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Yeast Sequencing Report

Identification of a triacylglycerol lipase gene family in *Candida deformans*: molecular cloning and functional expression

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Abstract

The yeast *Candida deformans* CBS 2071 produces an extracellular lipase which was shown to catalyse the production of various esters by the esterification of free fatty acids, even in the presence of a large molar excess of water. To clone the gene encoding this extracellular lipase, *Saccharomyces cerevisiae* was transformed with *C. deformans* genomic libraries and screened for lipolytic activity on a medium containing rapeseed oil emulsion and rhodamine B. Three members of a lipase gene family (*CdLIP1*, *CdLIP2* and *CdLIP3*) were cloned and characterized. Each deduced lipase sequence has a Gly–His–Ser–Leu–Gly–(Gly/Ala)–Ala conserved motif, eight cysteine residues and encodes an N-terminal signal sequence. MALDI–TOF mass spectrometry analysis of a proteolytic digest of the lipase produced was used to obtain experimental evidence that the *CdLIP1* gene encoded the extracellular lipase. Recombinant expression studies confirmed that the cloned genes encoded functional lipases. The three lipases are very similar to lipases from the related species *Yarrowia lipolytica*. Significant homologies were also found with several yeast and fungal lipases. As *C. deformans* CBS 2071 was previously considered to be synonymous with *Y. lipolytica*, the strains were compared for the extent of nucleotide divergence in the variable regions (D1/D2) at the 5'-end of the large-subunit (26S) ribosomal DNA (rDNA) gene. This rDNA region has diverged sufficiently to suggest that *C. deformans* is a separate species. The nucleotide sequences of the *CdLIP1*, *CdLIP2* and *CdLIP3* genes will appear in the EMBL nucleotide sequence database under Accession Nos AJ428393, AJ428394 and AJ428395, respectively. Copyright © 2003 John Wiley & Sons, Ltd.

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Introduction

Lipases or triacylglycerol lipases (E.C. 3.1.1.3) are enzymes capable of catalysing the hydrolysis of the ester bond between fatty acids and glycerol. They are, however, generally capable of hydrolysing esters of fatty acids and many alcohols other than glycerol. Triacylglycerols (triglycerides), having very low solubility in water, are their preferential substrates. Under natural conditions, they catalyse the hydrolysis of ester bonds at

the interface between an insoluble substrate phase and the aqueous phase in which the enzyme is dissolved. Therefore, they differ from esterases, which hydrolyse only soluble esters of fatty acids with short carbon chains. Under certain experimental conditions, some lipases can also catalyse ester synthesis via the esterification reaction (reversal of the hydrolysis reaction) or the transesterification reaction (transferring the acyl moiety of an ester to a suitable nucleophile other than water via alcoholysis or ester exchange). The amount of

water in the reaction mixture is a critical parameter that determines the direction of the lipase-catalysed reaction. In nearly anhydrous organic solvents, esterification and transesterification reactions are generally favoured while, in the presence of water, the reaction equilibrium is shifted towards hydrolysis and ester synthesis is limited.

Lipases are widespread in the animal, plant and microbial kingdoms. The first studies focused mainly on animal and plant lipases because of their physiological importance. Recently, lipases of microbial origin have attracted much interest due to their diverse properties, availability and relatively easy methods of preparation. Several review articles presenting comprehensive overviews of the biochemical properties and biotechnological applications of these enzymes have been published (Jaeger *et al.*, 1994; Jaeger and Reetz, 1998; Pandey *et al.*, 1998; Saxena *et al.*, 1999). Important uses in biotechnology include their significant role in the medical and therapeutic fields (supplements in patients with pancreatic insufficiencies, plasmatic triglyceride analysis, stereospecific synthesis of pharmacologically active products), chemistry of fats and oils (fat and oil hydrolysis, synthesis of esters with surfactant properties), cleaning (washing powders) and food technology (modification of flavour, improvements in conservation, acceleration of fermentation, formulation of oils).

Our laboratory previously reported that the yeast *C. deformans* CBS 2071 produces an extracellular 1,3-regiospecific lipase which was used for palm oil modification (Muderhwa *et al.*, 1985). Subsequently, our laboratory demonstrated that this enzyme was very effective in catalysing methyl ester synthesis from triacylglycerols or free fatty acids in a high water activity medium (Boutur *et al.*, 1994). In fact, it was determined that the enzyme catalysed ester production in aqueous media, not by alcoholysis but by the esterification of free fatty acids (Boutur, 1995; Boutur *et al.*, 1995).

In this study, in an attempt to clone the extracellular lipase gene from *C. deformans*, we used a complementation strategy employing several strains of *S. cerevisiae* transformed with *C. deformans* genomic libraries. By this approach, we have cloned and sequenced three *C. deformans* genes that imparted a positive response to *S. cerevisiae* cells.

Materials and methods

Strains and culture conditions

Strains used in this study are described in Table 1. The media and techniques used to grow the strains were as follows. *C. deformans* CBS 2071 (CBS stands for Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) was cultivated aerobically at 28 °C in Erlenmeyer flasks filled to one-tenth of their capacity. For lipase production, the strain was grown in synthetic G medium (Galzy, 1964) buffered to pH 6.5 with 100 mM NaH₂PO₄/Na₂HPO₄, and supplemented with 5 g/l rapeseed oil as the carbon source and inductor. Rich medium YEG, composed of yeast extract (5 g/l, Difco Becton Dickinson France SA, Le Pont de Claix, France) and glucose (10 g/l), was used for *C. deformans* chromosomal DNA extraction. Rich YPD medium, yeast extract (10 g/l), Bacto peptone (20 g/l, Difco), glucose (20 g/l), was used for *S. cerevisiae* cultivation at 30 °C. *S. cerevisiae* transformants were selected on YNB medium containing yeast nitrogen base without amino acids (6.7 g/l, Difco) and glucose (20 g/l). Adenine, histidine, tryptophan (20 mg/l) and leucine (30 mg/l) were added when required. For solid media, 20 g/l agar was added.

YNB-rhodamine B plates, used for lipase detection in *S. cerevisiae* transformants, were derived using the plate assay described by Kouker and Jaeger (1987). The medium contained glucose (5 g/l), rapeseed oil (5 g/l), agar (20 g/l) and was buffered to pH 6.5 with 100 mM NaH₂PO₄/Na₂HPO₄. The medium was autoclaved and cooled down to 60 °C. Then, 10 ml rhodamine B solution (1 mg/ml) and 100 ml yeast nitrogen base without amino acids (67 g/l, Difco) were added per litre. The medium was emulsified by mixing with Ultra-Turrax disperser (Janke & Kunkel KG, Staufen, Germany) and poured into Petri dishes. After incubation, strains producing extracellular lipase show, upon UV illumination, an orange fluorescent halo around the colony.

For lipase detection in *Y. lipolytica*, YNBH and YNBT plates were composed as follow: 50 mM KH₂PO₄/Na₂HPO₄ (pH 6.8), yeast nitrogen base without amino acids (6.7 g/l, Difco), agar (20 g/l), olive oil (YNBH, 10 g/l), tributyrine (YNBT, 10 g/l). Rhodamine B (10 mg/l) was added to YNBH for detection of free fatty acids. The media were emulsified as above.

