Analysis of the Gene Family Encoding Lipases in Candida rugosa by Competitive Reverse Transcription-PCR

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Synthesis of multiple extracellular lipases in Candida rugosa has been demonstrated. However, it is difficult to characterize the expression spectrum of lip genes, since the sequences of the lip multigene family are very closely related. A competitive reverse transcription-PCR assay was developed to quantify the expression of lip genes. In agreement with the protein profile, the abundance of lip mRNAs was found to be (in decreasing order) lip1, lip3, lip2, lip5, and lip4. To analyze the effects of different culture conditions, the transcript concentrations for these mRNA species were normalized relative to the values for gpd, encoding glyceraldehyde-3-phosphate dehydrogenase. In relative terms, lip1 and lip3 were highly and constitutively expressed (about 10 μg of total RNA) whereas the other inducible lip genes, especially lip4, showed significant changes in mRNA expression under different culture conditions. These results indicate that differential transcriptional control of lip genes results in multiple forms of lipase proteins.

Lipases (EC 3.1.1.3) from the nonsporogenic yeast Candida rugosa (formerly C. cylindracea) are very important industrial enzymes which have been widely used in biotechnological applications such as the production of fatty acid, synthesis of various esters, and kinetic resolution of racemic mixtures (10, 14, 22, 29, 40, 43, 44). Crude enzyme preparations are used in most applications, and enzymes from various suppliers have been reported to show variations in their catalytic efficiency and stereospecificity in various applications such as resolution of racemic 2-(4-hydroxyphenoxy) propionic acid (2). After our discovery of multiple enzyme forms with different substrate specificities and thermostabilities in a commercial C. rugosa lipase preparation (39), two to six enzyme forms were detected in subsequent studies (4, 9, 36, 37). More recently, we discovered that three commercial C. rugosa lipase preparations differed in protein composition, which accounted for the difference in their catalytic efficiency and specificity (5). This was related to the different culture conditions used. It was supported by the observation that different inducers in the culture media of C. rugosa changed the multiple form patterns and therefore the specificity and thermostability of crude lipase preparations (5).

The multiplicity of extracellular lipases in fungi (3a, 17–19, 32) has been attributed to a change in gene expression, variable glycosylation, partial proteolysis, or other posttranslational modification. After the cloning of five lipase genes (lip1 to lip5) from the C. rugosa genome (24, 26), a change in gene expression has been suggested to be the most probable mechanism for the enzyme multiplicity. However, the high similarity of same-sized deduced amino acid sequences in these mature proteins (66% identity and 84% similarity) makes it difficult to purify and identify the lipase gene products (27).

For highly related genes, the conventional methods in mRNA analysis are not specific and sensitive enough to distinguish and quantitate individual mRNAs. It is difficult to distinguish the transcription pattern of genes with a high degree of identity by Northern blot analysis. Although the nuclease protection assay has the ability to discriminate among closely related genes, this method, like Northern blotting, is not sensitive enough to detect small amounts of mRNA and permits only crude quantitation. The competitive reverse transcription-PCR (RT-PCR) technique (3, 12) may be a feasible alternative to obtain quantitative information on the highly related lip genes at the transcriptional level owing to its high sensitivity and specificity (35, 41).

Although the lip1 cDNA has been isolated from C. rugosa (21), there is no evidence that the other four genomic lipase-encoding sequences isolated are functional. Moreover, the molecular mechanisms of the individual lip gene regulation remain unclear. In this report, we describe a modification of the competitive RT-PCR method to detect and quantitate the five lip mRNA transcripts and thus confirm the functional expression of the five lip genes. By using this technique, it is possible to demonstrate the differential expression of the five lip genes in the presence of different inducers that are known to be able to increase C. rugosa lipase production (5, 13, 42).

MATERIALS AND METHODS

Microorganism and medium. C. rugosa ATCC 14830 was cultured in YM growth medium (0.5% yeast extract, 0.3% malt extract, 0.5% peptone, 1% dextrose) at 30°C for 24 h. The concentrations of additives are specified for the different experiments.

Bacterial transformation. Plasmid DNA was transformed into Escherichia coli TOP10 (Invitrogen) by the CaCl2 method and extracted from ampicillin-resistant colonies by the alkaline lysis method (38).

