The secreted aspartate proteinase of *Candida albicans*: physiology of secretion and virulence of a proteinase-deficient mutant

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It was established that *Candida albicans* grew rapidly in a simple medium containing yeast extract (0.2%, w/v) plus glucose (2%, w/v). These cultures were in or near to a state of nitrogen limitation and the concentration of secreted aspartate proteinase increased rapidly (within 3–4 h) on addition of BSA. Synthesis and secretion were apparently controlled both positively (induction by albumin or, more probably, the peptides produced from it) and negatively (repression by NH_4Cl). A small intracellular pool of the enzyme was detected during production of the enzyme and this pool decreased with the cessation of synthesis and secretion. A stable mutant, IR24, was isolated which secreted less than 0.3% of the amount of the proteinase exported by the parent strain ATCC 10261. The LD_{50} values for mutant IR24 and the parent strain administered intravenously to mice were $> 1.0 \times 10^9$ and 1.6×10^6 c.f.u. kg^{-1} respectively.

Introduction

Proposed virulence determinants of the opportunistic pathogen *Candida albicans* include adhesins, rapid phenotypic variation and antigenic modulations, dimorphism, toxins and secreted hydrolases (for a review see Odds, 1988). Of the secreted hydrolases, an aspartate proteinase is well characterized (Ruchel, 1981); the evidence for its involvement in pathogenesis is convincing but not definitive. Amongst the *Candida* spp. there is a correlation between secreted proteinase activity and relative virulence (MacDonald, 1984; Ruchel *et al.*, 1983). Schreiber *et al.* (1985) found no correlation between proteolysis and pathogenicity in a survey of *C. albicans* isolates from carriers and patients but Cassone *et al.* (1987) reported that the secreted proteinase activity of isolates from vaginitis patients was 1.7-fold higher than that of the isolates from carriers without symptoms. A correlation has also been reported between secreted proteinase activity and adherence (Ghannoum & Abu Elteen, 1986). Secreted proteinase has been detected in infected tissues by immunofluorescence (MacDonald & Odds, 1980; Ruchel, 1983) and antibodies to the enzyme have been detected in the serum of patients with disseminated candidosis (MacDonald & Odds, 1980).

The most convincing evidence for the role of the proteinase in virulence is that proteinase-deficient

mutants were found to be significantly less lethal to mice than the parental wild-type strains or a partial revertant of the mutant (MacDonald & Odds, 1983; Kwon-Chung *et al.*, 1985). In both studies the mutants were only partially deficient in proteinase activity (i.e. 10% and 19% of maximum activity of the parent strains) and in both studies proteinase revertants were isolated from mutant cultures. Recently Crandall & Edwards (1987) reported that the wild-type strain ATCC 28366 used by MacDonald & Odds (1983) is heterozygous for proteinase secretion and the proteinase-deficient mutant derived from this strain reverted at a high frequency.

It is well established (Staib, 1965; Rembold *et al.*, 1968; Ruchel *et al.*, 1982) that the proteinase is expressed and secreted when exogenous protein is the sole nitrogen source but it is not secreted when ammonium salts are the nitrogen source or during nitrogen starvation (Crandall & Edwards, 1987). In the present work we have established suitable conditions for the gratuitous expression of the proteinase (i.e. conditions for rapid growth in which proteinase production occurs but is not essential) and have used enzyme activity measurements and immunological assays to study the secretion of the enzyme. These growth conditions and assay systems were also used for the isolation of a stable proteinase mutant which was characterized for its biological and pathological properties.

Methods

Strains. *C. albicans* ATCC 10261, *C. albicans* CA-2, an agerminative isolate, *C. albicans* 3153A, the parent from which it was derived (Bistoni *et al.*, 1986) and *Saccharomyces cerevisiae* X2180-1A (Yeast Genetic Stock Center, Berkeley, Calif., USA) were used.

