Segregation of Proteinase-negative Mutants from Heterozygous *Candida albicans*

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(Received 17 February 1987; revised 22 April 1987)

The extracellular acidic proteinase (EC 3.4.23.6) produced by *Candida albicans* has been reported to be a virulence factor. In studying the role of this proteinase in human disease, we determined the optimum conditions for stimulating proteinase production in order to isolate proteinase-negative (Prt⁻) mutants. We found that in liquid medium containing bovine serum albumin (BSA) as the sole nitrogen source, at pH 4 and 27 °C, the sensitivity of proteinase detection was considerably greater than when assayed on BSA agar at 37 °C. This observation is due, in part, to temperature sensitivity of proteinase induction. Nitrogen starvation did not induce proteinase. Proteinase production on agar was increased by adding 0.01% yeast extract (YE) to BSA medium. Using BSA + YE agar to isolate mutants, it was discovered that *C. albicans* ATCC 28366 was heterozygous for a Prt⁻ mutation. Spontaneous Prt⁻ mutants occurred at a frequency of 2×10^{-3} . Ultraviolet light increased the mitotic segregation of Prt⁻ cells to a frequency of 1×10^{-2} . The Prt⁻ phenotype showed a large inoculum effect, Prt⁻ segregants reverted with a high frequency, and the revertants were unstable.

INTRODUCTION

An acidic proteinase, described in the yeast *Saccharomyces cerevisiae* by Dernby (1917), was classified as 'proteinase A' by Lenny (1956). It is located inside the *Saccharomyces* vacuole, an organelle functionally equivalent to the mammalian lysosome (Mechler *et al.*, 1982). Proteinase A was first described in *Candida albicans* by Staib (1964); however, in *Candida*, proteinase A is extracellular. *Candida* proteinase A is a carboxyl proteinase (EC 3.4.23.6) because its activity is inhibited by pepstatin-A (Ruchel *et al.*, 1982). The enzyme has optimum activity at pH 3.8–4.0 (Germaine & Tellefson, 1981).

Because the acidic proteinase from *C. albicans* is secreted, it may function as an 'aggressor' by digesting host proteins in acidic micro-environments. Evidence indicating that the extracellular acidic proteinase in *C. albicans* may be a virulence factor was reviewed by Odds (1985).

Staib (1969) reported that proteinase-positive (Prt⁺) strains of *C. albicans* were virulent in experimental murine infections whereas proteinase-negative (Prt⁻) isolates were avirulent. Macdonald & Odds (1983) found that a proteinase-deficient mutant, less virulent than its Prt⁺ parent in mice infections, was phagocytosed and killed by human and murine polymorphonuclear leukocytes to a greater extent than its Prt⁺ parent when both strains were grown in proteinase-inducing medium. The finding that mutants of *C. albicans* lacking extracellular proteinase were less virulent was corroborated by Kwon-Chung *et al.* (1985).

That proteinase secretion occurs *in vivo* was indicated by the observation of a halo around yeasts in infected animal tissue stained with fluorescent antibody to purified proteinase (Macdonald & Odds, 1980a). The presence of *Candida* secretory proteinase in a case of human acrodermatitis was also detected by immunofluorescence (Ruchel, 1983). Furthermore, precipitating antibodies reactive with purified proteinase were present in the serum of 75% of patients with disseminated candidiasis, but not in healthy individuals (Macdonald & Odds,

1980*b*). Use of purified proteinase as antigen in the serodiagnosis of invasive candidiasis by Macdonald & Odds (1980*b*) gave more specific results than those obtained by Staib *et al.* (1977) who used crude culture filtrate antigens and obtained only 39% positive tests in cases where deep *Candida* infection was suspected.

