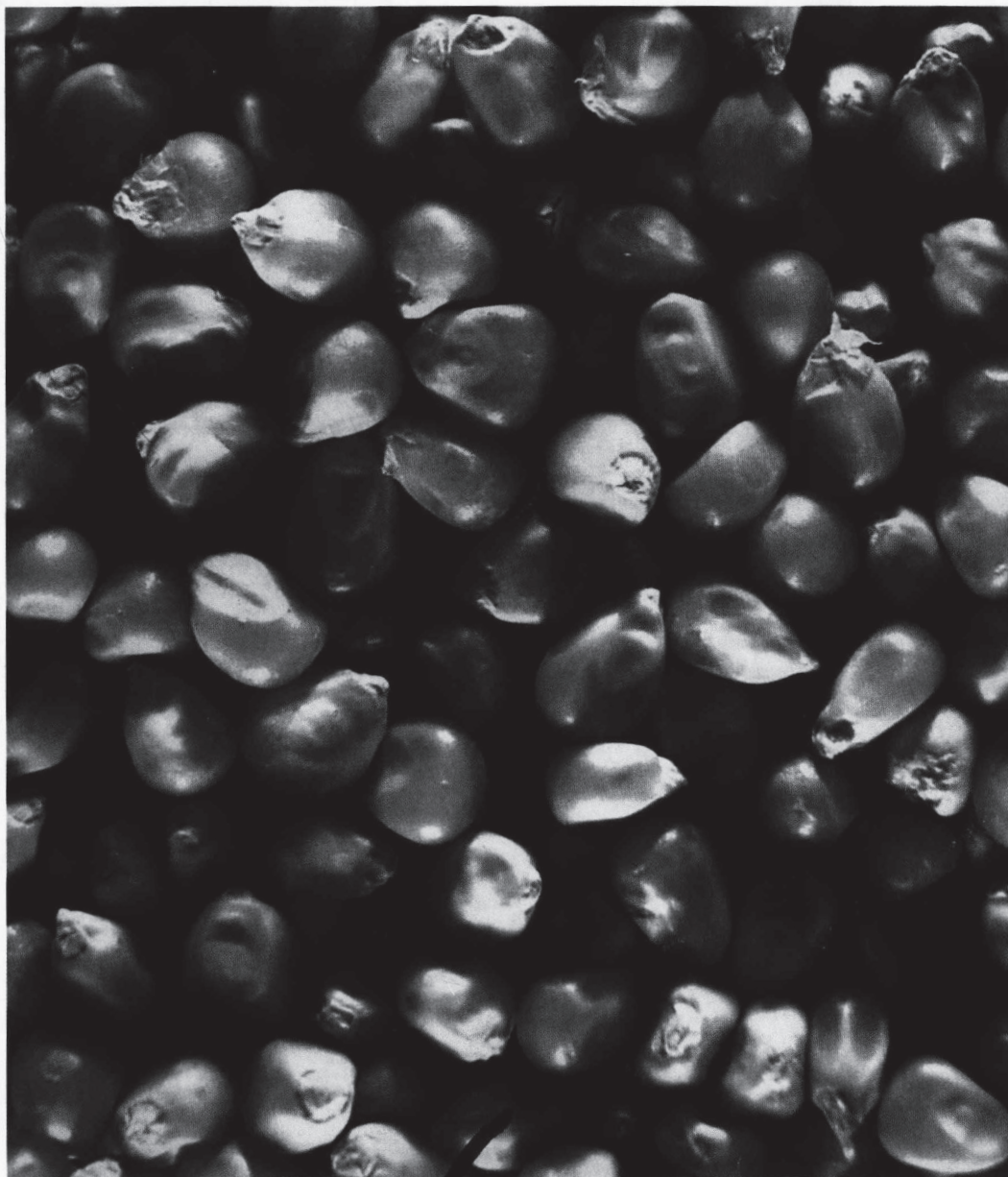




Journal of Bacteriology



AMERICAN
SOCIETY FOR
MICROBIOLOGY

MAY 2000, VOLUME 182, NUMBER 9

Published Twice Monthly

LCY Biotechnology Holding, Inc.

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Page 1 of 11

JOURNAL OF BACTERIOLOGY

VOLUME 182 • MAY 2000 • NUMBER 9

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JOURNAL OF BACTERIOLOGY

Volume 182

May 2000

No. 9

MINIREVIEW

Molecular Control of Expression of Penicillin Biosynthesis Genes in Fungi: Regulatory Proteins Interact with a Bidirectional Promoter Region

Juan F. Martín

2355-2362

STRUCTURE AND FUNCTION

Probing the CD Luminal Loop Region of the D2 Protein of Photosystem II in *Synechocystis* sp. Strain PCC 6803 by Combinatorial Mutagenesis

Anna T. Keilty, Svetlana Y. Ermakova-Gerdes, and Wim F. J. Vermaas

2453-2460

PLANT MICROBIOLOGY

Genetic Analysis of Functions Involved in Adhesion of *Pseudomonas putida* to Seeds

Manuel Espinosa-Urgel, Amparo Salido, and Juan-Luis Ramos

2363-2369

The *Sinorhizobium meliloti* Lon Protease Is Involved in Regulating Exopolysaccharide Synthesis and Is Required for Nodulation of Alfalfa

Michael L. Summers, Lina M. Botero, Scott C. Busse, and Timothy R. McDermott

2551-2558

GENETICS AND MOLECULAR BIOLOGY

Genetic Variation and Evolutionary Origin of the Direct Repeat Locus of *Mycobacterium tuberculosis* Complex Bacteria

J. D. A. van Embden, T. van Gorkom, K. Kremer, R. Jansen, B. A. M. van der Zeijst, and L. M. Schouls

2393-2401

Natural Genetic Competence in *Bacillus subtilis* Natto OK2

Sayaka Ashikaga, Hideaki Nanamiya, Yoshiaki Ohashi, and Fujio Kawamura

2411-2415

The Stability Region of the Large Virulence Plasmid of *Shigella flexneri* Encodes an Efficient Postsegregational Killing System

Sameera Sayeed, Lucretia Reaves, Lyndsay Radnedge, and Stuart Austin

2416-2421

Action of RNase II and Polynucleotide Phosphorylase against RNAs Containing Stem-Loops of Defined Structure

Catherine Spickler and George A. Mackie

2422-2427

A σ^{54} Activator Protein Necessary for Spore Differentiation within the Fruiting Body of *Myxococcus xanthus*

