GENETICS OF EXTRACELLULAR PROTEASE PRODUCTION IN *SACCHAROMYCOPSIS LIPOLYTICA*

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ABSTRACT

Mutants of *Saccharomycopsis lipolytica* with reduced ability to produce zones of clearing on skim-milk agar plates were isolated and their properties studied. For 18 mutants it was possible to score unambiguously segregants of crosses between these mutants and wild type for extracellular protease production. These mutants all produce reduced levels of extracellular protease in liquid culture. The mutations are recessive and are in nuclear genes. The 18 mutations define 10 or 11 complementation groups, no two of which are closely linked. Mutants in four of the complementation groups also produced reduced levels of extracellular RNAse, and the reduced levels of extracellular protease and RNAse production segregate together. Five of the mutants exhibited reduced mating frequency, and one mutant was osmotic remedial for extracellular protease production. These results demonstrate that many genes can affect extracellular protease production. Besides mutations in the structural gene and in regulatory genes, mutations are likely to be in genes involved in steps common to the production of several extracellular enzymes or in genes coding for cell wall or membrane components necessary for extracellular enzyme production.

STUDIES of mutants altered in extracellular protease production have yielded insights into both the regulation of extracellular protease production and, more generally, the components and processes involved in production of extracellular enzymes. For example, in *Bacillus subtilis* three classes of mutations overproducing extracellular proteases (*sacU*^{h}, *amy B* and *pap*) and mapping at the same genetic location (STEINMETZ, KUNST and DEDONDER 1976) also overproduce extracellular α-amylase and lavansucrase, are not transformable, are devoid of flagella (YONEDA and MARU 1975) and produce reduced levels of autolysin(s) (AYUSAWA *et al.* 1975). In *Aspergillus nidulans* a mutant (*xpr D1*) producing extracellular protease in the presence of ammonia (COHEN 1972) and a mutant (*xpr C1*) which has lost the ability to produce all three extracellular proteases (COHEN 1973) have been described. Mutations resulting in hyperproduction of extracellular proteases and with other pleiotropic effects have also

been described in *Aspergillus sojae* (NASUNO and OHARA 1971), *Staphylococcus aureus* (RYDEN, LINDBERG and PHILIPSON 1973) and *Proteus mirabilis* (HESSELWOOD 1974).

Extracellular protease production is quite common among the yeasts, but the two yeasts most suitable for genetic studies (Saccharomyces and Schizosaccharomyces) do not produce extracellular proteases (AHEARN, MEYERS and NICHOLS 1968; KAMADA *et al.* 1972). However, the hydrocarbon utilizing yeast, *Saccharomycopsis lipolytica*, offers a potentially valuable model system for the study of extracellular enzyme production in lower eucaryotes. *S. lipolytica* produces a single extracellular protease (TOBE *et al.* 1975; OGRYDZIAK and TANNEBAUM, unpublished results) and one or more extracellular RNAses (FRIBOURG, Master's Thesis, M.I.T. 1972) and lipases (PETERS and NELSON 1948). In addition genetic analysis is now possible in this organism (WICKERHAM, KURTZMAN and HERMAN 1969; GAILLARDIN, CHAROY and HESLOT 1973; OGRYDZIAK *et al.*, in preparation).

In this study, genetic analysis and preliminary characterization of mutants of *S. lipolytica* that produce reduced levels of extracellular protease are reported.

MATERIAL AND METHODS

Strains: The following strains were used: CX161-1B, genotype *ade1 A* (haploid) and DX7-70, genotype *trp1 ura1 ts1 B* (haploid). The mutants exhibiting reduced extracellular protease activity (*xpr*) were derived from CX161-1B. *A* or *B* denote the bipolar mating type (BASSEL and MORTIMER 1973).

Media, culture maintenance, and scoring: The following media were used for culture maintenance, mating, sporulation and scoring of auxotrophic markers:

YM, yeast extract-malt extract medium: 0.3% each of yeast extract and malt extract, 0.5% peptone, 1% glucose and 2% agar.

