

Characterization of an Extracellular Lipase Encoded by *LIP2* in *Yarrowia lipolytica*

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We isolated the *LIP2* gene from the lipolytic yeast *Yarrowia lipolytica*. It was found to encode a 334-amino-acid precursor protein. The secreted lipase is a 301-amino-acid glycosylated polypeptide which is a member of the triacylglycerol hydrolase family (EC 3.1.1.3). The Lip2p precursor protein is processed by the *KEX2*-like endoprotease encoded by *XPR6*. Deletion of the *XPR6* gene resulted in the secretion of an active but less stable proenzyme. Thus, the pro region does not inhibit lipase secretion and activity. However, it does play an essential role in the production of a stable enzyme. Processing was found to be correct in *LIP2*^Δ (multiple *LIP2* copy integrant)-overexpressing strains, which secreted 100 times more activity than the wild type, demonstrating that *XPR6* maturation was not limiting. No extracellular lipase activity was detected with the *lip2* knockout (KO) strain, strongly suggesting that extracellular lipase activity results from expression of the *LIP2* gene. Nevertheless, the *lip2* KO strain is still able to grow on triglycerides, suggesting an alternative pathway for triglyceride utilization in *Y. lipolytica*.

Several yeasts are able to utilize triglycerides (TGs) as their sole carbon source. One such yeast, nonpathogenic *Yarrowia lipolytica*, has potential for use as a model system for lipid utilization because classical and molecular genetic tools have been developed for this species (2, 3). Integrative and replicative vectors are available (16), and more recently, vectors for multiple integration have been developed (H. Wang and J.-M. Nicaud, unpublished data).

This yeast naturally secretes several proteins, depending on the growth conditions (21). For example, if the pH is higher than 6, it secretes an alkaline extracellular protease (AEP) (33, 38). Under optimal conditions, up to 1 g of AEP is secreted per liter (37). AEP is encoded by *XPR2* (11, 27, 33). This gene codes for a 454-amino-acid (aa) prepro enzyme precursor containing a 15-aa signal sequence and a stretch of nine X-Ala or X-Pro dipeptides, followed by a 124-aa pro region that includes a glycosylation site (Asn¹²³) and a Lys¹⁵⁶-Arg¹⁵⁷ processing site, and finally the mature form itself. The AEP precursor undergoes complex processing (27). A diaminopeptidase processes the stretch of nine dipeptides (X-Ala or X-Pro) (28, 29), and the endoprotease encoded by the *XPR6* gene is required to cleave the pro region, releasing the mature form (14). The pro region is involved in both the inhibition of protease activity and the folding of the propeptide into a conformation compatible with secretion, and secretion at 28°C depends on the glycosylation of the pro region (15, 28).

Several enzymes are secreted by *Y. lipolytica*, and lipase and esterase activities have been detected and analyzed in various studies. Lipase secretion was first reported in 1948 by Peters and Nelson (41, 42), who described a single type of glucose-repressible activity. An extracellular and two cell-bound types of activity corresponding to lipase I (39 kDa) and lipase II (44 kDa) were described by Ota and coworkers (39, 47). The extracellular lipase required oleic acid as a stabilizer-activator,

whereas the cell-bound lipases did not and differed in several properties from the extracellular enzyme (40). The rates of production of the extracellular and cell-bound enzymes were reported to depend on the carbon and nitrogen composition of the medium. Extracellular lipase was only detected in cultures grown with an organic nitrogen source (36, 48), and lipase levels were shown to be modulated by cell morphology. In minimal medium supplemented with *N*-acetylglucosamine or citrate buffer, both of which promote dimorphic growth, higher levels of cell-bound lipases were detected. However, no clear relationship was established between the dimorphic state and lipase production (35).

Recently, Ota and coworkers purified the 39-kDa extracellular lipase (called lipase A) and determined the N-terminal amino acid sequence (22). Destain and coworkers isolated *Y. lipolytica* strains overproducing an extracellular lipase. They used chemical mutagenesis to produce a first generation of mutants with levels of lipase production five times higher than that of the wild type. A second round of mutagenesis generated strains able to secrete 1,200 U of lipase per ml, 25 times the level of the wild-type strain. These mutant strains were used for large-scale fermentation (500 liters) to produce lipase (13). The secreted lipase was shown to have an apparent molecular mass of 38.5 kDa, giving three bands in isofocusing (pIs of 5.0, 5.2, and 5.4). The sequence of the first 49 aa of the N terminus was determined (12) and found to be identical to that of lipase A. This sequence is similar to that of cell-bound lipase I; however, the extracellular lipase and lipase I are considered to differ in amino acid composition (22).

This strongly suggests that several genes encoding lipase are expected in *Y. lipolytica*. This is supported by the lack of success of attempts to obtain mutants unable to grow on TG. The mutants isolated were affected only in lipase levels (32). Such multicomponent gene families have been observed in other yeasts. A lipase family of five genes, with 80% identity, has been isolated in *Candida rugosa* (4, 25), and two genes have been isolated in *Geotrichum candidum* (5).

In *Y. lipolytica*, two lipase genes of the carboxylesterase family have been identified by Dominguez and coworkers: *LIP1*,

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TABLE 1. Strains, plasmids, and oligonucleotides used in this study

Strain (host strain) or primer	Plasmid, genotype, or sequence (5'→3') ^c	Reference, source or location (bp)
<i>E. coli</i> strains		
JME225 (DH5 α)	pKS- <i>LIP2W29a</i>	This work
JME257 (DH5 α)	JMP5 (<i>ura3d1</i>)	This work
JME259 (DH5 α)	JMP6 (<i>ura3d4/pPOX2-LIP2</i>)	This work
JME276 (DH5 α)	JMP3 (<i>ura3d4</i>)	This work
JME260 (DH5 α)	JMP8 (<i>ura3d1/pPOX2-LIP2</i>)	This work
JME298 (DH5 α)	JMP13 (<i>ura3d1/pLIP2-LIP2</i>)	This work
JME300 (DH5 α)	JMP14 (<i>ura3d4/pLIP2-LIP2</i>)	This work
JME337 (DH5 α)	pIMR83	14
<i>Y. lipolytica</i> strains		
W29	<i>MAT</i>	3 (CLIB 89, ATCC 20460) ^a
POld	<i>MATa ura3-302 leu2-270 xpr2-322</i>	3 (CLIB 139)
E150	<i>MATa ura3-302 leu2-270 xpr2-322 his1</i>	3 (CLIB 122)
<i>Y. lipolytica</i> recombinant strains ^b		
JMY184 = POld-6-15	JMP6/pPOX2- <i>LIP2</i> ^Δ	This work
JMY279 = POld-6-17	JMP6/pPOX2- <i>LIP2</i> ^Δ	This work
JMY277 = POld- <i>lip2</i> KO	<i>p_{lip2} KO lip2 KO</i>	This work
JMY278 = POld-6-15- <i>xpr6</i> KO	<i>p_{xpr6} KO/pPOX2-LIP2^Δ xpr6 KO</i>	This work
JMY281 = POld-14-1	JMP14/pLIP2- <i>LIP2</i> ^Δ	This work
JMY282 = POld-14-2	JMP14/pLIP2- <i>LIP2</i> ^Δ	This work
JMY283 = E150-6-2	JMP6/pPOX2- <i>LIP2</i> ^Δ	This work
Primers		
YlipSA	GCi Cci Cci AGR GAR TGi CC	4313
YlipATG	CCC GGG CCG CAG TGG CCA CAA TGA <u>AGC TTT</u> CCA CCA TCC TTT TCA CAG CC	3733
YlipSTOP	GTT GGC GGC CGC GAA <u>TTC GGA TCC</u> TAA ATA AAC GAT ATT CAT TTA TTA AAG TAG ATA GTT GAG G	4806

^a CLIB, Collection de Levures d'Intérêt Biotechnologique, Thiverval-Grignon, France.