Lisa Gorski, Thomas Gronewold, and Dale Kaiser

2438-2444

Efficient Targeted Mutagenesis in *Borrelia burgdorferi*

James L. Bono, Abdallah F. Elias, John J. Kupko III, Brian Stevenson, Kit Tilly, and Patricia Rosa

2445-2452

Regions of RNase E Important for 5'-End-Dependent RNA Cleavage and Autoregulated Synthesis

Xunqing Jiang, Alexis Diwa, and Joel G. Belasco

2468-2475

Distribution of Twelve Linear Extrachromosomal DNAs in Natural Isolates of Lyme Disease Spirochetes

Nanette Palmer, Claire Fraser, and Sherwood Casjens

2476-2480

Genome Plasticity among Related *Lactococcus* Strains: Identification of Genetic Events Associated with Macrorestriction Polymorphisms

Pascal Le Bourgeois, Marie-Line Daveran-Mingot, and Paul Ritzenthaler

2481-2491

Effects of *bfp* Mutations on Biogenesis of Functional Enteropathogenic *Escherichia coli* Type IV Pili

Ravi P. Anantha, Kelly D. Stone, and Michael S. Donnenberg

2498-2506

Role of Ger Proteins in Nutrient and Nonnutrient Triggering of Spore Germination in *Bacillus subtilis*

Madan Paidhungat and Peter Setlow

2513-2519

Continued on following page

The Trimethylamine Methyltransferase Gene and Multiple Dimethylamine Methyltransferase Genes of *Methanosarcina barkeri* Contain In-Frame and Read-Through Amber Codons

DnaA, the Initiator of *Escherichia coli* Chromosomal Replication, Is Located at the Cell Membrane

Generation of Dominant Selectable Markers for Resistance to Pseudomonic Acid by Cloning and Mutagenesis of the *ileS* Gene from the Archaeon *Methanosarcina barkeri* Fusaro

The *yvaJ* Gene of *Bacillus subtilis* Encodes a 3'-to-5' Exoribonuclease and Is Not Essential in a Strain Lacking Polynucleotide Phosphorylase

H-NS Is a Repressor of the *Proteus mirabilis* Urease Transcriptional Activator Gene *ureR*

Negative Regulation of Expression of the *Bacillus megaterium bmlP1* Gene by the *bmlP1* 3' Flanking Region

Expression of UGA-Containing *Mycoplasma* Genes in *Bacillus subtilis*

Equivalence of Lauric Acid and Glycerol Monolaurate as Inhibitors of Signal Transduction in *Staphylococcus aureus*

A Common Regulator for the Operons Encoding the Enzymes Involved in D-Galactarate, D-Glucarate, and D-Glycerate Utilization in *Escherichia coli*

PLASMIDS AND TRANSPOSONS

A New IS4 Family Insertion Sequence, IS4*Bsu*1, Responsible for Genetic Instability of Poly- γ -Glutamic Acid Production in *Bacillus subtilis*

Dimers of π Protein Bind the A+T-Rich Region of the R6K γ Origin near the Leading-Strand Synthesis Start Sites: Regulatory Implications

High Spontaneous Mutation Rate in the Hyperthermophilic Archaeon *Sulfolobus solfataricus* Is Mediated by Transposable Elements

EUKARYOTIC CELLS

A Rac Homolog Is Required for Induction of Hyphal Growth in the Dimorphic Yeast *Yarrowia lipolytica*

Calcofluor Antifungal Action Depends on Chitin and a Functional High-Osmolarity Glycerol Response (HOG) Pathway: Evidence for a Physiological Role of the *Saccharomyces cerevisiae* HOG Pathway under Noninducing Conditions

An *n*-Alkane-Responsive Promoter Element Found in the Gene Encoding the Peroxisomal Protein of *Candida tropicalis* Does Not Contain a C₆ Zinc Cluster DNA-Binding Motif

CELL SURFACES

Functional Expression in *Escherichia coli* and Membrane Topology of Porin HopE, a Member of a Large Family of Conserved Proteins in *Helicobacter pylori*

Insertion Mutagenesis and Membrane Topology Model of the *Pseudomonas aeruginosa* Outer Membrane Protein OprM

Ligi Paul, Donald J. Ferguson, Jr., and Joseph A. Krzycki 2520-2529

Gillian Newman and Elliott Crooke 2604-2610

Paolo Boccazzi, Jun Kai Zhang, and William W. Metcalf 2611-2618

Irina A. Oussenko and David H. Bechhofer 2639-2642

Christopher Coker, Olubunmi O. Bakare, and Harry L. T. Mobley 2649-2653

Gwo-Chyuan Shaw, Yi-Huang Hsueh, Chi-Chang Sung, Yuan-Shou Chen, and Chang-Hsiesh Liu 2654-2659

T. R. Kannan and Joel B. Baseman 2664-2667

Alexey Ruzin and Richard P. Novick 2668-2671

Rafael Monterrubio, Laura Baldoma, Nuria Obradors, Juan Aguilar, and Josefa Badia 2672-2674

Toshiro Nagai, Lam-Son Phan Tran, Yasuhiro Inatsu, and Yoshifumi Itoh 2387-2392

Ricardo Krüger and Marcin Filutowicz 2461-2467

Erika Martusewitsch, Christoph W. Sensen, and Christa Schleper 2574-2581

Cleofe A. R. Hurtado, Jean-Marie Beckerich, Claude Gaillardin, and Richard A. Rachubinski 2376-2386

L. J. García-Rodríguez, A. Durán, and C. Roncero 2428-2437

Tamotsu Kanai, Akihiro Hara, Naoki Kanayama, Mitsuyoshi Ueda, and Atsuo Tanaka 2492-2497

Jim Bina, Manjeet Bains, and Robert E. W. Hancock 2370-2375

Kendy K. Y. Wong and Robert E. W. Hancock 2402-2410

Continued on following page

A Single Amino Acid Substitution in a Mannosyltransferase, WbdA, Converts the *Escherichia coli* O9 Polysaccharide into O9a: Generation of a New O-Serotype Group

A Novel Sphingoglycolipid Containing Galacturonic Acid and 2-Hydroxy Fatty Acid in Cellular Lipids of *Sphingomonas yanoikuyae*

Nobuo Kido and Hidemitsu Kobayashi

2567–2573

Takashi Naka, Nagatoshi Fujiwara, Eiko Yabuuchi, Matsumi Doe, Kazuo Kobayashi, Yoshiko Kato, and Ikuya Yano