Table 1. Strains and plasmids used in this study

Strains or plasmids	Description or relevant feature(s)	Reference or source
Strains		
<i>C. deformans</i>	(Zach) Langeron and Guerra; lipase-producing	CBS 2071
<i>S. cerevisiae</i> W303-1a	<i>MATα ura3-1 trp1-1 ade2-1 leu2-3,112 his3-11,15 can1-100</i>	
OLI	<i>MATα ura3-251,373 leu2-3,112 his3-15</i>	
LL-20	<i>MATα leu2-3,112 his3-11,15 can1</i>	
<i>Y. lipolytica</i>		
JMY277	<i>MATα ura3-302 leu2-270 xpr2-322 lip2::LEU2</i>	Pignede et al. (2000a)
JMY277(62)	JMY277 transformed to Ura ⁺ by integration of JMP62	This work
JMY277(62CdLIP1)	JMY277 transformed to Ura ⁺ by integration of JMP62CdLIP1	This work
JMY277(62CdLIP2)	JMY277 transformed to Ura ⁺ by integration of JMP62CdLIP2	This work
JMY277(62CdLIP3)	JMY277 transformed to Ura ⁺ by integration of JMP62CdLIP3	This work
Plasmids		
YEp352	Ap ^r <i>E. coli</i> - <i>S. cerevisiae</i> shuttle vector, 2 μm, <i>URA3</i>	Hill et al. (1986)
pRS425	Ap ^r <i>E. coli</i> - <i>S. cerevisiae</i> shuttle vector, 2 μm, <i>LEU2</i>	Christianson et al. (1992)
YEpC _W	YEp352 derivative, with 4.6 kb fragment from <i>C. deformans</i> containing <i>CdLIP1</i>	This work
YEpA _{C_O}	YEp352 derivative, with 5.2 kb fragment from <i>C. deformans</i> containing <i>CdLIP2</i>	This work
pRSA _L	pRS425 derivative, with 5.6 kb fragment from <i>C. deformans</i> containing <i>CdLIP3</i>	This work
JMP62	Ap ^r <i>E. coli</i> , <i>Y. lipolytica</i> integrative vector, <i>LIP2</i> promoter, <i>URA3</i>	Nicaud et al. (2002)
JMP62CdLIP1	JMP62 derivative, <i>CdLIP1</i>	This work
JMP62CdLIP2	JMP62 derivative, <i>CdLIP2</i>	This work
JMP62CdLIP3	JMP62 derivative, <i>CdLIP3</i>	This work

Escherichia coli XL1 Blue MRF' (Stratagene) used for plasmid preparation was grown in Luria-Bertani (LB) medium with ampicillin (100 mg/l) or kanamycin (40 mg/l).

Microbial growth was monitored by measuring culture absorbance at 600 nm.

Genomic DNA isolation

Cells from 2 ml *C. deformans* culture (ca 1.5×10^9 cells) were harvested by centrifugation and washed in 2 ml SDE buffer (0.9 M sorbitol, 0.1 M EDTA, 50 mM dithiothreitol, pH 7.5). Cells were resuspended in 0.5 ml SDE buffer containing 200 units of lyticase (Sigma) and incubated for 2 h at 37 °C. After centrifugation, the cell pellet was dispersed in 0.5 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 50 mM EDTA, 0.5 mg/ml proteinase K). Cell lysis was induced by adding 50 μl 10% (wt/vol) SDS. After overnight incubation at 55 °C,

proteins were extracted successively with one volume phenol (Tris-HCl saturated phenol, pH 8.0), one volume phenol:chloroform:isoamyl alcohol (25:24:1, vol:vol:vol) and one volume chloroform:isoamyl alcohol (24:1, vol:vol). DNA was precipitated with two volumes of ethanol and then dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Genomic library construction

Genomic DNA from *C. deformans* was partially digested with *Sau3A*, and fragments were separated by agarose gel electrophoresis. The resulting DNA fragments of 5–10 kb were recovered from agarose using the GeneClean kit (Bio 101 Inc., Vista, CA, USA), ligated into *Bam*HI-digested and dephosphorylated YEp352 and pRS425 plasmids, then introduced into *E. coli* XL1 Blue

MRF'. About 60 000 and 30 000 *E. coli* transformants were obtained for the YEp352 and the pRS425 libraries, respectively. For each library, analysis of 20 randomly selected transformants indicated that 85% of the plasmid contained inserts (mean size 5.7 kb). Plasmids from the pooled *E. coli* transformants were isolated and used to transform *S. cerevisiae* strains OL1, W303-1a (YEp352 library) and LL-20 (pRS425 library) to yield 18 000, 23 000 and 6200 transformants, respectively. After incubation for 4 days at 30 °C, *S. cerevisiae* transformants were recovered in saline solution (NaCl 9 g/l). The cells suspensions were diluted and spread on YNB-rhodamine plates and incubated at 30 °C in the dark.

Lipase activity assay and enzyme preparation

The production of extracellular and cell-bound lipases during the growth of *C. deformans* was analysed as follows: cells from 2 ml culture medium were harvested by centrifugation; the culture supernatant was conserved for extracellular lipase assay. The cell pellet was washed three times with 0.4 ml sodium phosphate buffer (20 mM NaH₂PO₄/Na₂HPO₄, pH 7.0); the washing supernatants were pooled. Lipase activity in both the culture supernatant and cell washes was determined. Lipase activity was determined by measuring the initial rate of triolein hydrolysis at 40 °C, pH 7.0, as described by Boutur *et al.* (1995).

Lipase preparation was performed as follows: cells from 0.75 l *C. deformans* culture grown in G medium were harvested at the early stationary phase by centrifugation. The cell pellet was extracted three times with 75 ml 20 mM sodium phosphate buffer (pH 7.0). Two volumes of cold acetone were added slowly to the supernatant. The mixture was kept at 4 °C for 30 min, and then centrifuged at 8000 × g for 15 min. The protein pellet was suspended in 40 ml 50 mM sodium phosphate (pH 7.0) and concentrated down to 0.7 ml on a regenerated cellulose ultrafiltration membrane (Millipore, YM10 membrane, cut-off 10 000 Da).

Protein analysis

When required, proteins were deglycosylated using endoglycosidase H (endo H, New England Biolabs Inc., Beverly, MA, USA) in accordance with

the manufacturer's instructions. Protein concentrations were determined using the Pierce BCA Protein Assay (Pierce, PerBio France, Bezons, France), with bovine serum albumin as the standard. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the method of Laemmli (1970). Low-range protein markers (Bio-Rad SA, Ivry sur Seine, France) and proteins were visualized by staining with Coomassie brilliant blue R-250.

For MALDI-TOF mass spectrometry analysis, proteins were separated by SDS-PAGE. The proteolytic protocol used for the trypsin digestion was adapted from Jensen *et al.* (1999). The 38 kDa band was excised following SDS-PAGE, washed, dehydrated in pure acetonitrile and dried under vacuum. After reduction of the disulphide bridges with dithiothreitol, the cysteines were alkylated with iodoacetamide. The proteins were digested overnight with 0.125 µg sequencing grade trypsin (Promega) at 37 °C. One volume of the medium, containing the peptides that diffused freely out of the gel slice, was mixed with an equal volume of saturated solution of α -cyano 4-hydroxycinnamic acid. A deposit of the mixture was made on a clean MALDI target and air-dried. Mass spectrometry analysis was performed on a MALDI-TOF BiFlex III spectrometer (Bruker, Breme, Germany). The masses were measured with an accuracy of 0.1 g/mol for a peptide of 1000 g/mol. Peptides are ionized [M + H]⁺ so the measured mass is increased by 1.0078 g/mol.

Lipase expression in *Y. lipolytica*

The expression vectors JMP62CdLIP1, JMP62-CdLIP2 and JMP62CdLIP3 (Table 1) were constructed as follows: the coding regions of *CdLIP1*, *CdLIP2* and *CdLIP3* were inserted into the expression vector JMP62 under the *POX2* promoter (Nicaud *et al.*, 2002). JMP62CdLIP1 was obtained by inserting together a 200 bp *HindIII*-*KpnI* fragment and a 1200 bp *KpnI*-*SphI* (*SphI* was blunt-ended with T4 DNA polymerase) fragment from plasmid YEpC_W (Figure 1A) into the *HindIII* and *EcoRI* (blunt-ended) sites of JMP62. JMP62CdLIP2 was obtained by inserting a 1500 bp *BamHI*-*EcoRI* PCR fragment into the corresponding sites of JMP62. The *BamHI* and *EcoRI* sites were created at the ATG initiation codon and in the 3'-untranslated region of *CdLIP2* by

