Genetic Analysis of Lipase Low-producing Mutants of *Yarrowia lipolytica*

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Fourteen lipase low-producing (Lip⁻) mutants of *Yarrowia lipolytica* were obtained by UV mutagenesis. Complementation studies showed that ten of these mutants belonged to one complementation group. Two other mutants, Lip⁻ 132 and 223, complemented those of the first group. Lip⁻ 1123 complemented mutants of the first and second groups. Lip⁻ 1351 did not complement Lip⁻ 742, 417, 529 and 1133, which belonged to the first group. Genetic recombination analysis of the Lip⁻ mutants by random ascospore analysis showed that mutants of the first and second groups have lesions in the same gene, which is proposed to be the lipase structural gene. Inter-allelic complementation occurs between certain mutants of these groups. Lip⁻ 1123 has a lesion in a different gene.

INTRODUCTION

Yarrowia lipolytica is a dimorphic yeast. Its alkane utilization, lysine metabolism and ability to secrete an alkaline protease and several acid proteases have been studied (Ogrydziak & Scharf, 1982; Ogrydziak *et al.*, 1982). DNA-mediated transformation systems for this yeast have been studied extensively by Davidow *et al.* (1985, 1987) and Gaillardin *et al.* (1985). Lipases, which hydrolyse triglycerides, have been isolated and purified from *Y. lipolytica* (Ota *et al.*, 1978, 1982). Ota *et al.* (1982) showed that *Y. lipolytica* produced an extracellular lipase and two cell-bound lipases.

Lipases are potentially of use in the palm-oil-based industries of Southeast Asia, for the hydrolysis of triglycerides, transesterification and the production of esters. In 1986, Malaysia produced 4.5 million tonnes of palm oil (Palm Oil Research Institute of Malaysia, 1986). To assist in understanding the genetic control of lipase biosynthesis in *Y. lipolytica* we isolated fourteen lipase low-producing mutants. We describe here the genetic analysis of these mutants.

METHODS

Strains of Yarrowia lipolytica. Strains A *his1* and B *leu2 ade1 xpr2* were from the collection of H. Heslot.

Culture conditions and media. Complex YEPD medium consisted of 1% (w/v) peptone, 2% (w/v) glucose and 1% (w/v) yeast extract. Difco Yeast Nitrogen Base (without amino acids) (YNB) was used as minimal medium. Supplementation of this medium when required was as follows: 2% (w/v) glucose, 1% (w/v) tributyrin, 100 mg each of histidine and leucine l⁻¹ and 50 mg adenine l⁻¹.

Mutagenesis. This was done by UV irradiation of cells of strain A *his1* from agar medium, suspended in 3 ml distilled water at about 5×10^7 cells ml⁻¹, giving 15–20% survival. Lipase low-producing (Lip⁻) mutants were selected by the nystatin procedure (Snow, 1966; Beckerich *et al.*, 1984). After UV treatment the cells were incubated in YEPD overnight at 28 °C with shaking. The cultures were harvested by centrifugation (3000 g, 5 min), resuspended in YNB medium containing glucose and incubated for 8 h for the starvation phase. The medium was then supplemented with 100 mg histidine l⁻¹, 10 mg tributyrin l⁻¹ and 50 mg yeast extract l⁻¹ and incubated for 6 h. Nystatin and dimethyl sulphoxide were then added to give final concentrations of 10 mg l⁻¹ and 1% (w/v) respectively and the culture incubated for 5 h.

Screening for *Lip*⁻ mutants. Colonies on YEPD plates were inoculated onto YNB supplemented with glucose and histidine. These colonies were then replica plated onto YNB medium supplemented with tributyrin and histidine. *Lip*⁻ mutants were not able to utilize tributyrin as the sole carbon source and were unable to grow on this medium.

Genetic studies and determination of mating type. Each of the *A his1* *Lip*⁻ mutants was crossed with the haploid strain *B leu2 ade1 xpr2*, using the method of Gaillardin *et al.* (1973). The resulting diploid strains were induced to sporulate (Barth & Weber, 1985), and ascospores were obtained by treating asci with cytohelicase (LKB) at 30 mg ml⁻¹ and cysteine at 2 mg ml⁻¹ in water for 4 h at 30 °C followed by sonication. Colonies grown from single ascospores were plated onto YEPD and subsequently replica-plated onto classification media to isolate haploid strains which were leucine-requiring and *Lip*⁻.