2660–2663

PHYSIOLOGY AND METABOLISM

Evidence for Na⁺ Influx via the NtpJ Protein of the KtrII K⁺ Uptake System in *Enterococcus hirae*

Kinetics and Substrate Specificity of Membrane-Reconstituted Peptide Transporter DtpT of *Lactococcus lactis*

Osmotic and Chill Activation of Glycine Betaine Porter II in *Listeria monocytogenes* Membrane Vesicles

Phosphorylation of HPr by the Bifunctional HPr Kinase/P-Ser-HPr Phosphatase from *Lactobacillus casei* Controls Catabolite Repression and Inducer Exclusion but Not Inducer Expulsion

Mutation of *ndh* Genes Leads to Inhibition of CO₂ Uptake Rather than HCO₃[−] Uptake in *Synechocystis* sp. Strain PCC 6803

ADP-Ribosylation of Variants of *Azotobacter vinelandii* Dinitrogenase Reductase by *Rhodospirillum rubrum* Dinitrogenase Reductase ADP-Ribosyltransferase

The GacS Sensor Kinase Regulates Alginate and Poly-β-Hydroxybutyrate Production in *Azotobacter vinelandii*

Heterologous Expression of Bacterial Epoxyalkane:Coenzyme M Transferase and Inducible Coenzyme M Biosynthesis in *Xanthobacter* Strain Py2 and *Rhodococcus rhodochrous* B276

Site-Specific Serine Incorporation by Lif and Epr into Positions 3 and 5 of the Staphylococcal Peptidoglycan Interpeptide Bridge

Miyuki Kawano, Ryoko Abuki, Kazuei Igarashi, and Yoshimi Kakinuma

2507–2512

Gang Fang, Wil N. Konings, and Bert Poolman

2530–2535

Paul N. M. Gerhardt, Linda Tombras Smith, and Gary M. Smith

2544–2550

Valérie Dossonnet, Vicente Monedero, Monique Zagorec, Anne Galinier, Gaspar Pérez-Martínez, and Josef Deutscher

2582–2590

Hiroshi Ohkawa, G. Dean Price, Murray R. Badger, and Teruo Ogawa

2591–2596

Sandra K. Grunwald, Matthew J. Ryle, William N. Lanzilotta, and Paul W. Ludden

2597–2603

Miguel Castañeda, Josefina Guzmán, Soledad Moreno, and Guadalupe Espín

2624–2628

Jonathan G. Krum and Scott A. Ensign

2629–2634

Kerstin Ehlert, Martin Tschierske, Claudio Mori, Werner Schröder, and Brigitte Berger-Bächi

2635–2638

ENZYMES AND PROTEINS

Peptidase E, a Peptidase Specific for N-Terminal Aspartic Dipeptides, Is a Serine Hydrolase

Purification and Characterization of the Alanine Aminotransferase from the Hyperthermophilic Archaeon *Pyrococcus furiosus* and Its Role in Alanine Production

Structural Modeling and Site-Directed Mutagenesis of the Actinorhodin β-Ketoacyl-Acyl Carrier Protein Synthase

A C-Terminal Disulfide Bridge in Pediocin-Like Bacteriocins Renders Bacteriocin Activity Less Temperature Dependent and Is a Major Determinant of the Antimicrobial Spectrum

Rachel A. L. Lassy and Charles G. Miller

2536–2543

Donald E. Ward, Servé W. M. Kengen, John van der Oost, and Willem M. de Vos

2559–2566

Min He, Mustafa Varoglu, and David H. Sherman

2619–2623

Gunnar Fimland, Line Johnsen, Lars Axelsson, May B. Brurberg, Ingolf F. Nes, Vincent G. H. Eijsink, and Jon Nissen-Meyer

2643–2648

Cover photograph (Copyright © 2000, American Society for Microbiology. All Rights Reserved.): Corn and the soil bacterium *Pseudomonas putida* KT2440 have been used as a model system to study the genetic basis of bacterial attachment to plant seeds. Isolation and characterization of *P. putida* mutants defective in adhesion to corn seeds have uncovered novel functions in this strain and suggest the existence of different, partially overlapping pathways for bacterial attachment to either biotic or abiotic surfaces. (See related article on page 2363.)

An *n*-Alkane-Responsive Promoter Element Found in the Gene Encoding the Peroxisomal Protein of *Candida tropicalis* Does Not Contain a C₆ Zinc Cluster DNA-Binding Motif

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Received 22 October 1999/Accepted 9 February 2000

When an asporogenic diploid yeast, *Candida tropicalis*, is cultivated on *n*-alkane, the expression of the genes encoding enzymes of the peroxisomal β -oxidation pathway is highly induced. An upstream activation sequence (UAS) which can induce transcription in response to *n*-alkane (UAS_{ALK}) was identified on the promoter region of the peroxisomal 3-ketoacyl coenzyme A (CoA) thiolase gene of *C. tropicalis* (CT-T3A). The 29-bp region (from –289 to –261) present upstream of the TATA sequence was sufficient to induce *n*-alkane-dependent expression of a reporter gene. Besides *n*-alkane, UAS_{ALK}-dependent gene expression also occurred in the cells grown on oleic acid. Several kinds of mutant UAS_{ALK} were constructed and tested for their UAS activity. It was clarified that the important nucleotides for UAS_{ALK} activity were located within 10-bp region from –273 to –264 (5'-TCCTGCACAC-3'). This region did not contain a CGG triplet and therefore differed from the sequence of the oleate-response element (ORE), which is a UAS found on the promoter region of 3-ketoacyl-CoA thiolase gene of *Saccharomyces cerevisiae*. Similar sequences to UAS_{ALK} were also found on several peroxisomal enzyme-encoding genes of *C. tropicalis*.

Candida tropicalis (strain pK233) is an asporogenic diploid yeast, which can utilize *n*-alkanes as the sole carbon and energy source. During utilization of *n*-alkanes or fatty acids, a profound development of peroxisomes occurs in the cells, which is a major characteristic of this yeast (26). Enzymes localized in peroxisomes, such as the enzymes of the fatty acid β -oxidation pathway and of the glyoxylate pathway, are also induced along with the peroxisome proliferation (14, 37).