RG, restrictive growth medium: 0.02% yeast extract and peptone, 0.1% glucose and 2% agar.

SM, synthetic minimal medium: 0.6% Difco yeast nitrogen base without amino acids, 2% glucose and 2% agar.

SC, synthetic complete medium: SM plus the following amino acids and bases, per liter: adenine 30 mg, uracil 20 mg, L-tryptophan 60 mg, L-threonine 37.5 mg, L-histidine 20 mg, L-lysine 40 mg, L-methionine 20 mg, L-phenylalanine 50 mg and L-tyrosine 20 mg. The threonine and tryptophan are filter sterilized and added after autoclaving.

OM, omission media: SC lacking one of amino acids or bases; *e.g.* —trp (SC minus tryptophan), — ade (SC minus adenine).

D, dissection medium: 5% glucose, 0.5% peptone, 0.3% beef extract, 1% yeast extract and 2.25% agar plus amino acids and bases as in SC.

Cultures were stored on YM at 5°. Scoring, mating and sporulation were done at 23°. Nutritional requirements were scored by replica plating on SC plates that lacked the needed supplement. The temperature sensitive (*ts*) marker was scored on YM at 34°.

Skim-milk plates were prepared by combining 60 ml of 10% Difco skim milk in 0.05 M phosphate buffer, pH 6.8 (autoclaved 10 min at 100°), 60 ml of a mixture of 24 g/l KH_2PO_4 and 35.5 g/l Na_2HPO_4 and 480 ml containing 1 g Difco Yeast Nitrogen Base without amino acids and without ammonium sulfate, 12 g agar and 15 mg adenine and 20 mg uracil.

RNA plates contain 10 g glucose, 4 g Difco Proteose-peptone, 2 g RNA (from Torula yeast RNA Type VI, Sigma), 0.145 g KH_2PO_4, 0.4 g $MgSO_4 \cdot 7H_2O$, 0.15 g $CaCl_2 \cdot 2H_2O$, 0.1 g NaCl, 1 mg thiamine, 20 g agar, 25 mg adenine and 32 mg uracil in 0.05 M citrate buffer, pH 5.0. Dropwise additions of 1N NaOH were made while dissolving the RNA in distilled water in order ot keep the pH between 5 and 6.

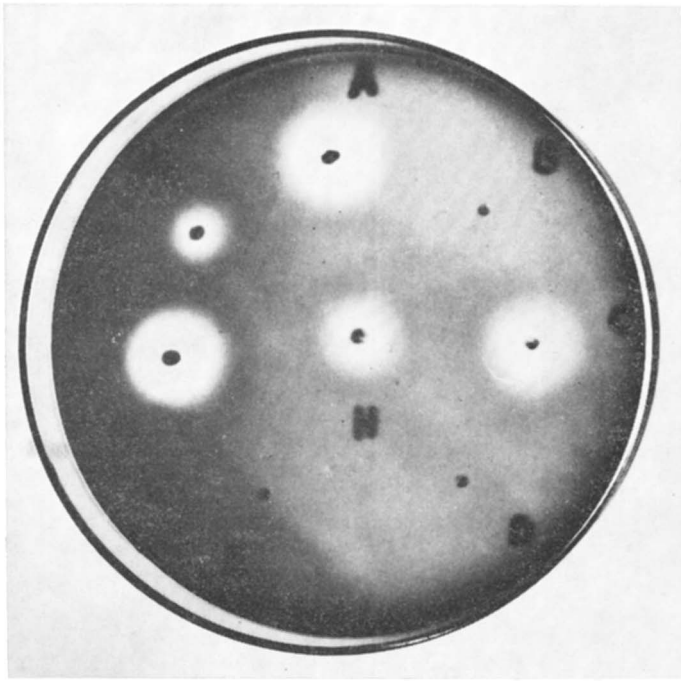

FIGURE 1.—Zones of clearing on a skim-milk plate produced by *Saccharomycopsis lipolytica*. The strains were toothpicked onto a 1% skim-milk plate and incubated at 23° for 48 hrs. The strains are as follows:

A. wild-type parental haploid, CX 161–1B *ade1 A*
B. *xpr* haploid derived from CX 161–1B *ade1 xpr1–5 A*
C. heterozygous diploid, PX 5 *ade1 xpr1–5 A x* DX 7–70 *trp1 ura1 ts1 B*
D. haploid *xpr* segregant of PX 5, *ura1 xpr1–5 B*
E. non-complementing diploid, *ade1 xpr6–25 A* x *ura1 xpr6–13 B*
F. complementing diploid, *ade1 xpr6–25 A* x *ura1 xpr3–23 B*
G. leaky *xpr* haploid derived from CX 161–1B, *ade1 xpr7–3 A*
H. leaky *xpr* haploid segregant, *ura1 xpr7–3 B*

Fifteen ml of medium per plate was used for the skim-milk and RNA plates. Inoculation with fresh cultures (1–2 days old) was done by making a small stab with a toothpick. Plates were incubated for 2 days at 23°. On 1% skim-milk plates quite distinct zones of clearing on a white background were formed (Figure 1). On 1% casein plates much less distinct cloudy zones on a clear background were formed. The RNA plates were scored using the technique of STREITFELD, HOFFMANN and JANKLOW (1962) in which the plates are flooded with a 0.1% solution of toluidine blue O (J. T. Baker). Extracellular RNAse activity appears as a bright pink halo against a blue background. Plates were scored "+" or "—" depending on relative zone sizes. Quantitative data were obtained by measuring the zone diameter and colony diameter to the nearest 0.5 mm. Plates for scoring osmotic remedial mutations were prepared as usual with the addition before autoclaving of sufficient KCl to give the desired final concentration.

Mutagenesis: Mutants were induced by UV irradiation from a germicidal lamp with a dose of 1450 ergs/mm^2 at the surface of the plates (OGRYDZIAK *et al.* in preparation). This treatment resulted in approximately 30% survival of treated cells. The treated cells were plated on skim-milk medium (in some cases, supplemented with glucose and ammonium sulfate or

glycerol, ammonium sulfate, glutamine, and cysteine) and colonies that produced no surrounding zone of clearing were selected.

Protease assay: Assay of extracellular protease production in liquid culture was done using the following medium: 6.7 g/l glycerol, 1.6 g/l Difco Proteose-peptone, 1.7 g/l Difco Yeast Nitrogen Base (YNB) without amino acids and ammonium sulfate, 30 mg/l adenine and 1 ml/l polypropylene glycol, MW 2000 (Polysciences, Inc.) in 0.16 M phosphate buffer pH 6.8. The Proteose-peptone was autoclaved separately in the phosphate buffer. Cultures were grown at 23° on a rotary shaker with 50 ml of medium in a 500 ml baffled flask. Samples were taken during exponential growth (at cell density of about 1 g dry wt/l), and protease activity of the supernatant was measured using the casein hydrolysis assay described by OGRYDZIAK, DEMAIN and TANNENBAUM (1977).

RNAse assay: Assay of extracellular RNAse production in liquid culture was done using the following medium: 6.7 g/l glycerol, 1.6 g/l Difco Proteose-peptone, 1.7 g/l Difco YNB without amino acids and ammonium sulfate, 30 mg/l adenine and 1 ml/l polypropylene glycol MW 2000 in 0.05 M citrate buffer pH 5.0. The Proteose-peptone was autoclaved separately in the citrate buffer. Cultures were grown at 30° on a rotary shaker with 50 ml of medium in a 500 ml baffled flask. Samples were taken during exponential growth (at cell density 1 g dry wt/l) and RNAse activity of the supernatant was measured using a modification of the RNA hydrolysis assay described by ANFINSEN *et al.* (1953). One tenth ml of enzyme was added to 0.9 ml substrate to give the following final concentrations per assay tube: 0.1% yeast RNA (Crestfield purif. Type XI, from Sigma Chemical Co.), 10^{-3} Mg++, 10^{-4} M Ca++ in 0.02 M citrate, buffer, pH 5.0. The mixture was incubated for 45 min at 40° and the reaction was stopped by cooling the assay mixture for 1 min and adding 1 ml of 12% perchloric acid solution containing 0.4% uranylacetate. The mixture was shaken, cooled 15 min on ice and centrifuged. The supernatant was diluted tenfold and the absorbance at 260 nm measured. A RNAse unit was defined as that amount of RNAse causing an increase in absorbance of 260 nm of 0.1 after 45 min at 40°. Samples were assayed in duplicate.