Media. YPD contained (l^{-1}) yeast extract (10 g), peptone (20 g) and glucose (20 g). YBD contained (l^{-1}) yeast extract (2 g), BSA (2 g) and glucose (20 g). YD contained (l^{-1}) yeast extract (2 g) and glucose (20 g). The BSA/glucose and haemoglobin/glucose media used for the isolation of proteinase mutants contained salts, vitamins and trace elements (Wickerham, 1946) except that the $(NH_4)_2SO_4$ was omitted, either BSA or haemoglobin ($2\text{ g } l^{-1}$) and glucose ($20\text{ g } l^{-1}$). Minimal medium (Shepherd & Sullivan, 1976) contained glucose, salts and biotin. Protein and vitamins were filter-sterilized as 20-fold and 200-fold concentrated stocks respectively. Media for plates contained agar ($30\text{ g } l^{-1}$).

Growth conditions. Cultures were stored on YPD slopes at 4°C and subcultured at 2 monthly intervals. Plates were incubated at 30°C . Liquid cultures were grown on a gyratory shaker (200 r.p.m.) at either 30 or 37°C as indicated. The initial volume was 20% of the nominal flask volume and the cultures were inoculated from overnight YPD or YD cultures. Growth was measured as OD_{600} with a Cecil CE 373 spectrophotometer ($1\text{ } OD_{600}\text{ unit} = 1 \times 10^8\text{ cells } ml^{-1}$).

Mutagenesis. Cells (5×10^8) from a YPD culture ($OD_{600} = 1.0$) were treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described by Poulter *et al.* (1981). After 60 min at room temperature the cells were washed three times with sterile water and stored at 4°C . The viable cell count on YPD plates was 2.5×10^7 cells (5% of the treated cells).

Morphological and biochemical characterization of the proteinase mutant. The mutant strain, IR24 (see Results), was subjected to the established morphological and biochemical tests for *C. albicans* identification: in particular, formation of germ-tubes and chlamydo-spores in serum and corn-meal agar respectively, and assimilation and fermentation tests as described by Meyer *et al.* (1984).

Purification of the proteinase. The proteinase was purified from YBD medium using a modification of previous methods (MacDonald & Odds, 1980; Ruchel, 1981). The purified enzyme gave one band, M_r 43000, on analysis by SDS-PAGE and had a specific activity of 10 to $14.5\text{ U (mg protein)}^{-1}$. Protein was determined by a modified Lowry procedure (Peterson, 1977) using BSA as a standard.

Antiserum. Purified proteinase [200 μg in 1 ml of saline (0.85% NaCl) and 1 ml of Freund's complete adjuvant] was administered subcutaneously to a New Zealand White rabbit. Further immunogen (100 μg in 1 ml saline and 1 ml incomplete adjuvant) was administered at 4 and 8 weeks. The serum was collected at 10 weeks and preabsorbed by treatment with a yeast suspension (Rothman *et al.*, 1986) to remove any nonspecific antibodies to carbohydrates. Antiserum to the vacuolar proteinase A of *S. cerevisiae* was kindly provided by Dr D. Wolf, Albert-Ludwigs Universität, Freiburg, FRG.

Preparation of samples for ELISA and Western blotting. Samples (1.0 ml) of the culture medium were treated with 0.1 ml 50% (w/v) TCA and the precipitates were redissolved in dissociation buffer (150 mM-Tris/HCl, pH 6.8, containing 3%, w/v, SDS, 6 mM-EDTA and 3%, v/v, β -mercaptoethanol) by heating at 100°C for 5 min. For ELISA, testing solutions were diluted 10-fold in 50 mM-sodium carbonate, pH 9.5. Cell extracts were prepared by incubating $1\text{ } OD_{600}$ unit of cells per 100 μl of 10 mM-potassium phosphate, pH 6.5, containing 1.2 M-sorbitol, 10 mM- NaN_3 , 2 mM- $MgCl_2$, 40 mM- β -mercaptoethanol, 4 μg Zymolyase 100 T (Seikagaku Kogyo) and 10 μg NovoZyme 234 (Novo Biolabs). After

30 min at 37°C the spheroplasts were harvested, suspended in 100 μl of dissociation buffer and prepared for ELISA.