Thiolase is an enzyme which catalyzes the final step of the β -oxidation pathway. There are three thiolase isozymes in *n*-alkane-grown *C. tropicalis*: two acetoacetyl coenzyme A (CoA) thiolases (thiolase I), one of which is localized in cytosol (Cs-thiolase I) and one of which is localized in the peroxisome (Ps-thiolase I), and one peroxisomal 3-ketoacyl-CoA thiolase (thiolase III) (17–19). Only Cs-thiolase I is found in the cells grown on glucose. Cs-thiolase I and Ps-thiolase I are encoded by the same pair of alleles (CT-T1A and CT-T1B) (9, 16), and expression of the genes is highly induced on *n*-alkane, whereas low but finite expression occurs in cells grown on glucose (10). Thiolase III is encoded by another pair of alleles (CT-T3A and CT-T3B) (10), and their expression is highly induced on *n*-alkane but completely repressed on glucose.

In *Saccharomyces cerevisiae*, induction of peroxisomal 3-ketoacyl-CoA thiolase (encoded by *FOX3/POT1*) is mediated via an upstream activation sequence (UAS) called the oleate response element (ORE) (3, 12, 30, 31). ORE also exists on the upstream regions of genes encoding enzymes relating to the β -oxidation pathway (*FOX1* and *FOX2*) and fatty acid metabolism (*SPS19* and *ECI1*) and proteins relating to peroxisomal biogenesis (*PEX1* and *PEX11*) (13). The transcriptional acti-

vation through ORE occurs by the binding of a heterodimeric protein complex consisting of Oaf1p and Oaf2p/Pip2p (12, 13, 22, 30, 31). Interestingly, *OAF2/PIP2* itself is also regulated by ORE whereas *OAF1* is not (12, 13, 31). However, the effect of this difference in the regulation mechanisms is not clear.

The molecular mechanism underlying the induction of peroxisomal enzymes or peroxisome itself is unclear for *C. tropicalis*, because no appropriate host-vector system has been available. Recently, using *ura3* derivatives of *C. tropicalis*, we have developed a transformation system for introducing exogenous DNA into the genomic DNA of *C. tropicalis* (9). We have also cloned an autonomously replicating sequence (ARS) from *C. tropicalis*, which enabled us to introduce exogenous DNA into *C. tropicalis* with a form of episomal vector (6).

In this study, using the transformation procedure and the episomal vector system developed for *C. tropicalis*, we have identified a UAS, which can induce transcription in response to *n*-alkane (designated UAS_{ALK}), on the promoter region of CT-T3A. In comparing its sequence with that of ORE, the possibility was suggested that the molecular mechanism inducing peroxisomal 3-ketoacyl-CoA thiolase in *C. tropicalis* was essentially different from the ORE-mediated induction mechanism in *S. cerevisiae*.

MATERIALS AND METHODS

Strains and media. *C. tropicalis* SU-2 (ATCC 20913) (*ura3a/ura3b*) (5), derived from *C. tropicalis* pK233 (ATCC 20336), was used as a host strain for transformation. *Escherichia coli* strain DH5 α (29) was used for gene manipulation.

C. tropicalis was cultivated aerobically at 30°C in a medium containing glucose (16.5 g/liter), *n*-alkane mixture (C₁₀ to C₁₃; 10 ml/liter), oleic acid (5 ml/liter), glycerol (20 g/liter), sodium acetate (13.6 g/liter), sodium propionate (10 g/liter), or sodium butyrate (11 g/liter) as the sole carbon source (15, 39). The pH was adjusted to 5.2 for glucose, *n*-alkane, oleic acid, and glycerol media or to 6.0 for acetate, propionate and butyrate media. Tween 80 (0.5 ml/liter) was added to the *n*-alkane and oleic acid media. The basic composition of the medium was as follows: 5.0 g of NH₄H₂PO₄, 2.5 g of KH₂PO₄, 1.0 g of MgSO₄ · 7H₂O, 0.02 g of FeCl₃ · 6H₂O, and 1.0 ml of corn steep liquor per liter of tap water (39).

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TABLE 1. Oligonucleotides used in this study

Name	Sequence
PRT3AJ-1	5'-CATGCTCGAGGTGTTGAATATGTGC-3'
T3(-550S)	5'-TAGGTCGACCCGCGGTATCAACCATCGTCC-3'
T3(-473S)	5'-TCTGTCGACATTTGGTGGGTCTGCCCCC-3'
T3(-407S)	5'-CACGTCGACACATCCCGCTTAGTTGCGGG-3'
T3(-382S)	5'-CGGGTCGACGAACGTGTATTTCCCGTAG-3'
T3(-343S)	5'-GTCGTCGACTAGTCACCCGCTTCTGCC-3'
T3(-310S)	5'-GGTGTGCACTCAAAGCTGGCATAAATG-3'
T3(-289S)	5'-ATAAGTCGACAAAAAAGCAGCATCCTGCACACAAC-3'
T3(-281S)	5'-CGAAGTCGACGCACAGCATCCTGCACACAAC-3'
T3(-270S)	5'-ACAGTCGACTGCACACAACCCTGCTCAG-3'
T3(-230S)	5'-TGGGTCGACAGAAAAACCTCGGCTTAAACC-3'
T3(-310X)	5'-CCAGTCGAGAGGCAACCCAGAATGGCAG-3'
T3(-289X)	5'-CTTCTCGAGCGTCATTTATGCCAGCTTTGA-3'
T3(-260X)	5'-CTGCTCGAGGTTGTGTGCAGGATGCTGTGC-3'
T3UAS(M1-2)	5'-GGGCTCGAGGTTGTGTGCAGGATGCTATGCTTTTTTTTG-3'
T3UAS(M2-2)	5'-GGGCTCGAGGTTGTGTGCAGGATAATGTGCTTTTTTTTG-3'
T3UAS(M3-2)	5'-GGGCTCGAGGTTGTGTGCAGGATAATGTGCTTTTTTTTG-3'
T3UAS(M4-2)	5'-GGGCTCGAGGTTGTGTGCAGGATAATGTGCTTTTTTTTG-3'
T3UAS(M5-2)	5'-GGGCTCGAGGTTGTGTGCAGGATAATGTGCTTTTTTTTG-3'
T3UAS(M6-2)	5'-GGGCTCGAGGTTGTGTGCAGGATAATGTGCTTTTTTTTG-3'
T3UAS(M1-3)	5'-AAAAGTCGACAAAAAAGCATAGCATCCTGCACACAAC-3'
T3UAS(M2-3)	5'-AAAAGTCGACAAAAAAGCATATATCCTGCACACAAC-3'
T3UAS(M3-3)	5'-AAAAGTCGACAAAAAAGCATATTTGCACACAAC-3'
T3UAS(M4-3)	5'-AAAAGTCGACAAAAAAGCATATCCTTTACACAAC-3'
T3UAS(M5-3)	5'-AAAAGTCGACAAAAAAGCATATCCTGCACACAAC-3'
T3UAS(M6-3)	5'-AAAAGTCGACAAAAAAGCATATCCTGCACACAAC-3'
T3UAS(WTB-1)	5'-AAAAGTCGACAAAAAAGCATATCCTGCACACAAC-3'
T3UAS(WTB-2)	5'-AGGGCTCGAGGTCGTGTGCAGGACGCCGTGTTTTTTTG-3'

