

Cloning and characterization of a gene (*LIP1*) which encodes a lipase from the pathogenic yeast *Candida albicans*

Yue Fu,¹ Ashraf S. Ibrahim,¹ William Fonzi,² Xiang Zhou,¹ Clarisa F. Ramos¹ and Mahmoud A. Ghannoum³

Author for correspondence: Mahmoud A. Ghannoum. Tel: +1 216 844 8580. Fax: +1 216 844 1076.
e-mail: MAG3@PO.CWRU.EDU

¹ Division of Infectious Diseases, St John's Cardiovascular Research Center, Department of Medicine, Harbour-UCLA Medical Center, Torrance, CA 90509, USA

² Department of Microbiology and Immunology, School of Medicine, Georgetown University Medical Center, Washington, DC 20007-2197, USA

³ University Center for Medical Mycology, and Mycology Reference Laboratory, Department of Dermatology, Case Western Reserve University and University Hospitals of Cleveland, 11100 Euclid Avenue, Stop LK5 5028, Cleveland, OH 44106-5028, USA

Extracellular phospholipases are demonstrated virulence factors for a number of pathogenic microbes. The opportunistic pathogen *Candida albicans* is known to secrete phospholipases and these have been correlated with strain virulence. In an attempt to clone *C. albicans* genes encoding secreted phospholipases, *Saccharomyces cerevisiae* was transformed with a *C. albicans* genomic library and screened for lipolytic activity on egg-yolk agar plates, a traditional screen for phospholipase activity. Two identical clones were obtained which exhibited lipolytic activity. Nucleotide sequence analysis identified an ORF encoding a protein of 351 amino acid residues. Although no extensive homologies were identified, the sequence contained the Gly-X-Ser-X-Gly motif found in prokaryotic and eukaryotic lipases, suggesting a similar activity for the encoded protein. Indeed, culture supernatants from complemented yeast cells contained abundant hydrolytic activity against a triglyceride substrate and had no phospholipase activity. The data suggest that *C. albicans*, in addition to phospholipases, also has lipases. Southern blot analyses revealed that *C. albicans* may contain a lipase gene (*LIP*) family, and that a lipase gene(s) may be present in *Candida parapsilosis*, *Candida tropicalis* and *Candida krusei*, but not in *Candida pseudotropicalis*, *Candida glabrata* or *S. cerevisiae*. Northern blot analyses showed that expression of the *LIP1* transcript, the cloned gene which encodes a lipase, was detected only when *C. albicans* was grown in media containing Tween 80, other Tweens or triglycerides as the sole carbon source, and not in Sabouraud Dextrose Broth or yeast/peptone/dextrose media. Additionally, carbohydrate supplementation inhibited *LIP1* expression. Cloning this gene will allow the construction of *LIP1*-deficient null mutants which will be critical in determining the role of this gene in candidal virulence.

Keywords: *Candida albicans*, *LIP1*, lipase

INTRODUCTION

Pathogenic fungi, including *Candida albicans*, are known to secrete various hydrolytic enzymes such as proteinases, phospholipases and lipases (Ogawa *et al.*, 1992). These extracellular hydrolytic enzymes may contribute to pathogenesis of infections by disrupting host cell membranes and extracellular matrices, thus aiding in dissemination and tissue invasion (Salyers &

Whitt, 1994). These enzymes may also allow microorganisms to utilize host cell macromolecules as a source of nutrients (Salyers & Whitt, 1994). *C. albicans* enzymes considered to be integral to pathogenesis are categorized into two main types: (1) proteinases that hydrolyse peptide bonds, and (2) phospholipases which hydrolyse phospholipids. The aspartyl proteinases have been extensively studied (for review see Ghannoum *et al.*, 1995; White & Agabian, 1995).

Experimental evidence implicating phospholipase as a virulence factor of *C. albicans* exists (Barrett-Bee *et al.*, 1985; Ibrahim *et al.*, 1995). Barrett-Bee *et al.* (1985)

The GenBank accession number for the sequence reported in this paper is U34807.

found that isolates of *C. albicans* with the highest extracellular phospholipase activity adhered most strongly to buccal epithelial cells and were most pathogenic in mice. In a recent study, our group obtained evidence that strongly suggests that extracellular phospholipases of *C. albicans* contribute to virulence of this yeast (Ibrahim *et al.*, 1995). Cloning of a gene(s) encoding extracellular candidal phospholipases is the essential first step to answer more definitively whether extracellular phospholipases contribute to the pathogenesis of *C. albicans*.

In this study, in an attempt to clone candidal extracellular phospholipases, we utilized a complementation strategy employing a lipase-deficient strain of *Saccharomyces cerevisiae* transformed with a *C. albicans* genomic library. The transformed organisms were screened for lipolytic activity using egg-yolk agar media. The egg-yolk agar assay has been used conventionally for the rapid screening of clinical isolates of *C. albicans* for the production of phospholipase (Price *et al.*, 1982; Williamson *et al.*, 1986). In this assay, phospholipase-producing strains are identified by the formation of a zone of turbidity around the colony. We have cloned and sequenced a *C. albicans* gene that imparted a positive response to *S. cerevisiae* cells grown on egg-yolk agar. Interestingly, nucleotide sequence analysis, and substrate specificity studies, utilizing specific phospholipid and triglyceride substrates, revealed that the cloned gene encodes a lipase and not a phospholipase. Although studies directed at investigating fungal lipases centre around their industrial applications, little attention has been paid to the contribution of lipases to fungal pathogenesis. Cloning a candidal gene encoding a lipase will allow determination of its role in the virulence of this important nosocomial pathogen. In this study, we have cloned and characterized a lipase gene (*LIP1*) from a clinical isolate of *C. albicans*. Additionally, Southern and Northern blot analyses were performed to search for the presence of homologous sequences in other *albicans* and non-*albicans* species, as well as to determine the level of expression of the *LIP1* gene.

METHODS

Strains and growth conditions. *Candida albicans* ATCC 36082 (a clinical isolate) and *Candida cylindracea* ATCC 14830 were obtained from the American Type Culture Collection. *Saccharomyces cerevisiae* strain LL-20, a lipase-deficient strain (*MAT α leu2-3,112 his3-11,15 can1*), was from William Fonzi (Georgetown University). *Escherichia coli* strain XL1-Blue was purchased from Stratagene. The following clinical *albicans* and non-*albicans* strains were used in this study to determine the universal distribution of the *LIP1* gene: *C. albicans* (six isolates), *Candida parapsilosis* (three isolates), *Candida tropicalis* (two isolates) and one isolate of *Candida krusei*, *Candida pseudotropicalis* and *Candida glabrata*.