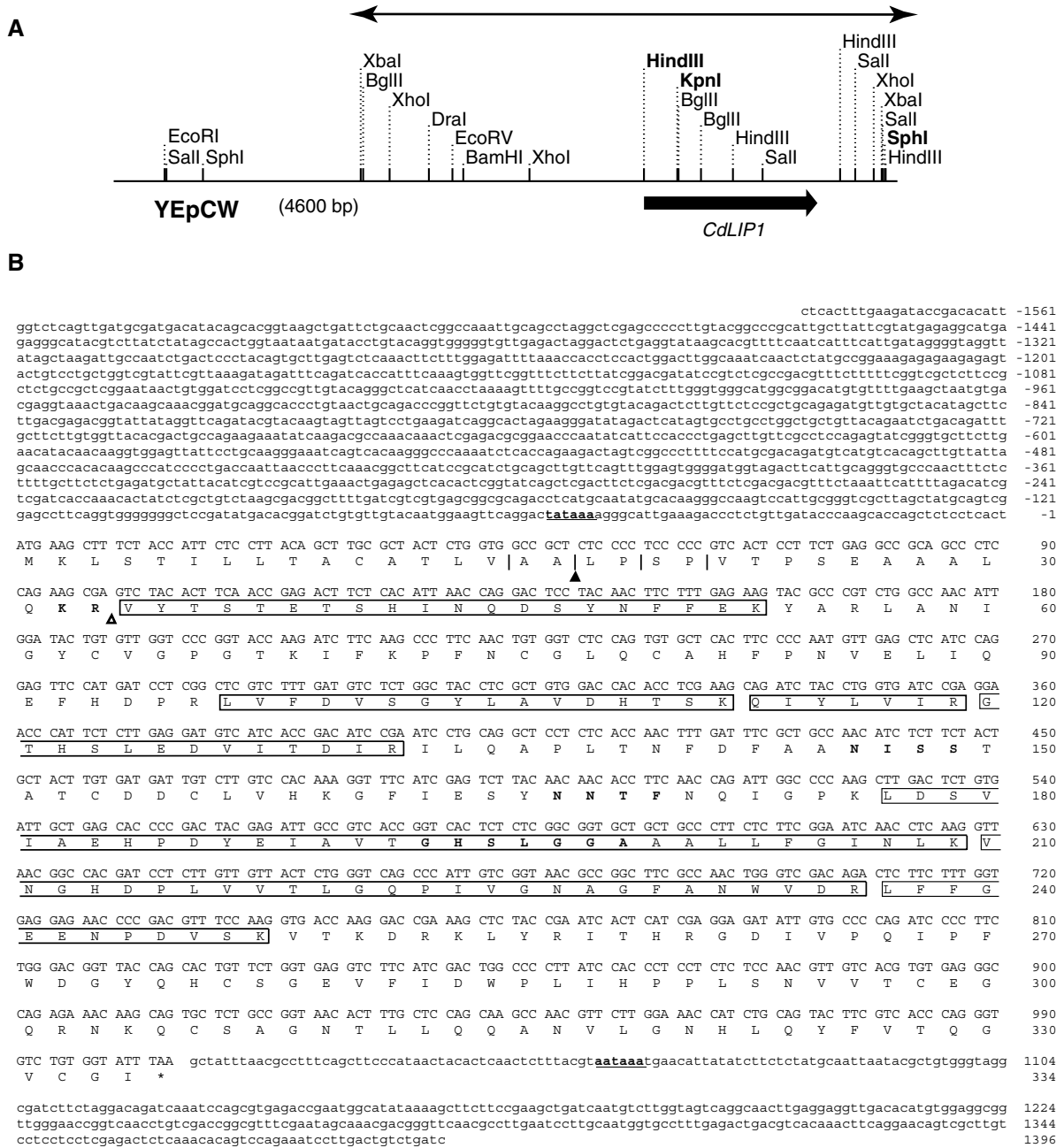


Figure 1. (A) Restriction map of the 4.6 kb fragment from plasmid YEpcw. The segment for which the 2981 nt sequence is reported is represented at the top. The ORF identified in the sequence is represented by a black arrow, and restriction sites used in the construction of expression vectors are in bold. (B) Nucleotide sequence of the *CdLIP1* gene and its derived amino acid sequence. Nucleotides and amino acids are numbered from the translation start codon. In the 5'-untranslated region, a potential TATA-box is in bold and underlined. The putative polyadenylation site at the 3'-untranslated region is in bold and underlined. The black triangle indicates a predicted signal peptidase cleavage site inside the three X–Ala or X–Pro dipeptides, and the open triangle a KEX2-like endopeptidase cleavage site after the Lys–Arg sequence. Potential N-glycosylation sites are shown in bold as well as the conserved GHSLGGA sequence containing the catalytic serine residue. Peptides identified by the MALDI–TOF mass spectrometry analysis of proteolytic digests of the secreted lipase produced by *C. deformans* are boxed

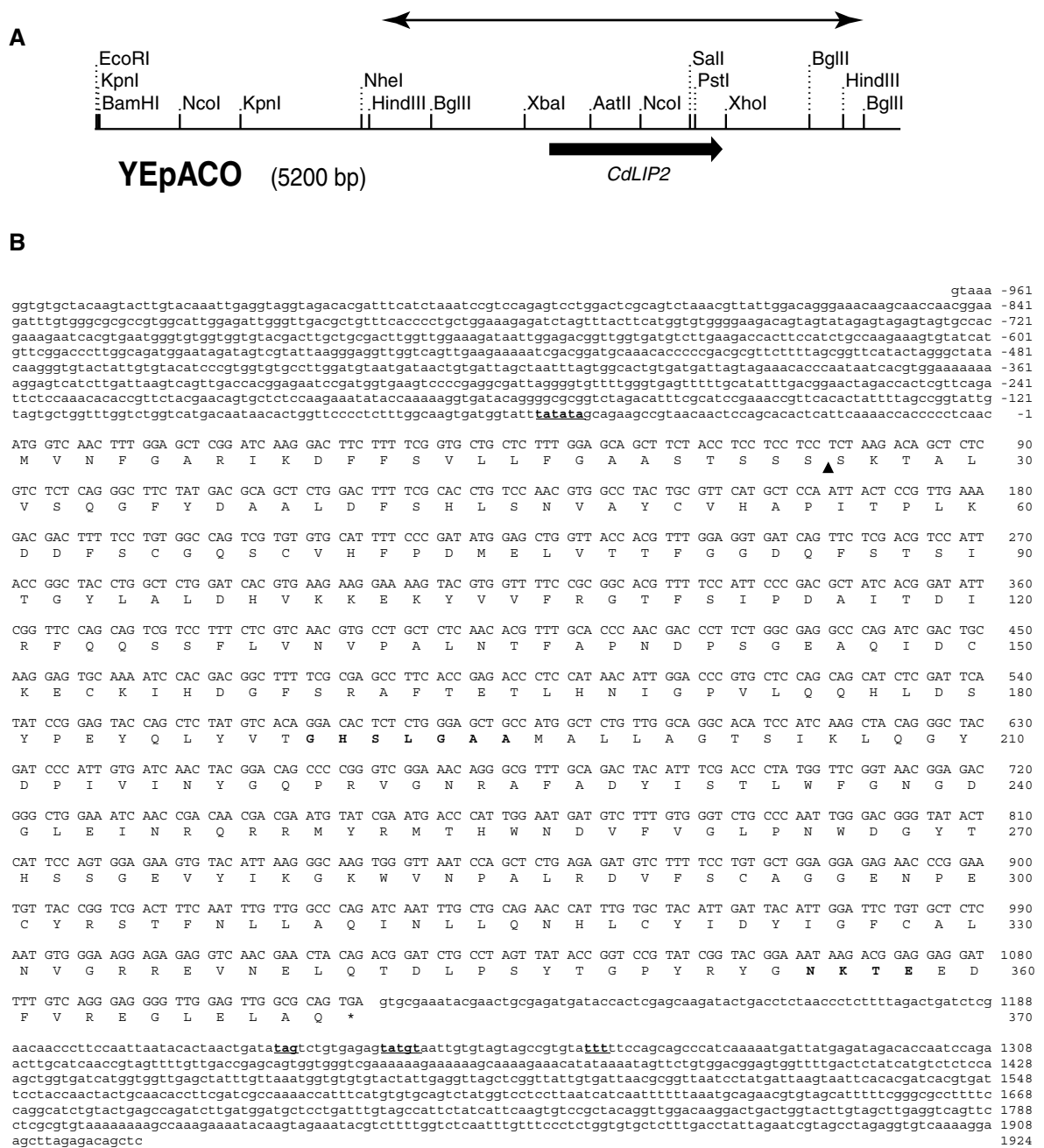


Figure 2. (A) Restriction map of the 5.2 kb fragment from plasmid YEpACO. The segment for which the 2889 nt sequence is reported is represented at the top. The ORF identified in the sequence is represented by a black arrow. (B) Nucleotide sequence of the *CdLIP2* gene and its derived amino acid sequence. The features are presented according to Figure 1

PCR amplification using YEpACO as the template. JMP62CdLIP3 was constructed by inserting a 1500 bp *NcoI* (blunt-ended)–*EcoRI* fragment from pRSA_L (Figure 3A) into the *BamHI* (blunt-ended) and *EcoRI* sites of JMP62.