Plasmid construction. *Lac4* encoding *Kluyveromyces fragilis* β -galactosidase was amplified using primers 5'-AACTGTCGACTATGCTTGCCTTATTCCTGA G-3' and 5'-CTGTCTCGAGCTTAACGGTCTAATCGTTAATCAG-3'. The genomic DNA of *K. fragilis* IFO1267 (ATCC8585) was used as a template DNA. The amplified *Lac4* fragment cut with *Sal*I and *Xho*I was inserted into the *Sal*I site of pUC-URA3, in which the 1.7-kbp *C. tropicalis* *URA3* was inserted into pUC19 (11), and the subclone was named pUL4. The ARS of *C. tropicalis* 1098 was amplified using primers 5'-AAAAGTCGACCAATTTCCCGGAAAAGT GCCAC-3' and 5'-AAAAGTCGACGGTTAATGTCTATGATAATAATGGT TTC-3', with pUCNUA1 (6) as a template DNA. Bluescript II(SK+) cut with *Ssp*I was filled in using T4 DNA polymerase and joined with an *Xho*I linker (named Bluescript-Xh), and the amplified ARS fragment cut by *Sal*I was inserted into the *Xho*I site of Bluescript-Xh (named Bluescript-ARS). Bluescript-ARS was cut with *Kpn*I, treated with T4 DNA polymerase (blunting), digested with *Sal*I, and a 1.4-kbp fragment containing ARS was eluted. This fragment was ligated with the *Sal*I-*Sma*I fragment of pUL4 containing *C. tropicalis* *URA3* and *LAC4*, to make pUAL4.

All deletion fragments were prepared either by PCR using pT37Bg (11) as a template or by annealing of two oligonucleotides. All the oligonucleotides used in this study are listed in Table 1. PCR was performed using primer PRT3AJ-1 and one of the following primers: T3(-550S), T3(-473S), T3(-407S), T3(-382S), T3(-343S), T3(-310S), T3(-289S), T3(-270S), or T3(-230S). Each amplified fragment was cut with *Sal*I and *Xho*I and inserted into the *Sal*I site of pUAL4 to construct plasmid pUTA550, pUTA473, pUTA407, pUTA382, pUTA343, pUTA310, pUTA289, pUTA270, and pUTA230, respectively. To construct plasmids pUTA311R, pUTA290R, and pUTA261R, PCR was performed with primer T3(-550S) and plus primer T3(-310X), T3(-289X), and T3(-260X), respectively. The amplified fragment cut with *Sal*I and *Xho*I was inserted into the *Sal*I site of pUTA230. Plasmids pUTA03F, pUTA03R, pUTA04F, and pUTA05F were constructed by the same method as above using the following set of primers: T3(-310S) and T3(-260X) for pUTA03F and pUTA03R, T3(-310S) and T3(-289X) for pUTA04F, and T3(-289S) and T3(-260X) for pUTA05F.

To construct pUTA11, pUTA12, pUTA13, pUTA14, pUTA15, pUTA16, pUTA17, and pUTA18, two complementary oligonucleotides [T3UAS(M1-2) and T3UAS(M1-3) for pUTA11, T3UAS(M2-2) and T3UAS(M2-3) for pUTA12, T3UAS(M3-2) and T3UAS(M3-3) for pUTA13, T3UAS(M4-2) and T3UAS(M4-3) for pUTA14, T3UAS(M5-2) and T3UAS(M5-3) for pUTA15, T3UAS(M6-2) and T3UAS(M6-3) for pUTA16, T3(-281S) and T3(-260X) for pUTA17, and T3UAS(WTB-1) and T3UAS(WTB-2) for pUTA18] were an-

nealed. The annealed fragments were filled in with the Klenow fragment, cut with *Sal*I and *Xho*I, and introduced into the *Sal*I site of pUTA230.

The nucleotide sequences of all the deletion fragments were checked using ABI DNA sequencer model 373.

β -Galactosidase assay. β -Galactosidase activity was determined by measuring the hydrolysis of 4-methylumbelliferyl- β -D-galactopyranoside (MUG; Molecular Probes) (2, 42). Enzyme solution (50 μ l) in Z buffer (940 μ l) (24) was incubated at 30°C for 1 min, 10 mM MUG solution (10 μ l) was added, and the increase in fluorescence was measured with a Hitachi fluorophotometer model 650-10S (excitation, 360 nm; emission, 449 nm). 7-Hydroxy-4-methylcoumarin (Molecular Probes) dissolved in 100 mM sodium phosphate buffer (pH 7.0) was used as the reference standard. All activities are the mean values of at least two experiments.

Other methods. Transformation of *C. tropicalis* was carried out by electroporation (1,000 V, 25 μ F, and 201 Ω) (11). The protein concentration was assayed by the Bradford method using bovine serum albumin as the standard (1).

Nucleotide sequence accession number. Nucleotide sequence data of the promoter region of *CT-3A* and *CT-3B* will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB025647 and AB025648, respectively.