Yeast cells of *C. albicans* were routinely grown at 30 °C in YPD medium (1%, w/v, yeast extract; 2%, w/v, peptone; 2%, w/v, dextrose; Difco). The medium used for selecting *S. cerevisiae* transformants was Yeast Nitrogen Base without

amino acids or ammonium sulfate (YNB; Difco) supplemented with 2% (w/v) dextrose, 0.5% (w/v) ammonium sulfate and 100 µg L-leucine ml⁻¹ (Sigma). Egg-yolk medium used to detect lipolytic activity consisted of (in 184 ml distilled water): Sabouraud Dextrose Agar (Difco), 13.0 g; NaCl, 11.7 g; CaCl₂, 0.111 g; and 10% (v/v) sterile egg yolk. The egg yolk was aspirated from the chicken eggs and centrifuged at 500 g for 10 min at room temperature to remove particulate matter. Twenty millilitres of the supernatant was added to the sterilized medium. Luria-Bertani (LB), SOB and SOC media used for growth of *E. coli* were prepared as described by Sambrook *et al.* (1989). Ampicillin (final concentration 80 µg ml⁻¹) was added as required. All media were solidified with 1.5% agar (Difco).

To study the expression of the *LIP1* gene by Northern analyses, fungi were grown in rich (Sabouraud Dextrose Broth and YPD) and chemically defined [YNB and Lee's (1975)] media under different growth conditions. Compounds used to supplement YNB included: Tweens 20, 40, 60 and 80, carbohydrate (glucose, maltose and galactose), phosphatidylcholine and α -naphthyl palmitate; these were purchased from Sigma. The triglycerides used in supplementing YNB were obtained from egg-yolk lipids and purified by TLC as described previously by Ghannoum *et al.* (1986b).

Plasmids and transformations. Two *E. coli*-*S. cerevisiae* shuttle vectors were used; pE20-H (kindly provided by Susan Sandmeyer, University of California at Irvine), a 2µ-based multicopy yeast plasmid containing the selectable *HIS3* gene, and pRS415 (Stratagene), a single-copy, centromere-based cloning vector. The sequencing plasmid used was pBluescript SK(+) (Stratagene). *E. coli* transformation was carried out according to the method of Hanahan (1983). Transformation of *S. cerevisiae* was performed using the protocol of Schiestl & Gietz (1989).

Genomic library construction. For genomic DNA isolation, *C. albicans* ATCC 36082 was grown to stationary phase in YPD medium. Genomic DNA was extracted by the method of Scherer & Stevens (1988), and partially digested with *Sau3AI* (Boehringer Mannheim). DNA fragments 10–15 kb in length were isolated by sucrose gradient centrifugation (Sambrook *et al.*, 1989) and then ligated into the *Bam*HI site of pE20-H. Analysis of random transformants indicated that 75% of the plasmids contained inserts of 10–15 kb. The resultant DNA library of about 8000 clones was amplified in *E. coli* strain XL1-Blue, and subsequently transformed into *S. cerevisiae* strain LL-20 to yield 24000 transformants.

Since the *C. albicans* *LEU2* gene is known to complement the *leu2* mutation of *S. cerevisiae* (Jenkinson *et al.*, 1988), the frequency of Leu⁺ transformants was determined to provide an indication of the completeness of the library and the frequency of single-copy genes within the library. By this test, 1/2200 *S. cerevisiae* transformants was Leu⁺, indicating that the library was representative and contained a high frequency of inserts.

RNA isolation. Fungal cells from various growth conditions were harvested by centrifugation for 5 min at 3000 g at 4 °C and the pellet was frozen in an ethanol/dry-ice bath precooled to -20 °C. The cells were resuspended in 1.5 ml phenol, 1.5 ml TE buffer (10 mM Tris/HCl, pH 7.6, 1 mM EDTA), containing 6 g glass beads and 75 µl 10% SDS, and vortexed for 4 min. Following centrifugation for 10 min at 3000 g, the aqueous phase was extracted three times with phenol (saturated with Tris buffer and 0.1% 8-hydroxyquinoline) and once with phenol/chloroform. The RNA was then precipitated with 0.1 vol. 3 M NaOAc/HOAc (pH 5.2) and two vols

100% ethanol, and stored at -20°C . Precipitated RNA was pelleted and resuspended in 200 μl diethyl-pyrocabonate-treated H_2O .

Southern (DNA) and Northern (RNA) blot analysis. Standard DNA electrophoretic techniques and formaldehyde RNA gels were performed according to the methods outlined by Sambrook *et al.* (1989). Blotting was carried out with nylon membranes (MSI) as described in the manufacturer's instructions. DNA fragments (1.1 kb), representing the lipase gene, used as hybridization probes were extracted from gels with the Gene-Clean kit (Bio101) and labelled with [α - ^{32}P]dATP, total count of 4×10^7 c.p.m., by using an NEBlot kit (New England Biolabs). DNA containing the *C. albicans* actin gene was used as a control in Northern analysis. Additionally, Southern blot analyses were used in other experiments to determine whether homologous sequences are present in clinical isolates of *albicans* and non-*albicans* species. Northern blot analyses were performed to monitor the expression of this gene in these isolates.

For low-stringency hybridization, genomic DNA was bound to nylon membranes. The membranes were then prehybridized at 42°C for 60 min in a solution containing $5 \times$ SSPE, $5 \times$ Denhardt's, 20% formamide (Fisher Biotech), 1% SDS and 100 μg denatured salmon-sperm DNA ml^{-1} . Hybridization was performed in the same solution containing the ^{32}P -labelled DNA probe. The membranes were exposed to X-ray film after three washes in $0.2 \times$ SSC, 0.1% SDS at 42°C . Similar conditions were used for high-stringency hybridization with the exception that the formamide concentration was changed from 20 to 50%. Hybridization was performed overnight in the same solution containing the ^{32}P -labelled random primed probe. The membranes were exposed to X-ray film after three washes in $0.2 \times$ SSC and 0.1% SDS at 42°C .

Selection and confirmation of clones exhibiting lipolytic activity. To screen for clones having lipolytic activity, transformants of *S. cerevisiae* LL-20 were spread on egg-yolk agar medium and incubated at 30°C for 96 h. Clones displaying lipolytic activity were identified by colonies surrounded by a halo of precipitation.

