INFECTION AND IMMUNITY, Sept. 1985, p. 571–575
0019-9567/85/090571-05$02.00/0
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Vol. 49, No. 3

Genetic Evidence for Role of Extracellular Proteinase in Virulence of *Candida albicans*

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Received 19 April 1985/Accepted 6 June 1985

The relationship between extracellular proteinase and the virulence for mice in *Candida albicans* was studied by using a set of three isolates. The set included a proteinase-producing parent (C9), a proteinase-deficient mutant derived from C9 by nitrous acid treatment (C9M1), and a spontaneous revertant (C9M1M) obtained by mouse passage of C9M1. The morphological markers and the carbon assimilation pattern were identical in these isolates. Isolate C9 produced a high level of proteinase in vitro and caused fatal infection (100%) within 21 days. The mutant produced no detectable enzymes in vitro, and all mice survived until day 22. Only 30% of the mice infected with C9M1 died between day 23 and 30. The isolates recovered from the dead mice were found to be proteinase sufficient, indicating that the mice died after the organism in tissue had reverted. The C9M1M isolate produced proteinase in vitro at 44% the level of C9 and induced fatal infection in 90% of the mice within 30 days. The number of CFU recovered from the kidneys correlated with the level of proteinase produced in vitro and, in turn, the rate of fatal infection produced by the isolates. These results support a previous observation indicating that proteinase activity is one of the virulence factors associated with C. albicans.

Candida albicans is the most common etiological agent of candidiasis (1). Its pathological manifestations range from vaginitis to systemic mycosis, and it is a particularly important consideration in the management of immunosuppressed patients (11). A major unanswered question about candidiasis is the role of the organism versus the role of the host in the contraction and progression of the infection. Can any strain of *C. albicans* infect a compromised host, or as with bacteria (6, 14), are there particular virulence factors?

One factor postulated to play a role in virulence is the extracellular proteinase activity that many *C. albicans* strains exhibit when incubated in medium lacking ammonia but containing a protein as the nitrogen source (15). Extracellular proteinase, which has been purified extensively by Rüchel (12), was shown to consist of three separate enzymes, depending on the strain used as the source. One of these enzymes was a complete proteinase; the other two were partially proteolytic. Although one of these latter enzymes differs from the complete proteinase enzyme in its substrate specificity and other properties, it cross-reacts immunologically (13). On the basis of the presence of serum antibodies to one of these enzymes in patients with systemic candidiasis (7) and the determination by indirect immunofluorescence of the enzyme in tissue lesions (8), MacDonald and Odds suggested a role for this enzyme in pathogenicity. They recently showed that a proteinase-deficient mutant derived from a proteinase-producing strain had reduced virulence in mice and was more easily phagocytized by both human and mouse polymorphonuclear leukocytes (9).

However, these experiments were not conclusive for several reasons. First, the proteinase-deficient strain appeared to grow more slowly than the parent at 26°C. Since the in vivo temperature of a mouse, except the testicles, is in the vicinity of 37°C (3), the difference in growth rates at that temperature could be even greater, and it could, therefore, account for any difference in virulence. No growth studies at 37°C with this mutant have been reported. Second, a more general criticism has to do with the mode of isolation of the proteinase-deficient strain. Nitrosoguanidine (NTG) is known to produce multiple mutations, and the extremely rigorous treatment given to the cells (exponential phase, 0.5 mg of NTG per ml, 37°C) seems certain to have caused multiple lesions. Indeed, 3 of the 10 properties measured were altered in the mutant strain (9). Finally, since at least one of the ways the mutant could have been produced was by mutation followed by mitotic crossing over in this diploid yeast (16), there is a possibility that the two strains differ not only in NTG-induced mutations but also in that the mutant is homozygous for some fraction of a chromosome for which the parent is heterozygous.

We report here a series of experiments that avoid some of these difficulties by examining the virulence of a proteinase-deficient mutant, its parent, and one proteinase-producing revertant. By the use of the revertant, we avoided the problem of multiple genetic differences. We conclude, in agreement with MacDonald and Odds (9), that the extracellular proteinase produced by *C. albicans* is one of the virulence factors associated with this organism and that the degree of virulence is correlated with the level of proteinase produced.

MATERIALS AND METHODS

Isolates. C9, the wild-type parent strain for these experiments, was obtained from D. Ahearn, Georgia State University, Atlanta. The proteinase-deficient mutant, C9M1, was obtained from C9 by nitrous acid mutagenesis as described. C9M1M, the proteinase-producing revertant, was isolated from a mouse that died 23 days after being inoculated with C9M1. These isolates all produced chlamydospores and germ tubes and had the same pattern for the assimilation of carbohydrates.