Proteinase enzyme assays

(a) **Spectrophotometric.** Each assay contained 0.6 ml 1.0% (w/v) BSA in 50 mM-sodium citrate, pH 3.2, and 0.15 ml of enzyme solution. After 30 min at 37°C 0.4 ml 10% (w/v) trichloroacetic acid was added, the tubes were stored on ice for 30 min and then centrifuged (1600 g for 10 min). The absorbance of the supernatant was read at 280 nm and corrected for background using a zero-time control in which the trichloroacetic acid was added prior to the enzyme. One unit of enzyme catalysed a ΔA_{280} of 1.0 min^{-1} . With the pure proteinase, the assay was proportional to enzyme concentration over the range ΔA_{280} 0.1–0.4 with a reliability of $\pm 12\%$ and a limit of detection of 1 μg .

(b) **Radioactivity.** BSA (6 mg) was labelled by reductive methylation (Jenthoft & Dearborn, 1979) with 10 μmol [^{14}C]HCHO [$12.3\text{ mCi mmol}^{-1}$ (455 MBq mmol^{-1}); Amersham]. The labelled protein was recovered by chromatography on a $10 \times 1.6\text{ cm}$ column of Sephadex G10, adjusted to pH 1.0 with HCl, dialysed for 12 h against 50 mM-sodium citrate, pH 3.2, and mixed with unlabelled BSA in the same buffer.

Each assay contained 300 μl 1.2% (w/v) [^{14}C]BSA (100000 c.p.m.) in 50 mM-sodium citrate, pH 3.2, and 75 μl of enzyme sample. After 20 min at 37°C , 0.2 ml 10% (w/v) trichloroacetic acid was added, the tubes were treated as described for the spectrophotometric assay and 100 μl of the supernatant was counted for 2 min in 10 ml of scintillation fluid (33%, v/v, Triton X-100, 67%, v/v, toluene and 0.6% butyl-PBD). One unit of enzyme activity, $5 \times 10^4\text{ c.p.m. min}^{-1}$ per total assay, was equivalent to the spectrophotometric assay unit. Measurement of activity with ^{14}C -methylated BSA increased the sensitivity to 0.2 μg of enzyme and the assays of crude enzyme samples were not complicated by a high background encountered with the A_{280} system. Both assays, however, were inhibited by peptides and constituents of the growth medium: 14% by 0.5% yeast extract, 10% by 0.2% peptone and 35% by 5 μl of the diffusate collected by ultrafiltration (cut-off filter M_r 10000) of YBD or YPD.

(c) **ELISA.** Samples (up to 30 μl) were placed in microtitre-plate wells, diluted to 90 μl with 50 mM-sodium carbonate, pH 9.5, and incubated overnight at 4°C . The supernatants were removed, the wells were washed five times with 100 μl of 0.5% casein in PBS (50 mM-potassium phosphate, pH 7.2, containing 150 mM-NaCl, 0.02% thiomersal and 0.01% Tween 20) and blocked by an incubation with 100 μl 1% (w/v) casein/PBS for 1 h at 37°C . Rabbit antiserum (100 μl of a 1 in 500 dilution in casein/PBS) was added and after 2 h at 37°C the wells were washed five times with casein/PBS. Goat anti-rabbit IgG peroxidase conjugate (Caltag Laboratories; 100 μl of a 1 in 8000 dilution in casein/PBS) was added followed by incubation for 1.5 h at 37°C and washing as above. The substrate mixture for the colour reaction (100 μl) consisted of 50 mM-citric acid, 100 mM- Na_2HPO_4 , 2 mM- H_2O_2 and 0.4 mg *o*-phenylenediamine ml^{-1} . After 15 min incubation in the dark 50 μl 2 M- H_2SO_4 was added and the plates were read at 492 nm. Each sample was measured in duplicate and corrected for background ($A_{492} = 0.03\text{--}0.08$) with no antigen. A standard curve (0–300 ng), determined with the purified proteinase for each plate, gave ΔA_{492} readings of 0–1.2.