^b Transformants were named according to the strain and plasmid used, and transformants were numbered as follows: for example; POld1-6-1 (strain-plasmid-clone number)

^c Underlined sequences correspond to introduced restriction enzyme sites as follows: for YlipATG, *Hind*III; for YlipSTOP, *Not*I, *Eco*RI, and *Bam*HI. The locations of the various oligonucleotides are based on the position in the EMBL sequence of the described *LIP2* locus.

which codes for a 486-aa lipase (17), and *LIP3*, which codes for a 498-aa lipase (9). Both are similar to the lipases of the fungi *Candida cylindracea* and *G. candidum* and belong to the carboxylesterase family (30). These are probably intracellular or cell-bound lipases, because no clear signal sequence was identified.

Here, we report on the isolation and characterization of the *LIP2* gene, which codes for the extracellular lipase Lip2p, a triacylglycerol hydrolase. We show that this gene is responsible for all of the extracellular lipase activity of *Y. lipolytica*. Lipase expression was repressed by glucose and induced by olive oil and oleic acid. We show that Lip2p processing is similar to AEP processing, involving cleavage of the signal sequence and processing by the endoprotease encoded by the *XPR6* gene. We also demonstrate that glycosylation and processing by the *XPR6* gene product are not essential for activity and suggest that they are required for stabilization of the protein.

MATERIALS AND METHODS

Strains and media. The *Y. lipolytica* strains used in this study were POld and derivatives (Table 1). *Escherichia coli* strain DH5 α was used for DNA propagation. The media and techniques used to grow and handle *Y. lipolytica* were described by Barth and Gaillardin (3), and those used for *E. coli* were described by Sambrook et al. (44). The compositions of the media used were as follows (per liter): YPD, 10 g of yeast extract (Difco), 10 g of Bacto Peptone (Difco), and 10 g of glucose; YPDH, 10 g of yeast extract, 10 g of Bacto Peptone, 10 g of glucose, and 10 g of olive oil; YTDH, 10 g of yeast extract, 10 g of Bacto Tryptone (Difco), 10 g of glucose, and 50 g of olive oil. The minimal medium, YNB, contained 1.7 g of yeast nitrogen base without ammonium and without amino acids (Difco), 4 g of ammonium chloride, and various carbon sources as follows: YNBD, 10 g of

glucose; YNBcas, YNBD with 5 g of Casamino Acids; YNBO, 10 g of oleic acid; YNBH, 10 g of olive oil; YNBTO, 10 g of triolein (To); YNBT, 10 g of tributyrin (T). Uracil (0.1 g/liter) and leucine (0.3 g/liter) were added to the media as required. Stock solutions of fatty acid (20% oleic acid, 0.5% Tween 40), oil (10% olive oil, 1% Tween 40), and tributyrin (20% tributyrin, 1% Tween 20) were subjected to sonication three times for 1 min each on ice. Agar (2%; Pastagar B; Institut Pasteur) was added for solid media. Media were buffered with 50 mM phosphate buffer, pH 6.8.

cDNA sequencing and *LIP2* gene isolation. The wild-type strain, POld, was grown in YPDH for 12 h. When cultures reached an optical density at 600 nm of 10, cells were harvested by centrifugation and washed twice with cold water and total RNA was extracted as previously described (1). mRNA was purified using the Quick Prep Micro mRNA kit (Pharmacia) and a cDNA library was constructed using the Marathon cDNA amplification kit (Clontech, Palo Alto, Calif.) in accordance with the manufacturer's instructions. A 5' rapid amplification of cDNA ends (RACE)-PCR amplification was performed with primer YlipSA and the linker primer AP1, using 50 ng of cDNA, giving a 620-bp fragment. This PCR product was isolated after electrophoresis in an agarose gel and was introduced into the *Eco*RV site of the pBluescript II KS⁺ plasmid (Stratagene, La Jolla, Calif.). The insert was then sequenced. New primers corresponding to the determined sequence were synthesized and used for a second round of 5' and 3' RACE amplification. PCR fragments were sequenced directly after gel purification. pKS-*LIP2W29a*, containing the *LIP2* cDNA, was constructed as follows. The cDNA was amplified by PCR using the YlipATG and reverse YlipSTOP primer pair (Table 1; see Fig. 2B). The PCR product was isolated and introduced into pKS⁺. Plasmids were checked by sequencing of the inserts.

Sequence determination and analysis of the *LIP2* gene. The six pools of the Xuan library (51) were tested by PCR, and it was found that a clone containing the lipase gene was present in pool 2. pINA-*LIP2* was isolated by colony hybridization using the 5' RACE-derived 620-bp fragment as a probe against pool 2 clones. *Pst*I (P1 to P4), *Bgl*II (B1), and *Eco*RV (E1) fragments (see Fig. 2C) were subcloned into the *Pst*I, *Bam*HI, and *Eco*RV sites of the Bluescript KS⁺ plasmid, respectively, and used for sequencing. We performed divergent PCR by digesting 100 ng of total genomic DNA with *Hind*III and ligating the digested product overnight at 16°C. We used 10 ng of the ligation product as a template for PCR

with the primer pair Ylip05-Ylip06 (see Fig. 2D). The 1.4-kb PCR fragment was cloned into the *EcoRV* site of pBluescript KS⁺, giving pKS-PCR-H (see Fig. 2D). Template preparation, sequencing, and nucleotide sequence analysis were performed as previously described (26). Proteins were compared using the GCG-gap program (version 9.1-UNIX, Sept. 97) and the BLOSUM 62 matrix (Genetics Computer Group, University of Wisconsin, Madison).

Southern blot analysis. Genomic DNA was prepared from cells grown overnight in 5 ml of YPD as previously described (3). DNA was digested, subjected to electrophoresis in a 1.2% agarose gel, and transferred to a Hybond-N nitrocellulose membrane (Amersham). DNA probes were radiolabeled using the Megaprime kit (Amersham). After hybridization, membranes were placed for 1 h against a Molecular Dynamics PhosphorImager screen and scanned using a Storm 860 PhosphorImager (Molecular Dynamics).