Cell density: Cell density was measured with a Klett-Summerson photoelectric colorimeter with a green filter. One gram dry weight of cells per liter yielded a value of 250 Klett units.

Genetic procedures: Genetic procedures are described in detail elsewhere (BASSEL, WARFEL and MORTIMER 1971, BASSEL and MORTIMER 1973, OGRYDZIAK *et al.*, in preparation). Mating was done on solid RG medium by mixing haploids with complementary nutritional markers and selecting diploids on minimal medium (SM). Possible diploids were restreaked on SM and then sporulated by heavily streaking on a YM plate and incubating 12–14 days at 23°. Concentrated suspensions of asci were prepared by washing sporulated cultures and collecting the mycelial clumps. For both random spore and tetrad analysis, glusulase was used to dissolve mycelia and ascal walls. Sonication was used to destroy diploids and to separate spores for random spore analysis. Micromanipulation was used for tetrad dissection. Linkage was determined using the standard calculation described by MORTIMER and HAWTHORNE (1975).

Complementation analysis: Segregants (*ura1 spr* or *trp1 xpr*) of *B* mating type from crosses of the *ade1 xpr A* mutants with DX 7–70 *trp1ura1 ts1 B* were selected for doing complementation analysis. The *xpr* haploids with complementary nutritional requirements were mated and possible diploids selected on SM medium. The possible diploids were restreaked on SM medium and then tested for extracellular protease production on skim milk plates. Those possible diploids showing no complementation (*i.e.*, small or no zone of clearing) were confirmed as diploids by sporulation.

RESULTS

Mutation frequency

After UV mutagenesis, 26 mutants producing small or no zone of clearing on skim milk plates (*xpr* mutants) were isolated from the 6587 colonies investigated.

Random spore analysis

The *xpr* mutants isolated in a CX161–1B *ade1 A* background were mated with DX7–70 *trp1 ura1 ts1 B*, which is wild type for extracellular protease production. For 18 mutants, it was possible to score unambiguously the random spore segregants of these crosses for extracellular protease production. For these 18 *xpr* mutants, the heterozygous diploids produced zones of clearing comparable in size to those produced by homozygous wild-type diploids, indicating that the mutations are recessive. Random spore analysis of most of the mutant by wild-type crosses showed 1:1 segregation for extracellular protease production. For some of the crosses a significant excess of protease producing spores were recovered. However, tetrad analysis of these crosses showed a 2:2 segregation for extracellular protease production. Therefore, all 18 mutations are in single nuclear genes.

One isolate producing a larger than average zone was mated with wild type. It was not possible to score unambiguously the segregants for overproduction of protease.

Extracellular protease activity

The results of assaying for extracellular protease activity in shake flask culture indicate that all the *xpr* mutants produce reduced levels of extracellular protease (Table 1). Except for mutants *xpr3–16* and *xpr3–23*, the plate and shake flask protease assay agree quite well.