RNA preparation. Total RNA from C. rugosa was isolated by a modification of the method of Köhler and Domdey (23). Cultured cells (5 ml) were collected by centrifugation (3,000 × g at 4°C for 5 min), resuspended in 300 μl of sodium acetate buffer (50 mM sodium acetate, 10 mM EDTA [pH 5.0]), and then transferred to a 1.5-ml microtube containing 0.3 g of glass beads (pretreated with 1 M HCl and autoclaved). After two cycles of hot-phenol extraction, total RNA was collected by ethanol precipitation.

To eliminate contaminating genomic DNAs, two purification methods were used. (i) RNase-free DNase I (20 U; Promega) was mixed with 50 μg of the total RNA in RT buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl2, 10 mM
dithiothreitol (pH 8.3) and incubated at 37°C for 30 min in a total volume of 50 μl. The RNA was purified by phenol-chloroform extraction followed by ethanol precipitation. (ii) The RNA pellets obtained from the hot-phenol extraction were dissolved in 500 μl of prechilled denaturing solution (4 M guanidine thiocyanate, 42 mM sodium citrate, 0.83% N-lauroylsarcosine, 0.2 mM 2-mercaptoethanol), and 50 μl of 2 M sodium acetate (pH 4.0) was then added to provide acidity. After 500 μl of a mixed organic solvent (phenol-chloroform-isoamyl alcohol, 25:24:1) was added, the mixture was vigorously vortexed for 1 min and chilled on ice for 3 min. After centrifugation at 10,000 × g and 4°C for 15 min, the aqueous phase was transferred to a new microtube and RNA was precipitated twice with an equal volume of isopropanol at −20°C for 30 min.

**RT-PCR.** 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.). To increase the efficiency of PCR initiation, RNase H (2 U; Life Technologies) was added and the mixture was incubated at 37°C for 20 min. PCR amplification was performed in a 25-μl reaction volume containing 0.5 μl of RT reaction solution, each deoxyribonucleoside triphosphate at 100 μM, 5 pmol each of 5′ and 3′ primer, 10 mM Tris-HCl (pH 8.8), 1.5 mM MgCl₂, 50 mM KCl, 0.01% (wt/vol) gelatin, and 0.25 U of DynaZyme (Finnzymes Oy, Espoo, Finland). The reagents for RT and PCR were always prepared as a single reaction mixture and then divided among different tubes. PCR was carried out in an Omnigene thermal cycler (Hybaid, Teddington, United Kingdom) on the following cycle program: one cycle of 94°C for 3 min and 72°C for 1 min; 40 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 90 s; and a final 10-min extension step at 72°C.

**Cloning of template DNAs.** The full-length lipase-encoding cDNA fragments were obtained by RT-PCR with specific primers based on the published sequences (24, 26). The PCR products with created flanking restriction sites were digested with restriction enzymes and ligated into pET-23a (Novagen) or pGEMT vector (Promega). A schematic presentation of the plasmids is given in Fig. 1.

To obtain the *C. rugosa* gpd gene, consensus sequences were determined by aligning multiple related DNA sequences from a database. The partial *C. rugosa* gpd DNA fragment was obtained by RT-PCR with degenerative primers designed from the consensus sequences indicated in Table 1. This DNA fragment was then cloned into the pGEMT vector (Promega), and its sequence (327 bp) was determined on both strands.

**Competitive RT-PCR.** Competitive RT-PCR was conducted as previously described for RT-PCR, except that known concentrations of competitor DNA, an exogenous template as an internal PCR standard, were spiked into a series of PCR tubes containing constant amounts of cDNA generated from total RNA. The competitor DNA has the same primer recognition sites as the target and thus competes with the target for the same primers during the amplification. It is important to select the appropriate lip competitor DNAs and the specific primer sets used for competitive RT-PCR, since one primer set must amplify only one lipase gene among the highly related gene family. Competitor DNA vectors (Fig. 1) were constructed by restriction digestion within each lipase-encoding region followed by self-ligation; therefore, after competitive PCR, the products of target and competitor DNA could be distinguished by their size. The competitor DNAs were obtained by PCR with primer pairs present in the vectors. To remove PCR template and primers, the competitor DNA fragment was eluted after agarose electrophoresis. The specific primer set, as shown in Table 1, had a unique and specific 5′ primer and a common 3′ primer for each lipase gene. These primer sets were tested for specificity and efficiency by PCR (data not shown).