The expression cassettes were introduced into the *Y. lipolytica* strain JMY277 by transformation using the lithium acetate method, as described previously (Le Dall et al., 1994). The vectors were digested by *NotI* to liberate the expression cassette

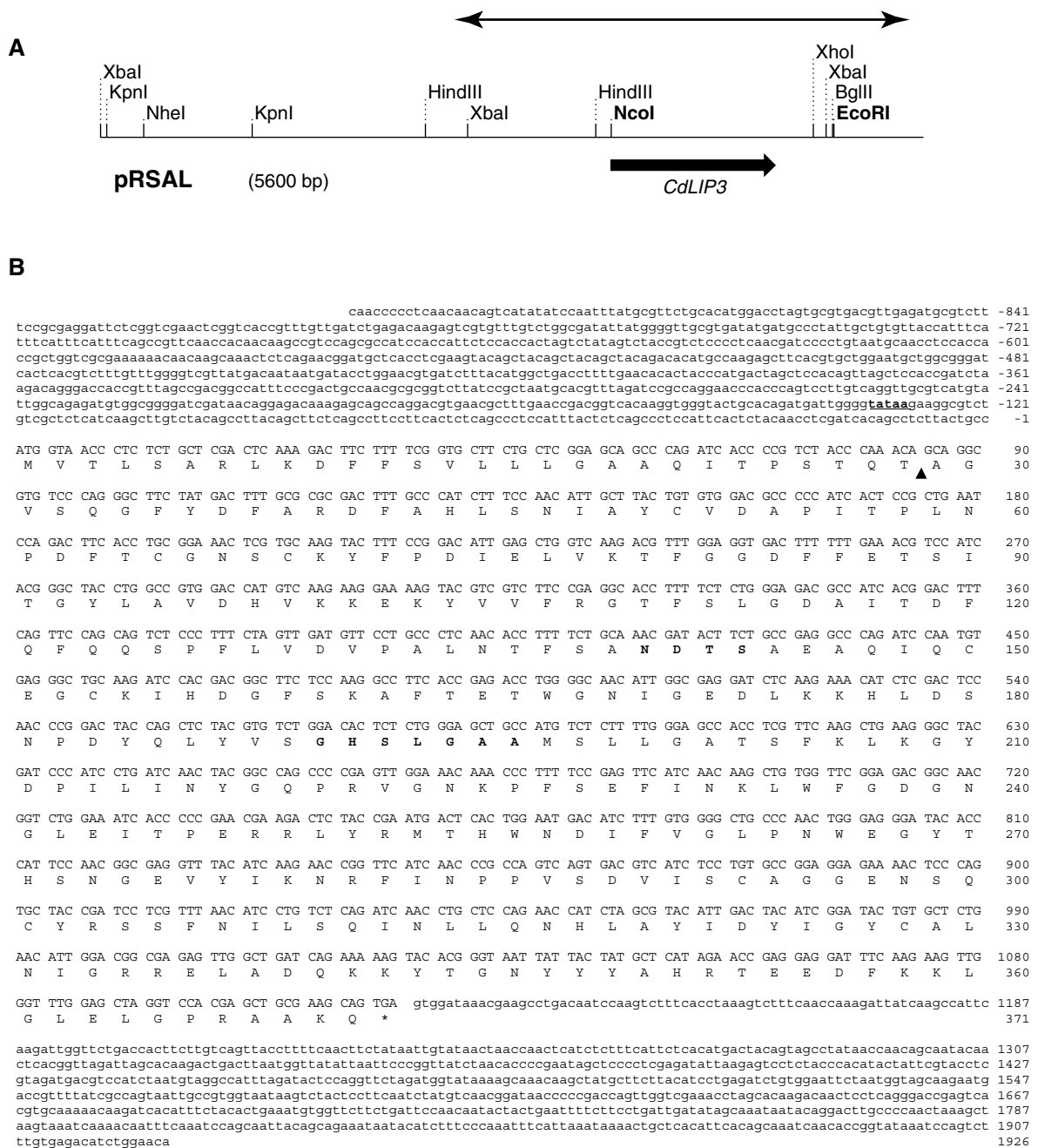


Figure 3. (A) Restriction map of the 5.6 kb fragment from plasmid pRSAL. The segment for which the 2845 nt sequence is reported is represented at the top. The ORF identified in the sequence is represented by a black arrow, and restriction sites used in the construction of expression vectors are in bold. (B) Nucleotide sequence of the *CdLIP3* gene and its derived amino acid sequence. The features are presented according to Figure 1

prior to transformation. Lipase production was analysed on YNBH and YNBT plates.

Other genetic methods, DNA sequencing and sequence analysis

Plasmid isolation, DNA manipulations, and plasmid transformation into *E. coli* XL1 Blue MRF' cells were performed as described by Sambrook *et al.* (1989).

For sequence determination, the fluorescence-based dideoxy DNA cycle sequencing method was used. Sequencing reactions were performed by Genome Express (Grenoble, France), using Big Dye terminator sequencing chemistry on Applied Biosystems automated sequencers (Applied Biosystems, Courtaboeuf, France). Sequence analyses were performed with the SeqLab interface of the Wisconsin Package (GCG package, version 10.1, Genetics Computer Group, Madison, WI, USA) and with the Staden package. For similarity searches, Swiss-Prot (release 40.0) and TrEMBL (release 18.0) databases were scanned with the FastA program (Pearson and Lipman, 1988). Sequence homologies were obtained after pairwise comparison with the Gap program (GCG). Multiple alignments of protein sequences were obtained by the program ClustalW (Higgins and Sharp, 1988).

Results

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

To clone the lipase encoding gene from *C. deformans*, we used a specific and sensitive rhodamine B plate assay, initially developed for bacterial lipases (Kouker and Jaeger, 1987). After incubation, by irradiating plates with UV light, lipase-producing colonies show orange fluorescent halos. Preliminary experiments showed that *S. cerevisiae* strains OL1, W303-1a and LL-20, unlike *C. deformans*, did not form a halo in this assay and therefore did not secrete detectable lipases. This suggested that the lipase plate assay could be useful in cloning the lipase gene from *C. deformans* through the ability of its DNA sequences to complement *S. cerevisiae* strains.

Two *Sau3A* genomic DNA libraries were constructed in the yeast vectors YEp352 and pRS425

(Table 1) as described in Materials and methods. The completeness of the YEp352 library was tested by complementation of *S. cerevisiae leu2* mutation. Twelve *Leu*⁺ transformants were obtained, corresponding to about 60 000 *Ura*⁺ *S. cerevisiae* OL1 transformants. This indicates that about 1/5000 of the transformants contained the *C. deformans LEU2* gene and that this gene is expressed in *S. cerevisiae*.

To screen for clones exhibiting lipolytic activity, approximately 6700, 4100 and 2900 *Ura*⁺ *S. cerevisiae* transformants were spread on YNB-rhodamine B plates for strains OL1, W303-1a and LL-20, respectively. Four lipase-positive colonies were isolated from strain W303-1a, and one each from strains OL1 and LL-20. Plasmid DNA from the positive clones were recovered and transformed into *E. coli*. Retransformation of W303-1a and LL-20 was performed to verify that the lipolytic phenotype was due to the plasmids. Transformants were tested on YNB-rhodamine plates and compared with transformants containing the parental plasmids (YEp352 and pRS425) as negative controls. In contrast to the cells containing the parental plasmids, all the transformants tested showed lipolytic activity.

The plasmid from the lipolytic OL1 transformant was named YEpAC_O and it contained a 5.2 kb genomic insert. The plasmids from the lipolytic W303-1a transformants shared common fragments (data not shown). One of these plasmids, named YEpC_W, which contained a 4.6 kb genomic insert, was selected for further analysis. The plasmid from the LL-20 transformant was named pRSA_L and it contained a 5.6 kb genomic insert. The genomic fragments from these plasmids were first single-strand sequenced by primer walking to detect open reading frames (ORFs), and sequenced on the second strand in regions which contained an ORF coding for proteins with high sequence similarities to yeast and fungal lipases (see below). Restriction maps, nucleotide sequences and amino acid translations are presented in Figures 1–3. The lipase coding genes from plasmids YEpC_W, YEpAC_O and pRSA_L were named *CdLIP1*, *CdLIP2* and *CdLIP3*, respectively.

