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Promoter Analysis and Differential Expression of the *Candida rugosa* Lipase Gene Family in Response to Culture Conditions

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Five lipase genes have been identified and sequenced from *Candida rugosa*. However, as the sequences of *LIP* multigene family are extremely closely related, it is difficult to characterize the expression spectrum of *LIP* genes. In the present work we have cloned, sequenced, and analyzed the promoters of these five *LIP* isoform genes, and several putative transcriptional elements including oleate response element (ORE) and upstream activation sequence 1 (UAS1) were identified. A quantitative real-time RT-PCR method was developed for determining the differential expression of *C. rugosa* lipase family genes in response to various environmental and nutritional factors. While all five *LIP* genes display significant changes in mRNA expression under oleic acid and/or olive oil culture conditions, *LIP2* showed the strongest induction (456-fold) in response to oleic acid. *LIP* transcription and promoter regulation were studied by assaying the β-galactosidase activities of promoter−*lacZ* fusions in *Saccharomyces cerevisiae*. Three of the *LIP* genes, *LIP3*, *LIP4*, and *LIP5*, showed significant induction by oleic acid, and their ORE and UAS1 elements are essential for induction by oleic acid. Together, this suggests that the multiple lipase expression profiles may be due to differential transcriptional regulation of the *LIP* genes in response to environment or nutritional factors.

KEYWORDS: ***Candida rugosa*; lipase; promoter; differential expression; real-time RT-PCR**

INTRODUCTION

Extracellular lipases (EC 3.1.1.3) produced by the nonsporgenic yeast *Candida rugosa* (formerly *Candida cylindracea*) are very important enzymes which have been frequently used in many biotechnological applications, including the production of fatty acids, synthesis of various esters, and kinetic resolution of racemic mixtures (*1–8*). Crude enzyme is widely used for biotransformations and biocatalysis (acylation and deacylation) reactions to produce useful materials. However, crude commercial CRLs obtained from various sources show remarkable variations in catalytic efficiency, substrate specificity, and enantioselectivity (*9*). Following our initial discovery of three distinct forms of lipolytic enzymes with different substrate specificities and thermostabilities in a commercial *C. rugosa* lipase preparation (*10*), other enzyme forms were detected in subsequent studies (*11–14*). We previously discovered that three commercial *C. rugosa* lipase preparations differed in protein composition, which accounted for the difference in their catalytic efficiency and specificity (*15*). These variations were related to the different culture conditions used in preparation, since the presence of different inducers in the culture media changed the pattern of enzyme forms and therefore the specificity and thermostability of crude lipase preparations (*15*).

Multiple forms of extracellular lipases in fungi (*16–20*) have been attributed to changes in gene expression, variable glycosylation, partial proteolysis, or other posttranslational modifications. Five lipase-encoding genomic sequences (*LIP1* to *LIP5*) from *C. rugosa* have been characterized (*21, 22*); regulation of gene expression has been suggested to be the most probable mechanism for the enzyme multiplicity. Because of the similarity in molecular size and high sequence homology among the five sequences, purification and identification of the lipase gene products remain challenging from the cultures of *C. rugosa* (*23*). We previously measured the differential expression level of the five lipase genes in YM media containing olive oil, oleic acid, or Tween 20 by competitive RT-PCR (*24*).

In the present work, a quantitative real-time RT-PCR method was developed for determining the differential expression of *C. rugosa* lipase (CRL) family genes in response to various

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Table 1. Primers Used for Lipase Promoter Cloning