Media. Isolates were maintained on YEPD (10 g of yeast extract, 20 g of peptone, 20 g of glucose, 20 g of agar per liter) or malt extract agar. For in vitro growth rate determi-

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nation, YEPD broth was used. The selection of mutants was made on bovine hemoglobin (BH) agar medium containing 1.45 g of yeast nitrogen base (YNB, no. 0335-15-9; Difco Laboratories, Detroit, Mich.) without ammonia and amino acids, 2 g of crystalline BH (Sigma Chemical Co., St. Louis, Mo.), 20 g of glucose, and 20 g of agar per liter. The YNB agar solution was autoclaved, and the filter-sterilized hemoglobin broth was added to the cooled (45°C) agar solution. The pH of the agar medium varied from 5.5 to 5.6. For measurement of proteinase, BH broth was used (pH 5.5 to 5.6).

Mutagenesis. Nitrous acid mutagenesis was carried out as described by Kakar et al. (2), except that the cells were incubated with the HNO_2 for 18 min at 25°C (resulting in a survival rate of 20%). Mutants were identified by transplanting (patching) onto YEPD and replicating onto the BH medium.

Proteinase production. Strains C9 and C9M1 were grown in YEPD broth for 4 h and centrifuged. The cell pellets were washed with sterile distilled water, and 10^6 washed cells were then inoculated into flasks (125 ml) containing 20 ml of BH broth and incubated on a shaker at 37°C for a total of 65 h. Culture (1 ml) was drawn periodically and centrifuged at 3,000 rpm in a clinical centrifuge for 10 min at 25°C to obtain the culture supernatant. The culture supernatant was the source of the proteinase. In multiple separate experiments, C9, C9M1M, and C9M1 were cultured for 18 h in BH broth to compare the amount of proteinase in the culture supernatant. At 18 h, the parent strain reached the end of the exponential growth phase in BH broth.

Enzyme assay. The extracellular proteinase was assayed in 0.1 M sodium citrate buffer, pH 3.2, containing 2 g of BH per liter. The culture supernatant and the assay medium were kept on ice. We allowed the reaction to start by adding 0.1 ml of the supernatant to 0.9 ml of assay medium and rapidly warming the mixture at 37°C. The reaction was stopped with the addition of an equal volume of 5% trichloracetic acid at various times. After an additional 10 min at 37°C, the reaction mixture was centrifuged in the clinical centrifuge, the supernatant was decanted, and the absorbance at 280 nm was read against a blank containing distilled water. BH broth culture filtrate treated with pepstatin (50 mg/ml) for 30 min at 37°C was used as the control. Enzyme units are expressed as the amount, in micromoles, of tyrosine released per minute either per milliliter of culture supernatant (see Fig. 1) or per 10^6 cells of *C. albicans* (see Table 1). Cell numbers were determined by hemacytometer count after being vortexed to break up clumps of cells.

Determination of growth rate. Growth rates of the isolates in YEPD broth at 34 and 37°C were compared. Cells were collected from 48-h malt agar slants, transferred into 50 ml of YEPD broth, and incubated for 15 h on a shaker. At the end of 15 h of incubation, 150 μl of the cultures was transferred into fresh medium (initial optical density, 0.03 to 0.05 at 600 nm with a Gilford 2600 spectrophotometer), and absorbency at 600 nm was measured at 30-min intervals.

Determination of virulence. The yeast cells from 48-h malt extract agar cultures of C9, C9M1, and C9M1M were suspended in saline, and serial dilutions were prepared. Dilutions containing either 2×10^6 or 2×10^3 cells per ml as determined by both hemacytometer and plate count were used as inocula. Female general purpose (National Institutes of Health) white mice weighing 19 to 21 g were infected with 0.5-ml injections of inocula into the lateral tail vein. Ten mice were injected with 10^6 cells of each inoculum and observed for up to 30 days to determine survival rates. Three other sets of 10 mice each were injected with 10^3 cells to test in vivo growth rates. The mice that received 10^3 cells were sacrificed periodically, and the homogenates of both kidneys were plated on malt extract agar as previously described (5). The colonies were counted after a 72-h incubation at 30°C.

