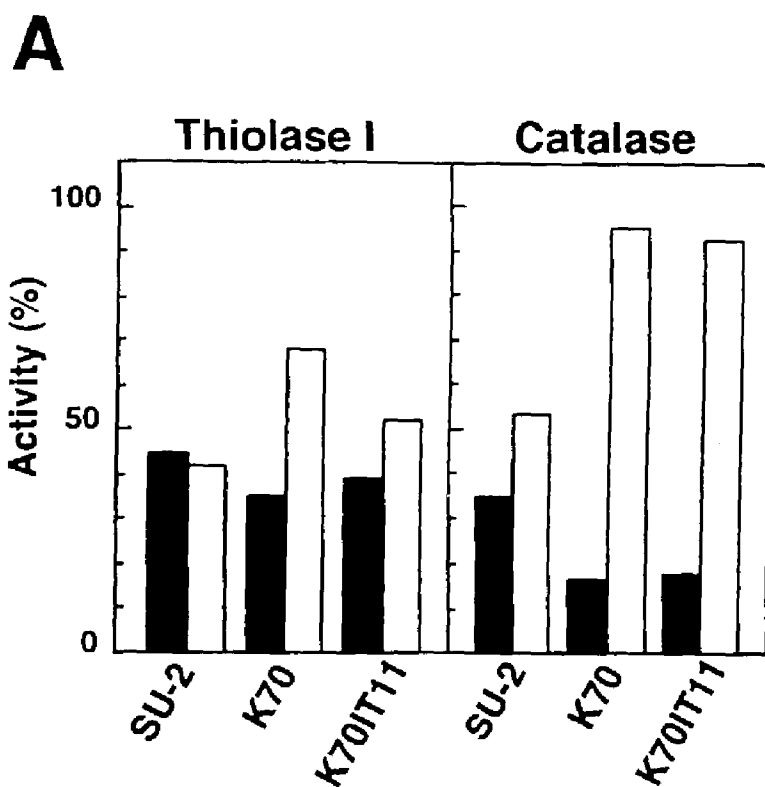
pUIT11); ▲, K70T31 (K70 + pUT31).

# A



Fig. 5.    Subcellular distribution of overexpressed Thiolase I.

(A) The butyrate grown cells of the strains SU-2, K70, and K70IT11 were fractionated by differential centrifugation and the activities of Thiolase I and catalase were determined in the 20,000 x *g* supernatant (solid bar, cytosolic and microsomal franction) and the 20,000 x *g* pellet (open bar, mitochondrial and peroxisomal fraction), respectively. (B, C) The butyrate-grown cells of the strain K70IT11 were observed by immunoelectron microscopy. The cells were labeled with anti-Ps-Thiolase I antiserum (B), and doubly labeled with anti-Ps-Thiolase I and anti-acyl-CoA oxidase antisera (C). Ten-nm gold particles represent acyl-CoA oxidase and 20-nm gold particles represent Thiolase I (C). M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole. Bar = 1 μm.