Disruption of *LIP2*. To construct a plasmid containing the lipase gene with its promoter, we inserted into *SalI*-*BamHI*-digested pKS⁺ a 2.7-kb *SalI*-*HindIII* fragment from pINA-*LIP2* containing the *LIP2* promoter together with the 1-kb *HindIII*-*BamHI* fragment from pKS-*LIP2W29a* carrying the lipase gene. The resulting plasmid was called pLIPpr-*LIP2*. To disrupt the *LIP2* gene, a 1,091-bp *BglII* fragment in pLIPpr-*LIP2* containing part of the promoter and half of the open reading frame (ORF) was deleted and replaced with the 2.7-kb *BglII*-*BamHI* *Y. lipolytica* *LEU2* gene isolated from pINA240 (see Fig. 5A). The resulting plasmid, *lip2::LEU2*, was digested with *NdeI* and *BamHI* to yield a linear fragment suitable for one-step gene disruption (43) (see Fig. 5A) and used to transform PO1d. Southern blots of *PstI*-digested DNA from Leu⁺ transformants were probed with *LIP2*. In the disrupted strain, the 3-kb *PstI* fragment was replaced with a 1.5-kb fragment (data not shown). The resulting strain, containing a *lip2::LEU2* deletion, was called JMY277.

Disruption of *XPR6*. pIMR83 contains an *xpr6A976::URA3* disruption cassette in which a 976-bp *BamHI*-*HindIII* fragment has been deleted and replaced with the 1.7-kb *Y. lipolytica* *URA3* gene (14). The derivative plasmid, *xpr6Δ976::LEU2* (JME337), was constructed by replacing the 1.7-kb *BamHI*-*HindIII* *URA3* fragment with the 2.7-kb *BamHI*-*HindIII* *LEU2* fragment from pINA1192 (8). The DNA was digested with *DraIII* and *SphI* prior to transformation of the JMY184 strain (PO1d-6-15) and selection of the Leu⁺ transformants. Gene disruption was checked by Southern blotting of *NcoI*-digested DNA from putative disruptants with an *XPR6* probe. Strain JMY278 had the two expected 1.1- and 1.3-kb *NcoI* fragments replacing the 2.7-kb wild-type signal (see Fig. 6B; data not shown).

Plasmid construction. We recently constructed vectors JMP3 and JMP5 for gene expression and amplification in the yeast *Y. lipolytica* (Wang and Nicaud, unpublished data). They are derived from the amplification vectors previously described (3, 24). These vectors contain the *ura3Δ1* (nondefective allele allowing single-plasmid integration) or *ura3Δ4* (defective allele allowing multicopy plasmid integration) marker for selection in *Y. lipolytica*, a polylinker for insertion of the expression cassette, an 812-bp zeta region, and, inserted into the middle of the zeta region at the *NotI* site, pHSS6, which confers replication and kanamycin resistance in *E. coli*. The zeta region corresponds to the long terminal repeat of the *Y. lipolytica* retrotransposon Ylt1, which has been shown to be present in the genome in about 60 copies (46).

Using JMP3 (defective) and JMP5 (nondefective) as parental vectors, we constructed the expression vectors JMP6, JMP8, JMP13, and JMP14 (Table 1). The promoter region from *LIP2* was rescued from pINA-*LIP2* by PCR and digested with *HindIII*. The resulting 1,361-bp *LIP2* promoter region was ligated together with the *HindIII*-*EcoRI* *LIP2* fragment and JMP3 or JMP5 digested with *HpaI* and *EcoRI*. The *POX2* promoter used (49) was a 2,163-bp *Clal*-*HindIII* fragment produced by PCR. The resulting *POX2* promoter was ligated together with the *HindIII*-*EcoRI* *LIP2* fragment and JMP3 or JMP5 digested with *Clal* and *EcoRI*. The resulting expression cassettes from the various plasmids described here were sequenced before transformation.

Transformation of *Y. lipolytica*. *Y. lipolytica* strains were transformed by the lithium acetate method as described previously (24). Expression vectors (5 μg) were digested with *NotI* and subjected to electrophoresis. The bands corresponding to the expression cassettes were extracted from the gel and used for transformation. Typically, 0.05 or 2 μg of expression cassettes was used for nondefective (transformation control) and defective vectors, respectively. Transformants were selected on YNBcas and appeared after 3 days at a frequency of 10⁵ transformants per μg of DNA for the nondefective vector (defective vectors: 5 to 15 days, 10 to 30 transformants).

Protein analysis. Protein concentration was determined as described by Bradford (7) using the Bio-Rad protein assay system with bovine serum albumin as the standard. Proteins were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (23), using prestained low-range protein markers (Bio-Rad) as molecular mass standards. Cell culture protein amounts equivalent to an optical density at 600 nm of 0.2 or to 5 μl of cell supernatant were loaded per lane.

Lipase activity detection and assay. For detection of lipase activity on agar plates, plates containing emulsions of either T or To were used. A halo (T) or a clearing zone (To) developed within 24 to 48 h of incubation at 28°C. Lipase activity was routinely measured by titrimetric assay as follows. The substrate emulsion was prepared with olive oil (50 ml; Sigma, Paris, France) and gum arabic (50 ml; 10%, wt/vol). The solution was emulsified in a Waring blender (three pulses of 2 min each at maximum speed). The supernatant of the cell

culture (20 μl), pure or diluted, depending on the quantity of lipase, was added to 5 ml of substrate emulsion and 2 ml of 50 mM phosphate buffer, pH 6.8 (Na₂HPO₄-KH₂PO₄). Samples were incubated for 20 min at 37°C with shaking (200 rpm). The reaction was stopped with 4 ml of acetone-ethanol (50/50, vol/vol) containing 0.09% thymolphthalein (Prolabo) as an indicator. Enzymatic activity was determined by titration of the fatty acid released with 50 mM sodium hydroxide. All lipase activity assays were performed at least in duplicate. One unit of lipase is the amount of enzyme that catalyzes the release of 1 μmol of fatty acid per min at 37°C.

N-terminal amino acid sequence of the lipase. The sequence of the N-terminal 7 aa of the lipase present in the cell culture supernatant was determined. The lipase was blotted onto a polyvinylidene difluoride membrane after SDS-PAGE, and the blotted protein, detected by staining of the membrane with Coomassie brilliant blue R-250, was used directly for Edman degradation in a protein sequencer. Automated Edman sequencing was performed using a Perkin-Elmer Applied Biosystems Procise 494A sequencer with the reagents and methods of the manufacturer.

Lipase modification. Deglycosylation of extracellular lipase using endoglycosidase H (endo H; NEB-Ozyme) was performed in accordance with the manufacturer's instructions. A 50-μl volume of cell culture supernatant (52 h) from lipase-overproducing strains JMY184 (PO1d-6-15) and JMY278 (PO1d-6-15-*xpr6* KO) was treated with 0.2% (wt/vol) SDS and 0.08 U of endo H per mg of protein at 37°C for 2 h (native condition) or heated for 5 min at 85°C and treated for 24 h (denaturing conditions).

Antibody preparation and detection on Western blots. Polyclonal antiserum directed against Lip2p was raised in a rabbit. Before use, the antibodies were immunopurified with the nitrocellulose membrane band containing the blotted Lip2p as previously described (19). For Western blot analysis, proteins were resolved by SDS-10% PAGE and transferred onto a nitrocellulose membrane (Schleicher & Schuell). Antigen-antibody complexes were detected using the ECL Kit (Amersham Pharmacia Biotech).