Sequencing. A 2.5 kb *XhoI*-*PstI* DNA fragment containing the putative lipase gene was cloned into plasmid pBluescript SK(+) in both orientations and unidirectional deletion constructs were generated by *ExoIII* digestion (Henikoff, 1984). The DNA sequence was determined, in both directions, using the dideoxy chain-termination method and the entire sequence was determined from both strands (Sanger *et al.*, 1977). Nucleotide and protein sequence analyses were performed with the Wisconsin Genetics Computer Group sequence analysis software package, version 7.0 (Devereux *et al.*, 1984). Homology searches of the GenBank database were conducted with the FASTA program (Pearson & Lipman, 1988).

Functional analysis of the cloned enzyme. To determine the functional activity of the encoded enzyme, transformed *S. cerevisiae* cells were grown for 24 h in 500 ml Sabouraud Dextrose Broth (Difco) at 30°C and then centrifuged at 4°C to remove particulate material. The supernatants were then collected and concentrated, about 10-fold, by ultrafiltration using Minitan-S filter sheets, 10000 NMWL (Millipore), and stored at -70°C . The activity of the encoded enzyme was determined by a radiometric assay (Ibrahim *et al.*, 1995). The lipase activity was assayed using triolein [$9,10$ - ^3H (N)] [specific activity 18.4 Ci mmol^{-1} (680 GBq mmol^{-1}); DuPont NEN] as

the substrate, while phospholipase activity was assayed using L-3-phosphatidylcholine, 1-palmitoyl-2[1- ^{14}C]palmitoyl [specific activity 157 mCi mmol^{-1} (5.8 GBq mmol^{-1}); DuPont NEN] as the substrate. To determine lysophospholipase activity, the substrate was L-lyso-3-phosphatidylcholine, 1-[1- ^{14}C]palmitoyl [specific activity 56.7 mCi mmol^{-1} (2.1 GBq mmol^{-1}); DuPont NEN].

An equal volume of the concentrated supernatant was added to 0.25 ml 0.1 M citrate buffer (pH 4.0) containing 0.1% (v/v) Triton X-100 and 48 nmol radiolabelled substrate. The mixture was incubated for 20 min at 37°C . The reaction was stopped by adding 2 ml chloroform/methanol (2:1, v/v) and the samples were extracted as described previously by Ibrahim *et al.* (1995). After drying under nitrogen, the lipid-containing phase was suspended in 0.1 ml chloroform and fractionated by TLC using a non-polar solvent system (Ghannoum *et al.*, 1986a). The lipids were visualized using iodine vapour and the bands were scraped from the plate into scintillation vials. The amount of radioactivity in each band was determined by scintillation spectroscopy and the results were expressed as a percentage of the total radioactivity in each lane. Authentic standards were chromatographed in parallel to determine the identity of the lipid fractions. All samples were tested in duplicate.

Verification that the egg-yolk assay can detect both phospholipase and lipase activities. For the egg-yolk assay to detect phospholipase and lipase activities it should contain the appropriate substrates (phospholipids and triglycerides, respectively). To demonstrate that egg yolk contains these substrates we obtained the egg yolk from one egg and extracted the lipids by suspending the yolk in chloroform/methanol (2:1, v/v). Total lipids were purified by established procedures (Folch *et al.*, 1957). The purified lipids were fractionated into individual phospholipids and neutral lipids by TLC as described previously (Ghannoum *et al.*, 1986b). Individual components were identified by comparison of their R_f mobility with those of commercially available standards.

RESULTS

Isolation of clones encoding *C. albicans* lipid-hydrolysing enzymes

To clone candidal genes encoding phospholipases it was necessary to use a screening method that is rapid, cheap and can resolve the enzyme activity in a large number of clones. The egg-yolk agar assay has been traditionally used for the rapid screening of clinical isolates of *C. albicans* for the production of extracellular phospholipase activity. In this assay, phospholipase-producing strains are identified by the formation of a zone of turbidity around the colony. Although some strains of *S. cerevisiae* produce extracellular phospholipase (Lee *et al.*, 1994), preliminary experiments showed that strain LL-20, unlike *C. albicans*, did not form a zone of turbidity in this assay, and, therefore, did not secrete detectable phospholipases. This finding suggested that the egg-yolk assay could be useful in cloning phospholipase genes from *C. albicans* by the ability of *Candida* DNA sequences to complement the *S. cerevisiae* LL-20 strain.

To screen for clones exhibiting lipolytic activity, approximately 2×10^4 *S. cerevisiae* transformants, con-

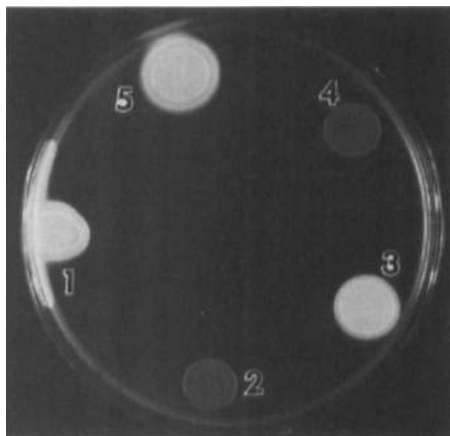


Fig. 1. Egg-yolk plate assay for detecting lipolytic activity. 1 and 3, *S. cerevisiae* LL-20 transformed with candidal DNA expressing lipolytic activity; 2, *S. cerevisiae* LL-20 transformed with the parent plasmid pE20-H showing no lipolytic activity; 4, *S. cerevisiae* LL-20 with no plasmid exhibiting no activity; 5, *C. albicans* ATCC 36082 secreting lipid-hydrolysing enzymes.

raining 10–15 kb candidal DNA fragments, were spread on egg-yolk agar medium. In this initial screen, two colonies positive for lipolytic activity were identified. Both isolates were $\text{Leu}^- \text{His}^+$, suggesting that they were derived from the original *S. cerevisiae* strain and were not contaminant *C. albicans*. Plasmid DNA was recovered from the two positive clones and used to transform *E. coli* strain XL1-Blue. To verify that the plasmids encoded the hydrolytic activity, *S. cerevisiae* LL-20 was transformed with the purified plasmids and screened again for activity on egg-yolk agar. *S. cerevisiae* containing the parent plasmid pE20-H was used as a negative control. Six isolates from each transformation were tested on egg-yolk agar. Each of the transformants was observed to possess lipolytic activity, while cells containing the parent plasmid pE20-H were negative in that they did not show any lipolytic activity (Fig. 1).

The *LIP1* gene was localized by subcloning portions of the insert DNA into pE20-H and subsequently screening the *S. cerevisiae* LL-20 transformants for lipolytic activity. Restriction mapping of the recovered plasmids indicated that each contained a common insert of approximately 9 kb. By this method, the gene was localized to a 2.3 kb *XhoI*–*PstI* fragment (Fig. 2a).