RESULTS

Isolation of a proteinase-deficient mutant and its revertants. A survey of the *C. albicans* strains available in the laboratory of one of the authors (P.T.M.) showed that of 10 strains tested, only 1 generated sufficient extracellular proteinase to produce a clearing zone on BH agar plates. This strain, C9, was used to isolate a proteinase-deficient mutant by nitrous acid mutagenesis, as described above. Testing on patch plates was necessary because on a spread plate a deficient colony may be masked by an adjacent producer. A strain that failed to produce a clearing zone after 6 days was defined as a mutant phenotype. Proteinase-deficient mutants appeared at a very high rate, of the 20% that survived the HNO_2 treatment, more than 1% were proteinase-deficient mutants. One mutant, C9M1, was chosen for further study. The revertant was isolated by mouse passage of C9M1. During experiments to test the virulence of C9M1 in mice, an isolate that had regained the ability to make proteinase was obtained from the kidney of a mouse that died on day 23. This revertant is referred to as C9M1M.

Growth rate and proteinase production by the isolates. The isolates, C9, C9M1, and C9M1M, were grown in YEPD broth at both 34 and 37°C. Growth rates of the mutant, C9M1, and the revertant, C9M1M, were identical at both temperatures, but they were slower than that of the parent strain. Strain C9 grew with a doubling time of 66 min at both temperatures, compared to 76 min and 80 min for the other strains at 34 and 37°C, respectively. We determined proteinase activity in the culture supernatants from cells grown in BH broth at 37°C. We found that proteinase activity reached a maximum at about 22 h after inoculation of 10^6 cells per ml into 20 ml of medium. After 22 h, the activity sharply decreased, possibly due to self-digestion of the proteinase (data not shown). Figure 1 shows the proteinase activity of the C9 and C9M1 isolates at various incubation periods. It can be seen that the C9M1 isolates produced no activity until they had been incubated for 40 h. The number of C9M1 cells in the BH broth culture showed only a fourfold increase at this time, whereas C9 increased by more than 100-fold after a 15.5-h incubation. Table 1 shows the proteinase activity (per 10^6 cells) of the C9, C9M1, and C9M1M after an 18-h incubation in BH broth. The mean of the enzyme unit of multiple separate experiments shows that the proteinase activity of the revertant was about 44% that of the wild type. The proteinase-deficient mutant showed no enzyme activity.

Virulence for mice. The mortality rate of mice injected with 10^6 cells and the in vivo growth rate of the isolates are shown in Fig. 2 and 3. Sixty percent of the mice that received C9 cells died within 10 days, and the remainder died within 21 days (Fig. 2). The average survival time for the 10 mice was 8.1 days. The mutant was shown to be markedly less virulent in that, after being inoculated with C9M1, all mice survived until day 20 and only 30% of the total had died by day 30. The average survival for the 30% was 25 days. The isolates recovered from the mice that died by infection with C9M1 were found to produce sufficient extracellular proteinase to produce a clearing zone on plates with BH agar. This indicated that the cultures had reverted during the growth in the kidney and that it was the revertants that produced the fatal infection. In fact, the C9M1 isolates reverted readily