Nucleotide sequence accession number. The nucleotide sequence of the *LIP2* gene will appear in the EMBL nucleotide sequence database under accession no. AJ012632.

RESULTS

Isolation and sequencing of the *LIP2* gene. We previously isolated the *LECE* gene coding for a 40-kDa extracellular triacylglycerol hydrolase from the yeast *Candida ernobii* (44). To isolate the corresponding gene from *Y. lipolytica*, we first compared the predicted amino acid sequences of secreted lipases from the yeasts *C. ernobii* and *Saccharomyces cerevisiae* (P47145) and from the fungi *Rhizomucor miehei* (P19515) (6), *Rhizopus delemar* (P21811) (18), *Fusarium heterosporum* (S77816) (31), and *Penicillium camembertii* (P25234) (52). Alignment of these protein sequences led to the identification of the highly conserved motif GHSLGG/AA, which contains the serine involved in the active site (Fig. 1). We designed the degenerate antisense oligonucleotide YlipSA (Table 1) for reverse transcription-PCR, based on the conserved core sequence, using available DNA sequences and on the basis of *Y. lipolytica* codon usage.

Lipase secretion in *Y. lipolytica* has been reported to be induced in rich complex media with olive oil as the carbon source (36). Therefore, a cDNA library was constructed using mRNA isolated from strain PO1d grown in the induction medium YPDH, used for 5' RACE-PCR, and sequenced as described in Materials and Methods. The complete cDNA sequence was determined, and it corresponds to coordinates 3737 to 4806 of the sequence with EMBL accession no. AJ012632. Plasmid pINA-*LIP2* was isolated from pool 2 of the Xuan library (51). It contains a 7.1-kb genomic insert that carries the promoter and part of the *LIP2* gene (Fig. 2C). Indeed, restriction analysis of pINA-*LIP2* and location of the *HindIII* and *BglII* sites (from the cDNA sequence) showed that only the promoter and part of the ORF were present in the *EcoRV*-*BamHI* region. This region was sequenced using six subclones (Fig. 2C). We used a divergent PCR strategy and genomic DNA to obtain the 3' end of the gene, which was absent from pINA-*LIP2*. First, we determined which restriction enzymes could be used by probing a Southern blot of genomic DNA digested with various restriction enzymes with

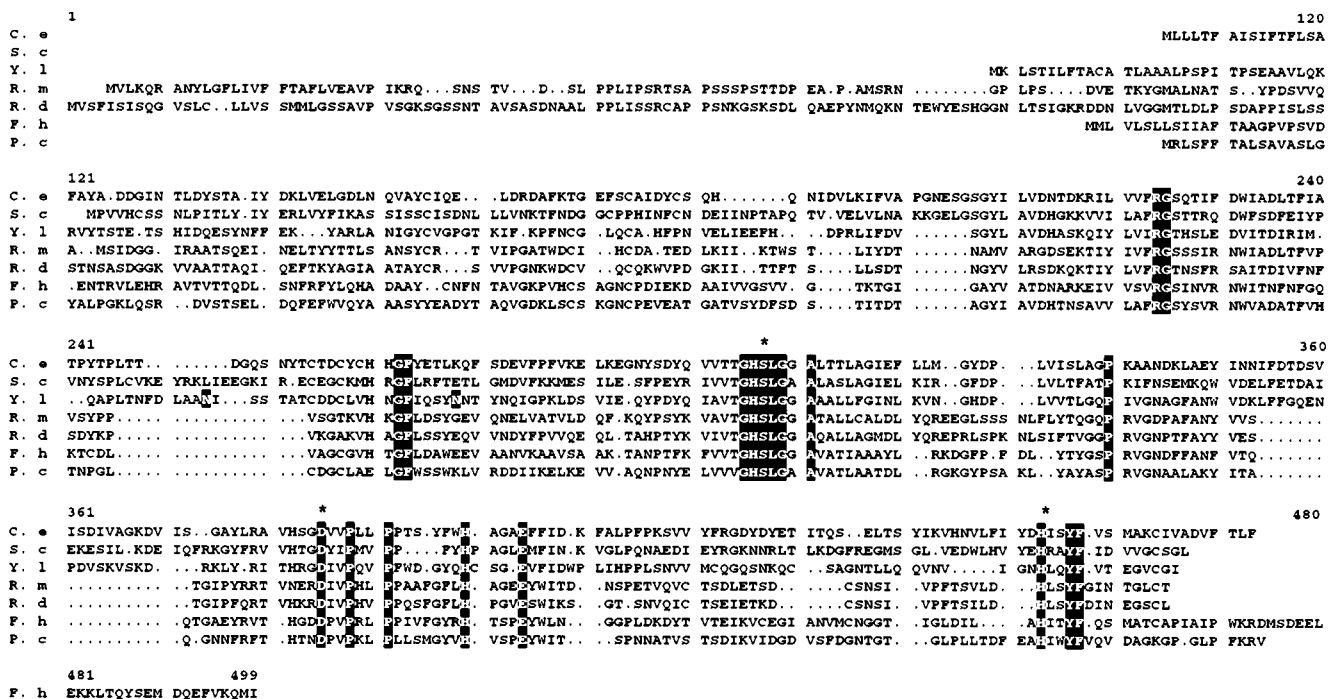


FIG. 1. Alignment of the predicted amino acid sequences of secreted lipases from the yeasts *C. enobii* (C. e) and *S. cerevisiae* (S. c; P47145) and from the fungi *R. miehei* (R. m; P19515), *R. delemar* (R. d; P21811), *F. heterosporum* (F. h; S77816), and *P. camembertii* (P. c; P25234). Conserved amino acids are highlighted. Potential N glycosylation sites in the *Y. lipolytica* sequence are in boldface type. Asterisks indicate amino acids involved in the active site.

the 5' RACE fragments, detecting 1.4-, 1.66-, and 3.7-kb fragments for the *Hind*III, *Pst*I, and *Sph*I digestions, respectively (data not shown). The 1.4-kb *Hind*III fragment was amplified, cloned, and sequenced. The 5,304-bp *Eco*RV-*Hind*III se-

quence will appear in the EMBL database under accession no. AJ012632.