Prior to subcloning and sequence analysis of the *LIP1* gene, the cloned DNA was hybridized to *C. albicans* genomic DNA to verify that the cloned fragment was derived from this organism. Furthermore, the cloned DNA fragment was able to hybridize only to sequences present in *C. albicans* genomic DNA and not to those present in *S. cerevisiae* (data not shown), confirming that the cloned gene is of candidal origin. In addition, Southern blot analysis of candidal genomic DNA digested with a number of restriction endonucleases was performed and demonstrated colinearity between the cloned DNA fragment and the genome.

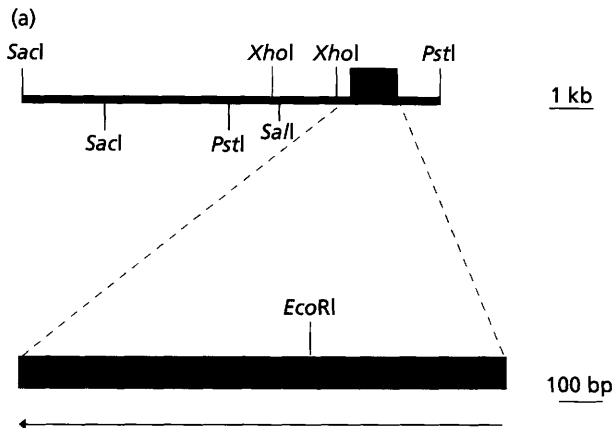
Nucleotide sequence determination of the cloned gene

The nucleotide sequence was determined for a 2053 bp region of the complementing 2.3 kb *XhoI*–*PstI* fragment (Fig. 2a). Analysis of this sequence revealed a single 1053 bp ORF encoding a putative peptide 351 amino acids in length with a predicted molecular mass of 38 kDa (Fig. 2b). The predicted protein had a hydrophobic amino terminus, suggesting that the protein may be secreted, but no obvious signal peptide cleavage site (von Heijne, 1986). MacPattern analysis for Prosite motifs (Fuchs, 1994) found *N*-glycosylation sites at amino acid positions 114, 202, 300, 305 and 334.

BLAST (Altschul *et al.*, 1990) and BLOCKS (Henikoff & Henikoff, 1991) homology searches found no significant homology to known proteins. However, it was noted that the putative protein contained a Gly-X-Ser-X-Gly motif characteristic of lipases (Antonin, 1988). The significance of this motif was investigated by multiple sequence alignment using Gibb's sampling strategy (Lawrence *et al.*, 1993) as instituted by the program MACAW (Schuler *et al.*, 1991). A significant block of homology ($P \sim 0$, $N = 1.491 \times 10^{40}$) which encompassed amino acids Ala-71 to Ser-82 of the *C. albicans* protein was identified when compared with 14 lipases of fungal and mammalian origin (data not shown). This block contains the Gly-X-Ser-X-Gly motif. When compared to fungal lipases only, an extended block of homology ($P \sim 0$, $N = 3.347 \times 10^{13}$) was identified between amino acids Pro-21 and Leu-108 (Fig. 3). A second extended region of homology was identified by visual alignment. This region, between Ala-121 and Asp-231 of the *C. albicans* gene, encompassed the Ser-His-Glu catalytic triad of the *Geotrichum candidum* lipase (Schrag *et al.*, 1991). A corresponding histidine was present in the *Candida* gene, but the glutamic acid was not. However, the position of the catalytic acid residue varies in lipases (Schrag *et al.*, 1992). Although the overall identity is small, 21%, the extended regions of conservation and preservation of potential active site residues suggested that the *C. albicans* gene encodes a lipase.

Gene dosage effects

Because extensive structural homologies were not found, to confirm that the cloned lipase gene is a structural gene and not a regulatory gene which activates expression of an *S. cerevisiae* lipase, we cloned the functional DNA fragment into a single-copy (pRS315) as well as a multicopy (pE20-H) vector. These constructs were then introduced into the *S. cerevisiae* LL-20 strain and assayed for their lipolytic activities. The yeast transformed with the multicopy vector expressed significantly higher lipolytic activity compared with the single-copy vector (Pz values of 0.92 and 0.58 for the single-copy and multicopy vectors, respectively). This zone (Pz) is calculated by dividing the colony diameter by the diameter of the cloudy zone plus colony diameter and is used to quantify the lipolytic activity. Since higher copies of the gene resulted in greater lipolytic activity,