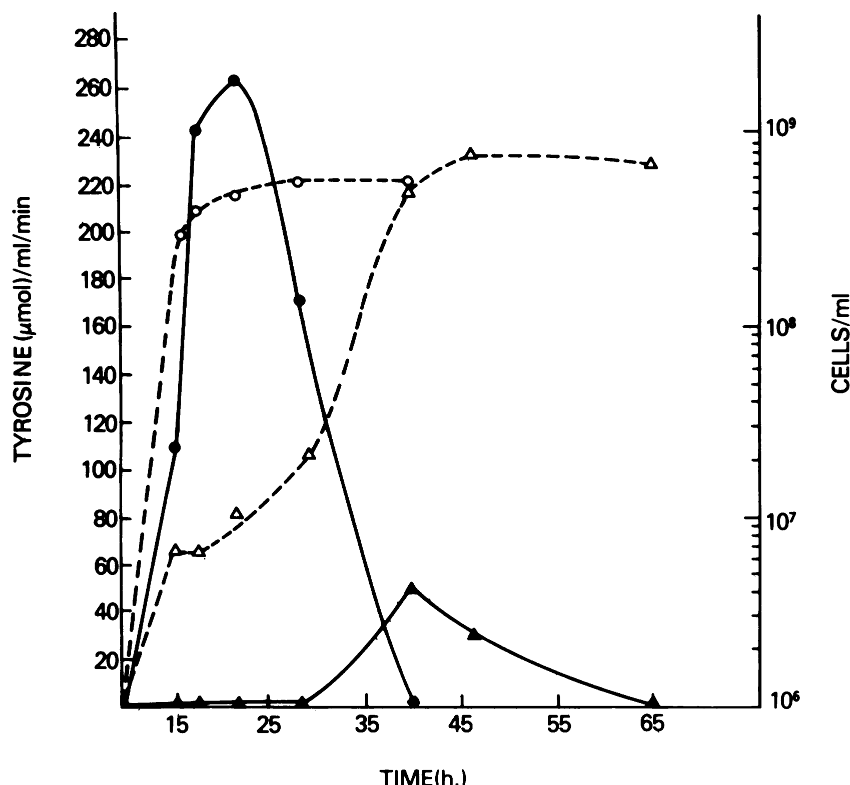

FIG. 1. Proteinase activity (solid lines) and the growth curve (dotted lines) for C9 and C9M1 in BH broth. Symbols: ● and ○, C9; ▲ and △, C9M1.

when maintained on agar medium through repeated subculturing. C9M1M was the revertant selected for the virulence study. By day 10, C9M1M had killed seven mice (70%), and two other mice were killed by day 30. The average survival time for all nine mice was 8.6 days. This result indicated that the virulence of C9M1M is only slightly lower than that of C9.

The results of the in vivo growth test (Fig. 3) were in concordance with the mortality rates (Fig. 2). With an inoculum of 10^3 cells per animal, C9M1 showed no evidence of growth and disappeared from the kidneys by day 20. Strain C9 grew rapidly in the kidneys, and the CFU count increased at least 100,000-fold in 8 days. The spontaneous revertant C9M1M also grew exponentially until day 8. The number of CFU of C9M1M slightly trailed that of C9 throughout the 20-day period, but the pattern of growth in both isolates was the same.

TABLE 1. Proteinase production by C9, C9M1, and C9M1M[a]

Isolates	Enzyme units[b]	Mean enzyme units from multiple experiments (±SD)
C9	0.704, 1.123, 0.805, 0.860, 0.848, 0.967, 1.030, 0.636	0.872 (±0.153)
C9M1M	0.312, 0.484, 0.384, 0.418, 0.379, 0.352	0.388 (±0.054)
C9M1	0.000, 0.000, 0.000, 0.000, 0.000	0

[a] From an 18-h (37°C) culture filtrate of BH broth.
[b] One enzyme unit equals one micromole of tyrosine released per minute per 10^6 cells under standard assay conditions.

DISCUSSION

The postulation that there is an association between extracellular proteinase and virulence in *C. albicans* has been presented by MacDonald and Odds (9). The mode of action of the enzyme is far from clear; however, it is possible that the enzyme uncovers sites on epithelial tissue where the cells adhere or that it has a cytopathic effect, assisting in spread of the infection. Although these authors showed that a proteinase-deficient mutant was less virulent and more

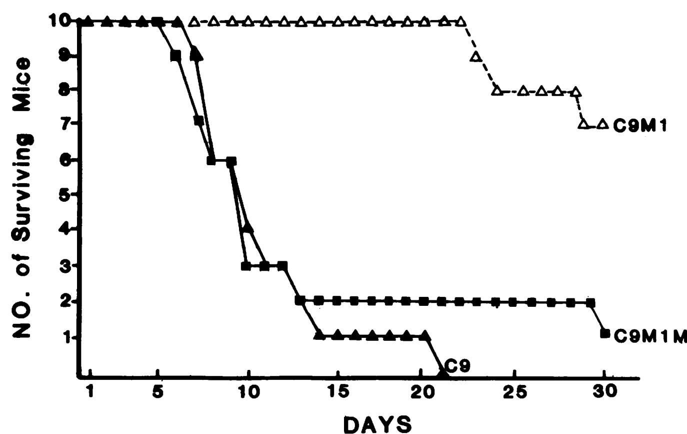

FIG. 2. Mortality rates of mice injected with *C. albicans* strains C9, C9M1, and C9M1M. Ten mice each were injected as described in the text with 10^6 cells, and the survival of mice was monitored for 30 days.