Determination of the quantity of the competitor DNA used to assess the amount of target cDNA is important for precise quantitation by competitive RT-PCR. An accurate quantity of the competitor DNA was determined by capillary gel electrophoresis (CGE). All CGE analyses were conducted on the
TABLE 1. Primers used for competitive RT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide sequence (5'-→3')a</th>
<th>Size (bp) of PCR productb</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIP3sp-5'</td>
<td>CGGTCGTCGTCGGAGCCAGGTTACAGC (180–207)</td>
<td>1,188 813</td>
</tr>
<tr>
<td>LIP2sp-5'</td>
<td>GAAGGTTGCGTCTGTTGTGGTCTGT (3–31)</td>
<td>1,365 782</td>
</tr>
<tr>
<td>LIP3sp-5'</td>
<td>TCCTGGCTCTGAGCGGCTGGCTGCC (20–45)</td>
<td>1,348 973</td>
</tr>
<tr>
<td>LIP4sp-5'</td>
<td>TACCTGGCTCTGTTGCGGTCGGCCGCG (17–45)</td>
<td>1,351 425</td>
</tr>
<tr>
<td>LIP5sp-5'</td>
<td>CCGCGAGCGAGCGGCGGCGGCGGCGG (152–179)</td>
<td>1,216 812</td>
</tr>
<tr>
<td>GPD-5'</td>
<td>GTGCGCGCGCGGCGGCGGCGGCGGCGG (1338–1367)</td>
<td>327 171</td>
</tr>
<tr>
<td>GPD-3'</td>
<td>GTARCCGCGGCGGCGGCGGCGGCGGCGG (305–327)</td>
<td></td>
</tr>
</tbody>
</table>

a Each specific 5' primer has a 3'-end nucleotide unique to a 3' sequence. The nucleotide numbers corresponding to the coding sequences of the lip genes are shown in parentheses. The portion of LIP-3' primer corresponding to lip2 is from bp 1335 to 1364. The symbols in the degenerate GPD primers are as follows: B for C, G or T; Y for C or T; S for C or G; and R for A or G. The GPD-5' and GPD-3' primers were used to clone the partial sequence of the gpd gene.

b The products were generated by using the respective 5' primers and the consensus 3' primer (LIP-3').

RESULTS

Validation of competitive PCR analysis. To ensure a successful analysis of gene expression within the highly conserved lip gene family, the quality of the RNA, the design of competitor DNAs and specific primer set, the choice of control gene, the quantitation of competitor DNAs, and the evaluation of the amplification efficiencies of the target and the competitor molecules, respectively, giving a 1.83-fold difference (very close to 2.0-fold). The correlation coefficients (r2) of the lines determined by least-square regression analysis were very close to 1. From mathematical considerations of competitive PCR (34), the slopes of the lines, ranging from 0.9 to 1.0, suggested that the amplification efficiencies of the target and the competitor were similar in the same reaction tube. The copy numbers of lip1 cDNA determined from the 125- and 62.5-ng total RNA were coamplified with twofold serial dilutions of the lip1 competitor. The copy numbers of lip1 transcript were calculated by determining the competition equivalence points in linear regression plots as described in Materials and Methods (Fig. 3B). The correlation coefficients (r2) of the lines determined by least-square regression analysis were very close to 1. From mathematical considerations of competitive PCR (34), the slopes of the lines, ranging from 0.9 to 1.0, suggested that the amplification efficiencies of the target and the competitor were similar in the same reaction tube. The copy numbers of lip1 cDNA determined from the 125- and 62.5-ng RNA plots (Fig. 3B) were 2.60 ± 0.02 × 106 and 1.42 ± 0.01 × 106 molecules, respectively, giving a 1.83-fold difference (very close to the predicted value of 2.0-fold). In conclusion, competitive PCR can be used to accurately analyze the lip gene expression in a quantitative manner.