SDS-PAGE, electrotransfer and immunoblotting. SDS-PAGE (Laemmli & Favre, 1973) was done using $10 \times 8 \times 0.2\text{ cm}$ gels (Matsudaira & Burgess, 1978) with a 12.5% (w/v) separating gel and a 5% (w/v) stacking gel. Electrophoresis was for 1–2 h at 120 V and 5 mA. The M_r markers used were β -galactosidase (116000), phosphorylase *b* (97000), BSA (66000), ovalbumin (45000), carbonic anhydrase (29000) and cytochrome *c* (12000). For protein staining, the gels were soaked in a solution of 7.5% (v/v) acetic acid plus 20% (v/v) methanol

for 20 min, stained for 1 h in 0.025% Coomassie blue in 7.5% (v/v) acetic acid and 50% (v/v) methanol and destained in 10% (v/v) acetic acid.

Electrotransfer to nitrocellulose (Towbin *et al.*, 1979) was done at 15 °C in 5 litres of precooled buffer consisting of 3 mM- Na_2CO_3 , 10 mM- NaHCO_3 and 20% (v/v) methanol. After 4 h at 40 V and 0.7 A, the transfer of the *M_r* markers was visualized with 0.01% Ponceau S in 0.05% acetic acid. The nitrocellulose was washed with distilled water and soaked overnight in 40 mM-Tris/HCl, pH 7.6, containing 150 mM-NaCl, 0.01% thiomersal, 0.05% Tween 20 and 1% (w/v) casein. The blocked nitrocellulose was probed with a 1 in 3000 dilution of antiserum in casein/PBS for 1.5 h at 37 °C, washed five times with casein/PBS and then similarly incubated in a 1 in 8000 dilution of goat anti-rabbit IgG peroxidase conjugate. After washing, the bound peroxidase was visualized by incubation in a solution freshly prepared by mixing 6 ml methanol containing 18 mg 4-chloronaphthol and 30 ml 50 mM-sodium citrate/100 mM-sodium phosphate, pH 5.4, containing 3 mM- H_2O_2 . The colour development was for 15 min to 2 h and the reaction was stopped by washing with water.

Assessment of the antiserum used. The specificity and sensitivity of the antiserum was assessed by Western blot analyses. With fresh preparations of pure proteinase, 1 ng was detected as one band with a 1 in 3000 dilution of antiserum. Older preparations of the enzyme exhibited multiple bands of proteolytic degradation products (Fig. 1*b*). No bands were detected with YBD medium, with a cell-free extract from cells grown in YPD or with 0.5 µg of the vacuolar aspartate proteinase from *S. cerevisiae* (Sigma). No bands were detected with preimmune serum. An antiserum to the *S. cerevisiae* aspartate proteinase at a 1 in 3000 dilution reacted specifically with the enzyme from this organism and there were no cross-reactions with the samples listed above.

Mouse infections. Mice were infected as described previously (Bistoni *et al.*, 1986). Briefly, groups of eight inbred male CD2F1 mice (18–21 g; Charles River Breeding Laboratories) were injected intravenously with different doses of *C. albicans* strains and the mortality was assessed over 30 d. In other experiments, mice were killed by cervical dislocation at intervals during the experimental infection, the organs removed aseptically and immediately fixed in 10% (v/v) formalin. Sections of paraffin-embedded organs were examined after treatment with periodic acid/Schiff (PAS) and Van Gieson stains.