1
AACTAAAGATTAAACATATGAATATCTCTCCACCCATGTTTGTAGTACACAGGATATATCTCTTTCCGGTGTAT
81
CAAAATAGTCCAAATTAATCAGAAATCTCTTTGACAAATGTCTCCGATTCGCCACTTCATTAACTAAAAGGAA
161
AATCTTGTGTCATCACCCTCTCATGTCTGCTACCTTGTGTTTAAAAAAGCACTAATTTGGTGTGACAGATCTGTGCA
241
GTAAAAATAAATCTCACCATATAATAGATACAAATTTGGCAAGATTTTAAACCTAGATTATATAAGTTTAAATCAAC
321
AATCCATTTCCATCAACTTCACCCCTCTCTCCACCATCACCACCAATATGAGAGGTATGCTGTTTCTGCTTTCAT
401
ATCATTAAATTTGCTCCCTCCCTGCTGCTAAGTACCCCTTGTGACGACCTCTATAGTCTGCTGATGGTATGAGT
481
CACCTAAATTTGGGAGAAATCTTAAAGTTGAGAAATCTCCCAAGTAACTCAGTAGATGTTTGTGAGTGAATATAAGA
561
ACTCTGGGAGCTTTTGTAGATCTGAAGATTCATTTGGAAATGCAACAGCAATTTGACACAGTATTCGAAACCATC
641
AAGCTGACCCATCCAAAGTCTTCTTATCAAACTTTTGAAGATTTGCAAAATATTGAATGTTCACTCTTACGGT
719/1
ATG CAA TAT GGT GCC CCT TGG TCA ACT GTG GCT ACT CAA ATT GAT ATG GCT TTA ATG GTA
Met gln tyr gly ala pro trp ser thr val thr gln ile asp met ala leu met val
779/21
CCT ATG TTG AAG CAA GGT TAT TAC GTT GTC AGT CCT GAT TAT GAG GGA CCA AAA TCC ACA
pro met leu lys gln gly tyr val val ser pro asp tyr glu gly pro lys ser thr
839/41
TTT ACT GTT GGT AGA CAA TCT GGG AAA GCC ACT TTA GAT TCC ATT AGA GCT ATT CTA AAA
phe thr val gly arg gln ser gly lys ala thr leu asp ser ile arg ala ile leu lys
899/61
TCC AAC AAA TTC ACT GGG ATC AAG AGT GAT GCA AAA GTC GCC ATG TGG GGA TAC TCT GGT
ser asn lys phe thr gly ile lys ser asp ala lys val ala met trp gly tyr ser gly
959/81
GGT TCA TTA GCT TCT GGT TGG GCT GCT GCA TTA CAA CCT AAG TAT GCT CCA GAA TTG AAG
gly ser leu ala ser gly trp ala ala leu gln pro lys tyr ala pro glu leu lys
1019/101
AAA AAT TTG ATT GGT GCT GCC CTT GGT GGT TTT GTC ACT AAT ATT ACT GCC ACT GCC GAA
lys asn leu ile gly ala ala leu gly gly phe val thr asn ile thr ala thr ala glu
1079/121
GCA ACC GAT GGT ACA TTA TTT GCG GGA TTG GTT CCA AAT GCC TTG AGT GGG TTA GCT AAT
ala thr asp gly thr leu phe ala gly leu val pro asn ala leu ser gly leu ala asn
1139/141
GAG TAT CCC GAA TTC AAA GAA ATA CTT TAC CAA AAA GTC TCC AAA GCT GCT ACT GAC AAT
glu tyr pro glu phe lys glu ile leu tyr gln lys val ser lys ala ala thr asp asn
1199/161
TTG GCT CAA GGT ACT GAA CAT TGT ATT GGA GGT GCT ATT CTT TAC TTT GCT GAA GAC CAA
leu arg gln gly thr glu his cys ile gly gly ala ile leu tyr phe ala glu asp gln
1259/181
TAC TTC ACT GGC GAT GAT AGA GCT TTC CCT GGA GGT TAT GGA TTA CTT AAA GAA GAG GTG
tyr phe thr gly asp asp arg ala phe pro gly gly tyr gly leu leu lys glu glu val
1319/201
GTG AAC AAG ACT ATT CTG GAA AAC AAT TTG ATG CAA ATG GAT AAA GAT TAT TTA CCA GAT
val asn lys thr ile leu glu asn asn leu met gln met asp lys asp tyr leu pro asp
1379/221
ATT CCA ATT TTT GTA TAC CAT GGT GCA TTG GAT TCC ATT GTT CCA ATC AGT AAC GTC CAT
ile pro ile phe val tyr his gly ala leu asp ser ile val pro ile ser asn val his
1439/241
GTC ACT TAT AAA AAT TGG TGT GAT TGG GGG ATC AAC TCA TTT GAA TTT TCT GAG GAT TTA
val thr tyr lys asn trp cys asp trp gly ile asn ser phe glu phe ser glu asp leu
1499/261
TTA AAT GGC CAT ATC ACC GAA ACT ATT GTT GGG GCC CCG GCA GCT ATA ACA TGG TTA GAA
leu asn gly his ile thr glu thr ile val gly ala pro ala ala ile thr trp leu glu
1559/281
GCA AGA TTT GAC GGT GAA CCA GTC GTC AAG GGA TGC AAA AAG ACT TCC AGA ATC ACC AAC
ala arg phe asp gly glu pro val val lys gly cys lys thr ser arg ile thr asn
1619/301
TTT TCT TAT CCA AAC ATT TCT GAT TCT ACT AGT AGT ATC TTC GAA GGG ATT CTC AAT TCT
phe ser tyr pro asn ile ser asp ser thr ser ser ile phe glu gly ile leu asn ser
1679/321
GTT ACT GGT TCC GAA TTG GGT CCA GGT GTC ACC TCT GAT AAC ATA ACG TTG GAT GGA TTA
val thr gly ser glu leu gly pro gly val thr ser asp asn ile thr leu asp gly leu
1739/341
ACC GGA TTC TTG GGG AAC TTT ATT GAC TTA AAA TAG ATGACAAACAGAAAAAACCCTAC
thr gly phe leu gly asn phe ile asp leu lys AMB
1806
TTACAGCCAAACCTAATCATATCCACACCTACCAAAATTTATTTTTCGTTGCCCTTTTCAATTTCTTACATACCAT
1886
AATCTCACTTTAAAGTGAATTAATTTTAAATACATATACAGTTAACTTACCAATAAACATTCATTAATAAAA
1966
TCCAAAGTTGGATCTCTCCCTGTTTGTGAGTGTATGTGCGTGTGACGCCACTGTTGAGGAAATCATGGTGGATTC
2046
CACT

Fig. 2. (a) Restriction map of the 2.3 kb *XhoI*-*PstI* genomic DNA fragment containing the *LIP1* gene. The boxed area in the lower portion of the figure indicates the fragment encoding the ORF of the *LIP1* gene. (b) Nucleotide sequence of the complementing DNA fragment and predicted amino acid sequence of the putative ORF. The numbers refer to the nucleotide position.

Calb. 21 PMLKQGY-VVVSF-DYEQPKSTFTVGRQSGKA 50
Gcan1 176 PVVVFVSINVRTPGYVGLGGDAITAEQ-NTNA 204
Gcan2 176 PVVVFVSINVRTPGYVGLGGDAITAEQ-NTNA 204
Crug1 183 PIHVSVNVRVSSWGFLAGDEIAEQ-SANA 192
Crug2 182 PVHVSVMNVRVSSWGFLAGDPDQNEQ-SIGNA 191

Calb. 51 TLDISIRAILK-SNKFTEGKSD-AKVAMWGY 79
Gcan1 205 GLHDQRKGLEWVSDNIANFGGDPDKVMIFGES 236
Gcan2 205 GLHDQRKGLEWVSDNIANFGGDPDKVMIFGES 236
Crug1 193 GLKDQRRLGMQWVAADIAAGGDPKVTIFGES 224
Crug2 192 GLHDQRRLAMQWVAADIAAGGDPKSVTIYGES 223

Calb. 80 GGSILASGWAAALQPKYAPELKKNLIGAAAL 108
Gcan1 237 AGAMSVAHQLVA YGGDNTYNGKQLFHSIAI 265
Gcan2 237 AGAMSVAHQLVA YGGDNTYNGKQLFHSIAI 265
Crug1 225 AGSMVMCHILWNDGNTYNGKPLFRAGAI 253
Crug2 224 AGSMSTFVHLVWNDGNTYNGKPLFRAGAI 252

Calb. 121 ATD-GTLEAGLVNALSGLANEYFEKIEILYQ 151
Gcan1 370 QDEDEGTILAPVA INATT-TPHVKKWLKYLICSE 400
Gcan2 370 QDEDEGTILAPVA INATT-TPHVKKWLKYLIFD 400
Crug1 353 ONDEGTFFGTSSLNVT-DIAQAREYFKQS FVH 383
Crug2 352 ONDEGTLEGLSSLNVT-DIAQARAYFKQSFIH 382