Of the seven major pathogenic *Candida* species, only the three species most virulent in animals (*C. albicans*, *C. tropicalis* and *C. parapsilosis*) secrete an acidic proteinase (Macdonald, 1984). Ruchel *et al.* (1983) also found the above three species to be the major proteinase producers. Malyszko *et al.* (1980) reported that 16 out of 38 patients with clinical signs of genito-urinary candidiasis carried yeasts which showed extracellular proteolytic activity on protein agar, and most of these isolates were *C. albicans*.

The standard published method for assaying secreted acidic proteinase activity was to grow the yeast at 37 °C on acidified minimal agar containing BSA as the sole nitrogen source, and then to measure the zone of clearing around colonies. Using a more sensitive liquid assay, Schreiber *et al.* (1985) found that 73/75 isolates of *C. albicans* were proteolytic. Ruchel *et al.* (1982) found that all of 108 *C. albicans* isolates tested produced detectable amounts of proteinase. More recently, Crandall and co-workers (unpublished) found that all of 46 *C. albicans* isolates secreted acidic proteinase. Because *C. albicans* acidic proteinase can degrade IgA₁, IgA₂ and sIgA (Ruchel *et al.*, 1982), it seems likely that this enzyme may be important to the survival of *C. albicans* as a commensal on mucosal surfaces as well as in the establishment of opportunistic infections.

The purpose of this study was to isolate Prt⁻ mutants in *C. albicans* to be used later in studies of pathogenicity. It was first necessary to determine the optimum conditions for inducing proteinase synthesis in Prt⁺ parental strains, and then to identify growth conditions for the isolation and assay of mutant derivatives lacking the extracellular, acidic proteinase.

METHODS

Strains. *C. albicans* ATCC 28366 (Macdonald & Odds, 1983) was purified by single colony isolation; subclone 28366a was used as the standard strain for most experiments.

Media. Stock cultures were maintained on agar slants of the following constitution: 0.7% (w/v) yeast extract (YE) (Difco) + 0.5% KH₂PO₄ + 2.0% (w/v) glucose + 2.0% (w/v) agar (YKDA). Slants inoculated with *Candida* strains were incubated overnight at 37 °C; other yeasts were incubated at 30 °C. Stock slants were stored at 5 °C. Minimal (MIN) agar contained 0.67% Yeast Nitrogen Base (YNB) without amino acids (aa) (Difco) + 2.0% (w/v) glucose + 2.0% (w/v) agar. Ammonium sulphate (AS) broth contained 0.2% (NH₄)₂SO₄ + 0.17% YNB (without aa or AS) + 1.0% (w/v) glucose, pH 4.7 (unadjusted). Bovine serum albumin (BSA) broth contained 0.2% BSA (Fraction V; Sigma) + 0.17% YNB (without aa or AS) + 1.0% (w/v) glucose (adjusted to pH 4.4 with 0.1 M-HCl). Concentrated stock solutions of each component for preparing BSA broth were filter sterilized and then diluted with sterile deionized water. Agar used to isolate Prt⁻ mutants was labelled BSA + YE; it contained 0.1% BSA + 1.17% Yeast Carbon Base (YCB) (Difco) + 0.01% YE + 2.0% (w/v) agar. BSA + YE agar was prepared as follows: a 4% BSA stock solution (filter sterilized) was diluted into YCB + YE agar which had been autoclaved and cooled to 55 °C; the medium was adjusted to pH 4.4 with 2 M-HCl. For viable counts, the following medium was used: 1.0% (w/v) YE + 2.0% (w/v) Bacto-peptone (Difco) + 2.0% (w/v) glucose + 2.0% (w/v) agar (YPDA).

Drop test for proteinase. Proteinase was assayed on agar by preculturing *Candida* cells in broth medium, then spotting 5 µl on BSA + YE agar. After 7 d at 37 °C, the zone of clearing around the drop inoculum was measured (in mm) from the edge of growth outward.