RESULTS

To evaluate the activity of promoter elements to induce transcription in *C. tropicalis*, pUAL4, a shuttle vector which can replicate in both *E. coli* and *C. tropicalis*, was first constructed by the method described in Materials and Methods. pUAL4 contains an ARS from *C. tropicalis* (6), *URA3* of *C. tropicalis* (11), and *LAC4* encoding β -galactosidase of *K. fragilis* (27). In *Candida* yeasts, *LAC4* instead of *LacZ* has usually been used as the source of the β -galactosidase gene (20, 21, 23), because several *Candida* yeasts translate the CUG codon as Ser instead of Leu, and *LAC4* contains fewer CUG codons than *LacZ* does (3 for *LAC4* and 53 for *LacZ*). A multicloning site was introduced before the translation initiation codon of

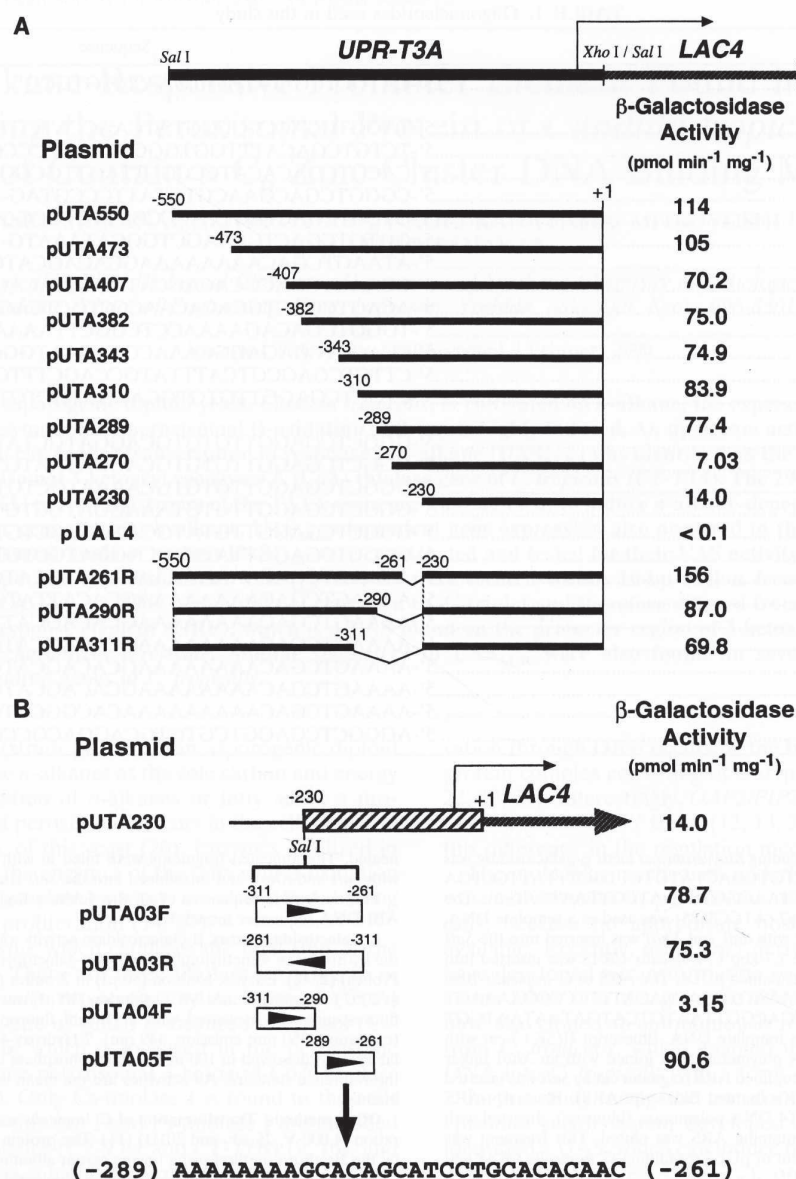


FIG. 1. Deletion fragments of the *CT-T3A* promoter and β -galactosidase activity in cells grown on *n*-alkane. The β -galactosidase activity after 24 h on *n*-alkane is shown (initial optical density at 570 nm = 0.2). Arrowheads indicate the direction of inserted fragments in panel B.

LAC4 so that the promoter sequence to be tested could be inserted.

The nucleotide sequences of the upstream regions of *CT-T3A* and *CT-T3B* (about 1.5 kbp) were determined. A 550-bp upstream region of *CT-T3A* (from -1 to -550; *UPR-T3A*) was introduced into pUAL4, and the resulting plasmid (pUTA550) was transformed into *C. tropicalis* SU-2. The transformant was then grown on either glucose or *n*-alkane as the sole carbon source, and the intracellular β -galactosidase activity was measured. β -Galactosidase activity in the glucose-grown cells was almost negligible (less than 0.1 pmol min⁻¹ mg⁻¹), while over 1,000 times more β -galactosidase activity was detected for the *n*-alkane-grown cells (Fig. 1A). This result indicated that a

550-bp *UPR-T3A* contained a sufficient region(s) to induce transcription in response to *n*-alkane.

A series of deletion fragments of *UPR-T3A* were constructed (pUTA550 to pUTA230), and their abilities to induce transcription by *n*-alkane were compared (Fig. 1A). When grown on glucose, all deletion mutants showed no detectable β -galactosidase activity (less than 0.1 pmol min⁻¹ mg⁻¹). In the cells grown on *n*-alkane, significant levels of β -galactosidase activity were detected from pUTA550 to pUTA289. The β -Galactosidase activity dropped sharply between pUTA289 and pUTA270, suggesting the existence of a UAS around -270 to -289. Three internal deletion mutants (pUTA261R, pUTA290R, and pUTA311R) were also constructed in which

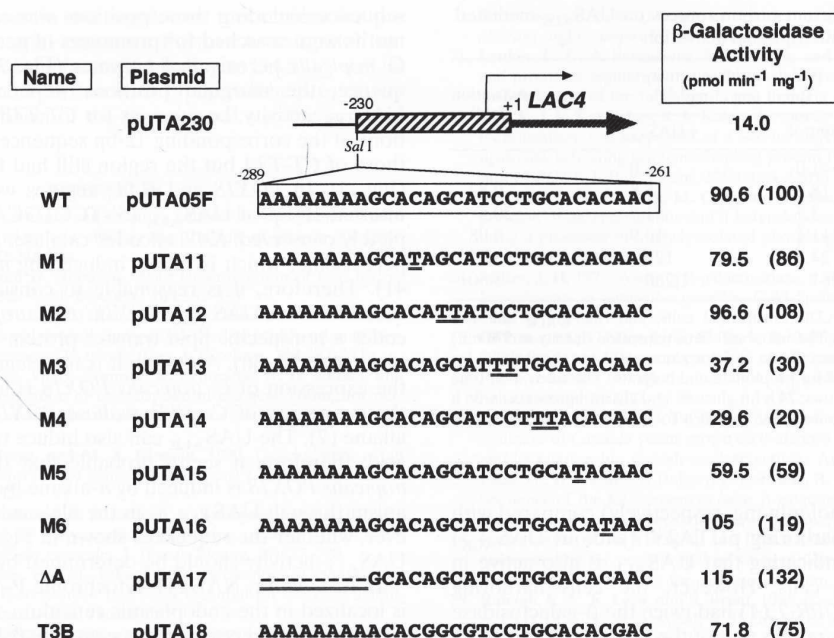


FIG. 2. UAS activity of UAS_{ALK} and its mutants on *n*-alkane. The nucleotides different from those of wild-type (WT) UAS_{ALK} are double underlined. The nucleotides in pUTA18 different from those of wild-type UAS_{ALK} are underlined. The number in parentheses indicates the UAS-dependent transcription inducing activity, where the activity of wild-type UAS_{ALK} was set as 100.