Differential expression of C. rugosa lip genes under different culture conditions. The expression of the different lip genes in various culture conditions was examined by competitive RT-PCR with specific primers and running for 40 PCR cycles. As shown in Fig. 4, abundant amounts of lip1, lip3, and gpd transcripts were observed. The copy numbers of lip1, lip3, and gpd transcripts were calculated by determining the competition
Expression of lip1 gene by RT-PCR. C. rugosa was cultured in YM alone (lanes 1 to 3) or containing 1% olive oil (lane 4), 1% oleic acid (lane 5), or 1% Tween 20 (lane 6). RNA was isolated or treated by different methods as indicated. (A) RNA samples (5 μg per lane) were electrophoresed on a 1% native agarose gel and stained with ethidium bromide. Arrows indicate the positions of the 25S and 17S rRNA bands. (B) Genomic DNA contamination was detected by RNA-PCR with dIII DNA size markers. (C) Gene expression of lip1 was analyzed by RT-PCR with cDNA generated from related RNA as the template. M, HinIII DNA size markers.

FIG. 2. Analysis of RNA integrity, genomic DNA contamination, and lip gene expression by RT-PCR. C. rugosa was cultured in YM alone (lanes 1 to 3) or containing 1% olive oil (lane 4), 1% oleic acid (lane 5), or 1% Tween 20 (lane 6). RNA was isolated or treated by different methods as indicated. (A) RNA samples (5 μg per lane) were electrophoresed on a 1% native agarose gel and stained with ethidium bromide. Arrows indicate the positions of the 25S and 17S rRNA bands. (B) Genomic DNA contamination was detected by RNA-PCR with prepared RNA as the template and the lipase-specific primers (LIP1sp-5' and LIP3'-in Table 1). The position of lip1 is indicated by the arrow. (C) Gene expression of lip1 was analyzed by RT-PCR with cDNA generated from related RNA as the template. M, HinIII DNA size markers.

FIG. 3. Validation of competitive PCR analysis. (A) Titer determination of lip1 competitor with a constant amount of cDNA generated from RNA isolated from YM-cultured C. rugosa. Fivefold (lanes 1 to 6) (4 to 0.00128 amol) and twofold (lanes 7 to 12) (0.8 to 0.025 amol) serial dilutions of lip1 competitor were coamplified with a constant amount of cDNA. After 40 cycles of amplification, the products were resolved on a 1% agarose gel and stained with ethidium bromide. The positions of the 1,188-bp lip1 target (T) and 813-bp competitor (C) PCR products are indicated. Lane M contains a HinIII digest of λ DNA as a size marker. (B) Analysis of relative changes in lip1 target levels by competitive PCR. cDNA samples generated from 125 and 62.5 ng of total RNA were amplified in the presence of twofold dilutions of lip1 competitor (the same as in panel A). A total of 40 cycles of PCR and electrophoresis on a 1% agarose gel were performed. The gels were stained with SYBR Green I. The DNA products visualized by chemiluminescence were scanned and quantitatively analyzed as described in Materials and Methods. The open and solid circles denote data derived from 62.5 and 125 ng of RNA, respectively.

Expression of lip1 at 2.08 × 10^5 ± 0.02 × 10^5 molecules per μg of total RNA was observed when C. rugosa was cultured in YM, and the amount did not change after adding 1% olive oil or Tween 20. However, addition of oleic acid (1%) to the medium did reduce the number of lip1 transcripts by 44%. lip3 expressing 1.42 × 10^5 ± 0.03 × 10^5 molecules per μg of total RNA (68% of lip1 expression) was detected when C. rugosa was cultured in YM. When olive oil or oleic acid was added to the cultures, the lip3 transcripts increased by a significant 2.1-fold and 1.53-fold, respectively. In contrast, the lip3 transcripts decreased 12% when Tween 20 was added to the YM.

The levels of lip2, lip4, and lip5 were much lower (Fig. 5). Since the quantities of these three lip genes were too low to reach the plateau phase within 40 PCR cycles, it may not be possible to accurately quantitate them in regression plots. The EPs of various culture conditions in individual gene could be estimated from lanes with similar target/competitor ratios (Table 2). When C. rugosa was cultured in YM, the amounts of lip2, lip4, and lip5 expressed relative to lip1 were estimated to be 0.5, 0.1, and 0.4%, respectively. Olive oil also enhanced lip2, lip4, and lip5 expressions by about twofold, and oleic acid promoted the mRNA expression of lip4 and lip5 by four- and twofold, respectively. Only lip4 was highly induced by Tween 20, by a dramatic 7.9-fold.