We obtained the complete lipase gene, a 1,118-bp fragment carrying the lipase ORF and the terminator right up to the

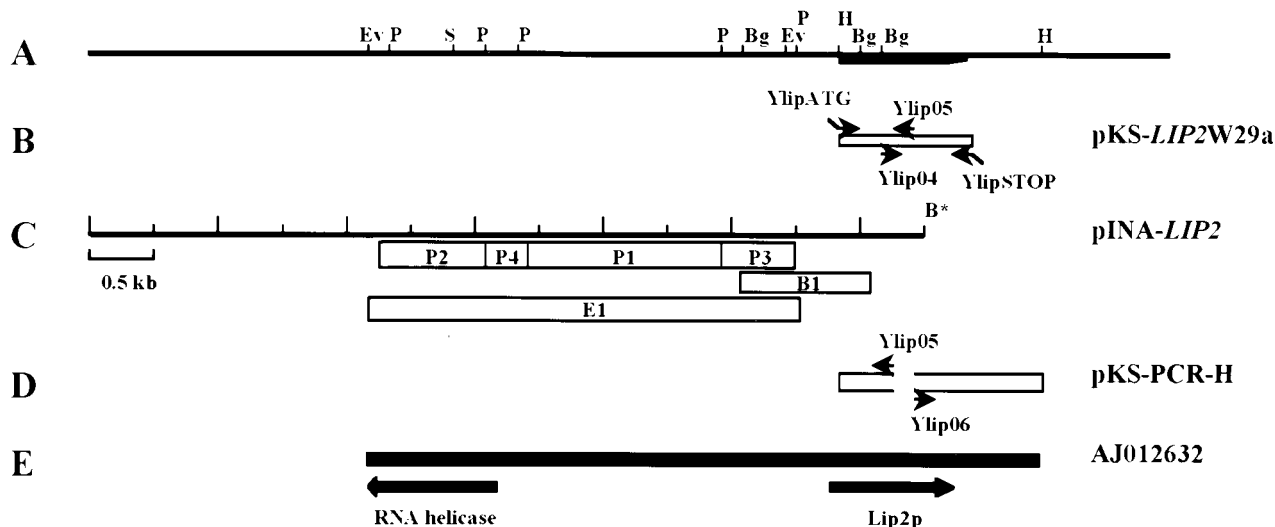


FIG. 2. Sequencing of the *LIP2* gene coding for the *Y. lipolytica* extracellular lipase. (A) Partial restriction map of the *LIP2* locus. Abbreviations: *Eco*RV, Ev; *Pst*I, P; *Bgl*II, Bg; *Hind*III, H. The location of the *LIP2* ORF is indicated by the thick line. (B) Location of the cDNA sequence and schematic representation of primers used for gene library screening and the oligonucleotide pair YlipATG-YlipSTOP used for amplification of the coding region of the lipase gene. The recombinant plasmid containing the *LIP2* gene was called pKS-*LIP2*W29a. (C) Schematic representation of the 6.5-kb insert contained within pINA-*LIP2* and location of the *Pst*I (P1 to P4), *Bgl*III (B1), and *Eco*RV (E1) subclones used for sequencing of the 5' region of the *LIP2* gene. (D) Sequence of the 1.4-kb *Hind*III genomic fragment. Genomic DNA was digested with *Hind*III, ligated, and amplified by PCR with the Ylip05-Ylip06 primer pair. The PCR fragment was cloned at the *Eco*RV site of pBluescript, giving pKS-PCR-H. (E) Location of the sequenced *Eco*RV-*Hind*III 5,304-bp region containing part of the DEAD box RNA helicase gene and the *LIP2* gene (AJ012632). All scales are the same.

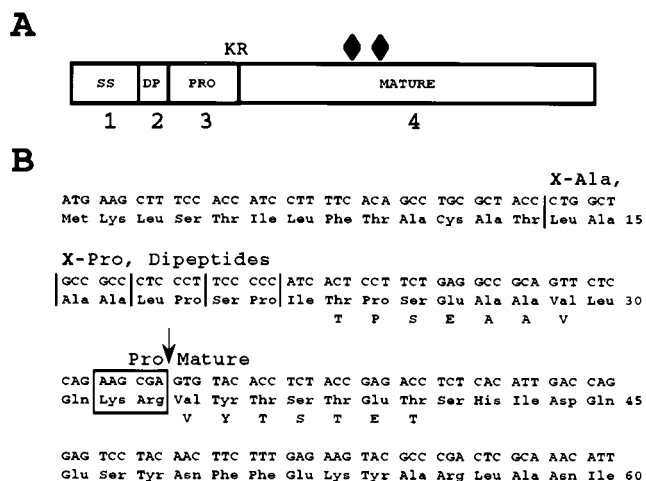


FIG. 3. Processing of *Y. lipolytica* Lip2p. (A) Schematic representation of lipase processing. Shown are the putative 13-aa signal sequence (SS), followed by a stretch of four dipeptides (DP), a short 12-aa pro region (PRO) including the Lys-Arg (KR) cleavage site of the *KEX2*-like *XPR6* endoprotease (14), and the mature 301-aa lipase. The diamonds indicate the positions of the potential signals for asparagine-linked glycosylation (Asn-X-Thr/Ser). (B) sequence of the preproregion and the first 27 N-terminal aa of the mature lipase encoded by the *LIP2* gene from *Y. lipolytica* strain W29. The DNA sequence is shown together with the predicted amino acid sequence (three-letter code) and the N-terminal amino acid sequences (one-letter code) of the lipases secreted by the JMY184 (*LIP2^Δ*; mature form) and JMY278 (*xpr6* KO; pro form) strains. The vertical arrow indicates the confirmed pro-mature lipase processing site, and the vertical lines represent the four X-Ala and X-Pro dipeptides which act as possible substrates for dipeptidyl aminopeptidase activity.

polyadenylation site by PCR using as a template either the cDNA library or genomic DNA from wild-type strain W29 and cloning the amplified fragments into pKS, giving rise to pKS-*LIP2*W29a (Fig. 2B). No sequence differences were observed between fragments of genomic and cDNA origin.

Sequence analysis. Two ORFs were identified in the 5,304-bp sequence (Fig. 2E). One corresponded to the NH₂ terminus of a protein with a sequence similar to the first 324 aa of the human putative DEAD box RNA helicase (45). The second ORF was 1,005 bp long and encoded a 334-residue polypeptide similar in sequence to the yeast and fungal secreted lipases. The comparison results gave levels of identity (similarity) of 30.3% (39.2) to the yeast *C. ernobii*, 28.6% (38.2) to the *S. cerevisiae*, 27.4% (34.1) to the fungus *R. miehei*, 31.5% (38.6) to the *R. delemar*, 29% (36) to the *F. heterosporum*, and 26.8% (33.3) to the *P. camembertii* lipases, as shown in Fig. 1. These lipases belong to the triacylglycerol hydrolase family (EC 3.1.1.3). The *LIP2* gene has a codon adaptation index of 0.426, indicating that it is a moderately expressed gene. The polypeptide encoded by the *Y. lipolytica* *LIP2* gene contains several processing motifs (Fig. 3A). The prepro protein presents 13 aa, followed by four X-Ala or X-Pro dipeptides, possible substrates of the diamino peptidase (29); a short proregion of 12 aa and a Lys-Arg dipeptide, a putative substrate of the *KEX2*-like endopeptidase encoded by the *XPR6* gene in *Y. lipolytica* (14); and the mature lipase of 301 residues. The signal sequence cleavage site is most probably located after aa 17, according to SignalP prediction (34), after the second potential X-Ala dipeptide, giving a 17-aa signal sequence, longer than that of AEP (28).