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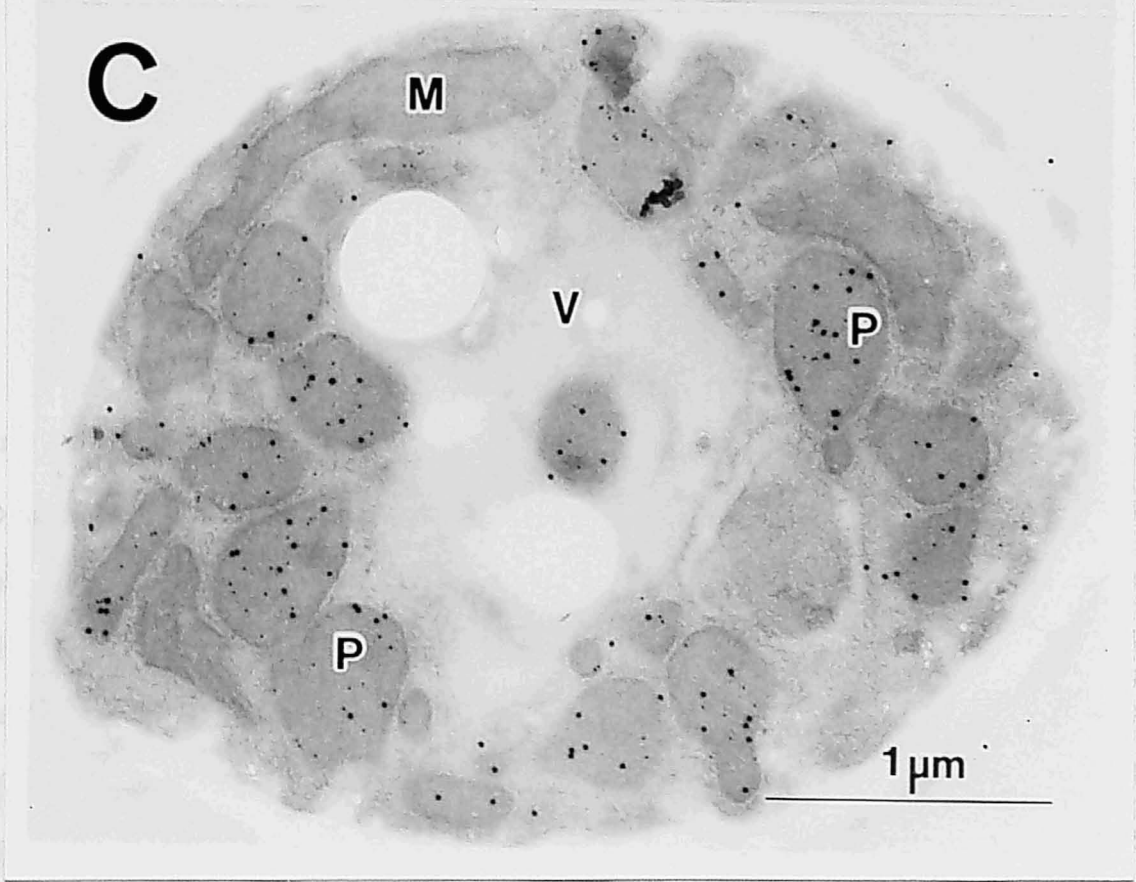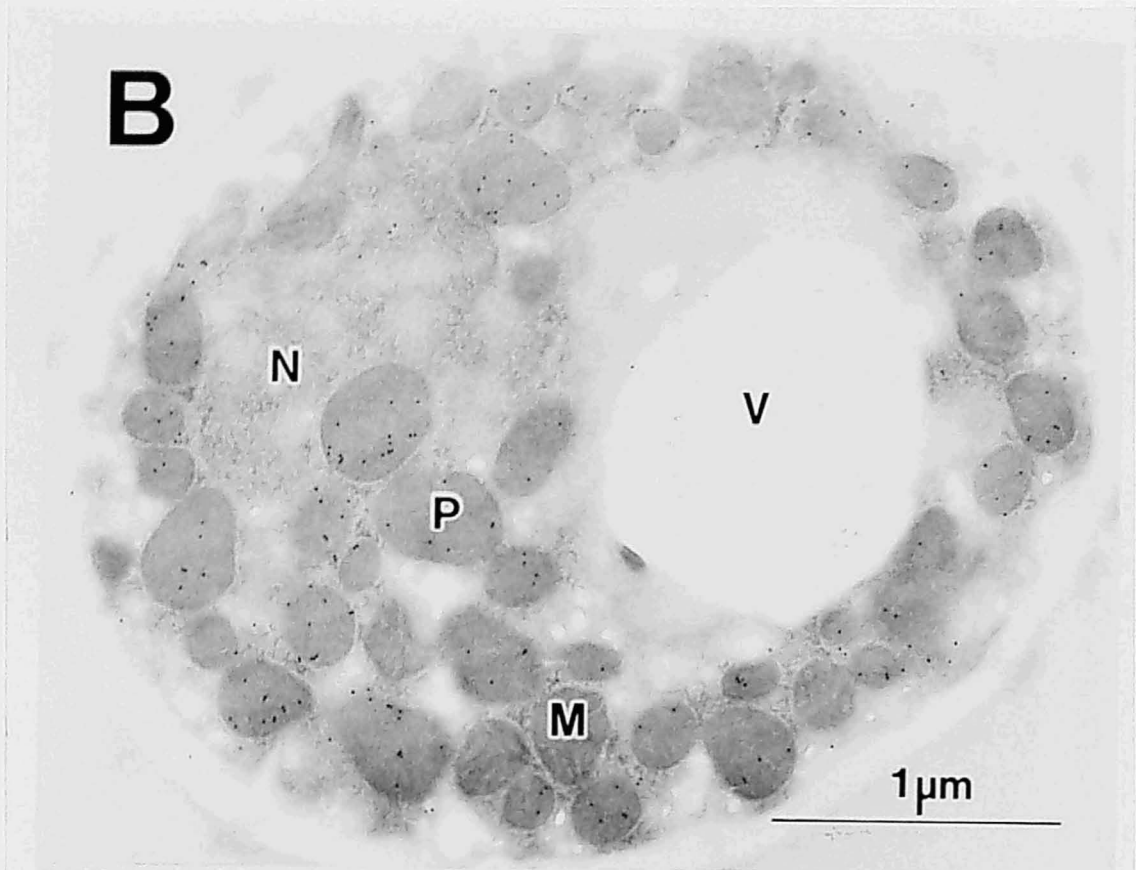
# DISCUSSION