Results

Conditions for proteinase synthesis and secretion

The *C. albicans* aspartate proteinase has commonly been produced by growth in minimal medium with BSA as the sole nitrogen source (MacDonald & Odds, 1980; Ruchel, 1981). These cultures have an extended lag phase (10–15 h) and proteinase secretion only reaches a maximum after 4–7 d. We therefore sought conditions for rapid growth and secretion. Fig. 1(*a*) shows that *C. albicans* ATCC 10261 grew rapidly in YBD medium ($2.5 \text{ OD}_{600} \text{ units h}^{-1}$) but this was interrupted by a decreased growth rate around OD_{600} 12–13 followed by a second phase of growth. Proteinase was secreted from 4 h and the concentration of the enzyme in the culture supernatant changed rapidly resulting in peaks (enzyme activity and ELISA) at 12 h and a peak (enzyme activity) at 20 h

(Fig. 1*a*). The amount of proteinase in the culture supernatant relative to the cell density was constant from 4 to 10 h [$5 \mu\text{g (OD unit)}^{-1}$]; it then rose to $14 \mu\text{g (OD unit)}^{-1}$ at 14 h, decreased to $2.3 \mu\text{g (OD unit)}^{-1}$ at 18 h and returned to $5 \mu\text{g (OD unit)}^{-1}$ at 20 h. These data were confirmed by a Western blot analysis of the culture medium (Fig. 1*b*) which showed changes in the concentration of the intact proteinase with growth and proteolytic degradation of the enzyme (8–14 h). Pure proteinase, freshly prepared, showed one band in Western blots but the pattern of multiple bands exhibited by stored preparations (Fig. 1*b, c*) was essentially the same as the pattern for the culture supernatant samples. The spheroplast extracts from cells grown in YBD medium contained a small pool of the proteinase (Fig. 1*c*) but there was no intracellular accumulation of the enzyme during growth and the pool decreased at 16 h prior to the second phase of growth and proteinase production.

When BSA was omitted from the medium (YD) *C. albicans* grew at $1.5 \text{ OD}_{600} \text{ units h}^{-1}$ with a low level of proteinase secretion (Fig. 2*a*); the growth rate was increased only slightly when 0.2% BSA was added at 4.5 h ($1.8 \text{ OD}_{600} \text{ units h}^{-1}$) but secretion of the proteinase increased rapidly between 7 and 8.5 h resulting in peaks of enzyme concentration at 8.5 and 10 h (Fig. 2*b*). Under these conditions secretion of the proteinase was repressed by NH_4Cl (Fig. 2*c*). L-Leucine (80 mM) also decreased secretion of the proteinase and the maximum concentration at 10 h was $22 \mu\text{g ml}^{-1}$ compared with $45 \mu\text{g ml}^{-1}$ for BSA added alone. When BSA was added at 4 h and NH_4Cl at 4.5 h there was only a small transient increase in the concentration of the secreted proteinase (Fig. 2*d*) compared with the culture supplemented with BSA alone (Fig. 2*b*).

Isolation of proteinase-deficient mutants

The proteinase indicator plates were initially tested with positive and negative controls for proteinase secretion. *C. albicans* ATCC 10261 produced 2–3 mm cleared zones around each colony after 3, 5 and 7 d growth on plates containing YBD, haemoglobin/glucose and BSA/glucose respectively. After 7 d growth *S. cerevisiae* X2180-1A produced minute colonies on BSA/glucose and good growth on the other two media; there were no cleared zones on any of the plates.