The leucine-requiring, *Lip*⁻ haploid strains were tested for mating with the haploid strain *A his1*. Prototrophic diploid strains were obtained when the *Leu*⁻ haploids were of *B* mating type.

Complementation studies. Diploid strains were obtained from crosses between specific *A his1* *Lip*⁻ haploid strains and *B leu2* *Lip*⁻ strains as above. These were replica-plated onto YNB medium supplemented with tributyrin to test for complementation between the *Lip*⁻ mutants.

Assay of extracellular lipase activity. The method used was that of Ota & Yamada (1967). To a mixture of 2 ml lipase assay buffer (0.2 M-Tris/HCl, 0.2 M-maleic acid, 0.2 M-NaOH, adjusted to pH 8.2), 1 ml sodium taurocholate (0.2%, w/v) and 1 ml distilled water in an Erlenmeyer flask was added 5 ml polyvinyl alcohol emulsion containing 0.5 ml tributyrin. After incubation at 37 °C for 10 min, 1 ml of the supernatant from a YEPD culture of the test strain which had been incubated for 30 h at 28 °C was added. The mixture was incubated for 2 h with shaking, then the reaction was stopped by adding 20 ml acetone/ethanol (1:1, v/v). This was then titrated against 0.05 M-sodium hydroxide with phenolphthalein as indicator. A blank was shaken with enzyme which was added immediately before titration, the reaction being stopped instantly. The titration value of the sample was corrected by subtracting the titration value of the blank sample from that of the corresponding test sample. Lipase activity was determined in lipase units. One lipase unit was defined as the amount of enzyme which liberated one microequivalent of free fatty acid per minute.

Strains were also assayed for extracellular lipase activity after growth in YEPD containing 1% (v/v) olive oil (Sigma). The lipase assay mixture in this case contained 0.5 ml olive oil instead of tributyrin.

Determination of cell dry weight. A 10 ml sample of an overnight culture was centrifuged in a pre-weighed screw-capped test tube. The pellet was dried to constant weight at 100 °C.

Reversion of *Lip*⁻ mutants. *Lip*⁻ mutants of strain *A his1* were inoculated onto YEPD agar and incubated for 3 d at either 28 °C or 16 °C. Cells from these cultures were suspended in 0.9% (w/v) saline, washed once in saline and resuspended in saline after centrifugation. Samples (0.1 ml) were then spread onto YNB supplemented with tributyrin and histidine and incubated at either 28 °C or 16 °C for 5 d. *Lip*⁺ revertants were isolated from this medium and supernatants from cultures of these strains were assayed for lipase activity.

Test for temperature-sensitive lipase of revertants. *Lip*⁺ revertants from *Lip*⁻ mutants were inoculated into two sets of flasks containing YEPD, incubated overnight at 28 °C for one set and 16 °C for the other. Samples of 1 ml of culture supernatant were used for assay for lipase activity at both 28 °C and 16 °C, with a reaction time of 2 h.

RESULTS AND DISCUSSION

*Characterization of *Lip*⁻ mutants*

Fourteen *A his1* *Lip*⁻ strains isolated as described in Methods and grown on unsupplemented YEPD medium were assayed for extracellular lipase activity with tributyrin as substrate (Table 1). All the mutants had low lipase activity, ranging from 0% to 4.2% of that of the control strain, *A his1*. *Lip*⁻ mutants 742, 1362, 936, 108 and 1324 were devoid of lipase activity in tests with olive oil as substrate, using samples of cultures grown in YEPD with olive oil (the other mutants were not tested). Lipase activity for *A his1* was 11.4 units (mg dry wt)⁻¹ under these conditions. In the case of samples from unsupplemented YEPD cultures, strain *A his1* and mutant 742 gave lipase activities of 7 units (mg dry wt)⁻¹ with olive oil as substrate, and mutants 1362, 936, 108 and 1324 showed activity of about 3.0 units (mg dry wt)⁻¹.

Each of the *A his1* *Lip*⁻ mutants was crossed with *B leu2 ade1 xpr2*. Sporulation of the resulting diploid strains gave ascospores which were plated onto YEPD media. Leucine-requiring *Lip*⁻ haploids corresponding to each of the 14 *A his1* *Lip*⁻ mutants were obtained. Confirmation of the *B leu2* character of these *Lip*⁻ haploid strains was achieved by mating with strain *A his1*.