The mature polypeptide contains the three conserved amino acids Ser¹⁶², Asp²³⁰, and His²⁸⁹, which have been shown to be part of the active site, and the conserved GHSLGGA motif (Fig. 1). A disulfide bridge may form between Cys⁷⁶ and

Cys¹²⁰. Two potential N glycosylation sites were also identified (N¹¹³IS and N¹³⁴NT).

Analysis of the nucleotide sequence and comparison with the cDNA sequence indicated that polyadenylation occurred at position 4806, 15 bp after the polyadenylation motif, AATA AA. The region between the lipase gene and the adjacent gene is 2,764 bp, larger than the mean intergenic region of the *S. cerevisiae* genome, although this is a general feature of intergenic regions in *Y. lipolytica*.

Lipase secretion, expression vectors, and strain construction. The lipase gene was expressed under the control of the *POX2* or *LIP2* promoter (Table 1). The expression vectors were constructed using three-way ligation as described in Materials and Methods. Plasmids JMP6, JMP8, JMP13, and JMP14 were introduced into a strain in which Ylt1 was absent (PO1d) or present (E150). For each transformation, 20 to 50 clones on YNBT plates were tested for lipase production with the defective vectors and 5 clones were tested for nondefective vectors. The strains used in this study are listed in Table 1. No difference in the size of the halo on YNBT was observed between strains transformed with nondefective vectors (JMP8 and JMP14) and the untransformed strain (data not shown). For the defective vectors, most (85%) of the transformants had larger halos and the rest (15%) corresponded to cases of *URA3* conversion (data not shown).

The copy number of integrated cassettes with the defective vectors was similar for all of the strains used, 10 copies, on average. However, the types of integration differed: mainly dispersed in the zeta-free strain (PO1d) and in tandem in strains containing zeta (E150). The copy number, the type of integration, and the stability of integrated expression cassettes will be presented elsewhere. We further studied lipase regulation and secretion using strains JMY184 and JMY279, which contained the multicopy cassette with the *POX2* promoter driving the *LIP2* gene, and JMY281 and JMY282, which contained the multicopy cassette with the *LIP2* promoter driving *LIP2*. The four strains presented similar growth kinetics on both glucose and olive oil media (data not shown), but JMY281 and JMY282 secreted 25 to 50% of the amounts of lipase secreted by JMY184 and JMY279. Both the *POX2* and *LIP2* promoters gave low levels of expression in glucose media and were induced by oleic acid and olive oil (see below and data not shown).

Overproduction of lipase does not affect Lip2p processing and secretion. Lipase secretion was compared in the wild-type (PO1d) and *LIP2^Δ* strains (JMY184 and JMY281; lipase gene expressed under control of the *POX2* and *LIP2* promoters, respectively). Typically, for the PO1d strain, lipase activity was detected at 20 U/ml in YNBH, 50 U/ml in YPDH, and about 100 U/ml in YTDH. In contrast, it was detected at 150 U/ml (200 U/ml) in YNBH, 400 U/ml (500 U/ml) in YPDH, and 1,500 U/ml (2,000 U/ml) in YTDH for JMY281 and JMY184, respectively. The kinetics of extracellular lipase production and protein accumulation in the culture supernatant during the growth of JMY184 in YTDH are presented in Fig. 4. Lipase production started 15 h after transfer into the inducing media when glucose was consumed and increased until 50 h of culture, when it reached a plateau. Activity and protein levels were stable over 40 h. In JMY281 and JMY184, 50- and 100-fold increases in lipase production were obtained, respectively. We investigated the nature of the Lip2p produced by *LIP2^Δ* strain JMY184 using immunoblotting to analyze the supernatant and extracts of cells induced in YTDH using anti-Lip2p antibodies. Only the 38.5-kDa mature form was detected in both fractions (data not shown). The N-terminal amino acid sequence of the secreted lipase (seven amino acids, Fig. 3B)

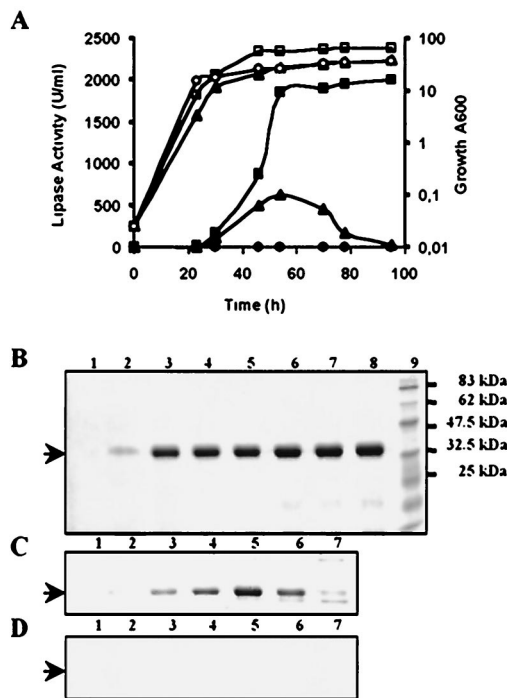


FIG. 4. Growth and lipase production of strains JMY184 (squares), JMY278 (triangles), and JMY277 (circles). (A) Growth (open symbols) and extracellular lipase production (closed symbols) in YTDH were monitored over time. Supernatant protein analysis during growth was done by SDS-PAGE and Coomassie blue staining for strains JMY184 (*LIP2^A*) (B), JMY278 (*LIP2^A, xpr6 KO*) (C), and JMY277 (*lip2*) (D). Lanes 1 to 8 correspond to the equivalent of 5 μ l of supernatant sampled after 0, 22, 28, 44, 52, 70, 78, and 94 h of culture, respectively. Sizes of prestained molecular mass standards (lane 9) are indicated on the right. The arrows mark the 38.5-kDa position.

corresponded to the mature form, indicating that precursor processing was not limiting (see below).

The *XPR6* gene product is involved in Lip2p processing. The *XPR6* gene has been shown to encode a dibasic processing endoprotease similar to that encoded by the *KEX2* gene in *S. cerevisiae* (14). In *Y. lipolytica*, Xpr6p is involved in AEP processing. *XPR6* is not an essential gene, but its disruption results in reduced growth and a defect in the yeast-to-mycelium switch (smooth colonies) (14). To confirm the role of Xpr6p in Lip2p processing, a *xpr6 Δ 976::LEU2* gene disruption cassette was introduced into strain JMY184 to give strain JMY278 (Fig. 5B). Small and smooth colonies corresponding to disrupted clones were formed by 60% of Leu⁺ transformants. Correct gene disruption was confirmed by Southern blotting.

The extracellular lipase activity of strain JMY278, which carried *xpr6 Δ 976*, was, at most, one-quarter of that of nondisrupted strain JMY184 (Fig. 4A). During growth, extracellular lipase accumulates in JMY184 (Fig. 4B) whereas in JMY278, it increases and then decreases, in parallel with protein levels (Fig. 4C). This decrease may result from the production of a less stable pro protein or degradation by a protease released by the cells.