promoter	primer	oligonucleotide sequence (5′ to 3′)
LIP1	L1P-GSP1	CTGCATGCAAGACGGGCCGTAGAAGTGAACTTC
	L1P-GSP2	AATGAGCAGGAGCGCAAGAGCGAGCTC
	F-L1P[a]	GGAATTCCCCACACTTGCACATGC
	R-L1P[a]	CAAGCTTGGGGAGCGAGTGGGGAG
LIP2	L2P-GSP1	CTGCATGCAAGACGGGCCGTAAGAGGTAAACTGC
	L2P-GSP2	GTGGCCGTGGGGGCTGCCGCCACCGCA
	F-L2P[a]	TCGAATTCGGGTCTTTTTGGAGAT
	R-L2P[a]	CAAGCTTGGAGAGACTGGAGTGAG
LIP3	F-LIP5-3′	TGTAGACAATGTACATGTGC
	R-LIP3-5′	AGCGCAAGAGCGAGCTTCAT
	F-L3P[a]	AGAATTCTAGACAATGTACATGTGC
	R-L3P[a]	TCAAGCTTGGGGAGCGAGCAGGTGAG
	F-L3PORE-1[a]	TGAATTCAATACCGGACCGCCAGACAG
	F-L3PORE-2[a]	TTCCCGCGTGCAGGA▼GAGTTTGCAACTGTT
	R-L3PORE-2[a]	AACAGTTGCAAACTC▼TCCTGCACGCGGGAA
	F-L3PUAS1-1[a]	ATGCACCCCTTGGAC▼CGTGCGCGCATCCAC
	R-L3PUAS1-1[a]	GTGGATGCGCGCACG▼GTCCAAGGGGTGCAT
LIP4	F-LIP3-3′	TGTAGTTGTGTATGTGCCAG
	R-LIP4-5′	AGTACAAGAGCGAGCTTCAT
	F-L4P[a]	CTGAATTCTAGTTGTGTATGTGCC
	R-L4P[a]	TCAAGCTTGGGGAGTGAGCTGGAGC
	F-L4PUAS1-1[a]	GGCACATTGGGCAGT▼AGCACCCGGGGGCAT
	R-L4PUAS1-1[a]	ATGCCCCCGGGTGCT▼ACTGCCCAATGTGCC
	F-L4PUAS1-2[a]	ACACCATATCTACCA▼TAGCAATCAGAGCCC
	R-L4PUAS1-2[a]	GGGCTCTGATTGCTA▼TGGTAGATATGGTGT
	F-L4PORE[a]	AATGATCACGCGCCG▼TAAAAGCCCGGGCTA
	R-L4PORE[a]	TAGCCCGGGCTTTTA▼CGGCGCGTGATCATT
LIP5	L5P-GSP1	CCTTGGGGAGGTTTGGCTCGTAGGTGCC
	L5P-GSP2	GTTCTGCTGCATGCAAGACGGACCGTACGC
	F-L5P[a]	TGAATTCAACGACGTGAGGTTGACG
	R-L5P[a]	CAAGCTTGGGGAGCGAGCAGGTGAG
	F-L5PORE[a]	TCAGGCACACGCAAA▼ATGATCCGCACATGT
	R-L5PORE[a]	ACATGTGCGGATCAT▼TTTGCGTGTGCCTGA

[a] Primers used for cloning promoters into β-gal reporter vector Yep356. The introduced *Eco*RI/*Hin*dIII restriction sites are underlined. The symbols (▼) indicate the positions of deleted ORE or UAS1 elements.

environmental and nutritional factors. We have cloned, sequenced, and analyzed the promoters of the five LIP isoform genes. Putative oleate response element (ORE) and upstream activation sequence 1 (UAS1), which are related to oleic acid induction, were found in CRL promoters. Elements in several *LIP* promoters were sufficient to confer oleate induction of a promoterless *lacZ* reporter gene in *Saccharomyces cerevisiae*.

MATERIALS AND METHODS

Microorganism and Medium. *C. rugosa* (ATCC 14830) were cultured in basal mineral solution (*25*) at 30 °C for 24 h in a orbital shaker at 250 rpm. Cells were then collected and cultured under various conditions (1% Tween 20, 1% oleic acid, 1% olive oil, or 1% ethanol instead of glucose as a carbon source) or subjected to nutrient starvation (without nitrogen or carbon source) at 30 °C for 24 h (*24, 26*). *S. cerevisiae* INVSc1 (Invitrogen, Carlsbad, CA) were cultured in synthetic complete medium (6.7 g/L yeast nitrogen base without amino acids, 25 μg/mL tryptophan, 25 μg/mL histidine, 25 μg/mL leucine, 25 μg/mL uracil, and 2% glucose) or synthetic drop-out medium (6.7 g/L yeast nitrogen base without amino acids, 25 μg/mL tryptophan, 25 μg/mL histidine, 25 μg/mL leucine, and 2% glucose) for selection of transformed cells.

Cloning and Sequencing of LIP Isoform Promoters. Genomic DNA was extracted from *C. rugosa* using the Wizard Genomic DNA purification kit according to the manufacturer's instructions (Promega, Madison, WI). According to the localization of lipase genes on *C. rugosa* chromosomes, *LIP3*, *LIP4*, and *LIP5* are clustered together (*27*). The *LIP3* promoter was cloned by PCR from genomic DNA using the forward primer annealing to the 3′ end of *LIP5* (F-LIP5-3′) and the reverse primer annealing to the 5′ end of *LIP3* (R-LIP3-5′); the *LIP4* promoter was cloned using the forward primer to the 3′ end of *LIP3* (F-LIP3-3′) and the reverse primer to the 5′ end of *LIP4* (R-LIP4-5′) (**Table 1**).