In Part II, Chapter 1, the author suggested that in *Candida tropicalis* peroxisomes, both Ps-Thiolase I and Thiolase III participate in the fatty acid β-oxidation system. Since *Candida tropicalis* with either a lack of Ps-Thiolase I or that of Thiolase III can grow on butyrate, the author investigated the contribution of these peroxisomal thiolase isozymes to the β-oxidation system. The present study revealed that a lack of Thiolase III resulted in the increased protein and mRNA levels of peroxisomal proteins and that phenotypes of Thiolase III deficiency could not be compensated by the overexpression of Ps-Thiolase I. These results provide evidence that Thiolase III contributes to the β-oxidation system more greatly than Ps-Thiolase I in *Candida tropicalis* even grown on a $C_4$ substrate, butyrate.

The overexpression of Ps-Thiolase I could not compensate the function of Thiolase III in butyrate-grown cells despite greater specific activity toward acetoacetyl-CoA. The finding indicates that the difference in the amount in peroxisomes and the kinetic properties of these two isozymes is not a determinant for the contribution to the β-oxidation system in *Candida tropicalis*. Therefore, in order to overcome the kinetic disadvantage of Thiolase III to Ps-Thiolase I, Thiolase III may be organized with other enzymes of the peroxisomal fatty acid β-oxidation system, for example, the multienzyme complex which performs the channeling of metabolite intermediate (36). The intermediate channeling of the peroxisomal β-oxidation system has been applied to multifunctional enzyme (formerly bifunctional enzyme) from rat liver (37). However, the channeling has not been well characterized in the peroxisomal β-oxidation system. The detail discrimination of Ps-Thiolase I and Thiolase III functions can provide the implication of the intermediate channeling from the third step to the fourth step of the peroxisomal β-oxidation system in *Candida tropicalis*.

The up-regulation of peroxisomal proteins in the butyrate-grown cells without Thiolase III leads the author to the idea that a lack of Thiolase III resluts in the decreased flux of the β-oxidation and directs cells to respond to feed back the decrease of flux to the level expression of the β-oxidation system. On the other hand, a disruption of either of two acyl-CoA oxidase genes (*POX4* and *POX5*) (11) resulted in the up-regulation of acyl-CoA oxidase activity in

the n-alkane-grown cells of *Candida tropicalis* (38). In this case also, cells may respond to the decreased flux of the β-oxidation. In both cases of thiolase and oxidase isozymes, the disruption of either of isozyme genes did not result in severe growth defect on the respective carbon sources, butyrate and n-alkane, and the resultant up-regulation appeared in growing cells. Therefore, it is implied that *Candida tropicalis* possesses a regulatory mechanism of the β-oxidation system in a feedback manner.