Sequence analysis of the three lipase encoding genes

From plasmid YEpC_W, we determined the sequence on the two strands of a 2981 bp

fragment (Figure 1A). It contained *CdLIP1*, which is 1005 bp long. Analysis of the 5'-untranslated regions revealed a potential TATA-box, identical to the most optimal yeast consensus TATAAA (Chen and Struhl, 1988), at position -56 bp relative to the ATG codon (Figure 1B). The 3'-untranslated region of *CdLIP1* contained the sequence AATAAA (50 bp downstream of the stop codon) usually found upstream to the polyadenylated 3' terminus in most eukaryotic mRNAs. The resulting 334 amino acid (aa) sequence shared 91.6% identity with YILIP2, the extracellular lipase produced by *Y. lipolytica* (Pignede *et al.*, 2000a). The two proteins shared similar primary structure organization (Pignede *et al.*, 2000a), as shown in Figures 1B and 4A, composed of a prepro region with a 15 aa peptide, followed by three X-Ala or X-Pro dipeptides (four for YILIP2), substrates of a diamino peptidase (Matoba and Ogrydziak, 1989); a short 12 aa pro region ending at a Lys-Arg (KR) site, the substrate of the KEX2-like endopeptidase encoded by the *XPR6* gene in *Y. lipolytica* (Enderlin and Ogrydziak, 1994); and finally the 301 aa mature protein coding for a 33.4 kDa protein (Figure 1B). A potential cleavage site of a signal peptide is most likely to occur between aa positions 17 and 18, according to SignalP prediction (Nielsen *et al.*, 1997), after the first X-Ala dipeptide, to give a 17 aa signal sequence. The mature CdLIP1 presents two potential N-glycosylation sites at amino acids 146 and 167 (Figure 1B). The predicted pI of CpLIP1 is 5.6.

From plasmid YE_pACO, we determined the sequence on the two strands of a 2889 bp fragment (Figure 2A). It contained *CdLIP2*, which is 1113 bp long. Analysis of the 5'-untranslated region revealed a TATATA sequence at position -56 bp relative to the ATG codon that could work as an effective TATA-box in yeast (Figure 2B). The 3'-untranslated region of *CdLIP2* contains the sequence TAG...TATGT...TTT (108, 121 and 146 bp downstream of the stop codon), corresponding to the tripartite element that is found close to the transcription termination and polyadenylation sites in many yeast genes (Zaret and Sherman, 1982). The resulting 370 aa sequence contains several processing motifs (Figure 2B). A potential signal sequence cleavage site was detected between positions 25 and 26, according to SignalP, resulting in a 345 aa (38.6 kDa) mature protein with an

estimated pI of 5.2. One potential N-glycosylation site has been identified at aa position 355.

From pRSA_L, we determined the sequence on the two strands of a 2845 bp fragment (Figure 3A). *CdLIP3* is 1116 bp long and encodes a 371 aa sequence. In the 5'-untranslated region of *CdLIP3*, a potential TATA-box (sequence TATAAG) at position -135 bp relative to the ATG codon is present (Figure 3B). Several processing motifs were detected in the deduced aa sequence (Figure 3B). No clear signal peptide cleavage site could be predicted according to SignalP. Using SignalP-HMM (Nielsen and Krogh, 1998), a potential 28 aa signal sequence was detected, giving a 343 aa (38.5 kDa) mature protein with an estimated pI of 5.5. One potential N-glycosylation site has been identified at aa position 140.

The three genes constitute a lipase family in *C. deformans*

The three *C. deformans* genes encode lipases with similarities to each other and with the same overall structure, suggesting that these three lipase genes are members of a gene family (Figure 4A). In particular, a high degree of conservation was found in the middle of the lipase sequences, where a conserved Gly-His-Ser-Leu-Gly-(Gly/Ala)-Ala motif matches the consensus Gly-X-Ser-X-Gly, in which the serine of the active site is enclosed in most lipolytic enzymes. The sequences also contain eight conserved cysteine residues, which may form disulphide bridges contributing to a similar three-dimensional structure.

Searching through databases for proteins similar to *C. deformans* lipases revealed, in addition to YILIP2, several yeast and fungal lipases (Figure 4B). The highest homologies were also found with other lipases (YILIP4, YILIP5, YILIP7 and YILIP8) produced by *Y. lipolytica* (J.-M. Nicaud, unpublished data). The highest amino acid sequence identities were 91.6% (between CdLIP1 and YILIP2), 86.9% (between CdLIP2 and YILIP7) and 89.2% (between CdLIP3 and YILIP8). When the *C. deformans* lipase sequences were compared with the remaining yeast and fungal lipase sequences from databases (Figure 4B), similarities in conserved regions were found, even if overall similarities were minor. The greatest similarities were found with the lipases from *Candida ernobii* (Accession No. Q9HDQ8) and

A

CdLIP2	-----MVFNGARIKDFP-----S-----VLLFGAASTS-----SSSKTALVSQ-----GFYDA-----	38
CdLIP3	-----MVTLSARLKDFP-----S-----VLLLGAQAIT-----PSTQTAGVSQ-----GFYDF-----	38
YLLIP2	---MKLSTILFTACATLAAALP-----SP-----ITPSEAAVLQKRVTSTETSHIDQ-----ESYNF-----	50
CdLIP1	---MKLSTILLTACATLVAALP-----SP-----VTPSEAAVLQKRVTSTETSHINQ-----DSYNF-----	50
RmLIP	MVLKQRANVYLGFLIVFFTAFLV--EAVPIKRQS--NSTVDS--LPPLIPSRTSAPSSSPSTTDPEAP--AMSRN--	76
RdLIP	MVSFISISQGVSLCLLVSMMLGSSAVPVSGKSGSNTAVSASDNAALPPLISSRCAPPSSNKGSKSDLQAEPYMNQKNT	100
TLLIP	-----MRSSL-----VLFVSAWTA--LASPIRREVQ-----DLFNQ-----	31

	D1	D2	D2	
CdLIP2	-----ALDFSHLSNWAYCVHAPITPLKDDFSCG-QSCV--HFPDMELVTFGGDQFSTSIITGYLAL	96		
CdLIP3	-----ARDFAHLSNIAYCVDAPITPLNPDFTCG-NSCK--YFPDIELVKTFFGGDFPETSITGYLAV	96		
YLLIP2	-----FEKYARLANIGYCV-GPGTKIFKPFNCG-LQCA--HFPNVELIEEFHDPRLIFDVSGLYAV	107		
CDLIP1	-----FEKYARLANIGYCV-GPGTKIFKPFNCG-LQCA--HFPNVELIQEFHDPRLIFDVSGLYAV	107		
RmLIP	YGMALNATS--YPDSVVQAMS--IDGG--IRAATSQEIHELTYTTLSANSYCR--TVIPGAT-WDC--IHCD--	162		
RdLIP	GMATLDPDAPPISLSSSTNSASDGGKVAATTAIQEFQTYAGIAATAVCR--SVVPGNK-WDC--VQCQK-WVPD	192		
TLLIP	-----FNLFAQYSAAAYCGKNNDAPAGTNICTGNACPEVEKADATFLYSFEDS--GVGDVTFGLAL	91		

	D3	D3	*	
CdLIP2	DHVKKEKYVFRGTFSPDAITDIRFQQSSFLVNPALNTPANPDSGEAQIDCKECKIHDGFSRAFTETLHNIGPVLQ	HLSLGGAA	*	196
CdLIP3	DHVKKEKYVFRGTFSLGDAITDFQFQQSPFLVNPALNTPSANDTSARAQIQCEGCKIHDGFSKAFETWGNIGEDL	HLSLGGAA	*	196
YLLIP2	DHASKQIYLVIRGTHSLEDVITDIRIMQAP-LTNF--DLAAN-ISS'TA--TCDCLVHNGPIQSYNNYFNQIGPKL	HLSLGGAA	*	199
CdLIP1	DHTSKQIYLVIRGTHSLEDVITDIRILQAP-LTNF--DFAAN-ISS'TA--TCDCLVHNGPIESYNNYFNQIGPKL	HLSLGGAA	*	199
RmLIP	GDSEKTIYIVFRGSSIRNWIADLTFVPSV--YPPVS--CTKVHKGPLDSYGEVQNELVATVLDQPKQYPSYKVA	HLSLGGAA	*	242
RdLIP	SDKQKTIYLVFRGTSNFRSAITDIVNFS--YKPVK--GAKVHAGFLSSYEQVNDYFPVQEQQLTAHTYKVI	HLSLGGAA	*	272
TLLIP	DNTNKLIVLSFRGSRSENWIENLNFDLKE--INDI--CSGCRGHGDTSSRSVADTLRQKVEDAVREHPDYRVV	HLSLGGAA	*	172