LIP1, *LIP2*, and *LIP5* promoters were cloned using the Universal GenomeWalker kit (Clontech, Palo Alto, CA). Total genomic DNA was separately digested with different restriction enzymes, and the digested products were ligated to a GenomeWalker adaptor and used as templates for PCR according to the manufacturer's instructions. The primary PCR was performed with adaptor primer AP1 and gene-specific primer GSP1. The secondary PCR was performed with primers AP2 and GSP2 (**Table 1**). The PCR products of five *LIP* promoters were cloned into pGEMT easy vector (Promega) and sequenced by a DNA sequencing kit (Perkin-Elmer Applied Biosystems).

RNA Isolation. *C. rugosa* cells were collected by centrifugation (3000*g* at 4 °C for 5 min) and frozen in liquid nitrogen. RNA samples were extracted with the Trizol extraction kit (Gibco BRL, Long Island, NY) following the manufacturer's instructions. No genomic DNA contamination was observed in the RNA samples with the RNA-PCR method (*24*).

Real-Time PCR and Data Analysis. Total RNA (5 μg) was reverse transcribed into first-strand cDNA in a 20 μL reaction mixture by using oligo(dT) primers, deoxyribonucleoside triphosphates, and SuperScript II enzyme as specified by the manufacturer (Life Technologies, Gaithersburg, MD). After reverse transcription, RNase H (2 units; Life Technologies) was added, and the mixture was incubated at 37 °C for 20 min. The sequences of *C. rugosa LIP1*, *LIP2*, *LIP3*, *LIP4*, and *LIP5* (*21, 22*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) genes (*24*) were evaluated using the Primer Express software (Perkin-Elmer Applied Biosystems, Foster City, CA) to design appropriate primer sets for real-time PCR. The oligonucleotide primers were synthesized by GENSET Singapore Biotech. Pte Ltd. Real-time PCR was performed on an ABI PRISM 7700 sequence detector and analyzed using the ABI PRISM 7700 sequence detector software 1.7 (Perkin-Elmer Applied Biosystems). The amplifications of all five *LIP*s and *GAPDH* were carried out in 50 μL reactions containing 1× SYBR Green PCR master mix with AmpliTaq Gold DNA polymerase (Perkin-Elmer Applied Biosystems), 3 μL of cDNA reaction, and *LIP* specific primer sets. The amplification program was 40 cycles of a two-step PCR (95 °C for 15 s and 60 °C for 60 s) after an initial activation (95 °C for 10 min) of DNA polymerase. The PCR reactions were subjected to heat dissociation protocol to verify that the SYBR green dye detected only one PCR product. Following the final cycle of the real-time PCR the melting temperature profile was as follows: 95 °C for 15 s, 60 °C for 20 s, ramped from 60 to 95 °C in 19 min 59 s, and 95 °C for 15 s. Triplicate cDNAs from each sample were amplified using primers for five *LIP* genes and *GAPDH*. Three independent assays with the same cDNA samples and primers for five *LIP*s and *GAPDH* were performed and the values measured for each individual experiment.

After SYBR Green PCR amplification, data acquisition and subsequent data analyses were performed using the ABI PRISM 7700 sequence detector software 1.7. The PCR cycle at which a statistically significant increase in the ΔR_n (the fluorescence of SYBR Green relative to that of internal passive dye, ROX) is first detected is called the threshold cycle (C_T). The ΔC_T is the difference between the mean C_T value of the *LIP* and the endogenous control, *GAPDH*. The $\Delta\Delta C_T$ is the difference between the mean ΔC_T value under the specific culture condition and the calibrator culture condition in glucose. The amount of target, normalized to an endogenous reference and relative to a calibrator, is given by $2^{-\Delta\Delta C_T}$. Derivation of the $2^{-\Delta\Delta C_T}$ equation has been described in Applied Biosystems User Bulletin No. 2 (P/N 4303859). Thus, experimental samples could be expressed as an *n*-fold difference relative to the calibrator. For the real-time assays with the $2^{-\Delta\Delta C_T}$ method, the amplification efficiency of the target gene and internal control gene was tested by plotting the amount of the input template versus the ΔC_T, where a slope of approximately zero demonstrates that the efficiencies were equal.