TABLE 1

Extracellular protease production by extracellular protease-nonproducing mutants of Saccharomycopsis lipolytica

Isolate	Protease		Protease
	Zone diameter (mm)	Colony diameter (mm)	U/mg dry weight
Wild type (CX 161–1B *ade1 A*)	11	3	131
xpr1–5	N.D.†	2	0.0
xpr1–14	N.D.	2	1.0
xpr2–7	4.5	2.5	6.7
xpr2–9	N.D.	2	0.1
xpr3–16	N.D.	2	39
xpr3–23	3	2.5	67
xpr4–18	N.D.	2	1.6
xpr4–21	N.D.	2	13
xpr5–2	N.D.	2.5	1.3
xpr5–11	3.5	3	7.9
xpr5–22	N.D.	2	6.1
xpr6–13	N.D.	2	0.1
xpr6–25	N.D.	2	0.5
xpr7–3	5	3	74
xpr8–10	N.D.	2	3.8
xpr9–8	N.D.	2	3.4
xpr10–4	3	2.5	12
xpr–15	N.D.	2	1.6

† N.D. No zone of clearing on skim-milk medium could be detected surrounding the colony.

In liquid culture (Proteose-peptone was the sole nitrogen source), the growth rate of several of the *xpr* mutants slowed considerably at a cell density of 0.6–0.8 g dry wt cells/l, while the wild type was still growing exponentially at a cell density of 1.0 g dry wet cells/l. The effect of reduced extracellular protease production by the *xpr* mutants on their growth with proteins as a substrate was also seen in the reduced colony diameter of the *xpr* mutants growing on the skim-milk plates (Table 1).

Complementation analysis

The 18 *xpr* mutations can be classified into at least 8 complementation groups based on complementation analysis (Table 2). Attempts were made to cross all the mutants with each other. However, for several of the mutant combinations mating was not obtained even after numerous attempts using the standard mating procedure and after several attempts using modifications such as liquid mating.

The complementation results are clear cut (see Figure 1) and internally consistent. Mutants *xpr-3,4,15* can be excluded from the first 7 complementation groups; since they did not mate with each other, they possibly are all at the same locus. Mutant *xpr-8* could also be at this locus or it could be at the *xpr5*, *xpr6* or *xpr8* loci.

TABLE 2

Intergenic complementation of xpr⁻ *mutants*

Isolate number	2	3	4	5	7	8	9	10	11	13	14	15	16	18	21	22	23	25
2	—	+	+	+	+		+	+	—	+	+	+	+	+	+	—	+	+
3				+	+		+	+		+	+		+	+	+		+	+
4				+	+		+	+	+	+	+		+	+	+		+	+
5				—	+		+	+	+	+	—	+	+	+	+	+	+	+
7					—	+	—	+	+	+	+	+	+	+	+	+	+	+
8							+				+			+			+	
9							—	+	+	+	+	+	+	+	+	+	+	+
10								—	+	+	+	+	+	+	+	+	+	+
11									—	+	+	+	+	+	+	—	+	+
13										—	+	+	+	+	+	+	+	—
14											—	+	+	+	+	+	+	+
15													+	+			+	+
16													—	+	+	+	—	
18														—	—	+	+	+
21															—	+	+	+
22																—	+	+
23																	—	+
25																		

Complementation groups

xpr1–5, 14 *xpr2–7,9* *xpr3–16,23*
xpr4–18,21 *xpr5–2,11,22* *xpr6–13,25* *xpr8–10*
Unassigned:
xpr–3 *xpr–4* *xpr–8* *xpr–15*

Linkage data

Several of the unassigned *xpr* mutants can be assigned to independent loci based on mapping data (Table 3). Mutation *xpr-8* is linked to *ade1* and it complements *xpr2–7,9*—the only other markers linked to *ade1*. Therefore, *xpr-8* can be assigned to the independent locus *xpr9–8*. Mutations *xpr-4,15* are linked to *ura1* and *xpr-3* is not; *xpr-3* is assigned to the independent locus *xpr7–3*. By combining the complementation and mapping data, it was possible to assign the 18 *xpr* mutations to a minimum of 10 and a maximum of 11 complementation groups, depending on whether or not *xpr10–4* and *xpr-15* are alleles.