	*	
CdLIP2	MALLAGTISI--KLQGYDP--IVINYQPRVGNRAFADYISTLWFG--NGDGLEINRQRMRYRMTHWNDFVGLPNWD	288
CdLIP3	MSLLGATSF--KLKGYDP--ILINYQPRVGNKPPSEFINKLWFG--DNGLEITPERRLYRMTHWNDFVGLPNWE	288
YLLIP2	AALLFGINL--KVNQHDP--LVVTLGQPIVGNAGFANWVDRLLFFGGENPDVSKVSKDRKLYRITHRGDIPVQI	293
CdLIP1	AALLFGINL--KVNQHDP--LVVTLGQPIVGNAGFANWVDRLLFFGGENPDVSKVSKDRKLYRITHRGDIPVQI	293
RmLIP	TALLCALDLYQREBGLSSNLFYTLQGGPRVGDPAFANYVSTGIP--YRRTVNERDIVPHLPPAAGFLHAGEEY	324
RdLIP	QALLAGMDLYQREPRLSPKNLSIFTVGGPRVGNPTFAFYVESTGIP--FQRTVHKRDIVPHVPPQSGFLHGP	353
TLLIP	LATVAGADL--RNGYDI--DVFSYGAPRVGNRAFAEFLTVQTGG--TLYRITHNDIVRPLPPREFYSHSPEYWI	252

	D4	D4	*	D1
CdLIP2	DVFSKAG-GENPECYRSTFN-LLAQINLLQNHLCYIDY-IGFCALNVGRREVNELQTDLPSTGYPYRGNKTEEDFV	REGLELAQ----		
CdLIP3	DVISCAG-GENSQCYRSSFN-ILSQINLLQNHLYIDY-IGYCALNIGRREL--ADQKKTGNYYYAHRTEEDFKKL	GLELGPRAAKQ		
YLLIP2	NVVMCQG-QSNKQC--SAGNTLLQANVNLGNHLQYFVT-EGVCGI-----			
CdLIP1	NVVTCBG-QRNKQC--SAGNTLLQANVNLGNHLQYFVT-QGVCGI-----			
RmLIP	TVQVCTSDLETSDC--SNSI--VPFTSVL-DHLSYFGINTGLCT-----			
RdLIP	NVQICTSEIETKDC--SNSI--VPFTSVL-DHLSYFDINEGSCL-----			
TLLIP	TRNDIVK-IEGIDA--TGSN-NQPNIPDIPALHLWYFGL-IGTCL-----			

B

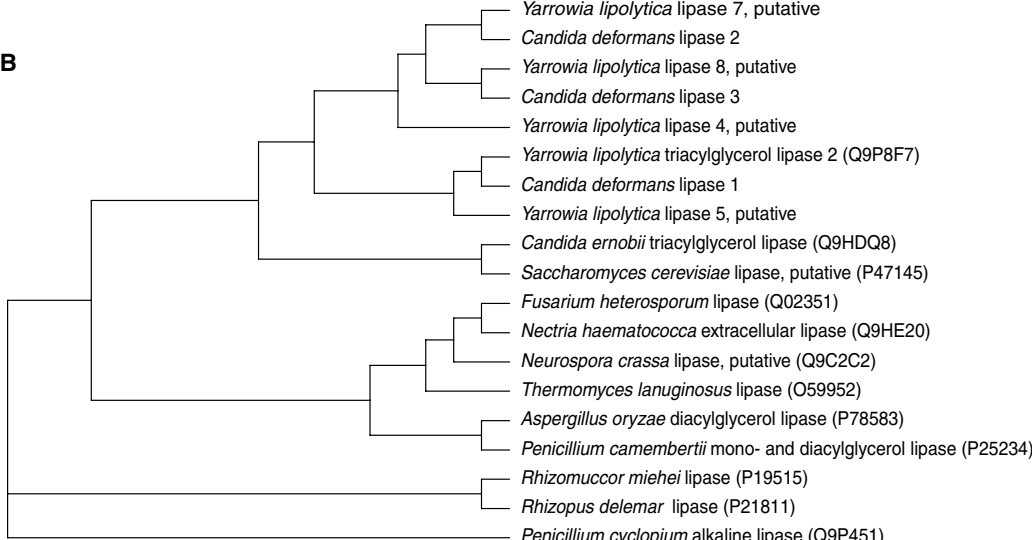


Figure 4. (A) Multiple alignment obtained by the ClustalW program of the lipases from *C. deformans* and *Y. lipolytica* with three lipases from the fungal lipase family: *Rhizomucor miehei* (RmLIP lipase, Accession No. P19515), *Rhizopus delemar* (RdLIP lipase, P21811) and *Thermomyces lanuginosus* (TLLIP lipase, O59952). The three amino acids of the catalytic triad are marked by asterisks, and the eight cysteines that may form four disulphide bridges are labelled according to sequence conservation with the fungal lipase family (D1, D2, D3, D4). The conserved GHSLGG motif containing the Ser residue of the active site is boxed. (B) Dendrogram (guide tree) obtained by the ClustalW program (gap opening, 10; gap extension, 0.1; scoring matrix, identity) showing the homologies of the lipases from *C. deformans* with those found in protein databases. Accession Nos are in brackets except for the putative lipases from *Y. lipolytica* (J.-M. Nicaud, unpublished data)

S. cerevisiae (Accession No. P47145). According to the multiple alignment on Figure 4A, the lipases from *C. deformans* and *Y. lipolytica* belong to the family of fungal lipases (from *Rhizopus delemar*, *Rhizomucor miehei* and *Thermomyces lanuginosus*). The aspartic and histidine residues that belong to the catalytic triad of lipases, together with the serine in the active site, were found at perfectly conserved positions.

Evaluation of the relatedness of *C. deformans* and *Y. lipolytica*

Amino acid comparison of CdLIP1 and YILIP2 (see above) suggested that *CdLIP1* would be the homologue of *YILIP2* in *C. deformans*. Nucleotide sequence comparison between the coding regions

of these two genes revealed 88.4% sequence identity, while sequence homology is 60% and 67.4% in the 5'- and 3'-untranslated regions respectively. In order to evaluate the relatedness of *C. deformans* to *Y. lipolytica*, we determined the nucleotide sequence of the variable region (D1/D2) at the 5'-end of the large-subunit ribosomal DNA (LSU rDNA) gene for *C. deformans*. After pairwise comparison of the DNA sequence obtained from *C. deformans* to that of *Y. lipolytica* (Accession No. U40080), 14 nucleotide differences were found, indicating a high degree of genetic divergence between the two species (Figure 5). Searching through databases for more homologous nucleotide sequences revealed the *Y. lipolytica* sequence only.