DISCUSSION

Studies on the differential expression of C. rugosa lip gene have been hampered by the difficulties of quantitation of individual mRNAs due to the highly related DNA sequences among this gene family. In the present study, we developed a sensitive and specific competitive RT-PCR method to quantitate the individual mRNA transcript of the five C. rugosa lip
genes. The results demonstrated that the amount of lip transcripts follows the descending order of lip1, lip3, lip2, lip5, and lip4. lip1 and lip3 achieve higher expression, whereas expression of lip2, lip4, and lip5 is only 0.1 to 0.5% of the expression of lip1 transcript under YM culture conditions. These expression profiles are consistent with the findings that LIP1 and LIP3 are the major lipase proteins obtained by purification methods (9, 21, 36).
mRNA Tween 20 had a significant inducing effect on lip5, even in the presence of glucose, which previously was reported to be a repressing carbon source (28). Obviously, lip5 isozyme production (Lipase type VII [Sigma, St. Louis, Mo.]) were deduced to be products of different genes based on partial peptide sequencing (9, 36). However, LIP3, purified by Rúa et al. (36) as a major component of this lipase preparation, was not detected in any of the purified lipase preparations obtained by Diczfalussy et al. (9). This may be explained by our conclusion that the expression profile of C. rugosa lip genes can be altered by different culture conditions and even by batch-to-batch culture differences. We previously reported that different lipase isoforms from C. rugosa displayed quite different substrate specificities and thermostabilities (5, 39). Therefore, the production of different lipase isoforms in response to different growth conditions is physiologically important for C. rugosa, enabling its growth on various substrates and in different environments. Traditionally, the culture conditions in fermentation are optimized for the 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.

The multiplicity of genes encoding isozymes has been reported for many other yeast species (1, 3a, 16, 30, 31). The competitive RT-PCR method developed in this study can be used to examine the possible differential regulation of other