The mutations in all 14 *Lip*⁻ strains were recessive, as the diploids formed between each of them and *B leu2 ade1 xpr2* were *Lip*⁺. Analysis of the colonies derived from ascospores of these

Table 1. Lipase activities of *Lip*⁻ mutants

All the experiments were performed at least three times and the results presented are means. Standard deviation was less than 10% of the given figures except in the case of *Lip*⁻ strain 1123, for which the standard deviation was 18% of 2.6. The lipase activity of the control strain, A *his1*, taken as 100%, was 298 units (mg dry wt)⁻¹.

A <i>his1</i> <i>Lip</i> ⁻ strain	Lipase activity*	A <i>his1</i> <i>Lip</i> ⁻ strain	Lipase activity*
742	0 (0%)	1133	2.9 (1.0%)
1362	12.8 (4.2%)	350	6.6 (2.2%)
936	0 (0%)	1432	0 (0%)
108	0 (0%)	132	0 (0%)
417	10.6 (3.6%)	223	6.6 (2.2%)
1324	3.5 (1.2%)	1351	3.5 (1.2%)
529	6.6 (2.2%)	1123	2.6 (0.9%)

* The results are expressed as units (mg dry wt)⁻¹; the values in parentheses represent the percentage of control activity.

Table 2. Complementation tests between lipase low-producing mutants

Eight A *his1* *Lip*⁻ strains, 742, 1362, 936, 108, 417, 1324, 529 and 1133, were crossed with each of the corresponding eight B *leu2* *Lip*⁻ strains, and the resulting diploid strains were replica-plated onto YNB medium plus tributyrin to test for complementation. No complementation occurred. Lipase activities were measured for certain other diploid strains, as shown below.

Diploid: A <i>his1</i> <i>Lip</i> ⁻ /B <i>leu2</i> <i>Lip</i> ⁻	Lipase activity [units (mg dry wt) ⁻¹]
417/1324	26
742/1432	22
529/132	182
1324/132	171
223/132	21
132/1123	238
1324/1123	252
A <i>his1</i> <i>Lip</i> ⁺ /B <i>leu2</i> <i>Lip</i> ⁺	316

diploids showed that each gave a 1:1 pattern of segregation for *Lip*⁺:*Lip*⁻. This result indicated that the mutation in the *Lip*⁻ strains was chromosomal and monogenic.

Complementation analysis

Eight of the A *his1* *Lip*⁻ mutants, 742, 1362, 936, 108, 417, 1324, 529 and 1133, were crossed with each of the eight corresponding B *leu2* *Lip*⁻ strains to obtain diploid strains. Diploid strains obtained from these crosses, tested for complementation on YNB medium containing tributyrin, showed that these eight *Lip*⁻ mutants belonged to one complementation group (see Table 2). Diploid strains obtained from crosses between the B *leu2* *Lip*⁻ strains 350 and 1432 and each of the above A *his1* *Lip*⁻ strains showed that *Lip*⁻ 350 and 1432 did not complement these eight mutants. *Lip*⁻ 1351 did not complement *Lip*⁻ 742, 417, 529 and 1133. B *leu2* *Lip*⁻ 132, 223 and 1123 complemented A *his1* *Lip*⁻ 936, 1324 and 529. The diploid strain A *his1* *Lip*⁺/B *leu2* *Lip*⁺ and those constructed between B *leu2* *Lip*⁻ 1123 and each of A *his1* *Lip*⁻ 936, 1324 and 529 were able to grow on YNB containing tributyrin and had a distinctly faster growth rate on this medium than the diploids formed between B *leu2* *Lip*⁻ strains 132 and 223 and the same A *his1* *Lip*⁻ strains. *Lip*⁻ 132 did not complement *Lip*⁻ 223. *Lip*⁻ 1123 complemented *Lip*⁻ 132 and 223.

Genetic recombination analysis

In order to determine if the *Lip*⁻ mutants had lesions in the same gene, genetic recombination studies using random ascospore analysis were carried out for the crosses shown in Table 3.