Number of xpr *genes*

Linkage between the complementation groups was investigated to determine if possibly some of the complementation was intragenic. All the *xpr* mutants were mapped against *ade1*, *ura1*, *trp1*, and *ts1* (Table 3). Mutants *xpr2–7,9* and *xpr9–8* were linked to *ade1*, and *xpr10–4* and *xpr-15* were linked to *ura1*. None of the other mutants was linked to any of the four markers. Therefore, none of the seven other complementation groups was tightly linked to *xpr2–7,9*, *xpr9–8*, *xpr10–4*, or *xpr-15*. Linkage between the seven groups was determined by random spore analysis of crosses between representatives of each complementation group (Table 4). For unlinked groups, 25% of the segregants should be XPR^+. A difficulty with random spore analysis was that some of the diploids survive the glusulase treatment and sonication. To insure that diploids were excluded, the calculation was done by using only segregants that segregated at least one marker (not including *xpr*). The results based on these segregants should yield a true estimate of the percentage of XPR^+ spores if, as was true in this case, none of the *xpr* mutants was linked to the other markers segregating in the cross. Both *xpr7* and *xpr5* were linked to *xpr1* and to each other. Rough estimates of map distance

TABLE 3

Linkage data for xpr *mutants of* Saccharomycopsis lipolytica

Gene pairs	Tetrads PD	NPD	T	Random spores P	NP
xpr2–7 · ade 1				35	9
xpr2–9 · ade 1	32	4	35	173	75
xpr9–8 · ade 1	6	0	3*	159	58
xpr10–4 · ura 1				108	13
xpr–15 · ura 1				187	46

Final assignment of loci based on mapping and complementation data

Complementation groups		
xpr1–5,14	*xpr2–7,9*	*xpr3–16,23*
xpr4–18,21	*xpr5–2,11,22*	*xpr6–13,25*
xpr7–3	*xpr8–10*	*xpr9–8*
xpr10–4	*xpr–15*	

Parentals (PD or P) significantly exceed nonparentals (NPD or NP) at the 1% level, unless indicated with * where $2.5\% > P > 1\%$.

TABLE 4

Random spore analysis of linkage between xpr *complementation groups*

	xpr1	*xpr3*	*xpr4*	*xpr5*	*xpr6*	*xpr7*	*xpr8*
xpr1	x	16:31	20:31†	9:61**	19:36	4:69***	33:55†
xpr3		x	22:58	32:61†	12:33	22:56	15:35
xpr4			x	17:36	21:51	23:48	21:46
xpr5				0:116***	24:69	23:114*	27:58
xpr6					x	18:54	34:53†
xpr7						x	22:60
xpr8							x

Data reported as follows: no. of *XPR+* spores: no. of *xpr⁻* spores.
* Linkage—significantly less than 25% *XPR+* **P*<0.05; ***P*<0.025; ****P*<0.001 (χ^2; df=1).
† Significantly more than 25% *XPR+* spores.

(*xpr1–xpr5*, 26 cM; *xpr1–xpr7*, 11 cM; *xpr7–xpr5*, 34 cM) indicates that *xpr1* lies between *xpr7* and *xpr5*.

Both *xpr2–7,9* and *xpr9–8* were linked to *ade1*, but random spore analysis (results were based on total segregants due to the linkage to *ade1*) indicated that they were unlinked, and therefore they must be on opposite sides of *ade1*.

Significantly more than 25% *XPR+* spores were found for four cases listed in Table 4. Most probably this results from selection against recovery of *xpr⁻* spores during the random spore procedure.

Extracellular RNAse activity

In order to determine which *xpr* mutants were not specific for extracellular protease production but affected extracellular enzyme production more generally, extracellular RNAse production was measured in shake flasks and on RNA plates. There is reasonable agreement between the plate and shake flask assays (Table 5). Mutants *xpr1*, *xpr3*, *xpr4* and *xpr8* showed significantly reduced levels of extracellular RNAse. In all cases, low extracellular RNAse production and the *xpr* mutation were shown to segregate together in clones isolated by tetrad dissection.