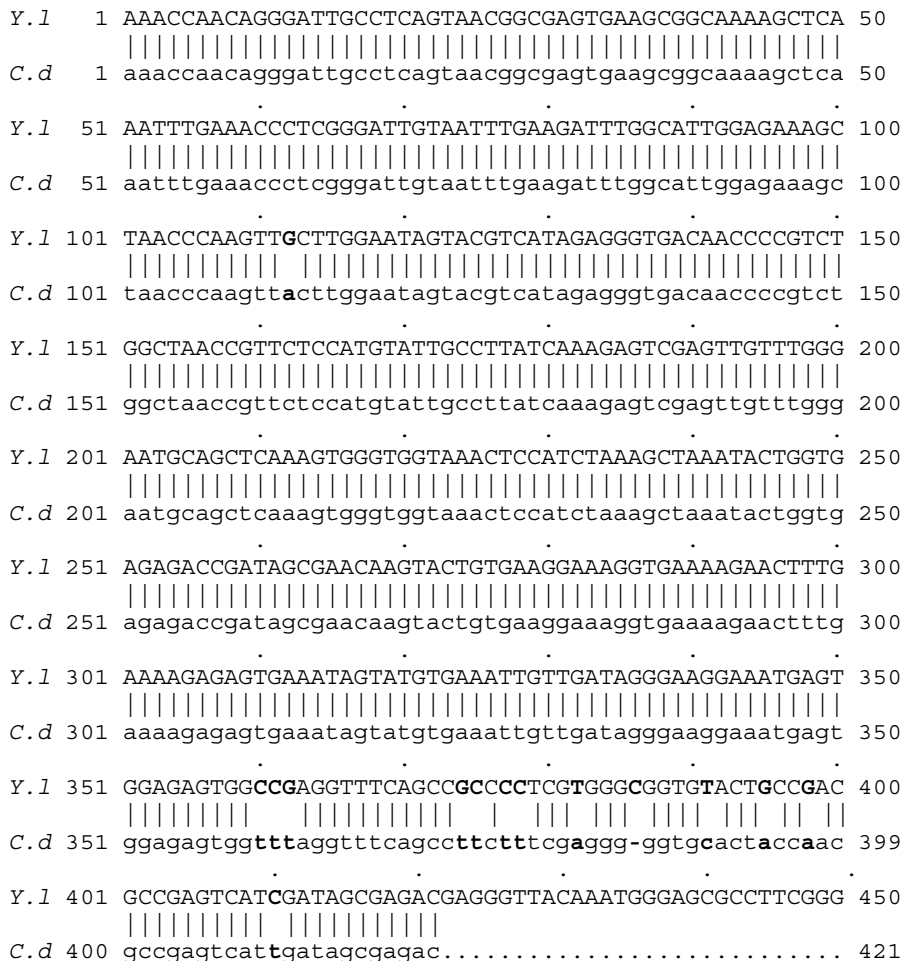


Figure 5. Nucleotide divergence between *C. deformans* and *Y. lipolytica* (Genbank Accession No. U40080) in the variable region at the 5'-end of the large-subunit (26S) ribosomal DNA gene

Analysis of the secreted lipase of *C. deformans*

Cell growth and triolein hydrolysis activity were monitored during growth on G medium (Figure 6A). Lipase production was maximal when cell growth reached the stationary state and then decreased rapidly. Lipase activity released by washing cells with buffer represented 75% of total culture activity, suggesting that a large proportion of the lipase was bound to the cell wall, in contrast to the lipase from *Y. lipolytica*, which was secreted into the extracellular medium (Pignede et al., 2000a).

When the cell-bound lipase was concentrated and analysed by SDS-PAGE, a single protein band of apparent molecular mass of 38.0 kDa was detected (Figure 6B, lane a). Deglycosylation with endoglycosidase H under denaturing conditions resulted in a 4.2 kDa decrease in the apparent molecular mass, suggesting that the protein was glycosylated (Figure 6B, lane b). The apparent molecular mass of 33.8 kDa is close to the predicted 33.4 kDa calculated for the mature polypeptide of CdLIP1 (301 aa). The prepared lipase was subjected to mass spectrometric peptide mapping (data

not shown). This map was compared with the theoretical map that one should obtain after trypsin digestion of the three lipases, CdLIP1, CdLIP2 and CdLIP3. Seven peptides in the spectrum (Table 2) had a mass that was expected for peptides obtained from CdLIP1, resulting in a sequence coverage of 42.2% (127 aa for a total size of 301 aa for the mature CdLIP1 lipase) and suggesting that the main extracellular lipase produced by *C. deformans* is encoded by *CdLIP1*.

Expression of *C. deformans* lipase genes in *Y. lipolytica*

To investigate whether *CdLIP2* and *CdLIP3* genes encoded for functional extracellular lipases, we used a previously described *Y. lipolytica* expression system (Pignede et al., 2000a; Nicaud et al., 2002). This system consists of an integrative vector (JMP62) and a host strain (*Y. lipolytica* JMY277) in which the extracellular lipase gene *YLIP2* has been disrupted. The vector contains a *URA3* marker for selection in *Y. lipolytica* and the *POX2* promoter for driving gene expression upon induction by triglycerides.

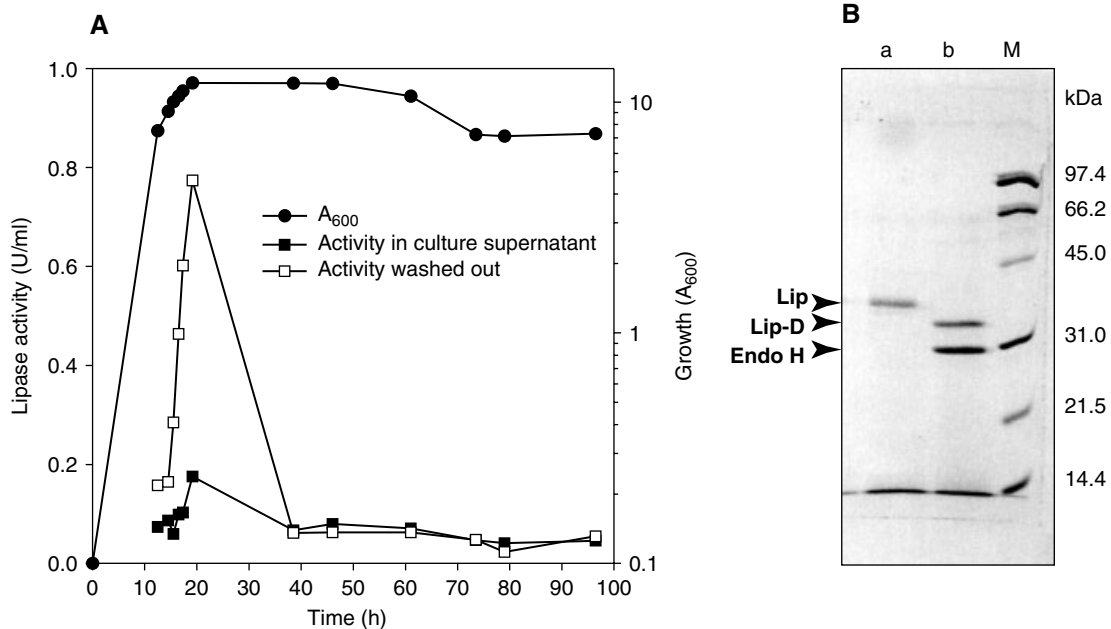


Figure 6. (A) Growth and lipase production of *C. deformans* on G medium were monitored over time. Production of lipase was assayed on the culture supernatant and washing supernatant of the cells. (B) SDS-PAGE analysis of a concentrated lipase preparation. Untreated proteins (lane a) and proteins deglycosylated with Endo H (lane b) were separated by SDS-PAGE (12% wt/vol acrylamide) and stained with Coomassie brilliant blue R-250. The positions of Endo H and the untreated (Lip) and deglycosylated (Lip-D) lipases are indicated on the left. Lane M contains molecular weight markers

Table 2. CdLIP1 peptides identified by MALDI-TOF mass spectrometric peptide mapping

Amino acid position	Peptide sequence	Monoisotopic mass [M + H] ⁺	
		Calculated	Observed
113–119	QIYL VI R	904.6	904.6
237–248	LFFGEENPDV SK	1381.7	1381.8
120–132	GTHSLEDVITD IR	1455.7	1455.9
97–112	LVFDVSGYLAVDHT SK	1750.9	1751.0
34–53	VYTSTETSHINQDSYN FFEK	2410.1	2410.3
210–236	VNGHDPLVVTLGQPIVGNAGFANW WDR	2845.5	2845.8
177–209	LDSVIAEHPDYEIAVTGHS LGAAALLFGINLK	3391.8	3392.3