Analysis of LIP Promoter Activities. The five *LIP* promoters were obtained from genomic DNA by PCR with their specific primers F-L1P−L5P and R-L1P−L5P primers (**Table 1**). The internal deletions of ORE or UAS1 in LIP3, LIP4, and LIP5 promoters were conducted by using the overlap-extension PCR method. Taking LIP3 promoter ΔUAS1 for example, two fragments were first amplified using primer sets F-L3P/R-L3PUAS1-1 and F-L3PUAS1-1/R-L3P, and then the LIP3 promoter ΔUAS1 fragment was generated by overlap-extension PCR using the mixture of these two fragments as templates. LIP3 promoter ΔORE-1 was generated directly by PCR using primers

F-L3PORE-1 and R-L3P. PCR products were digested on the flanking *Eco*RI/*Hin*dIII restriction sites that were introduced via PCR and ligated into the shuttle vector Yep356 (*28*). Vector DNA was transformed into *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA) by the $CaCl_2$ method and extracted from ampicillin-resistant colonies by the alkali lysis method (*29*). The plasmids (10 μg) harboring the *LIP* promoters were then transformed into *S. cerevisiae* INVSc1 by electroporation. High-voltage pulses (0.54 kV) were delivered to 100 μL samples in 0.2 cm electrode gap cuvettes using a Gene Pulser apparatus supplied with the Pulse Controller (Bio-Rad Laboratories, Hercules, CA). Transformants were plated on synthetic drop-out medium plates for selection.

Transformed cells were cultured in synthetic drop-out medium at 30 °C for 24 h in an orbital shaker at 250 rpm. Cells were then collected, and culture conditions were modified to a synthetic drop-out medium containing 2% glucose or 2% oleic acid as a carbon source for β-gal induction. The time course of β-gal activity in *S. cerevisiae* has also been done (see Supporting Information, Supplementary 3). It revealed that the β-gal expression reached to the highest level at 12−24 h induction. Therefore, we chose the single time point (24 h) for β-gal induction. Cells were then assayed for β-galatosidase activity using a β-gal assay kit (Invitrogen) according to the manufacturer's instructions.

RESULTS

Cloning, Sequencing, and Bioinformatic Analysis of Five LIP Isoform Promoters. The five *LIP* promoters were cloned by the genome-walking method (for *LIP1*, *LIP2*, and *LIP5* promoters) or PCR method using specific primers (for *LIP3* and *LIP4* promoters). The nucleotide sequences of the *LIP1* promoter (852 bp), *LIP2* promoter (1216 bp), *LIP3* promoter (1156 bp), *LIP4* promoter (1459 bp), and *LIP5* promoter (1625 bp) were submitted to the GenBank under accession numbers DQ984519, DQ984520, DQ984521, DQ984522, and DQ984523, respectively. Analysis of 3′ regions indicates the location of TATAA and CAAT-like boxes as previously reported (*30*) (see Supporting Information, Supplementary 1). To identify further putative *cis*-acting regulatory elements, we submitted these five promoter sequences to the Kyoto Encyclopedia of Genes and Genomes (KEGG) web service (http://motif.genome.jp/). Several putative elements were found including stress-related and nutrition-related elements, such as stress response element (STRE) and response elements for pH-response regulator (PacC), positive-acting nitrogen regulator (NIT2), and alcohol dehydrogenase gene regulator 1 (ADR1).

Notably, each of the five *C. rugosa LIP* promoters harbors many putative ADR1 response elements (20–32 elements), and particularly interesting is the role for ADR1 in fatty acid degradation and peroxisome proliferation in *S. cerevisiae* (*3, 31*). Two ADR1 elements, named upstream activation sequence (UAS1), are required to be in inverted orientation for transcriptional activation to occur (*32*) and act synergistically with another transcriptional regulative element, the oleate response element (ORE) (*3, 31, 33*). UAS1 and ORE govern the upregulation of many related genes in the presence of oleic acid in *S. cerevisiae*, and their consensus sequences have been refined (*33, 34*). We analyzed *LIP* promoters and identified putative ORE elements in all five *C. rugosa* lipase promoters and putative UAS1 elements in *LIP2*, *LIP3*, and *LIP4* promoters (**Table 2**). To further demonstrate the *LIP* transcription level, a quantitative real-time RT-PCR method was performed for determining the differential expression.

Validation Analysis for Real-Time PCR. To validate real-time PCR for the quantitation of the mRNA of *C. rugosa* lipases, *C. rugosa* total RNA was extracted and reverse transcribed to cDNA for testing. To detect the expression level of the five *LIP* genes, we designed specific primers which amplify the *LIP1*, *LIP2*, *LIP3*, *LIP4*, *LIP5*, and *GAPDH* to result in amplification products of 72, 88, 110, 84, 74, and 101 bps, respectively. No amplification was observed in the control which has no template or primer, and no cross-reactivity with any other *LIP* homologue was observed for each primer set by analyzing the heat dissociation of PCR products. The amplification efficiencies of all amplicons are similar (see Supporting Information, Supplementary 2).