Alleles 18 and 21 of *xpr4* are of interest. The RNAse production is much less sensitive to the mutational defect than is the protease production. For *xpr4–18*, protease production is about 1% of wild type and RNAse production is significantly decreased, while for *xpr4–21*, protease production is about 10% of wild type and RNAse production is essentially unaffected.

Temperature sensitivity and osmotic remediability

None of the *xpr* mutants was temperature sensitive for growth. Mutant *xpr6–13* was shown to be osmotic remedial on skim-milk plates with 0.5 M KCl. Preliminary experiments indicate that *xpr6–13* either produces an altered protease that requires the presence of the KCl during synthesis for the active conformation to be established or that a KCl sensitive inhibitor of the protease is produced (unpublished results).

Mating frequency

Several of the *xpr* mutants (*xpr7–3*, *xpr10–4*, *xpr9–8*, *xpr-15*, and *xpr5–22*) exhibit greatly reduced mating frequency, and *xpr11–20* would not mate at all. However, several of the *xpr* mutants (*xpr1–5,14*, *xpr2–9*, *xpr5–13* and *xpr4–18*) producing very low levels of extracellular protease (less than 2% of wild type) exhibit normal mating frequency. The low mating frequency and the inability to produce extracellular protease were shown to segregate together for the mutant *xpr9–8*—the only *xpr* mutant for which the segregation of mating frequency was investigated.

DISCUSSION

Regulation of extracellular protease production was studied in *Candida lipolytica* NRRL Y-1094 by OGRYDZIAK, DEMAIN and TANNENBAUM 1977. Crosses involving this strain had very low spore viability making it unsuitable for a genetic study. Therefore, a genetically useful strain (*Saccharomycopsis lipolytica* CX 161–1B *ade1 A*) which had been developed by inbreeding was used instead (MORTIMER and BASSEL 1973). The biochemistry of extracellular protease production by *S. lipolytica* CX 161–1B has not been done and the number of extracellular proteases produced is not known. However, both *Candida lipolytica* AJ 4555 (TOBE *et al.* 1976) and *Candida lipolytica* NRRL Y-1094 (OGRYDZIAK,

TABLE 5

Extracellular RNAse production by extracellular protease—nonproducing mutants of Saccharomycopsis lipolytica

Isolate	RNAse Zone diameter (mm)	RNAse Colony diameter (mm)	RNAse U/mg dry weight
Wild type (CX 161–1B *ade1 A*)	8	3	103
xpr1–5	4.5	3	38
xpr1–14	3.5	3	42
xpr2–7	8	3	101
xpr2–9	8	3.5	79
xpr3–16	5.5	3.5	35
xpr3–23	4	3	37
xpr4–18	4	3	21
xpr4–21	7	2.5	96
xpr5–2	8	3	88
xpr5–11	8	3	84
xpr5–22	8.5	3	88
xpr6–13	6.5	3.5	83
xpr6–25	7.5	3.5	
xpr7–3	8	3	74
xpr8–10	4	3	40
xpr9–8	7.5	3	80
xpr10–4	7.5	3	81
xpr–15	7	3	73

unpublished results) seem to produce only one extracellular protease, based on isolation and purification studies. And the existence of several classes of *xpr* mutants derived from *S. lipolytica* CX 161–1B that produce essentially no extracellular protease while producing wild type levels of extracellular RNAse indicates that *S. lipolytica* CX 161–1B probably also produces only one extracellular protease.

The agreement between extracellular protease production in shake flasks and the size of the zones of clearing on skim-milk plates indicates that in most cases the size of the zone of clearing is a good indication of a strain's potential for extracellular protease production. The reason for the lack of agreement for mutants *xpr 3–16,23* is unclear.

The essentially wild-type growth rate of the *xpr* mutants in liquid medium at lower cell densities is probably supported by the low molecular weight nitrogen sources present in Proteose-peptone. The slowing of growth at higher cell densities exhibited by several of the *xpr* mutants most likely reflects a nitrogen limitation due to the reduced degradation of high molecular weight nitrogen sources present in Proteose-peptone.