Mutagenized cells were grown on YPD plates and 3000 colonies were selected for screening on proteinase indicator plates. Of the isolates, 93 (3.1%) showed no zone of clearing in an initial screening on haemoglobin/glucose. After more than ten replatings on the various indicator plates with passages onto YPD plates the collection was reduced to 23 with the elimination of

Fig. 1. Growth of *C. albicans* and secretion of the proteinase. YBD medium was inoculated to OD₆₀₀ = 0.2 with a 12 h YPD culture (OD₆₀₀ = 10.0) of *C. albicans* ATCC 10261. At the times indicated (a) 1.0 ml samples of the culture were measured for growth (●) and culture supernatant proteinase by enzyme activity (○) and ELISA (■). SDS extracts of the culture supernatant (b) and cell lysates (c), equivalent to 37.5 µl of the culture at the times indicated, were analysed in Western blots for the proteinase. Each blot shows a lane containing 1 µg of the purified proteinase and the characteristic pattern of autodegradation. Std, stored preparation.

Fig. 2. Regulation of proteinase secretion. Four flasks (a-d) of YD medium were inoculated to OD₆₀₀ = 1.0 with a 12 h YD culture of *C. albicans* ATCC 10261 and measured for growth (●) and culture supernatant proteinase by ELISA (○). The four cultures were treated as follows: (a) no additions; (b) BSA added to 0.2% at 4.5 h; (c) BSA and NH₄Cl added to 0.2% and 40 mM respectively at 4.5 h; (d) BSA added to 0.2% at 4.5 h and NH₄Cl added to 40 mM at 5 h.

isolates that showed proteinase secretion or altered growth characteristics (colony size and morphology).

These 23 isolates were then screened on minimal medium plates and various liquid media. Six isolates finally selected from the screening included three proteinase-deficient mutants, an unusual mutant that secreted the proteinase at 37 °C but not at growth temperatures below 30 °C and two revertants that arose late in the screening process. One isolate, IR24, exhibited the most suitable phenotype: no detected reversion, and a growth rate in minimal medium comparable with that of the parent strain; growth in YBD and YD was slightly impaired. Isolate IR24 developed germ-tubes in serum, chlamydospores in cornmeal agar medium and it also formed mycelia *in vivo* to an extent comparable to that of the parental strain ATCC 10261 (see also Fig. 4). In fermentation and

assimilation tests the parent and mutant strains were identical.

With isolate IR24 no proteinase was detected in YBD medium by either the enzyme assay or ELISA but a faint band was detected by immunoblotting a sample of the culture medium (Fig. 3). The limit of detection of pure proteinase by immunoblotting was 1 ng (data not shown) and on this basis the immunoreactive proteinase secreted by IR24 was less than 0.3% that of *C. albicans* ATCC 10261 (Fig. 3, lanes 2 and 4).

Virulence for mice of IR24 and wild-type strains of C. albicans

The experimental pathogenicity of *C. albicans* ATCC 10261 and its proteinase-deficient derived mutant IR24 were assessed in a systemic infection of

Fig. 3. Proteinase secretion by *C. albicans* ATCC 10261 and IR24. Flasks of YBD medium were inoculated to $OD_{600} = 0.2$ with 12 h YD cultures of *C. albicans* ATCC 10261 and the proteinase mutant IR24. The culture supernatants were analysed for secreted proteinase after 2 h and 7 h growth by ELISA and in a Western blot (2 h, lanes 1 and 3; 7 h, lanes 2 and 4). Each lane contained 15 μ l of medium and the amounts of proteinase per lane as estimated by ELISA are given in parentheses. Lanes 1 and 2, ATCC 10261 (1.3 μ g and 1.9 μ g); lanes 3 and 4, proteinase mutant IR24 (no proteinase detected).

Table 1. Mortality of mice challenged intravenously with different strains of *C. albicans*

The results are shown as the LD_{50} , the median survival time (MST) in days and as dead mice at 30 d over total number of animals tested (D/T). Groups of eight mice were used for each dose of cells administered. The experiments for the calculation of the mortality after a challenge with 10^6 cells were done separately from the experiment to determine LD_{50} value.