Induction of proteinase in broth cultures. To induce the proteinase, cells were precultured in AS broth until they reached stationary phase (2×10^8 cells ml⁻¹), then washed, and diluted to 2×10^4 cells ml⁻¹ BSA broth in Erlenmeyer flasks filled to 1/5th capacity. Cultures were aerated at 200 r.p.m. in a rotary incubator shaker (New Brunswick) for 2 d at 37 °C. Growth was monitored by measuring the optical density at 550 nm of an appropriate dilution. The pH decreased from 4.4 to 3.8 during growth in BSA broth.

Proteinase assay in liquid. After induction of the enzyme in BSA broth, cells were harvested and the culture filtrate (CF) was assayed for proteolytic activity using the procedure of Macdonald & Odds (1980*a*): the CF (0.5 ml) was added to 2.0 ml 1% (w/v) BSA dissolved in 0.1 M-sodium citrate buffer, pH 3.8, and the mixture was incubated at 37 °C for 30 min. The reaction was stopped by addition of 5.0 ml ice-cold 5% (w/v) trichloroacetic acid (TCA). Controls were prepared as follows: 0.5 ml of the CF was incubated for 30 min at 37 °C, then 5.0 ml 5% (w/v) TCA and 2.0 ml substrate solution were added. The BSA precipitate was removed by filtration through 0.45

μm Acrodiscs (Gelman Sciences). The amount of proteolysis was determined by measuring the absorbance at 280 nm. Controls were subtracted from enzyme reactions to obtain the ΔA_{280} .

Isolation of *Pr^t-* mutants after UV irradiation. A lawn of *C. albicans* 28366 was grown on YPDA at 37 °C for 3 d; the cells were resuspended in sterile deionized water and the concentration was adjusted to 10^3 cells ml^{-1} . This cell suspension consisted of 89% single cells and the rest were budded; cell viability of the unirradiated control was 56%. Samples (10 ml) were added to glass Petri dishes and irradiated with the cover off for 0, 5, 10, 15 and 20 s. The survival percentage was determined by spreading 0.1 ml samples of the unirradiated and irradiated suspensions on BSA + YE agar plates and incubating at 37 °C for 7 d. Colonies were screened for the presence or absence of a zone of clearing which indicated proteinase production. The protocol and UV lamp used were the same as reported in Crandall (1983). The dose rate at the agar surface was $5 \text{ J m}^{-2} \text{ s}^{-1}$.

Detection of auxotrophs and resistant mutants. Irradiated cells (from above) were also plated on YPDA and then replica plated on MIN agar and MIN agar + $50 \mu\text{g}$ 5-fluorocytosine ml^{-1} .

RESULTS

Stimulation of proteinase production

When 0.01% YE was added to BSA agar, the zone of clearing was increased by about 70% and clearing was observed 1–2 d sooner. The iron content of YE is quite high ($150 \mu\text{g g}^{-1}$) (Grant & Pramer, 1962) so to determine if iron might be responsible for the stimulation of proteinase production, *C. albicans* 28366a was grown in BSA broth containing FeCl_3 at various concentrations. Cultures were aerated at 27 °C for 4 d, then assayed. Proteinase activity was increased by about 50% at $20 \mu\text{M-FeCl}_3$.

Glucose concentration for optimum proteinase induction

The effect of glucose concentration in BSA broth was studied to determine the conditions for obtaining maximum yield of enzyme activity. Proteinase activity was proportional to the optical density (OD_{550}) (Fig. 1) with the optimum glucose concentration being 1% or greater. If the proteinase activity (ΔA_{280}) was divided by the OD_{550} at each concentration of glucose studied, maximum specific activity was achieved at 0.2% glucose. However, since maximum levels of proteinase were obtained when the culture density was higher, a concentration of 1% (w/v) glucose was chosen for routine growth media.