Table 2. Analysis of the Promoter Regions of *C. rugosa LIP* Genes

promoter	ORE (CGGN$_3$TNRN$_{8-12}$CCG)	USA1 (CYCCRDN$_{4-36}$HYGGRG)
LIP1	−591[a] CGGN$_{11}$CCAGGCCCG	not found
LIP2	−205 CCGN$_{11}$TAACCACCG	−108 CCCCGTN$_{26}$TTGGAG
LIP3	−1103 CCGN$_8$TGAGCCCCG	−945 CTCCAAN$_{20}$CTGGGG
	−329 CGGN$_9$TGACTGCCG	
LIP4	−285 CGGCTGTTGN$_9$CCG	−1043 CCCCAGN$_{32}$ACGGGG
		−664 CCCCAAN$_{20}$CTGGGG
LIP5	−441 CGGCATTTGN$_{10}$CC	not found

[a] Number of bp 5′ to the ATG start codon.

Real-Time PCR Analysis. The ΔC_T values of *LIPs* were obtained by normalizing the mean C_T value of each *LIP* to that of an internal control *GAPDH* gene and were represented as the mRNA expression levels of *LIP*s. Many inducers, such as natural oils, fatty acids, fatty esters, sterols, bile salts, Tween, and Span, have been used as additives for *C. rugosa* lipase production. Since glucose is a carbon source in common culturing medium, culture medium containing glucose was used as a control to calibrator for culture conditions. The $\Delta\Delta C_T$ values indicate the fold change in mRNA expression relative to the control cells cultured in glucose. Significant changes in expression were detected when *C. rugosa* was cultured under different conditions (**Table 3**). *LIP1* and *LIP2* were expressed at higher levels when cultured in Tween 20 (8.7-fold and 44.1-fold) compared to glucose-containing medium. This supports our previous report that Tween 20 induces *C. rugosa* lipases (*15*). All five *LIPs* were induced by olive oil and oleic acid in mineral solution (without glucose). Remarkably, *LIP2* was induced in oleic acid (455.7-fold) and olive oil (253.1-fold) conditions and showed higher expression than other *LIPs*. The magnitude degree of fold changes in the present study could be due to the stringent regulation of LIP2 expression (see further discussion in latter section). *LIP2*, *LIP4*, and *LIP5* were induced under carbon starvation but not in nitrogen starvation. Together, the diverse expressional profiles reveal the differential transcription regulation of the *LIP* gene family.

Analysis of CRL LIP Isoform Promoters in *S. cerevisiae.* Since all *LIPs* were transcriptionally induced by oleic acid, we next analyzed if the ORE and/or UAS1 promoter elements in *LIP*promoters contribute to this transcriptional regulation. We first cloned each of the five *LIP* promoters upstream of a lacZ reporter gene and transformed the vectors to *S. cerevisiae* to analyze the induction of transcription in response to oleic acid and olive oil by measuring β-galatosidase activity. β-Galatosidase regulated by *LIP3*, *LIP4*, and *LIP5* promoters was induced 1.6-, 2-, and 10-fold when cells were cultured in media containing oleic acid as compared with glucose (**Figure 1**). These three *LIP* genes were also induced when cultured with olive oil (2.4-, 2.2-, and 6.7-fold) compared with glucose. These results suggest that, except for *LIP1* and *LIP2*, the transcriptional regulation of *LIP* genes in *C. rugosa* also occurs in *S. cerevisiae*.

To further analyze if the putative transcriptional elements found in *LIP*promoters were necessary for induction, deletion

Table 3. Expression Levels of *C. rugosa* Lipases by Using the Comparative C_T Method