Strain	$10^{-6} \times LD_{50}$ (c.f.u. kg^{-1})*	Mortality after challenge with 10^6 cells	
		MST (d)	D/T
3153A	0.60 ± 0.12	3	5/5
CA-2	158 ± 2.7	> 30	0/5
ATCC 10261	1.6 ± 0.1	5	5/5
IR24	> 1000	> 30	0/5

* $\pm$ Standard error.

CD2F1 mice given graded numbers of *Candida* cells by the intravenous route. For comparison, two additional strains of *C. albicans* were included in the experiment: strain 3153A and its derivative CA-2, previously characterized as an agerminative proteinase producer with low virulence (Bistoni *et al.*, 1986). The data in Table 1 show that strains 10261 and 3153A of *C. albicans* were highly pathogenic for mice with LD_{50} values of

Fig. 4. Stained sections of heart from mice infected with 10^6 cells of *C. albicans* ATCC 10261 (a) and isolate IR24 (b). The organs were taken from mice killed on day 2 after the challenge. The arrows point to clusters of yeast cells with hyphal elements. Bars, 30 μ m.

about 10^6 cells kg^{-1} and a median survival time of less than 1 week after an intravenous injection of 1×10^6 cells. In contrast, mutant IR24 was much less pathogenic, being unable to kill any animal after a challenge with 10^6 cells (the LD_{50} was about three orders of magnitude higher than for the wild-type strains). Table 1 also shows that the proteinase mutant was less pathogenic than the agerminative CA-2 strain. Strain CA-2, however, produces secreted proteinase at approximately 50% of the amount secreted by strain 3153A. Fig. 4 shows heart sections of animals infected with the parent strain ATCC 10261 and the mutant IR24. Extensive hyphal development was noted with both strains. *C. albicans* isolates were also recovered from kidneys and

hearts of the infected mice. The isolates from mice infected with mutant IR24 showed no proteinase activity on YBD plates but the isolates from mice infected with ATCC 10261 were all proteinase-positive.

Discussion

Previous studies suggested that the production of the *C. albicans* secreted proteinase is induced by protein (MacDonald & Odds, 1981; Ruchel *et al.*, 1982; Crandall & Edwards, 1987), stimulated by 0.01% yeast extract (Germaine *et al.*, 1978) or the iron content therein (Crandall & Edwards, 1987), stimulated by glucose (Samaranayake *et al.*, 1984) and repressed by $(\text{NH}_4)_2\text{SO}_4$ (Crandall & Edwards, 1987). The enzyme was not secreted during nitrogen starvation (Crandall & Edwards, 1987) and the evidence for induction and repression has not been clearly documented.

In the present work we found that YD medium (yeast extract plus glucose) contained sufficient nutrients for rapid growth of *C. albicans* but the cultures were apparently in or near to nitrogen limitation. In YD cultures the concentration of the secreted proteinase was low (2–5% of the maximum) but this amount of the enzyme could contribute to the provision of nitrogen for growth. The growth rate did not change markedly on the addition of other nitrogen sources but the concentration of the proteinase increased 20 to 50-fold within 4 h after addition of BSA. This response was strongly repressed by NH_4Cl and to a lesser extent by leucine. The repression by leucine may have been mediated via other metabolites such as NH_4^+ and further experiments are needed to establish whether other amino acids and low-molecular-mass nitrogen metabolites also repress production of the proteinase.

Proteinases of eukaryotic micro-organisms (North, 1982) and in particular *S. cerevisiae* (Achstetter & Wolf, 1985) have been extensively reviewed. The production of extracellular proteinases is often controlled by catabolite repression only, e.g. *Aspergillus nidulans* (Cohen, 1981) and *Schizophyllum commune* (Sessoms & Lilly, 1986). In these organisms proteinase secretion increases under conditions of nitrogen limitation and in the absence of exogenous protein. The production of exocellular proteinases in *Neurospora crassa* (Cohen & Drucker, 1977) and *Mucor miehei* (Lasure, 1980) is also repressed by NH_4^+ and amino acids but nitrogen limitation does not result in proteinase production unless protein is present in the growth medium. The production of the *C. albicans* proteinase is apparently also controlled by repression and induction. It is noteworthy that in *N. crassa* exocellular proteinase activity is required before induction can occur (Drucker, 1973) and a similar requirement

in *C. albicans* would account for the extended lag phase encountered when the organism is transferred to media with BSA as the sole nitrogen source.