Table 3. *Genetic recombination analysis of Lip⁻ mutants*

Diploid: A <i>his1</i> Lip ⁻ /B <i>leu2</i> Lip ⁻		Ascospore colonies of diploid strains					
		His ⁻	Ability to grow on YNB medium plus histidine and tributyrin		Leu ⁻	Ability to grow on YNB medium plus leucine and tributyrin	
			+	-		+	-
417/1324	12	0	12	9	0	9	
529/1432	13	0	13	8	0	8	
529/132	16	0	16	9	0	9	
936/1133	14	0	14	12	0	12	
742/1123	19	5	14	13	3	10	
529/1123	16	4	12	11	3	8	

Leucine- and histidine-requiring colonies from ascospores of each of the diploids were tested for their ability to grow on YNB medium supplemented with tributyrin. The ascospore-derived colonies from each of the A *his1* Lip⁻/B *leu2* Lip⁻ diploids 417/1324, 529/1432, 529/132, 936/1133, 742/1123 and 529/1123 were inoculated onto YNB medium containing histidine or leucine and tributyrin. Except for the colonies from the 1123 diploids, all the histidine- and leucine-requiring colonies were unable to grow on appropriately supplemented YNB media with tributyrin. The diploid 742/1123 gave 14 His⁻ colonies which were Lip⁻ and 5 which were Lip⁺; diploid 529/1123 gave 12 colonies which were Lip⁻ and 4 which were Lip⁺. Leucine-requiring colonies from these diploids also gave a ratio of Lip⁻:Lip⁺ colonies of close to 3:1. When considered together, these results and those of the complementation studies showed that the Lip⁻ mutants 742, 1362, 936, 108, 417, 1324, 529, 1133, 350 and 1432 had lesions in one lipase gene, that mutants 132 and 223 had lesions in the same gene and inter-allelic complementation occurred between 132 and 223 on the one hand and 936, 1324 and 529 on the other. Lip⁻ 1123 had a lesion in a different gene affecting lipase production.

Reversion studies of Lip⁻ mutants

To establish whether the Lip⁻ mutants had lesions in the putative structural gene for lipase in *Y. lipolytica*, spontaneous revertants of the A *his1* Lip⁻ mutants 1362, 108, 223, 1324, 350, 1432 and 1123 were obtained to determine the temperature sensitivity of their extracellular lipase. The results are shown in Table 4 (the samples for lipase assay were from cultures grown at 28 °C). Except for strain Lip⁻ 1123, all the mutants gave a number of revertants with temperature-sensitive lipase. We conclude that these mutants are affected in the lipase structural gene. No temperature-sensitive lipase was obtained from Lip⁺ revertants of mutant 1123 in a total of 102 revertants assayed, suggesting that in this mutant the lesion might have affected a different gene, perhaps one of the genes responsible for the secretion of lipase.

Ota *et al.* (1973) described an extracellular lipase which required activator A from culture supernatant of *Y. lipolytica* or an unsaturated fatty acid like oleic acid (Ota & Yamada, 1967) for its lipolytic activity at pH 7.0 in an unemulsified shaken reaction system. Sugiura *et al.* (1976) reported that cell-bound lipase was active without the addition of an activator. The latter authors also showed that cell-bound lipase and extracellular lipase had an optimum pH of 8.0 when olive oil was the substrate. The optimum pH for cell-bound lipase was 7.0 with tributyrin as the substrate, while extracellular lipase hydrolysed tributyrin most effectively at pH 8.2 in the presence of oleic acid. Ota *et al.* (1982) reported the isolation of two cell-bound lipases and an extracellular lipase. They suggested, from a study of the enzymological properties of cell-bound lipases I and II, that one enzyme was probably a modified form of the other. According to Ota *et al.* (1982), cells grown in the absence of lipid produced lipase which hydrolysed tributyrin but not olive oil. In the present study, cells grown in YEPD without olive oil produced lipase which hydrolysed both tributyrin and olive oil. In the presence of olive oil, *Y. lipolytica* produced an extracellular lipase which hydrolysed olive oil. It seems possible that the Lip⁻ mutants have

Table 4. Lipase activities of revertants of Lip⁻ mutants

A his1 Lip ⁻ strain	No. of Lip ⁺ revertants	No. of temperature-sensitive revertants	Lipase activity at 16 °C/lipase activity at 28 °C*	
			Temperature-sensitive revertants	Other revertants†
1362	76	3	0.3	0.7 -1 (11)
108	81	3	0.3	0.7 -1 (5)
223	74	3	0.25	0.75-1 (11)
1324	79	2	0.33	0.66-1 (8)
350	80	2	0.33	0.8 -1 (6)
1432	75	2	0.27	0.7 -1 (12)
1123	102	0		0.7 -1 (14)

* Samples for lipase assay were from YEPD cultures incubated at 28 °C.

† The number of revertants tested is shown in parentheses.

lesions that affect the gene for lipase which hydrolyses both tributyrin and olive oil, but at different levels of activity.

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