target mRNA	treatment	C_T	ΔC_T[a]	$\Delta\Delta C_T$[b]	fold induction[c]
lip1	glucose	25.88 ± 0.37	9.89 ± 0.65	0	1
	Tween 20	24.00 ± 0.22	6.77 ± 0.10	−3.13 ± 0.10	8.7 (8.2 ± 9.4)
	oleic acid	27.53 ± 0.15	8.85 ± 0.15	−1.05 ± 0.15	2.0 (1.9 ± 2.3)
	olive oil	23.84 ± 0.42	5.48 ± 0.20	−4.42 ± 0.20	21.3 (18.6 ± 24.5)
	nitrogen starvation	25.82 ± 0.19	8.20 ± 0.38	−1.69 ± 0.38	3.2 (2.5 ± 4.2)
	carbon starvation	28.57 ± 0.47	8.77 ± 0.11	−1.02 ± 0.11	2.0 (1.9 ± 2.2)
lip2	glucose	27.99 ± 0.44	12.01 ± 0.77	0	1
	Tween 20	23.77 ± 0.21	6.54 ± 0.38	−5.47 ± 0.38	44.1 (33.9 ± 57.6)
	oleic acid	21.86 ± 0.24	3.17 ± 0.23	−8.83 ± 0.23	455.7 (389.0 ± 534.0)
	olive oil	22.38 ± 0.60	4.02 ± 0.38	−7.98 ± 0.38	253.1 (194.3 ± 329.6)
	nitrogen starvation	27.78 ± 0.33	10.16 ± 0.19	−1.85 ± 0.19	3.6 (3.2 ± 4.1)
	carbon starvation	26.61 ± 0.94	6.92 ± 0.50	−4.44 ± 0.50	34.1 (24.2 ± 48.0)
lip3	glucose	32.71 ± 0.33	16.72 ± 0.74	0	1
	Tween 20	33.46 ± 0.45	16.23 ± 0.38	−0.5 ± 0.38	1.4 (1.1 ± 1.8)
	oleic acid	31.39 ± 0.49	12.70 ± 0.27	−4.02 ± 0.27	16.2 (13.5 ± 19.5)
	olive oil	30.29 ± 0.48	11.93 ± 0.38	−4.79 ± 0.38	27.7 (21.3 ± 36.0)
	nitrogen starvation	32.91 ± 0.43	15.29 ± 0.33	−1.43 ± 0.33	2.7 (2.1 ± 3.4)
	carbon starvation	34.05 ± 0.60	14.36 ± 0.33	−2.37 ± 0.33	5.2 (4.1 ± 6.5)
lip4	glucose	29.60 ± 0.93	13.62 ± 0.62	0	1
	Tween 20	32.66 ± 0.17	15.43 ± 0.26	1.18 ± 0.26	0.3 (0.2 ± 0.3)
	oleic acid	30.39 ± 0.29	11.70 ± 0.18	−1.92 ± 0.18	3.8 (3.3 ± 4.3)
	olive oil	31.75 ± 0.38	13.39 ± 0.16	−0.23 ± 0.16	1.2 (1.1 ± 1.3)
	nitrogen starvation	29.01 ± 0.28	11.39 ± 0.14	−2.23 ± 0.14	4.7 (4.3 ± 5.2)
	carbon starvation	30.04 ± 0.28	10.35 ± 0.32	−3.27 ± 0.32	9.7 (7.8 ± 12.0)
lip5	glucose	30.21 ± 0.46	14.23 ± 0.85	0	1
	Tween 20	32.69 ± 0.31	15.46 ± 0.49	1.24 ± 0.49	0.4 (0.3 ± 0.6)
	oleic acid	29.61 ± 0.18	10.92 ± 0.31	−3.30 ± 0.31	9.9 (8.0 ± 12.3)
	olive oil	28.75 ± 0.07	10.39 ± 0.29	−3.83 ± 0.29	14.3 (11.7 ± 17.5)
	nitrogen starvation	30.66 ± 0.47	13.04 ± 0.44	−1.18 ± 0.44	2.3 (1.7 ± 3.1)
	carbon starvation	30.34 ± 0.29	10.64 ± 0.18	−3.59 ± 0.18	12.0 (10.6 ± 13.7)
gapdh	glucose	15.98 ± 0.41			
	Tween 20	17.23 ± 0.18			
	oleic acid	18.69 ± 0.22			
	olive oil	18.36 ± 0.22			
	nitrogen starvation	17.62 ± 0.22			
	carbon starvation	19.70 ± 0.45			

[a] The ΔC_T value is determined by subtracting the average gapdh C_T value from the average lip C_T values. The standard deviation of the difference is calculated from the standard deviations of the lip and gapdh values. [b] The calculation of $\Delta\Delta C_T$ involves subtraction by the glucose treatment ΔC_T value. This is subtraction of an arbitrary constant, so the standard deviation of $\Delta\Delta C_T$ is the same as the standard deviation of the ΔC_T value. [c] The range given for fold induction relative to different treatment to glucose was determined by evaluating the expression: $2^{-\Delta\Delta C_T}$ with $\Delta\Delta C_T + s$ and $\Delta\Delta C_T - s$, where s = the standard deviation of the $\Delta\Delta C_T$ value.

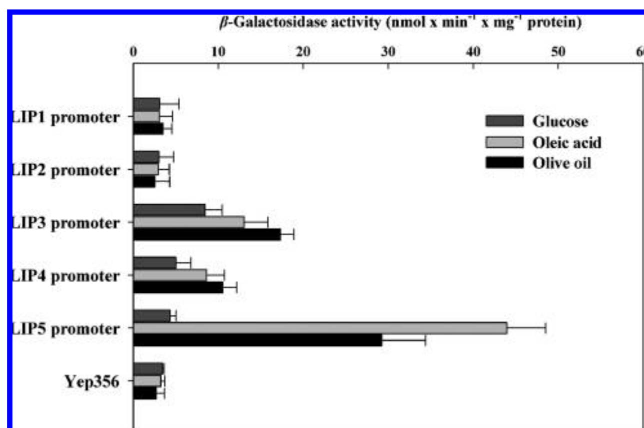

Figure 1. *LIP* promoter activities in response to culture conditions. Promoter activities of *LIP* genes were tested under glucose, oleic acid, and olive oil culture conditions. *S. cerevisiae* was transformed with reporter vectors derived from Yep356 plasmids, containing *lacZ* driven by individual promoters of *LIP* genes. Activities of the promoter are expressed in nanomoles of *o*-nitrophenyl β-D-galactopyranoside (ONPG) hydrolyzed per minute per milligram of protein.