Measurement of the concentration of the secreted proteinase was difficult because of the relative insensitivity of the enzyme activity assays, the inhibition of the enzyme by constituents of the growth medium and the inherent inability of the ELISA to distinguish between intact and degraded forms of the enzyme. Enzyme activity was not directly proportional to enzyme concentration as measured by ELISA but three methods of analysis (activity, ELISA and Western blotting) showed that the concentration of the secreted proteinase oscillated during growth in YBD (Fig. 1). Several factors could have contributed to this result. The changes in proteinase concentration coincided with the decreased growth rate in YBD around OD_{600} 12–14, the cell density at which YD cultures entered stationary phase (Fig. 2a). This suggests that the YBD cultures undergo a nutritional adaptation at this time. The synthesis of the proteinase decreased during this phase as judged by the decreased intracellular pool. Western blot analysis of the culture supernatant revealed a pattern of proteinase degradation similar to that observed with the purified enzyme. These data suggest that autodegradation contributed to the decreased concentration and enzyme activity in the culture supernatant. It is not known whether any of the proteolytic breakdown products have enzyme activity.

YBD was a suitable medium for screening proteinase-deficient mutants because the organism was not dependent on the secreted enzyme for growth but the proteinase synthesis was not repressed. Only a trace of immunoreactive proteinase was secreted by the mutant IR24 and this was well below the levels of detection of the ELISA and enzyme activity assays. The nature of the mutation in IR24 has not been determined. It is possible that the mutagenesis procedure used could have produced other undetected mutations but IR24 was not auxotrophic for any nutrient and the slight decrease in the growth rate in YD and YBD could be due to the proteinase deficiency itself. The mutant isolated by MacDonald & Odds (1983) showed an initial lag phase but then grew rapidly in a medium with BSA as the sole nitrogen source. Initial growth of the mutant isolated by Kwon-Chung *et al.* (1985) was rapid in a synthetic medium with haemoglobin as sole nitrogen source (possibly due to low-molecular-mass nitrogen compounds in the medium); this was followed by a lag phase (from 15–20 h) and then by growth to the same cell density as the parent strain.

Importantly, IR24 and the parent strain grew at comparable rates at 37 °C; both formed germ tubes *in vitro* and *in vivo* and the mutation affecting proteinase secretion was stable.

The pathogenicity experiments showed that strain IR24 was 1000-fold less virulent than the wild-type proteinase-secreting strains and about 10-fold less virulent than a low virulence, proteinase-positive, agerminative strain. MacDonald and Odds (1983) reported a 10-fold difference in the LD₅₀ values of a proteinase mutant and its parent strain. The proteinase mutant IR24 can be regarded as a non-pathogenic strain of *C. albicans*. In contrast, the proteinase mutants isolated by MacDonald & Odds (1983) and Kwon-Chung *et al.* (1985) retained appreciable pathogenicity for mice, interpreted as due to a partial *in vivo* reversion to proteinase production. The lack of pathogenicity of mutant IR24 can be accounted for by the stability of the proteinase mutation *in vivo*. It may, however, differ from strain ATCC 10261 in virulence-related properties other than proteinase secretion. Our data indicate more strongly than the previous studies (MacDonald & Odds, 1983; Kwon-Chung *et al.*, 1985) that the secreted aspartate proteinase plays a role in the pathogenicity of *C. albicans* but unequivocal proof will require the construction of a proteinase-negative mutant by a specific gene disruption.

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