Nitrogen starvation

Since proteinase is not produced when low- M_r nitrogen sources are available for growth, the possibility that nitrogen starvation might trigger proteinase synthesis was investigated. Cells of *C. albicans* 28366a were precultured in MIN broth at 27 °C for 3 d at 200 r.p.m., 5 ml were

Fig. 1. Glucose concentration for optimum proteinase production. $\circ$, Growth of *C. albicans* 28366a in 0.17% YNB (without aa or AS) + 0.1% BSA containing different concentrations of glucose at 27 °C for 4 d; $\bullet$, proteinase activity (ΔA_{280} per 0.5 ml for 30 min at 37 °C); ---, specific activity ($\Delta A_{280}/\text{OD}_{550}$).

Fig. 2. Isolation of Prt⁻ mutants after UV irradiation. ○, Survival percentages calculated from viable counts on BSA + YE agar; ●, frequency of Prt⁻ colonies per 10³ survivors.

harvested, washed twice in 0.17% YNB (without aa or AS), and resuspended in 25 ml 0.17% YNB (without aa or AS) containing 1.0% (w/v) glucose but no nitrogen source. After 4 d aeration at 27 °C, there was no change in the optical density of this nitrogen-starved culture (OD₅₅₀ 1.25), and no proteinase was present in the CF. Control cultures grown in this same basal medium but containing 0.2% BSA as the sole nitrogen source were maximally induced for proteinase after only 2 d. These results indicate that protein is both the substrate and the inducer of this enzyme.

Isolation of Prt⁻ mutants after UV irradiation

As mutants lacking an extracellular proteinase would be unlikely to grow on media containing protein as the sole nitrogen source, 0.01% YE was added to BSA agar to serve as a source of low-*M_r* nitrogen thereby allowing Prt⁻ mutants to form a small colony; 0.01% YE also served to stimulate proteinase in the Prt⁺ parent.

C. albicans 28366 was chosen as the Prt⁺ parent for these genetic studies because proteinase-deficient mutants had been isolated from this wild type strain by Macdonald & Odds (1983). *C. albicans* 28366 was irradiated with UV light to determine whether recessive Prt⁺ genes were present in the heterozygous condition. Several laboratories have reported that clinical isolates of *C. albicans* are diploid and naturally heterozygous for certain genetic markers (Crandall, 1983).

Spontaneous Prt⁻ mutants were found in the unirradiated stock culture at a frequency of 1.8×10^{-3} . Prt⁻ mutants were identified as small colonies lacking a zone of clearing when isolated on BSA + YE agar after 7 d at 37 °C. After 20 s irradiation (100 Jm⁻²) the survival was 63% and the frequency of Prt⁻ mutants was increased about 10-fold to 11.6 per 10³ survivors (Fig. 2). A total of 44 putative Prt⁻ colonies were streak purified on YPDA and assayed for proteinase activity on BSA + YE agar at 37 °C using the drop test. Only 12 out of the 44 Prt⁻ isolates were completely negative mutants; these were reported in Fig. 2 (2/1138 at zero time, 2/437 at 10 s, and 8/689 at 20 s). Of these 12 negative mutants, only four did not grow in BSA broth at 37 °C; these were labelled 0-1S, 10-10S, 20-21M and 20-42S. The other mutants either reverted or had partial proteinase activity.

In contrast to this high frequency of Prt⁻ mutants, no auxotrophic or 5-fluorocytosine-resistant mutants were isolated after UV irradiation of this strain out of a total of 1604 colonies screened. Hence, these recessive genetic markers are apparently not present in the heterozygous condition in strain 28366.

Temperature sensitivity of proteinase induction in BSA broth

The four Prt⁻ mutants described above were re-tested in BSA broth at 27 °C and 37 °C, to compare enzyme levels with their parent and other selected strains (Table 1). Temperature sensitivity of proteinase induction was seen for all strains; only about 10% of the activity was

Table 1. *Temperature dependence of induction of proteinase activity in C. albicans isolates during growth in BSA broth**

Isolate	Activity† at 27 °C	Percentage activity (37 °C/27 °C)
28366a	0.179	9
0-1S	0.074	11
10-10S	0.141	NG
20-21M	0.160	NG
20-42S	0.153	NG
372	0.131	10
PY17-1AS	0.113	19
PY6	0.153	8

NG, No growth.