The N-terminal sequences of the secreted lipases produced in YPDH by strains JMY184 and JMY278 after 52 h of cell growth (Fig. 4B and C, lanes 5) were determined. For JMY278, the amino acid sequence obtained was TPSEAAV. The Thr residue corresponds to aa 23 (Fig. 3B). For JMY184, the sequence was VYTSTET, corresponding to the N-terminal sequence of the mature lipase (aa 34), as determined by Destain

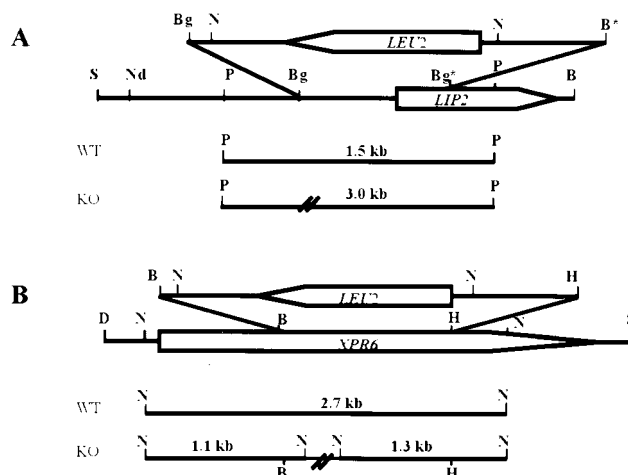


FIG. 5. Disruption of the *LIP2* and *XPR6* genes. Physical maps of the lipase (*LIP2*, A) and endoprotease (*XPR6*, B) genes and disruption cassettes are shown. ORFs are represented by arrow boxes indicating the direction of transcription. (A) For *LIP2* gene disruption, the *Bgl*III fragments were replaced with a *Bgl*III-*Bam*HI *LEU2* gene and the *Nde*I-*Bam*HI linear fragment was used for transformation. (B) For *XPR6* gene disruption, the *Bam*HI-*Hind*III *LEU2* gene and the *Dra*I-*Sal*I linear fragment was used for transformation. The sizes of the *Pst*I (A) and *Nco*I (B) fragments obtained by Southern blotting of the wild-type (WT) and disrupted (KO) strain DNAs using *LIP2* (A) and *XPR6* (B) probes are shown. Restriction sites: B, *Bam*HI; Bg, *Bgl*III; D, *Dra*I; H, *Hind*III; N, *Nco*I; Nd, *Nde*I; P, *Pst*I; S, *Sal*I.

(12). The specific activity of the lipases secreted at 52 h by JMY184 (Lip2p) and JMY278 (pro-Lip2p) were compared and found to be similar, indicating that pro-Lip2p was as active as Lip2p (data not shown).

Lip2p is a glycosylated protein. Many extracellular enzymes produced by yeast are glycosylated. Two potential N glycosylation sites were detected in the mature form (Fig. 1). As a difference was observed between the experimentally determined (SDS-PAGE) molecular mass (38.5-kDa) and the calculated molecular mass (33,384 Da) of Lip2p, we investigated whether N glycosylation was responsible for this difference. Both lipases, Lip2p and pro-Lip2p, were treated with endo H under native and denaturing conditions. The decrease in the apparent molecular masses of both proteins shows that they

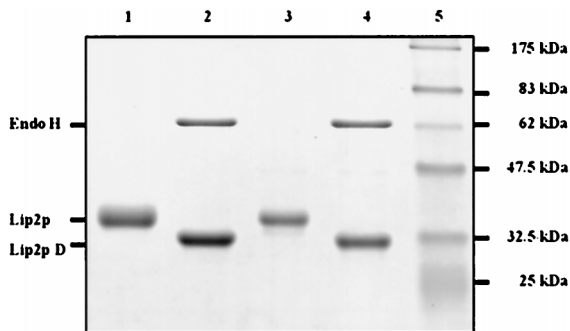


FIG. 6. Analysis of the extracellular lipase from JMY184 and JMY278 by SDS-PAGE. The equivalent of 5 μ l of the supernatant (52 h) from strains JMY184 (*LIP2^A*; mature form) (Fig. 4B, lane 5) and JMY278 (*xpr6 KO*; pro form) (Fig. 4C, lane 5) was treated with endo H. Untreated (lanes 1 and 3) and deglycosylated (lanes 2 and 4) proteins were separated by SDS-12% PAGE and stained with Coomassie blue. The positions of endo H and the untreated (Lip2p) and deglycosylated (Lip2p D) lipases are indicated on the left. Prestained molecular mass markers were used as standards (lane 5).

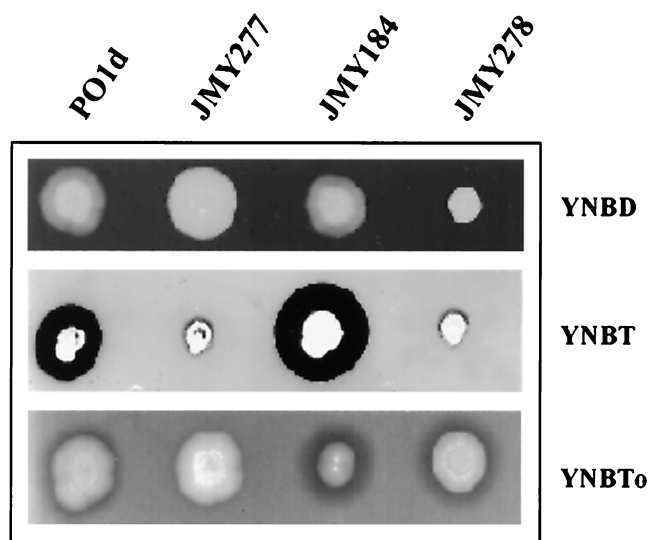


FIG. 7. Growth and lipase activity of *Y. lipolytica* strains on plates. Cells growing exponentially in YNBD at 28°C were diluted, and 10^5 cells in $10 \mu\text{l}$ were used to inoculate YNBD, YNBT, and YNBTo plates. Lipase secretion was detected as a halo after 3 days of incubation.

were glycosylated and contained 10 to 15% sugar (Fig. 6, lanes 2 and 4). Lip2p, pro-Lip2p, and their corresponding deglycosylated forms presented similar specific activities (data not shown), indicating that glycosylation is not required for activity.

A single gene accounts for extracellular lipase activity. Several lipase genes have been identified in the yeasts *C. rugosa* and *G. candidum*. We investigated if there were also several lipase genes in *Y. lipolytica* by digesting genomic DNA with five restriction enzymes (*Pst*I, *Hind*III, *Bam*HI, *Bgl*II, and *Cla*I) and probing at low stringency with the *LIP2* gene. For each enzyme, a single band was observed (data not shown) indicating that the *LIP2* gene was unique in *Y. lipolytica*. We also constructed strain JMY277, in which the *LIP2* gene was deleted (*lip2* knockout [KO], Fig. 5A). We compared lipase production in cell supernatant after 40 h of culture for PO1d (wild type) and JMY277 (*lip2* KO) in three different media. In YNBD, for PO1d and JMY277, less than 0.5 U/ml was detected. Under inducing conditions, YNBH and YNBO, we detected 19 and 10 U/ml for PO1d and less than 0.5 U/ml for JMY277. Comparison of YNBH values show the loss of at least 97% of the extracellular lipase activity. Even in the rich YTDH medium, no lipase activity or extracellular protein was detected (Fig. 4D). Finally, during *LIP2* gene isolation, a single and unique 5' RACE-PCR fragment was isolated using the YlipSA primer. Thus, the extracellular lipase is encoded by the single *LIP2* gene, in contrast to previous reports suggesting the presence of several extracellular lipase-encoding genes.