It is of great interest what messengers lead to the up-regulation of peroxisomal proteins. A lack of Thiolase III would result in the decrease of acetyl-CoA production and in accumulation of substrates and intermediates of the β-oxidation system, because the growth retardation on butyrate was observed for cells without Thiolase III as shown in the present and previous chapters. For the yeast *Saccharomyces cerevisiae*, recent studies showed that mitochondrial functions are important for gene expression of peroxisomal proteins. Rtg1p and Rtg2p which participate in the communication between mitochondria and the nucleus are required for full expression of nuclear genes for peroxisomal proteins (39, 40). Respiration is also required for high-level expression of peroxisomal thiolase gene (41). In *Candida tropicalis* also, it is likely that acetyl-CoA production level may affect functions of mitochondria communicating with the nucleus. On the other hand, for mammalian cells, fatty acids were recently identified as candidates of ligands for peroxisome proliferator-activated receptor (PPAR) which mediates gene expression of the peroxisomal β-oxidation enzymes (4, 42, 43). Furthermore, in liver of mice with a disrupted peroxisomal fatty acyl-CoA oxidase gene (ACOX), the expression levels of other genes of the peroxisomal β-oxidation system were increased, suggesting that ACOX gene disruption leads to transcriptional activation mediated by PPAR with increased endogenous ligands (7). In *Candida tropicalis* without Thiolase III, the up-regulation of peroxisomal proteins would be also mediated by transcriptional activating factors. Investigation of the factors will make clear the reguratory mechanism of the perxisomal β-oxidation system and whether the up-regulation of peroxisomal proteins is related to the induction of peroxisomal proteins in response to the n-alkane-utilization of *Candida tropicalis*.

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# SUMMARY

In the yeast *Candida tropicalis*, two thiolase isozymes, peroxisomal acetoacetyl-CoA and 3-ketoacyl-CoA thiolases, participate in the peroxisomal fatty acid β-oxidation system. In the previous chapter the author showed that the disruption of peroxisomal 3-ketoacyl-CoA thiolase genes resulted in the growth retardation on butyrate, suggesting that peroxisomal 3-ketoacyl-CoA thiolase contributes to the β-oxidation system more greatly than peroxisomal acetoacetyl-CoA thiolase. In the present study, the individual contribution of peroxisomal acetoacetyl-CoA and 3-ketoacyl-CoA thiolases to the β-oxidation system was investigated in cells grown on butyrate, on which *Candida tropicalis* can grow with either the absence of peroxisomal acetoacetyl-CoA thiolase or that of peroxisomal 3-ketoacyl-CoA thiolase. A lack of peroxisomal 3-ketoacyl-CoA thiolase protein resulted in the induced expression of acetoacetyl-CoA thiolase and other peroxisomal proteins, whereas a lack of peroxisomal acetoacetyl-CoA thiolase did not. Overexpression of acetoacetyl-CoA thiolase gene could not suppress the induction of peroxisomal proteins and the growth retardation on butyrate in cells without peroxisomal 3-ketoacyl-CoA thiolase, although much amount of overexpressed acetoacetyl-CoA thiolase was detected in most peroxisomes of butyrate-grown cells. These results provide evidence of the greater contribution of 3-ketoacyl-CoA thiolase to the peroxisomal β-oxidation system than acetoacetyl-CoA thiolase, and implies that components in the peroxisomal fatty acid β-oxidation system are organized in *Candida tropicalis* peroxisome matrix and that the expression of the peroxisomal β-oxidation system is regulated in a feedback manner.

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# GENERAL CONCLUSION

The present study has been carried out to elucidate the physiological functions of thiolase isozymes in an n-alkane-assimilating yeast, *Candida tropicalis*.

Two transcribed genes encoding peroxisomal 3-ketoacyl-CoA thiolase were isolated. Both these genes were composed of 1,224 nucleotides, corresponding to 408 amino acids, and they showed extremely high similarity each other. Peroxisomal 3-ketoacyl-CoA thiolase contained a putative peroxisomal targeting signal in the amino-terminal region and exhibited 35% identical to peroxisomal acetoacetyl-CoA thiolase form *Candida tropicalis*. Expression of these thiolase isozymes was different in the manner on various carbon sources, suggesting that the biosynthesis of them was differently regulated, although both of them were the enzymes in the final step of the peroxisomal fatty acid β-oxidation system.