the region between -231 to -260, between -231 to -289, or between -231 to -310 was deleted, respectively (Fig. 1A). The relatively higher activities detected for pUTA261R than for pUTA290R and pUTA311R might be explained by the existence of upstream repression sequence (URS) in the region between -231 and -260. The putative URS between -231 and -260 and the UAS between -270 and -289 would be present. A noticeable sequence for URS could not be detected in the sequence between -231 and -260: 5'-CCTGCTCAGT GTGACAGGTGGTGGTGTAAAT-3'.

To determine the region functioning as the UAS, the following plasmids were constructed in which the sequence between -311 and -261 was inserted into the *Sal*I site of pUTA230 (pUTA03F) (Fig. 1B). pUTA230, which contained the TATA sequence of *UPR-T3A*, did not have UAS activity by itself (Fig. 1A). β-Galactosidase activity in pUTA03F-transformed cells grown on *n*-alkane was significantly higher than that in pUTA230-transformed cells (Fig. 1B). Moreover, pUTA03R, in which the same sequence was inserted in the opposite direction to pUTA03F, also showed a significant increase in β-galactosidase activity. On the other hand, when grown on glucose, neither pUTA03F-transformed nor pUTA03R-transformed cells, together with pUTA230-transformed cells, showed β-galactosidase activity (data not shown). These results demonstrated the presence of an *n*-alkane-responsive UAS (designated UAS_{ALK}) in the region between -311 and -261. Further deletion analysis indicated that the 29-bp region between -289 and -261 contained sufficient sequences for UAS_{ALK} (pUTA05F in Fig. 1B).

To find the important nucleotide sequences inside this 29-bp region, a series of point mutations were introduced. First, six kinds of mutants (M1 to M6) were made in which one or two adjacent guanine and/or cytosine nucleotides were changed into thymine nucleotides, and their UAS activities were compared (Fig. 2). Mutants M1, M2, and M6 had almost compa-

table (over 80%) UAS activity to the wild-type UAS_{ALK}. On the other hand, mutants M3, M4, and M5 had lower UAS activity, showing 30, 20, and 59% of the wild-type activity, respectively. Moreover, a mutant, in which the adenine stretch located between -289 and -282 was deleted (ΔA) had a UAS_{ALK} activity comparable to the wild-type activity. These results indicate that nucleotide positions changed in mutant M4 (positions -268 and -269) are particularly important for UAS_{ALK} activity. Furthermore, the upstream sequence of *CT-T3B* corresponding to the region of UAS_{ALK} was tested for its UAS activity, and the result indicated that this region also had sufficient UAS activity (75% of that of *CT-T3A*).

Expression of thiolase III is induced not only by *n*-alkane but also by other carbon sources, such as butyrate (10, 17). Therefore, it is of interest to examine whether UAS_{ALK} induces gene expression by other carbon sources. Cells transformed with pUTA05F were cultivated on glucose, glycerol, *n*-alkane, acetate, propionate, or butyrate as the sole carbon source, and intracellular β-galactosidase activities were compared (Table 2). Cells transformed with pUTA230 were used as a control for estimating UAS_{ALK}-independent transcriptional activation. When the cells were cultivated on glucose, glycerol, or acetate, no UAS_{ALK}-dependent increase of β-galactosidase activity was observed. On the other hand, in cells grown on propionate or butyrate as well as on *n*-alkane, a UAS_{ALK}-dependent increase of β-galactosidase activity was observed. These results demonstrate that induction of the expression of the thiolase III gene in the propionate- or butyrate-grown cells occurs, at least in part, by a common mechanism that acts through UAS_{ALK}.

In *S. cerevisiae*, the transcription of 3-ketoacyl-CoA thiolase encoded by *FOX3/POT1* is induced by oleic acid (4, 8). Accordingly, UAS_{ALK} was tested to find whether it can induce transcription by oleic acid. β-Galactosidase activity was increased in the oleic acid-grown cells harboring pUTA05F (with UAS_{ALK}) or pUTA17 (with ΔA derivative of UAS_{ALK}) (activ-

TABLE 2. Effect of different carbon sources on UAS_{ALK}-mediated transcriptional activation

Carbon source	β-Galactosidase activity (pmol min ⁻¹ mg ⁻¹) ^a		Activation (fold)
	Control	+UAS _{ALK}	
Glucose	<1.0	<1.0	
Glycerol	18.2	15.8	0.87
<i>n</i> -Alkane	3.5	71.7	20.5
Acetate	11.1	10.9	0.98
Propionate	24.7	127	5.15
Butyrate	381	1,260	3.30

^a Control indicates pUTA230-transformed cells, and +UAS_{ALK} indicates pUTA05F-transformed cells. The initial cell density (optical density at 570 nm) for each carbon source was as follows: 0.05 for glucose, 0.1 for glycerol, 0.2 for *n*-alkane and acetate, and 0.4 for propionate and butyrate. The cultivation time for each condition was as follows: 24 h for glucose- and glycerol-grown cells, 36 h for *n*-alkane- and acetate-grown cells, and 88 h for propionate- and butyrate-grown cells.

ity of 22.4 and 26.0 pmol/min/mg, respectively) compared with the activity in those harboring pUTA230 (without UAS_{ALK}) (2.51 pmol/min/mg), indicating that UAS_{ALK} is also active in the oleic acid-grown cells. However, the cells harboring pUTA550 (with total *UPR-T34*) had twice the β-galactosidase activity (45.0 pmol/min/mg) as that of the pUTA05F-harboring cells, demonstrating the possible existence of another UAS(s) in addition to the UAS_{ALK} in response to oleic acid on *UPR-T34*.