* Cells were precultured in Sabouraud's dextrose broth and diluted 10^{-4} (without washing) into 1.17% YCB (contains amino acids) + 0.2% BSA, adjusted to pH 4.4.

† ΔA_{280} per 0.5 ml for 30 min at 37 °C.

produced at 37 °C compared with that achieved at 27 °C. While higher levels of enzyme activity are obtained in BSA broth at lower temperatures, proteinase production on agar must be measured at 37 °C because no zone of clearing is obtained at 27 °C.

In this one experiment, the spontaneous Prt^- mutant, 0-1S, grew to the same density as the parent whereas in other experiments it was completely negative. Variability in growth from one experiment to the next was probably due to a high reversion rate with selection of Prt^+ cells during incubation in BSA broth. Hence, stock cultures of Prt^- mutants were stored on nonselective medium (YKDA slants) to try to prevent the accumulation of revertants.

Inoculum effect in the assay of Prt^- mutants

The Prt^- phenotype was difficult to assay in BSA broth until the inoculum effect was recognized. Precultures must be diluted 10^{-3} or 10^{-4} into BSA broth (without YE) in order to distinguish between Prt^- and Prt^+ strains. For example, after 3 d incubation with aeration at 37 °C, the Prt^- mutant at a 10^{-3} dilution of the inoculum remained at a low cell density but it grew up in 4 d whereas the 10^{-4} inoculum did not grow up until much later (Fig. 3). This inoculum effect on growth of the Prt^- mutant in BSA broth may be attributed to a 'leaky' phenotype, i.e., a partial defect in proteinase production and/or the presence of revertants which secrete sufficient quantities of proteinase to digest the BSA to low- M_r nitrogen compounds allowing the proteinase-deficient cells to grow.

Reversion frequency of the Prt^- mutation

Cultures of mutant 0-1S were grown to stationary phase in AS broth or BSA broth at 37 °C, then diluted and spread on BSA + YE agar. After 7 d incubation at 37 °C, colonies with a zone of clearing (Prt^+ revertants) were counted. A high frequency of revertants was observed ($16/518 = 3.1 \times 10^{-2}$ in the AS broth culture and $53/1173 = 4.5 \times 10^{-2}$ in the BSA broth culture).

Ten Prt^+ revertant colonies were picked from BSA + YE agar plates inoculated from the AS broth culture of mutant 0-1S. The revertant clones were inoculated into AS broth (nonselective medium) and, after overnight growth at 37 °C, were spotted onto BSA + YE agar which was incubated at 37 °C for 7 d along with control cultures and subclones. All 10 Prt^+ revertants gave a zone of clearing equal to the Prt^+ parent (1–2 mm); a fresh subclone (0-1Sb) of the Prt^- mutant was negative but the AS and BSA broth cultures (each of which contained about 4×10^{-2} revertants) gave small zones of clearing indicating the presence of revertants. To test the stability of Prt^- subclone 0-1Sb, the AS broth culture was diluted and spread on BSA agar. After 7 d at 37 °C, no revertants were found out of 613 colonies scored ($< 1.6 \times 10^{-3}$), in agreement with the observation that this subclone was negative in the drop test.

Fig. 3. Inoculum effect in the assay of the *Prt*⁻ phenotype. ●, *Prt*⁺ *C. albicans* 28366a; ▲, *Prt*⁻ mutant 0-1S. Both strains were precultured in MIN broth, diluted 10^{-1} , 10^{-2} , 10^{-3} or 10^{-4} into YCB + 0.2% BSA broth, pH 5.0, and aerated at 37 °C for 4 d. Growth was monitored by measuring the optical density of the culture (OD₅₅₀).

To test the stability of the revertants, 1R1, 1R3 and 1R4 (derived from 0-1S) were grown in AS broth to stationary phase, then diluted 2×10^{-4} into AS or BSA broth, and aerated at 37 °C for 2 d. During these two subculturings, all three revertants back mutated to the *Prt*⁻ state.