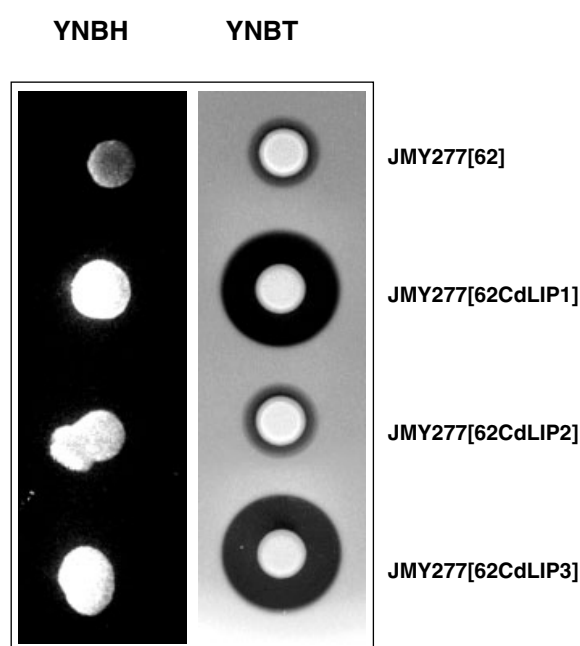


Figure 7. Lipase production by Ura⁺ transformants. Lipase detection was performed by spotting 5 μ l of each culture (ca. 10^5 cells) onto YNBH and YNBT plates. The plates were incubated for 2 days at 28 °C. On the YNBH plate, lipase production is revealed by irradiating the plate with UV light, with colonies showing orange fluorescence, and by the appearance of a clearing zone on the YNBT plate

The production of extracellular lipase by Ura⁺ transformants was tested on YNBT and YNBH plates (Figure 7). In YNBT plates, tributyrine was chosen as a substrate because it yields water-soluble products upon hydrolysis; thus, emulsions clear relatively quickly in the presence of lipase. Tributyrine, however, is not a lipase-specific substrate, and additional evidence that lipolytic enzymes were produced was required. YNBH

plates, containing triglycerides from olive oil that meet the strict definition of a lipase substrate, were used to distinguish between lipases and esterases. After 2 days of incubation, lipase production was detected on YNBH plates and was particularly evident on YNBT plates for strains expressing *CdLIP1* and *CdLIP3*. Although lipase expression was recorded on the triglyceride containing medium YNBH, tributyrine hydrolysis was not observed for the *CdLIP2*-containing strain growing on YNBT plates. The control strain, JMY277, transformed with the vector JMP62 alone, did not show any fluorescence on YNBH plates, whereas a very small clearing zone was observed on YNBT plates, indicating the absence of extracellular activity in this strain.

We further studied the production of extracellular lipase by the recombinant strains growing in liquid medium (data not shown). The supernatant of the culture medium exhibited lipase activities of 15 U/ml, 0.5 U/ml and 2 U/ml for the strains expressing *CdLIP1*, *CdLIP2* and *CdLIP3*, respectively. This demonstrates that *CdLIP2* and *CdLIP3*, along with *CdLIP1*, were expressed as functional lipases.

Discussion

The production of extracellular lipase activity by *C. deformans* has previously been reported (Muderhwa *et al.*, 1985). We have demonstrated that this lipase could be useful for methyl ester production (Boutur *et al.*, 1994). However, no study describing the encoding genes has been published to date. To produce large amounts of this enzyme, we used the genetic engineering approach rather than the selection of hyper-producer mutants.

At the very beginning of the work, the cloning of two genes encoding lipases (*YILIP1* and *YILIP3*) was reported for the related species *Y. lipolytica* (Choupina *et al.*, 1999). These lipases are similar to lipases from *Candida rugosa* and *Geotrichum candidum* and belong to the carboxyesterase family. They do not show clear signal peptides and therefore may be intracellular or membrane-bound. Since no information was available concerning an extracellular lipase, we decided to use a complementation strategy employing several strains of *S. cerevisiae*. This strategy has previously been reported for the cloning of a lipase gene from *C. albicans* by screening *S. cerevisiae* transformants on egg-yolk medium (Fu *et al.*, 1997), a medium predominantly used to screen for the production of extracellular phospholipase.

Two *C. deformans* genomic libraries were constructed corresponding to the equivalent of 20 genomes (ca 440×10^6 bp). These libraries were used to transform three different non-lipase-producing *S. cerevisiae* strains. Six clones that developed an orange fluorescent halo (upon UV illumination) on a medium containing rhodamine B and emulsified rapeseed oil were isolated among the 14 000 Ura^+ transformants tested. Restriction mapping and sequencing of the genomic inserts revealed three different lipase-encoding genes, which were named *CdLIP1*, *CdLIP2* and *CdLIP3*. This indicates that about 1/2300 of the transformants contained *LIP* genes, suggesting that all *C. deformans* lipase encoding genes that are accessible by the method have been isolated. However, we could not exclude the possibility that genes may be present but not expressed in *S. cerevisiae*, or that the protein was not secreted or detected during the screening. Nevertheless, these results indicate that this screening, which was predominately used for the detection of bacterial lipase activity (Kouker and Jaeger, 1987), can also be used to detect lipase production in yeast.

The discovery of a lipase gene family in *C. deformans* is not surprising, since it had already been reported for other yeasts; e.g. *C. rugosa* and *C. albicans* are known to contain five and ten distinct lipases genes, respectively (Lotti *et al.*, 1993; Fu *et al.*, 1997; Hube *et al.*, 2000). The three cloned lipase genes encode for proteins that are related to the filamentous fungi lipase family. These lipases share in common the Gly–His–Ser–Leu–

Gly–(Gly/Ala)–Ala motif that encloses the serine of the active site. They present a putative signal sequence indicating that they correspond to secreted lipases. This is confirmed by their secretion when expressed in *Y. lipolytica*. The highest homology (91.6%) is observed between *CdLIP1* and the extracellular lipase *YILIP2* from *Y. lipolytica* (Pignede *et al.*, 2000a). Furthermore, alignment of the two protein sequences revealed the same organization of their primary structures, indicating that *CdLIP1* is synthesized as a pre-pro protein with a 33 aa prepro region ending at a Lys–Arg (KR) site, similarly to *YILIP2* (Pignede *et al.*, 2000a). MALDI–TOF mass spectrometry analysis after trypsin digestion of the *C. deformans* extracellular lipase demonstrated that *CdLIP1* encodes the extracellular lipase. However, contrary to *YILIP2*, which is secreted into the culture supernatant, *CdLIP1* was mainly bound to the cell wall but could easily be released by washing the cells with phosphate buffer. Currently, despite the high homology between the two proteins, we have no explanation for the protein being predominately localized in the cell wall in *C. deformans*, whereas it is predominately in the culture supernatant in *Y. lipolytica*. The mature protein (301 aa) has a calculated 33.4 kDa molecular mass. This estimation is consistent with the 33.8 kDa obtained from the SDS–PAGE analysis of the deglycosylated lipase. Deglycosylation results in a 4.2 kDa decrease in the apparent molecular mass, suggesting that at least one and possibly two of the two possible N-glycosylation sites are glycosylated.

In *The Yeasts: a Taxonomic Study*, 4th edn (Kurtzman and Fell, 1998), *C. deformans* (Zach) Langeron and Guerra CBS 2071 is considered to be a synonym of *Candida* (*Yarrowia*) *lipolytica*. However, a close inspection of the nucleotide sequences in the coding regions of *CdLIP1* and *YILIP2* reveals only 88.4% sequence identity, while sequence homology falls to 60% and 67.4% in the 5'- and 3'-untranslated regions respectively. The differences found could have occurred either by natural genetic divergence between the two strains or because another extracellular lipase encoding gene is present in *C. deformans* and remains to be discovered. The latter hypothesis is unlikely, since Southern blot hybridization using *YILIP2* as the probe failed to reveal another lipase-encoding gene with higher sequence identity (data not shown). The former hypothesis is

strengthened by the finding of 14 nucleotide differences in the LSU rDNA region D1/D2 between *C. deformans* and *Y. lipolytica* (Figure 5). Kurtzman and Robnett (1997) have observed that members of a same species show no more than two nucleotide substitutions. These sequences have sufficiently diverged to allow the reliable separation of the two yeast species.

Maximum lipase production in *C. deformans* reaches 1 unit/ml, with 80% being cell-bound. When *CdLIP1* was expressed in *Y. lipolytica*, we were able to obtain 15 U/ml in the supernatant. This corresponds to a 15-fold increase in lipase production when integrated in a single copy. Further improvement could be expected if multicopy integrants are isolated using the amplification method developed by Pignede *et al.* (2000b). We could therefore compare the enzymatic properties of *CdLIP1* expressed in *Y. lipolytica* with the enzyme produced by *C. deformans* for ester production.

In conclusion, we have successfully isolated three genes from *C. deformans* encoding proteins belonging to the lipase family. The genes were functionally expressed in *Y. lipolytica* and lipase activities encoded by the genes could be detected in the extracellular medium. Recombinant production of the newly described lipases are in progress, with the aim of studying their enzymatic properties.

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