The finding of so many complementation groups (10 or 11) for 18 *xpr* mutants was at first somewhat surprising. The complementation groups seem to spread over the genome. With the possible exception of *xpr10–4* with *xpr-15*, the groups were not tightly linked, and therefore the complementation could not be intragenic. An obvious question is "What kinds of mutations other than in the structural gene affect extracellular protease production?" Some possibilities are mutations in (1) regulatory proteins, (2) enzymes involved in glycosylation of the protease (the protease of *C. lipolytica* NRRL Y-1094 is a glycoprotein, OGRYDZIAK and TANNENBAUM, unpublished results), (3) enzymes involved in activation of an apo-protease or in protease release. The mutations might be in cell membrane or cell wall structural components or the mutation may result in the release of a protease inhibitor, as may be the case for *xpr6–13*.

Extracellular protease regulation in *C. lipolytica* NRRL Y-1094 was complex (OGRYDZIAK, DEMAIN and TANNENBAUM 1977). The function of the protease seemed to be the degrading of proteins in the environment to supply nutrients for the cell. Protein in the medium induced the protease, and there seemed to be at least three different signals that reflected the carbon, nitrogen and sulfur nutritional status of the cells that were involved in protease regulation. This regulatory complexity suggests that there would be numerous regulatory elements that when mutated could result in the *xpr* phenotype. Several of the *xpr* mutants could be in the more general carbon, nitrogen or sulfur regulatory circuits as was found in *Neurospora crassa* (HANSON and MARZLUF 1975).

Several of the *xpr* mutations could be involved in steps specific for the eventual appearance of active protease external to the cell. However, some could also affect the production of other extracellular enzymes. To determine if some of the *xpr* mutants were not specific for extracellular protease, extracellular RNAse production was investigated. Mutants in four of the complementation groups showed significantly reduced levels of extracellular RNAse production. The decreases in

RNAse production were not as dramtic as for protease production. *C. lipolytica* NRRL Y-1094 produces at least two extracellular RNAses (J. L. FRIBOURG, M.I.T. Master's thesis, 1972). The number produced by *S. lipolytica* CX 161–1B has not been investigated. It would be interesting to determine in these mutants if the level of more than one RNAse was decreased or if only one RNAse was decreased and the remaining activity was due to the presence of other RNAses.

The cosegregation of the *xpr*⁻ and RNAse⁻ phenotypes for four of the *xpr* mutants indicates that in each case both defects result from mutation in a single gene.

It is possible that the decrease in extracellular RNAse production by some *xpr* mutants could reflect regulation of RNAse production. Most *xpr* mutants grow more slowly than wild type with protein as a sole nitrogen source. Perhaps when nitrogen is limiting and phosphorus is not and there is no RNA in the medium, then *S. lipolytica* shuts off wasteful extracellular RNAse synthesis. However, the high levels of extracellular RNAse production by some of the protease non-producers argue against this interpretation. More likely, the decrease in RNAse production by some *xpr* mutants is a pleiotropic effect due to a defect in a step common to the production of extracellular enzymes. These mutations are probably not in the structural gene for the extracellular protease.

The normal mating frequency of several *xpr* mutants that produce little or no extracellular protease activity indicates that extracellular protease is not required for mating. The decrease in mating frequency exhibited by some mutants is probably due to some cell wall alteration. Other evidence linking cell wall alterations with the *xpr* phenotypes comes from results with *alk* mutants (unable to grow on decane but able to grow on decanol) of *S. lipolytica*. Several *alk* mutants score as *xpr* (J. BASSEL, unpublished results). All of the 18 *xpr* mutants used in this study grew on decane.

S. lipolytica presents a potentially valuable model system for the study of extracellular production in lower eukaryotes. *S. lipolytica* produces at least 3 types of extracellular enzymes—protease, RNAse and lipase—and it is amenable to genetic and biochemical studies.

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