***LIP2* is not essential for growth on TG media.** We tested whether *LIP2* was essential for growth on TG media by comparing the growth on solid and liquid media of wild-type strain PO1d and modified strains JMY277 (*lip2* KO), JMY184 (*LIP2^A*), and JMY278 (*xpr6* KO). In each case, we compared growth on YNBD, YNBT, and YNBTo (Fig. 7). No differences in growth were observed on YNBD plates, except for JMY278. Similarly, no growth differences were observed on YNBTo (Fig. 7, bottom) and YNBH (data not shown) plates. This was confirmed by the similar growth of these strains in liquid media (data not shown), demonstrating that *LIP2* was not essential

for growth on long-chain TGs. Surprisingly, JMY277 and JMY278 grew poorly on short-chain TGs (Fig. 7, middle) but normally in liquid media (data not shown). We have no explanation for this effect.

Extracellular lipase activity was detected on YNBH and YNBTo plates and was particularly evident on YNB plates. Halos were present on YNBTo plates for the wild-type strain and were larger for the amplified strain, JMY184. No halos were observed for JMY277 (Fig. 7, bottom), indicating the absence of any other extracellular lipase activity, which was confirmed by the absence of extracellular protein in this strain. This suggests that the growth of JMY277 on YNBH and YNBTo results from TG metabolism by another pathway, probably involving membrane-bound lipases I and II or the intracellular *LIP1* and *LIP3* gene products. This demonstrates that *LIP2* is not essential for growth under these conditions.

DISCUSSION

Several lipases have been detected in *Y. lipolytica*, including intracellular, membrane-bound, and extracellular enzymes (for reviews, see references 2 and 21). The sequences of only two genes encoding lipases were determined by Dominguez and coworkers i.e., the *LIP1* and *LIP3* genes. These lipases are similar to the lipases of the fungi *C. cylindracea* and *G. candidum* and belong to the carboxylesterase family. They may be intracellular or membrane bound, because no clear signal sequence was detected (9). In this study, we isolated the *LIP2* gene, which codes for the extracellular lipase Lip2p, which belongs to the triacylglycerol hydrolase family.

Lip2p is synthesized as a prepro protein with 13 aa followed by four dipeptides (X-Ala or X-Pro), a short 12-aa pro region ending in a Lys-Arg (KR) site, and finally the mature protein. This is very similar to the structure of the prepro AEP precursor of the AEP encoded by the *XPR2* gene (28). This precursor has 13 aa followed by 10 dipeptides (X-Ala or X-Pro), a large 122-aa pro region ending in a KR site, and finally the mature protein. For Lip2p, SignalP predicted cleavage at Ala¹⁷, whereas for AEP, SignalP predicted cleavage at Ala¹⁵, the observed cleavage site (29). Whatever the location of the signal peptide cleavage site, two to three X-Ala or X-Pro dipeptides follow that are potential substrates for dipeptide aminopeptidase processing.

The two signal sequences had similar hydrophobicity characteristics (10, 34), indicating that Lip2p may be secreted via the signal recognition particle-dependent pathway (20). Lip2p maturation is dependent upon KEX2-like processing. In strain JMY278, which carries the *xpr6* KO deletion, the secreted precursor started at Thr²³ rather than Ile²², as expected from diaminopeptidase processing. Based on SignalP prediction, signal peptide cleavage after Ile²² is very unlikely. Although Lip2p was overproduced in JMY278, there was no evidence that X-Ala or X-Pro dipeptides were present at the N terminus of the precursor, whereas this was the case for the AEP precursor produced in an *xpr6* KO strain. Thus, there is no direct evidence that dipeptidyl aminopeptidase processing occurs and the nature of the processing event removing Ile²² is unclear. Mature Lip2p and its corresponding deglycosylated form have similar specific enzymatic activities. Similarly, pro-Lip2p produced in strain JMY278 and its corresponding deglycosylated form also have specific activities similar to that of the mature form, but these pro proteins were shown to be less stable. Assuming that the pro protein observed in the *xpr6* KO corresponds to the pro peptide in vivo, these results indicate that if the pro peptide is not removed: (i) secretion is not greatly affected, as lipase production in JMY278 is only one-quarter of

that in JMY184; (ii) there is no evidence that the pro peptide inhibits enzymatic activity, as has been shown for AEP; and (iii) the precursor seems to be less stable, indicating that the pro peptide may destabilize the protein. We do not know whether N glycosylation is involved in the secretion of Lip2p, but it may contribute to the stability of the protein and its resistance to protease, as has been shown for the human gastric lipase (50). Wild-type strains secrete about 30 to 50 U of lipase per ml. Mutant strains producing 25 times more lipase than the wild type were isolated by two-step mutagenesis. These mutants produced 1,200 U/ml under optimized conditions in a 500-liter fermentor (13). We used the *POX2* promoter to drive *LIP2* gene expression and multicopy integration of this expression cassette and obtained stable transformants that produced 2,000 U/ml, under nonoptimized conditions, corresponding to about 0.5 g of lipase per liter of supernatant. The production of large quantities of protein will facilitate the detailed analysis of the biochemical characteristics of the enzyme and provide material for crystallization and structural analysis. The secreted lipase had an apparent molecular mass of 38.5 kDa, 5.2 kDa more than the expected size. This suggests that the two potential glycosylation sites (Asn¹¹³ and Asn¹³⁴) are N glycosylated with core oligosaccharide.

Our PCR and DNA hybridization results show that unlike some yeasts, including *C. rugosa* (4, 25) and *G. candidum* (5), in which several highly homologous genes are present, *Y. lipolytica* probably contains a single gene encoding extracellular lipase. This is supported by the low residual extracellular lipase activity (less than 0.5 U/ml, the lower limit of detection of our assay) in strain JMY277, in which *LIP2* was deleted. We cannot totally exclude the possibility that there is another extracellular lipase encoding a gene with a low level of sequence similarity that would not be detected by hybridization. If such a gene is present, it is only weakly expressed under the growth conditions tested.

A surprising result was that *lip2* KO strain JMY277 grew with the same growth rate and cell yield as a *LIP2* strain on TG media. This suggests either that a very low level of activity (0.5 U/ml) is sufficient to sustain normal growth or that TGs may enter the cells directly via an unknown process and be hydrolyzed by intracellular and/or cell-bound lipases. Preliminary results of partial sequencing indicate that a third carboxylesterase and two short sequences containing the motif GH-SLGG/AA characteristic of the triacylglycerol lipase family are present in *Y. lipolytica*. Further analyses of these genes are in progress to determine the localization of the gene product and their function in the TG metabolism of this yeast.

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