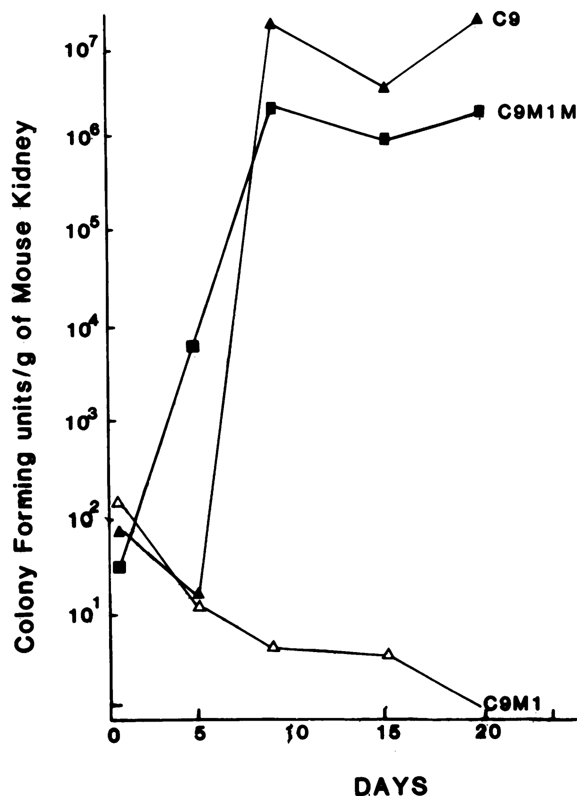

FIG. 3. CFU of *C. albicans* found in the kidneys of mice injected with C9, C9M1, and C9M1M. Each isolate was inoculated into mice at 1,000 cells per mouse. At the times shown, two mice of each set were sacrificed, and the kidneys were homogenized and plated on malt extract agar. The CFU per gram of kidney were determined after the plates had been incubated for 72 h.

easily phagocytized than the wild type, the way in which they isolated their mutant, using a very strong mutagenesis with an agent (NTG) known to induce multiple lesions, did not eliminate the possibility that the altered virulence was due to genetic changes separate from the proteinase gene. The fact that 3 of 10 properties unassociated with the proteinase were altered in the mutant as compared to the parent is another indication that the correlation between the amount of proteinase and the degree of virulence could be fortuitous.

We attempted to avoid the above objections by testing the virulence of a proteinase-deficient strain that is as nearly isogenic to the positive strains as possible. We constructed these strains by isolating a proteinase-deficient mutant, reverting it by mouse passage, and then testing the virulence of the parent, the mutant, and the revertant in mice. There was a strong correlation between proteinase production and virulence. The in vitro doubling time of the proteinase-deficient mutant was 14 min slower than that of C9 at 37°C. The doubling time of the revertant was identical to that of the mutant, and yet the revertant showed virulence in mice which was close to that of C9. It is clear that a 14-min-slower generation time in vitro does not significantly affect the virulence in *C. albicans* as long as the other properties are not altered. The recovery of the proteinase activity in the revertant was only about 44% of that seen in the wild-type parent, although the two isolates showed only a slight difference in virulence. This may indicate that there is a threshold level of proteinase that contributes to the virulence of *C. albicans*. The growth of three isolates in the mouse kidney correlated with their level of proteinase activity in vitro. With the initial dose of 10^3 cells, the enzyme-deficient mutant failed to grow, but the parent and the revertant multiplied rapidly in the kidneys. As MacDonald and Odds found in the in vitro system, the proteinase-deficient cells may be phagocytized more readily in vivo. These results indicate that the proteinase is important in virulence, although there might be a number of additional factors that play important roles in pathogenesis. Among the other factors that may be important are the adherence of the cells (10), the yeast-to-hyphal transition in the host tissue, the nutritional characteristics (4) of the isolate, the growth rate at 37°C, and the metabolic parameters, which are as yet unidentified.

Crandall et al. (M. Crandall, L. Hirano, and J. E. Edwards, Jr., Abstr. Annu. Meet. Am. Soc. Microbiol., 1985 F62, p. 374) showed that the neutral pH conditions which induce hyphal formation in *C. albicans* preclude the production of extracellular acidic proteinase. Also, the acidic conditions (pH 4) that induce the production of proteinase are inhibitory for germ-tube formation. The pH of the medium that was used in this experiment ranged from 5.5 to 5.6. The germ-tube test for our isolates was not performed in this pH range.

While other factors may exist, our present results, which confirm those of MacDonald and Odds (8), show that extracellular proteinase is an important factor in the degree of virulence, at least in the mouse model. As more of such factors are identified, one may be able to estimate the potential virulence of clinical isolates without going through cumbersome, slow, and expensive animal tests, thus providing information that can aid in the management of candidiasis.

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