### TABLE 2. Comparison of specific mRNA expression under various culture conditions by competitive RT-PCR

<table>
<thead>
<tr>
<th>Target mRNA</th>
<th>Culture conditions</th>
<th>Slope (r²)</th>
<th>EP (no. of copies per µg of total RNA)</th>
<th>No. of copies per gpd mRNA</th>
<th>Fold induction</th>
<th>Amt relative to lip1</th>
</tr>
</thead>
<tbody>
<tr>
<td>lip1</td>
<td>YM</td>
<td>0.98 (0.97)</td>
<td>(2.08 ± 0.02) × 10⁵</td>
<td>1.05 × 10⁻²</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>YM-oil</td>
<td>0.81 (0.96)</td>
<td>(2.19 ± 0.13) × 10⁵</td>
<td>1.12 × 10⁻²</td>
<td>1.07</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>YM-oleic acid</td>
<td>0.89 (0.97)</td>
<td>(1.46 ± 0.06) × 10⁵</td>
<td>6.0 × 10⁻²</td>
<td>0.56</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>YM-Tween 20</td>
<td>0.88 (0.97)</td>
<td>(2.38 ± 0.09) × 10⁵</td>
<td>1.13 × 10⁻²</td>
<td>1.08</td>
<td>1</td>
</tr>
<tr>
<td>lip2</td>
<td>YM</td>
<td>0.85 (0.98)</td>
<td>(1.04 ± 0.09) × 10⁵</td>
<td>5.2 × 10⁻⁴</td>
<td>1</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>YM-oil</td>
<td>0.81 (0.96)</td>
<td>(2.80 ± 0.29) × 10⁵</td>
<td>1.43 × 10⁻⁴</td>
<td>2.69</td>
<td>0.013</td>
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<tr>
<td></td>
<td>YM-oleic acid</td>
<td>0.89 (0.97)</td>
<td>(1.29 ± 0.12) × 10⁵</td>
<td>5.3 × 10⁻⁴</td>
<td>1.24</td>
<td>0.009</td>
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<tr>
<td></td>
<td>YM-Tween 20</td>
<td>0.88 (0.97)</td>
<td>(1.22 ± 0.03) × 10⁵</td>
<td>5.8 × 10⁻⁴</td>
<td>1.17</td>
<td>0.005</td>
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<tr>
<td>lip3</td>
<td>YM</td>
<td>0.85 (0.98)</td>
<td>(1.42 ± 0.03) × 10⁵</td>
<td>7.2 × 10⁻²</td>
<td>1</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>YM-oil</td>
<td>1.01 (0.97)</td>
<td>(2.90 ± 0.15) × 10⁵</td>
<td>1.48 × 10⁻²</td>
<td>2.07</td>
<td>1.32</td>
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<tr>
<td></td>
<td>YM-oleic acid</td>
<td>0.90 (0.95)</td>
<td>(2.68 ± 0.15) × 10⁵</td>
<td>1.10 × 10⁻²</td>
<td>1.53</td>
<td>1.83</td>
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<tr>
<td></td>
<td>YM-Tween 20</td>
<td>0.85 (0.95)</td>
<td>(1.33 ± 0.02) × 10⁵</td>
<td>6.3 × 10⁻²</td>
<td>0.88</td>
<td>0.56</td>
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<tr>
<td>lip4</td>
<td>YM</td>
<td>0.85 (0.98)</td>
<td>(2.45 ± 0.16) × 10²</td>
<td>1.2 × 10⁻⁴</td>
<td>1</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>YM-oil</td>
<td>0.81 (0.96)</td>
<td>(5.22 ± 0.42) × 10²</td>
<td>2.07 × 10⁻⁴</td>
<td>2.13</td>
<td>0.002</td>
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<td></td>
<td>YM-oleic acid</td>
<td>0.90 (0.95)</td>
<td>(9.92 ± 0.32) × 10²</td>
<td>6.41 × 10⁻⁴</td>
<td>4.05</td>
<td>0.007</td>
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<tr>
<td></td>
<td>YM-Tween 20</td>
<td>0.85 (0.95)</td>
<td>(1.93 ± 0.08) × 10³</td>
<td>9.0 × 10⁻⁶</td>
<td>7.89</td>
<td>0.008</td>
</tr>
<tr>
<td>lip5</td>
<td>YM</td>
<td>0.95 (0.99)</td>
<td>(1.98 ± 0.07) × 10⁷</td>
<td>95.3</td>
<td>95.3</td>
<td>95.3</td>
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<tr>
<td></td>
<td>YM-oil</td>
<td>0.89 (0.99)</td>
<td>(1.95 ± 0.09) × 10⁷</td>
<td>89.2</td>
<td>89.2</td>
<td>89.2</td>
</tr>
<tr>
<td></td>
<td>YM-oleic acid</td>
<td>1.04 (0.98)</td>
<td>(2.44 ± 0.09) × 10⁷</td>
<td>166.5</td>
<td>166.5</td>
<td>166.5</td>
</tr>
<tr>
<td></td>
<td>YM-Tween 20</td>
<td>1.20 (0.99)</td>
<td>(2.11 ± 0.01) × 10⁷</td>
<td>88.7</td>
<td>88.7</td>
<td>88.7</td>
</tr>
</tbody>
</table>

- The log of the ratio of the corrected fluorescence intensity of the competitor PCR product to that of the target PCR product was plotted against the log of the copy numbers of competitor originally added. These plots were linear, and the slopes and correlation coefficients (r²) are shown.
- The EP for each mRNA is the copy number of competitor DNA required at which the corrected fluorescence intensity of the competitor PCR product is equal to the measured intensity of the target PCR product. The values are averages from three independent experiments, and standard deviations are given.
- The absolute copy numbers for each lip mRNA were normalized relative to the copy numbers of gpd mRNA before the fold induction or repression was calculated.
- Relative amount is defined as the amount of individual mRNA relative to lip1 under each culture condition.
yeast gene families (11, 20). The expression level of the mRNA transcript may be affected by many factors, such as promoter activity, upstream regulatory elements, and stability of the mRNA. Although elements such as the CAAT and TATAA boxes characteristic of eukaryotic promoters for transcriptional initiation have been found in the conserved regions upstream from the lip genes (26), we anticipate that transcriptional regulatory elements of C. rugosa lip promoters should be localized upstream from the conserved regions. Recently, nutrient-related transcriptional controlling elements have been identified in Saccharomyces cerevisiae (7). By assaying the β-galactosidase activities of promoter-lacZ fusions in S. cerevisiae, our unpublished data showed that the promoter activities of lip3 genes under various culture conditions were much higher than those of lip4 promoter. These findings suggest that the expression profile of lip genes could be accompanied by different regulation of the lip promoter activities. Studies of the transcriptional controlling elements of C. rugosa lip genes to further elaborate the mechanism of differential regulation of lip genes by various inducers are under way.

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