constructs were generated and β-galatosidase activity was measured (**Figure 2**). Deletion of the upstream ORE and UAS1 regions in the *LIP3* promoter abolished the induction by oleic acid. Similarly, the upstream ORE and UAS1 in

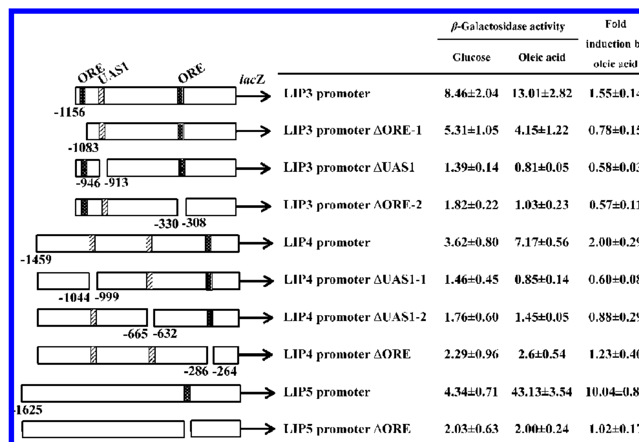

Figure 2. Deletion analysis of *LIP* promoter activity. The left panel depicts schematics of the wild-type and internal deletion constructs of *LIP* promoter fused to *lacZ* (narrow line arrow). The black and hatched bars represent putative ORE and UAS1 elements, respectively. The number above each line indicates the position with respect to the ATG start codon (A designated as +1). Each construct was transformed to *S. cerevisiae* grown under glucose or oleic acid conditions. Promoter activity was determined by assaying the β-galactosidase activity (nmol × min^{-1} × mg^{-1} of protein), and fold induction by oleic acid was calculated by comparing with the glucose culture condition.

LIP4 promoter were essential for full responsiveness and transcriptional induction. The *LIP5* promoter, with one putative ORE, was potently responsive to oleic acid in *S. cerevisiae*. As shown in **Figure 2**, deletion of ORE element compromised the transcriptional induction of the *LIP5* promoter in response to oleic acid.

DISCUSSION

Although many reports on the structure, function, and activities of the *C. rugosa* lipases have been published, little information regarding the factors and regulations that control their biosynthesis is available. Lipid-related substrates (oleic acid, for example) have been reported as an efficient and critical inducer on lipase production (*35*). The understanding of the oleic acid-induced mechanism, however, and expression profiles of five *LIP* genes is not complete. Here we sequenced *C. rugosa* lipase promoters and found many putative transcriptional control elements in these promoters. Likely, a complex mechanism involved in lipase production might exist, in which several aspects (such as inducers and fermentation conditions) may play an important role in terms of both quantity and quality.

Although the expression level of the mRNA transcript could be affected by many factors, such as promoter activity, upstream regulatory elements, and stability of the mRNA, changes of gene expression are more direct for a prolonged response. To further analyze the possible regulatory effects of these control elements on five *C. rugosa LIP* genes, we used the real-time RT-PCR method to measure the differential expression of five *LIP* mRNA transcripts. As shown in **Table 3**, the induction levels of five *LIP* genes were variant in different cultured conditions. Differential expression profiles demonstrate their distinct enzymatic functions in physiological metabolism. Especially, the lipid induction of *LIP* genes could be important in utilizing lipid nutrient for metabolism and gluconeogenesis.

In general, the expression levels of *LIP* genes in a medium containing glucose were lower than those in a medium containing other carbon sources (**Table 3**). Interestingly, the induction level in a medium without glucose was much higher than that we previously reported by adding an inducer in a glucose-containing medium (*24*). This suggests the depletion of glucose, a repressor for *LIP* genes, could activate the lipase expression. A rich glucose-containing medium has been reported to repress the production of lipase and affected the detection of induction levels (*36*). Furthermore, repression by glucose was dominant over oleic acid induction (*37*). This catabolite repression of glucose was especially significant for *LIP2* in the present work. We previously reported that *LIP2* was not induced significantly by oleic acid or olive oil in a glucose-containing YM medium (*24*), and high production of LIP2 has been shown in a basal medium without glucose (*38*). We demonstrated in the absence of glucose *LIP2* can be greatly induced by oleic acid or olive oil. These data suggest that gene expression of *LIP2* must be stringently regulated by glucose. The greater induction magnitude of *LIP2* than other *LIP*s could be due to the presence of some unknown strong enhancer elements in the *LIP2* promoter region. The basal medium we used in the present work has the advantage to control the culture condition and exclude the glucose effect for detecting the differential expression of five *LIP* genes.