DISCUSSION

Several laboratories have reported a relationship between the ability of *C. albicans* to secrete an extracellular acidic proteinase and the virulence of this opportunistic pathogen. As a first step in studying the *in vivo* function of this enzyme, we investigated various *in vitro* parameters of proteinase production including the optimum conditions for induction, and the genetics of proteinase in a diploid isolate of *C. albicans*.

Proteinase secretion was induced when *Prt*⁺ strains were grown on BSA as the sole nitrogen source, but not when cells were starved for nitrogen or grown on ammonium sulphate. Addition of ammonium sulphate to BSA medium repressed the synthesis of proteinase (M. Crandall and co-workers, unpublished).

Optimum growth parameters for proteinase induction in liquid medium were found to be: 0.2% BSA broth, pH 4.4, room temperature, aeration and 1% (w/v) glucose. The pH optimum for enzyme activity was also found to be pH 4.4 (M. Crandall and co-workers, unpublished). In later experiments, when 46 clinical isolates of *C. albicans* were grown with aeration at 22 °C in minimal broth medium (yeast carbon base) containing 7% (w/v) bovine calf serum, at pH 4, all secreted an acidic proteinase (M. Crandall and co-workers, unpublished).

Proteinase induction was stimulated by the addition of 0.01% YE to BSA agar, corroborating the results of Germaine *et al.* (1978). Proteinase secretion was also stimulated by serum, or by the addition of 20 μ M-FeCl₃ to BSA broth. Thus, the observed stimulation of proteinase production by YE or serum may be due, in part, to their iron content. Iron has been considered an important

factor for growth and virulence of microbial pathogens (Weinberg, 1978), and has been shown to stimulate germination of *C. albicans* in serum (Landau *et al.*, 1965).

A high frequency of spontaneous Prt^- mutants was found in cultures of *C. albicans* ATCC 23866, and this frequency was increased by UV irradiation, in the absence of significant killing. This result indicates heterozygosity at the genetic locus (*prtA*) coding for this enzyme. The basis for this conclusion comes from reports which present evidence that clinical isolates of *C. albicans* are diploid and naturally heterozygous (+/−) at various genetic loci (Whelan & Magee, 1981; Poulter *et al.*, 1982; Crandall, 1983). Segregation of homozygous recessive (−/−) diploids from these strains results from mitotic recombination which occurs spontaneously at a high frequency, and is increased in rate by UV light. Thus, the diploid nature of *C. albicans* is corroborated by our results.

All Prt^- mutants isolated were small colonies lacking a zone of clearing. However, when assayed in BSA broth, the Prt^- phenotype could only be demonstrated when stationary-phase precultures of mutants were diluted 10^{-4} to 10^{-3} . This inoculum effect may be explained by the presence of high frequencies of revertants in cultures of Prt^- mutants, perhaps because Prt^- mutant cells grow more slowly allowing Prt^+ revertants to overgrow the population. The Prt^+ revertants were characterized by a high rate of back mutation to Prt^- , so it was not possible to isolate a stable revertant. Macdonald & Odds (1983) found that the mutants they isolated from this same strain of *C. albicans* were proteinase deficient, i.e., they retained a small percentage of secreted proteolytic enzyme activity; however, these authors did not publish the percentage of revertants in mutant cultures.

The techniques and mutants developed in this paper have been used in later investigations of the relationships between the acidic proteinase of *C. albicans* and germination, adherence and vaginitis.

This research was supported by a National Institutes of Health (NIH) Biomedical Research Support Grant awarded to the Research and Education Institute Inc. of Harbor-UCLA Medical Center, and by a National Science Foundation Visiting Professorship for Women in Science and Engineering to Dr Crandall. This research was completed while Dr Crandall was a Senior Fellow of the NIH National Research Service Award. Dr Edwards was funded by Research Grant AI-19990 from the NIH.

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