Calb. 152 KVSKAATDNLRQGTGHECIG-G- -AILYFAE 178
Gcan1 401 -ASDAISLDRVLSLYPGSWSEGAFFRTGILNAL 431
Gcan2 401 -ASEAISLDRVLSLYPQTLVSGSPFRTGILNAL 431
Crug1 384 -ASDAEIDTLMATAYPGDTTQGSFDTGILNAL 414
Crug2 383 -ASDAEIDTLMATAYTSDTQGSFDTGIFNAI 413

Calb. 179 DOYFTGGDDRAFP-GYGLKEEVVNKTILENN 209
Gcan1 432 TPQFKRIAAIFTDLLFQSPRRVMLNATKDVNR 463
Gcan2 432 TPQFKRVAAIFDMLFQSPRRVMLNATKDVNR 463
Crug1 415 TPQFKRISAVLGLDGLFLAARYFLNHYTGGTK 446
Crug2 414 TPQFKRISALLGLDGLFLAARYFLNYYQGGTK 445

Calb. 210 LMQMDKDYLPDIPIF-VHGAALD 231
Gcan1 464 WTYLATQLHNLVPLFLGTFHGS-D 485
Gcan2 464 WTYLSTHLNLVPLFLGTFHGS-E 485
Crug1 447 YSFLSKQLS-GLPVLGTFHGS-D 467
Crug2 446 YSFLSKQLS-GLPVLGTFHGS-D 466

Fig. 3. Multiple sequence alignment of *C. albicans* putative lipase with lipases from *G. candidum* and *C. cylindracea* (*rugosa*). Catalytic residues are marked with asterisks. The region conserved in fungal mammalian lipases is underlined. The boxed regions in the figure contain identical amino acid residues.

the results are consistent with the cloned *C. albicans* gene encoding this activity.

Functional analysis of the enzyme encoded by the candidal gene

During the initial isolation of this gene, we used egg-yolk agar which has been the traditional screen for phospholipase activity (Samaranayake *et al.*, 1984; Schuler *et al.*, 1991). However, there are substantial amounts of triglycerides in the egg yolk, suggesting lipase activity might also be detected (see below). Because the cloned gene lacked detectable homology with known phospholipases, but exhibited some similarity to lipases, we developed a more specific assay to determine if the candidal enzyme was a phospholipase or a lipase.

To examine the type of enzyme activity encoded by the candidal gene, the concentrated supernatants from cultures of transformed *S. cerevisiae* were assayed for

acid/amino acid residues. The numbers specifying the nucleic acid residues are derived from the entire genomic fragment sequenced, whereas the amino acid numbers refer to the first methionine defining the coding region of the *LIP1* gene.

Table 1. Enzymic activity of the encoded protein

Values are the mean \pm SD of analyses of three separate experiments. ND, No detectable activity.

Strain	Activity [units (mg protein) ⁻¹]		
	Phospholipase*	Lysophospholipase†	Lipase‡
<i>S. cerevisiae</i> (Candida gene)	ND	ND	10.84 \pm 0.14
<i>S. cerevisiae</i> (empty plasmid)	ND	ND	ND
<i>C. cylindracea</i> §	ND	104.9 \pm 2.3	14.04 \pm 3.96
Purified phospholipase standard	4.7 \pm 0.34	FCSnd	ND

*Unit is defined as the activity of the enzyme required to liberate 0.1 nmol fatty acid from phosphatidylcholine ml⁻¹ h⁻¹.

†Unit is defined as the activity of the enzyme required to liberate 0.1 nmol fatty acid from lysophosphatidylcholine ml⁻¹ h⁻¹.

‡ Unit is defined as the activity of the enzyme required to liberate 0.01 nmol fatty acid from trioline ml⁻¹ h⁻¹.

§ Positive control of lipase activity.

|| Positive control of phospholipase activity.