When we transformed *S. cerevisiae* with five *LIP* promoters individually fused to the *lacZ* reporter gene, only those with the *LIP3*, *LIP4*, and *LIP5* promoters were activated by oleic acid or olive oil (**Figure 1**). The reason why *LIP1* and *LIP2* promoters do not show activity in *S. cerevisiae* may be that the regulatory pathways, which govern *LIP1* and *LIP2* promoters in *C. rugosa*, are not intact in *S. cerevisiae*. Moreover there might be additional factors required for the induction of *LIP1* and *LIP2* in *C. rugosa* that are not present in *S. cerevisiae*. *C. rugosa* and *S. cerevisiae* may use different regulatory systems to control *LIP1* and *LIP2* promoters. It was noted that the differential responses of *LIP3* and *LIP5* in *S. cerevisiae* are similar to real-time RT-PCR data in *C. rugosa*, while *LIP4* did not show a higher induction level by oleic acid in the β-gal assay. Therefore, the *LIP3*, *LIP4*, and *LIP5* promoters were functional in *S. cerevisiae*, and these reporter systems can be used to identify the potential control elements in *LIP* promoters.

The ORE and UAS1 regulatory elements found in *LIP3*, *LIP4*, and *LIP5* promoters are presumably responsible for regulating gene expression when oleic acid is the sole carbon source (**Figure 1**). The element-deletion experiments indicate that the ORE and UAS1 elements in the *LIP* promoters are essential for regulating their transcriptional responses to oleic acid (**Figure 2**). The exact induction mechanism of oleic acid remains unclear. A specific oleic acid binding domain of *S. cerevisiae* Oaf1p (ORE binding protein) was revealed (*39*). Although no such homologous transcription factors have been identified in *C. rugosa*, it is reasonable to expect that *C. rugosa* could have similar factors that activate *LIP* promoters in the presence of oleic acid. We also found that the *LIP5* promoter, which lacks a UAS1, was induced by oleic acid to a greater extent than *LIP3* and *LIP4* promoters, both of which contain ORE and UAS1 elements (**Table 2**). *PEX14*, *PEX25*, *IDP3*, and *DCI1*, all of which lack a UAS1, are all induced by oleic acid (*3, 40, 41*). Although it is still unclear how ADR1 (UAS1 binding protein) participates in the induction of UAS1-dependent ORE-regulated genes, the pleiotropic activator ADR1 is recognized as a regulator for carbon source metabolism-related genes. UAS1 might play an important role in modulating the induction of *LIP3* and *LIP4* promoters by oleic acid. *LIP3* promoter ΔUAS1 and *LIP4* promoter ΔUAS1 lost oleic acid-induced activities, and this suggests that they are UAS1-dependent ORE-regulated genes (**Figure 2**). The SPS19 and POX1 genes, which are similar to *LIP3* and *LIP4*, are UAS1-dependent ORE-regulated because they were not induced in an *adr1*Δmutant supplied with oleic acid (*3, 33*).

ORE transcriptional elements have been identified in genes functioning in the β-oxidation pathway and peroxisomal biogenesis. Here we identify a novel transcriptional regulation of ORE and UAS1 elements in controlling lipase production. These findings could elucidate the mechanism by which lipidic substrates induced lipase production in *C. rugosa.* The present work clearly showed that the expression profiles of *C. rugosa LIP* genes are altered by different culture conditions and are due to their differences in promoters with unique and various *cis* elements. Traditionally, the culture conditions in fermentation are optimized for maximal production of enzyme activity units. Our results indicate that quality is as important as quantity in enzyme preparations, since different culture conditions might result in production of heterogeneous compositions of the isozymes, which display different catalytic activities and specificities. By engineering the culture conditions, we can obtain enzyme preparations enriched in selected isozymes for particular biotechnological applications. In addition, the strong oleic acid-inducible promoters with ORE elements discovered in the present work could be very useful for driving other genes with industrial applications. Further studies on the promoter

regulatory mechanism and the promoter engineering are currently under way.

Supporting Information Available: Three figures showing nucleotide sequences and phylogenetic analysis of five CRL promoters, validation analysis of real-time PCR, and time course of LIP3, LIP4, and LIP5 promoter activities in *S. cerevisiae*. This material is available free of charge via the Internet at http://pubs.acs.org.

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