DISCUSSION

We have identified the UAS sequence that responds to *n*-alkane (UAS_{ALK}) in the *n*-alkane-assimilating yeast *C. tropicalis*. Deletion analysis delimited the sequence of UAS_{ALK} within 29 bp (from positions -289 to -261 of *UPR-T34*). Further mutation analysis showed that the nucleotides that were changed in the M4 mutant (positions -268 and -269) were the most critical for the UAS_{ALK} activity. The 12-bp

sequence including these positions was selected, and similar motifs were searched for promoters of genes encoding several *C. tropicalis* peroxisomal enzymes (Fig. 3). In this 12-bp sequence, the marginal positions were not crucial for the UAS_{ALK} activity, because, as for *CT-T3B*, the marginal positions of the corresponding 12-bp sequence were different from those of *CT-T3A* but the region still had the UAS_{ALK} activity (Fig. 2). In *POX18* and *KAT*, regions were found in which internal 10 bp of UAS_{ALK} (5'-TCCTGCACAC-3') was completely conserved. *KAT* encodes catalase, a marker enzyme of peroxisome, which is highly induced by *n*-alkane (28, 34, 40, 41). Therefore, it is reasonable to consider that this region functions as a UAS_{ALK}. *POX18* of *C. tropicalis* (*POX18*) encodes a nonspecific lipid transfer protein which is induced by oleic acid (35, 36). Although it is not clear at present whether the expression of *C. tropicalis* *POX18* is induced by *n*-alkane, the expression of *Candida maltosa* *POX18* is inducible by *n*-alkane (7). The UAS_{ALK} can also induce transcription by oleic acid; therefore, it seems probable that the expression of *C. tropicalis* *POX18* is induced by *n*-alkane by the common mechanism through UAS_{ALK} as in the oleic acid-grown cells. However, whether the sequences shown in Fig. 3 actually have the UAS_{ALK} activity should be determined by experiments.

In *C. maltosa*, NADPH-cytochrome P-450 reductase which is localized in the endoplasmic reticulum, is highly induced by *n*-alkane. By a reporter gene assay, the 0.47-kbp 5' noncoding region of the gene was shown to be sufficient for the induction on *n*-tetradecane (25). We compared this region with UAS_{ALK}. Although no region closely homologous to UAS_{ALK} was detected, there were two CACAT motifs, the pentanucleotide often found in the 5'-noncoding regions of P-450alk genes, encoding cytochrome P-450, of *C. maltosa* (25). UAS_{ALK} of *C. tropicalis* contains a CACACA sequence at its 3' end. Physiological and genetic evidence suggests that *C. tropicalis* and *C. maltosa* are closely related strains. Therefore, it is likely that the similar activation mechanisms are present in these yeasts, in which the CACA motif sequence might play an important role.

Besides *n*-alkane and oleic acid, UAS_{ALK}-dependent transcription also occurred with butyrate and propionate. These carbon sources can also induce the proliferation of peroxisomes in *C. tropicalis* (17, 39). *n*-Alkane or long-chain fatty acids incorporated in *C. tropicalis* are degraded through the fatty acid β-oxidation system localized in peroxisomes and are ultimately converted into butyryl- or propionyl-CoA. Therefore, the results of this study show that these short-chain fatty acids and/or their derivatives might be a true inducer(s) that causes UAS_{ALK}-dependent transcriptional activation.

In *S. cerevisiae*, the consensus sequence of ORE is suggested as inverted repeats of the CGG triplet with a spacing of 15 to 18 nucleotides (CGG_N₁₅₋₁₈CCG) (13, 30). The CGG triplet repeat is the common consensus sequence for the C₆ zinc cluster family of fungal transcriptional regulators, such as Gal4p (32, 38). In fact, a heterodimeric protein complex consisting of Oaf1p and Oaf2p/Pip2p, both of which have a C₆ zinc cluster motif, was identified as the factor that binds to ORE (12, 22, 30, 31). On the other hand, UAS_{ALK} does not contain the CGG triplet repeat. This fact strongly suggests that the ORE-like regulation mechanism does not exist in *C. tropicalis*. Sloots et al. (33) investigated the regulation mechanism of the gene (*HDE*) encoding the peroxisomal bifunctional enzyme of *C. tropicalis* by introducing its upstream region into *S. cerevisiae*. By deletion analysis, they identified an oleic acid-responsive region located between positions -393 and -341. When this region was compared with UAS_{ALK}, no homologous sequence was observed, which supports our notion that these two

		M3	M4	M5	M6	Score
CT-T3A	[-274]	ATCCTGCACACA				[-263] (12/12)
POX18	[-297]	CTCCTGCACACA				[-308] (11/12)
CT-T3B	[-268]	CTCCTGCACACG				[-257] (10/12)
KAT	[-998]	CTCCTGCACACt				[-1009] (10/12)
POX4	[-264]	TTTtTGCACACA				[-253] (09/12)
BFE	[-226]	ACACTGCACACA				[-237] (09/12)
CAT	[-272]	CTGCTGCACACA				[-261] (09/12)
POX2	[-432]	CTCCTGCtCtCC				[-443] (08/12)
BFE	[-253]	CACTTGCACACt				[-242] (08/12)
KAT	[-378]	GCCAGCACAAC				[-367] (07/12)
POX2	[-251]	GGCAATGCACGCT				[-240] (07/12)

FIG. 3. Nucleotide sequences similar to UAS_{ALK} found on promoters of *C. tropicalis* peroxisomal enzyme genes. *POX2* and *POX4* encode acyl-CoA oxidase (accession numbers for *POX2* and *POX4* are M18259 and M12160, respectively); *BFE* encodes the bifunctional enzyme (X57854); *CAT* encodes carnitine acetyltransferase (D84549) (unpublished data); *KAT* encodes catalase (X13978, E01922) (unpublished data); *POX18* encodes nonspecific lipid transfer protein (X53633 and M24440). The score indicates the number of the nucleotides that are the same as those of *CT-T3A*. The positions of changed nucleotides in the UAS_{ALK} mutants (M3, M4, M5, and M6) are indicated above the sequences. Numbers on both sides of the sequences indicate the distance relative to the translational start codon.

yeasts have differences in the regulation mechanism for the induction of peroxisomal enzymes. Further investigation involving the isolation of the factor(s) binding to UAS_{ALK} and its characterization by comparison with Pip2p will help to clarify the activation mechanism of the peroxisomal enzyme genes in *C. tropicalis*.

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