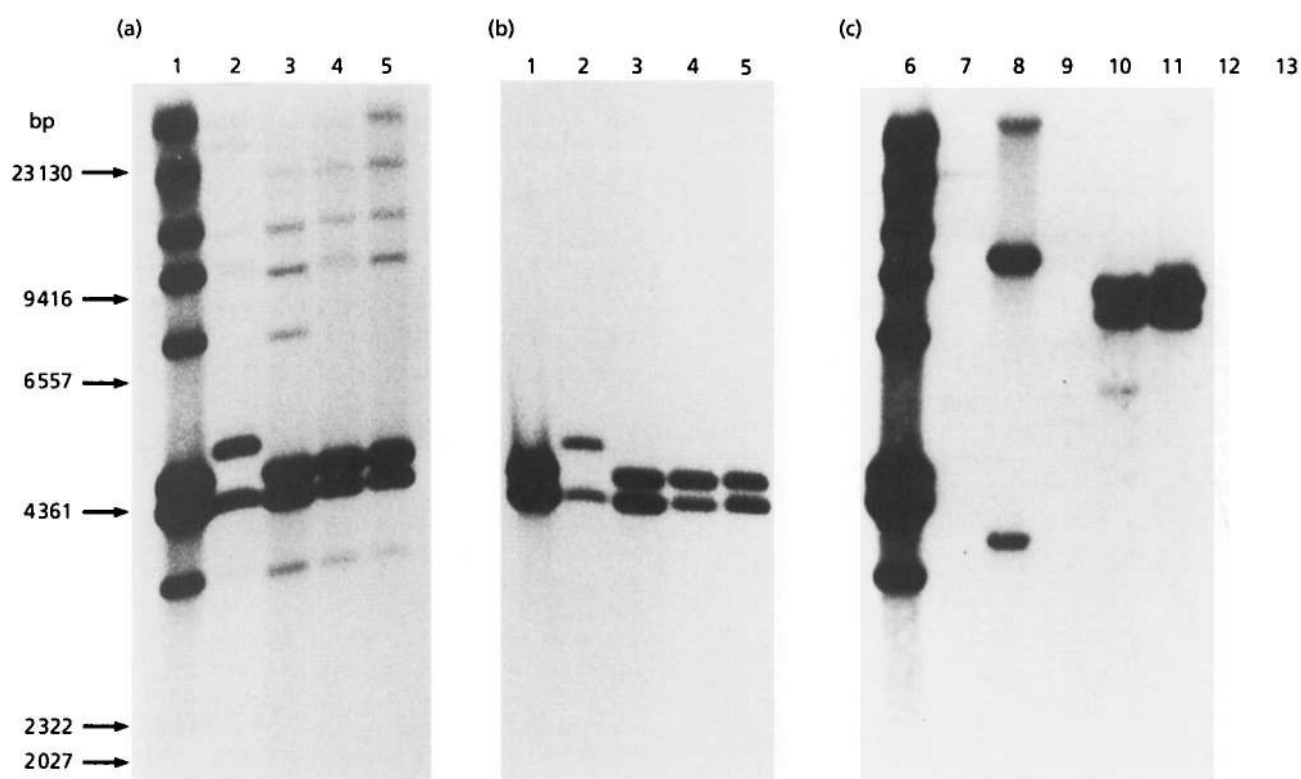


Fig. 4. (a, b) Southern blot analysis of lipase genes in different strains of *C. albicans*. The yeast genomic DNA was digested with the restriction enzyme *Pst*I and hybridized with *LIP1* DNA under (a) low-stringency and (b) high-stringency conditions. Lanes: 1, strain 36082; 2, strain 15153; 3, strain 16427; 4, strain 17236; 5, strain 17737. (c) Southern blot analysis of lipase genes in different species of yeast. Lanes: 6, *C. albicans* 36082; 7, *C. krusei*; 8, *C. tropicalis*; 9, *C. pseudotropicalis*; 10, *C. parapsilosis* 1110; 11, *C. parapsilosis* 1112; 12, *S. cerevisiae*; 13, *C. glabrata*. The yeast genomic DNA was digested with *Pst*I and hybridized with *LIP1* DNA. DNA isolation and Southern blot hybridization were performed as described in Methods.

lipase, lysophospholipase and phospholipase activities by using purified substrates in a radiometric assay. Culture supernatants from *S. cerevisiae* transformed with the candidal gene were able to degrade triolein but not phosphatidylcholine nor lysophosphatidylcholine (Table 1). No hydrolytic activities against any of the substrates were observed with the control strain (*S. cerevisiae* transformed with an empty plasmid). Table 1 shows that lipase activity was detected in concentrated filtrates from *C. cylindracea*. This yeast is known for its ability to produce extracellular lipases (Lotti *et al.*, 1993) and was used as a positive control. Additionally, commercially available phospholipase (Sigma) was used as a positive control for phospholipase activity. These results indicate that the cloned candidal gene encodes a lipase.

Evidence that egg yolk contains substrates for both phospholipase and lipase

To substantiate that egg yolk contains substrates for both lipases and phospholipases, we extracted the total lipid content from one egg yolk and analysed the extracted lipids using solvent systems that resolve polar (e.g. phospholipids, the substrates for phospholipases) and non-polar (e.g. triglycerides, the substrates for lipases) lipids. Our data demonstrated that egg yolk contains, in addition to cholesterol, triglycerides, phosphatidylcholine and phosphatidylethanolamine (data not shown). This finding confirms that egg yolk contains substrates for both phospholipases and lipases.

Southern blot analysis suggests that *C. albicans* may contain a *LIP1* gene family, and that this gene may be present in other non-*albicans* species

*Pst*I-digested genomic DNA of five different clinical *C. albicans* strains was blotted on nylon membranes and hybridized at low stringency with a 1.1 kb oligonucleotide probe, located in the *Xho*I-*Pst*I fragment, containing the whole *LIP1* gene. Between seven and eight distinct bands were identified depending on the strain tested (Fig. 4a). Two of these bands (Fig. 4b) were detected after hybridization at high stringency with the *LIP1* probe, suggesting that these bands are characteristic of the *LIP1* gene encoding a lipase in *C. albicans*. These data suggest that a lipase gene family may exist in *C. albicans*. The presence of two bands instead of one, following digestion with *Pst*I, is most likely a reflection of restriction site polymorphism between the two *LIP1* alleles.

In other experiments, *Pst*I-digested genomic DNAs of *C. tropicalis*, *C. krusei*, *C. pseudotropicalis*, *S. cerevisiae* and *C. parapsilosis* were hybridized, under low stringency, using the *C. albicans* *LIP1* oligonucleotide probe. Three bands from *C. tropicalis* and *C. parapsilosis* and two bands from *C. krusei* were detected (Fig. 4c). The same *Pst*I band pattern was found using eight *C. parapsilosis* strains isolated from different patients (data not shown). In contrast, no hybridization signals were observed when the *C. albicans* *LIP1* probe

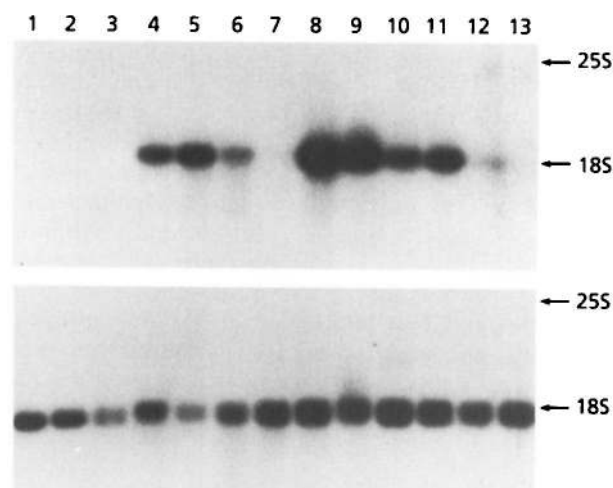


Fig. 5. Northern blot analysis of lipase mRNA levels. Various *Candida* species were grown in YNB supplemented with Tween 80 as sole carbon source. Lanes: 1, *C. parapsilosis* 1125; 2, *C. parapsilosis*; 3, *C. tropicalis*; 4, *C. albicans* 16240; 5, *C. albicans* 16727; 6, *C. albicans* 17336; 7, *C. albicans* 17737. *C. albicans* 36082 cells were grown in YNB supplemented with 2.5% Tween 80 (lane 8), Tween 20 (lane 9), Tween 40 (lane 10) or Tween 60 (lane 11). Expression was also monitored for this *C. albicans* strain following growth in YNB supplemented with either triglycerides (lane 12) or α -naphthyl palmitate (20 μ g ml⁻¹) (lane 13). RNA isolation and Northern blot hybridization were performed as described in Methods. The upper panel displays the results of hybridization with *LIP1* DNA and the lower panel shows the results of hybridization with actin DNA. The positions of rRNA are marked on the right.

was used in Southern hybridization analyses with DNA fragments from *C. glabrata*, *C. pseudotropicalis* or *S. cerevisiae* (Fig. 4c).