The evolutional distance was estimated from the comparison of thiolases from various organisms. The result suggests that the coexistence of acetoacetyl-CoA thiolase and 3-ketoacyl-CoA thiolase in peroxisomes is important in order to consider the molecular evolution of the fatty acid β-oxidation system and the biogenesis of peroxisomes.

Cytosolic acetoacetyl-CoA thiolase was first purified from glucose-grown *Candida tropicalis*. Cytosolic acetoacetyl-CoA thiolase was virtually identical to peroxisomal acetoacetyl-CoA thiolase in molecular mass, kinetic and immunochemical properties, and primary structure at the N-terminus. The recombinant thiolases expressed from peroxisomal acetoacetyl-CoA thiolase genes in the yeast *Saccharomyces cerevisiae* also exhibited practically identical properties to cytosolic and peroxisomal acetoacetyl-CoA thiolases from *Candida tropicalis*. These results revealed that cytosolic and peroxisomal acetoacetyl-CoA thiolases were encoded by the same gene.

The physiological functions of thiolases isozymes in *Candida tropicalis* have been examined by the gene disruption of respective genes and the site-directed mutagenesis deleting a putative peroxisomal targeting signal of acetoacetyl-CoA thiolase. The disruption of

acetoacetyl-CoA thiolase genes abolished the activity of acetoacetyl-CoA thiolase and resulted in mevalonate auxotrophy. The disruption of 3-ketoacyl-CoA thiolase genes abolished the activity of 3-ketoacyl-CoA thiolase and resulted in growth deficiency on long-chain $n$-alkanes. The C-terminus-truncated acetoacetyl-CoA thiolase was solely present in cytoplasm. Whereas growth on all carbon sources employed was not affected by a lack of peroxisomal acetoacetyl-CoA thiolase, growth on butyrate was retarded by a lack of peroxisomal 3-ketoacyl-CoA thiolase. A defect of both peroxisomal isozymes completely abolished growth on butyrate. These results demonstrated that cytosolic acetoacetyl-CoA thiolase was indispensable for the mevalonate pathway, and that both peroxisomal acetoacetyl-CoA thiolase and 3-ketoacyl-CoA thiolase could participate in the peroxisomal β-oxidation system, although, 3-ketoacyl-CoA thiolase greatly contributed even to the β-oxidation of a $C_4$ substrate, butyrate, in addition to the essential contribution to the β-oxidation of longer-chain fatty acids.

The contribution of peroxisomal acetoacetyl-CoA thiolase and peroxisomal 3-ketoacyl-CoA thiolase to the peroxisomal fatty acid β-oxidation system was investigated in cells grown on butyrate, on which *Candida tropicalis* can grow with either the absence of peroxisomal acetoacetyl-CoA thiolase or that of peroxisomal 3-ketoacyl-CoA thiolase. A lack of peroxisomal 3-ketoacyl-CoA thiolase protein resulted in the induced expression of acetoacetyl-CoA thiolase and other peroxisomal proteins, whereas a lack of peroxisomal acetoacetyl-CoA thiolase did not. Overexpression of acetoacetyl-CoA thiolase gene could not suppress the induction and growth retardation in cells without peroxisomal 3-ketoacyl-CoA thiolase, although much amount of acetoacetyl-CoA thiolase was detected in most peroxisomes of butyrate-grown cells. These results gave evidence of the greater contribution of 3-ketoacyl-CoA thiolase to the β-oxidation system than acetoacetyl-CoA thiolase, and implies that components of the peroxisomal fatty acid β-oxidation system are organized in *Candida tropicalis* peroxisomes and that the β-oxidation system is regulated in a feedback manner.

# PUBLICATION LIST

Part I

Chapter 1

Comparison of Molecular Structures and Regulation of Biosynthesis of Unique Thiolase

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