Expression of the *LIP1* gene

When the expression of the *LIP1* gene was monitored using Northern blot analysis, it was shown that the *LIP1* transcript was expressed only in YNB minimal media supplemented with Tweens 80, 60, 40 and 20 as the sole carbon source (Fig. 5). This expression was observed when the cultures were grown at either 30 or 37 °C. Expression of the *LIP1* transcript was also detected when *C. albicans* was grown in YNB supplemented with triglycerides as the only carbon source, albeit the signal detected was weaker than the signals observed when candidal cells were grown in YNB supplemented with Tweens (Fig. 5). In contrast, no expression was observed when either rich (Sabouraud Dextrose Broth, YPD) or chemically defined (YNB, Lee's) media were used to grow the yeast. Additionally, supplementing YNB with carbohydrates (including glucose, maltose or galactose), phosphatidylcholine, α -naphthyl palmitate, K₂HPO₄ or K₂HPO₄ plus KCl did not induce expression of the *LIP1* transcript (data not shown). Furthermore, altering the pH and the temperature of incubation for cells grown in Lee's medium, supplemented with glucose (1.25%, v/v), did not induce the expression of the *LIP1* gene (data not shown).

To determine whether the cloned lipase gene is expressed in other *C. albicans* and non-*albicans* species, total RNA was isolated from yeast cells grown in YNB supplemented with 2.5% Tween 80 as the sole source of carbon. A single 1.7 kb transcript that hybridized to the *LIP1* gene was detected in four clinical *C. albicans* isolates. However, no signal was detected in one clinical *C. albicans* isolate (Fig. 5). Interestingly, although significant hybridization was observed with Southern blot hybridization using DNA fragments from *C. parapsilosis* and *C. tropicalis* (Fig. 4c), indicating that homologous sequences were present, we were unable to detect a lipase expression signal from these non-*albicans* species grown under these conditions (Fig. 5).

DISCUSSION

A gene encoding a candidal lipase was cloned by complementation in *S. cerevisiae*. Comparison of the predicted protein sequence of the candidal lipase revealed significant homology with the lipid-binding site of lipases from *C. cylindracea* and *Candida antarctica* (Uppenberg *et al.*, 1994), and other fungi, such as *G. candidum* (Sidebottom *et al.*, 1991). There was also significant homology with the lipid-binding region of mammalian lipases (Lowe *et al.*, 1989) (Fig. 3). The conserved residues of this lipid-binding site include Gly-X-Ser-X-Gly. The serine of the active site has been shown to be enclosed in this highly conserved domain (Lotti *et al.*, 1993). This consensus domain represents the only feature shared by all determined lipase sequences (Antonin, 1988) including those from *C. cylindracea* and *G. candidum*. The remainder of the lipase structure is very heterogeneous from species to species.

Lipase genes cloned from *C. cylindracea* encode proteins of approximately 57 kDa and 534 amino acids (Lotti *et al.*, 1993). These enzymes are larger than the cloned *C. albicans* lipase that we have identified. The deduced sequence of the lipase from *C. albicans* cloned in this study contained five potential N-glycosylation sites. Previously identified lipases have had one to five potential N-glycosylation sites (Lotti *et al.*, 1993; Uppenberg *et al.*, 1994). Additionally, the lipase from *C. albicans* contained nine cysteine residues, while other cloned lipases contain between five and nine cysteine residues (Lotti *et al.*, 1993; Uppenberg *et al.*, 1994).

Our Southern hybridization findings suggest that *C. albicans* may contain a *LIP* gene family. This is expected since lipase gene families have been reported for other *Candida* spp.; for example, *C. cylindracea* is known to contain five distinct lipase genes (Lotti *et al.*, 1993).

In this study, detection of mRNA levels specific for a *LIP1* gene was only possible under a limited set of conditions. The presence of either a Tween compound or a triglyceride, and the lack of a carbohydrate source were found to be critical for the expression of this gene. Tweens are water-soluble mixtures of polyoxyethylene-sorbitan compounds containing different types of fatty

acids. Fatty acids incorporated in Tweens are: oleate in Tween 80, stearate in Tween 60, palmitate in Tween 40 and laurate in Tween 20. Our results showed that the lipase gene is expressed in cells grown in YNB supplemented with any of the Tween species, irrespective of the carbon chain length or degree of saturation. Thus, the expression is not dependent on the number of carbons or double bonds making up the fatty acids incorporated. The difference in expression signal observed between cells grown in the presence of Tween or triglycerides, the latter inducing a relatively weaker level of expression, could be due to the difference in solubility between these compounds. The poor solubility of triglycerides may have provided limited availability to *C. albicans*. The fact that carbohydrate supplementation inhibited *LIP1* expression is not entirely surprising as glucose is known to repress the transcription of a number of genes in *Saccharomyces*, including *FOX1* which encodes acyl-CoA oxidase, involved in the β -oxidation of fatty acids (Stanway *et al.*, 1995). Also, growth media containing low levels of glucose have been shown to induce increased activity of the *SNF1* product, a protein kinase responsible for the derepression of many glucose-repressed genes. *SNF1* is also known to inactivate a key enzyme (acetyl-CoA carboxylase) involved in fatty acid biosynthesis (Woods *et al.*, 1994). Interestingly, we found that the presence of glucose represses *LIP1* expression. This finding is in contrast with our observations regarding *PLB1*, a gene encoding candidal extracellular phospholipase B, where maximum expression was observed in medium containing a high concentration of glucose (Ibrahim *et al.*, 1996). These findings suggest that these two genes are expressed under different environmental conditions which may reflect different biological functions of these enzymes.

The *LIP1* gene from *C. albicans* was identified by screening transformed *S. cerevisiae* for clones that produced a precipitate when grown on egg-yolk medium. This medium has predominantly been used to screen for the production of extracellular phospholipase (Price *et al.*, 1982; Samaranayake *et al.*, 1984; Williamson *et al.*, 1986). Additionally, we discovered that egg yolk contains substantial amounts of triglycerides as well as phospholipids. The fact that egg-yolk medium cannot distinguish between the secretion of lipase and phospholipase activity represents an important finding originating from this study.

The specific role of lipases in the pathogenesis of candidal infections is unknown and has received limited attention. We are aware of only one study attempting to find a correlation between pathogenicity of *Candida* species and production of lipases. These authors reported that candidal species considered to be more pathogenic had higher levels of lipase activity compared to less pathogenic ones (Ogawa *et al.*, 1992). To determine the contribution of lipases to candidal virulence with greater certainty, it is necessary to use targeted disruption to delete the lipase gene. Our cloning and future disruption of the lipase gene should contribute to our understanding of the role of these enzymes

in candidal pathogenesis. These studies are currently underway.

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