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Genetics

Septum formation, cell division, and sporulation in mutants of yeast deficient in proteinase B

(protein turnover/posttranslational modification/chitin synthase)

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ABSTRACT **Mutants of *Saccharomyces cerevisiae* that carry lesions in the structural gene for proteinase B, *prb1*, have been isolated. Proteinase B was thought to be involved in activation of chitin synthase zymogen to allow septum formation during budding and to be involved in sporulation. The *prb1* mutants are able to grow and divide and they form apparently normal septa that contain chitin. Most diploids homozygous for *prb1* mutations are unable to complete sporulation.**

In yeast, the primary septum, which forms between mother and daughter cells during budding and which remains with the mother cell as a bud scar upon cell separation, is composed of chitin (1–4). Chitin synthesis is catalyzed by chitin synthetase (chitin synthase; UDP-acetamido-2-deoxy-D-glucose:chitin 4-β-acetamidodeoxyglucosyltransferase, EC 2.4.1.16), an enzyme present in the cell membrane as a zymogen (5). Ulane and Cabib (6) have postulated that proteinase B (EC 3.4.22.9), located in vacuoles (7–9), is responsible *in vivo* for activation of chitin synthetase zymogen by fusion of vesicles containing proteinase B with selected sites on the plasma membrane (10). Proteinase B has also been implicated in catabolite inactivation of gluconeogenic enzymes upon medium shift (11–13) and in turnover of proteins when cells sporulate (4). We have isolated mutants deficient in proteinase B in order to determine the physiological function of this proteinase.

METHODS AND MATERIALS

Materials. Hide powder azure (HPA), cycloheximide, and azocoll were purchased from Calbiochem; sodium dodecyl sulfate ($NaDodSO_4$), canavanine sulfate, and hemoglobin, from Sigma; ethyl methanesulfonate, from Eastman; Triton X-100, from Baker; Diafine from Acufine (Chicago, IL); *N*-acetylphenylalanine β-naphthyl ester (AcPheNap), from Schwarz/Mann; and Fast Garnet GBC, from Roboz Surgical Instrument Co., Inc. (Washington, DC). Calcofluor ST was a gift from American Cyanamid Company (Bound Brook, NJ).

Strains. Mutants of *Saccharomyces cerevisiae* deficient in proteinase B were isolated in two genetic backgrounds: M16-14C (*a leu1-1 ser1-171*) and X2180-1B (α *gal2*). The parent strains were s126 (α *trp1 gal2* from X2180-1B) which carries a lysis mutation resulting in lysis of a fraction of the cells in a colony and which was isolated by the procedure of Cabib and Durán (15) and ape23 (*a leu1-1 ser1-171 pep15-1*) which also carries a lysis mutation. Outcross parents for each isogenic series were derived from M16-14C (the parent of ape23) and X2180-1B by mutation and crosses within a series and had the genotypes α *met*$^-$ *ser1-171 thr1* and *a ade6 his5 gal2*, respectively. Gene symbols are: *a* and α, mating type alleles; *ade6*, *his5*, *leu1*, *met*$^-$, *ser1*, *thr1*, and *trp1*, requirements for adenine, histidine, leucine, methionine, serine, threonine, and tryptophan, respectively; *gal2*, inability to ferment galactose; and *pep15-1* (16), decreased ability to cleave AcPheNap.

Media. YEPD, KAc, SC, and omission media were as described (17). In YEPG broth, 3% glycerol replaced the glucose in YEPD. In YEPG agar, 5% glycerol replaced the glucose in YEPD. Canavanine-containing medium was arginine omission medium to which canavanine sulfate was added at 60 mg/liter.

Genetic Methods. Procedures for sporulation, dissection, and scoring of nutritional markers were as described (18). The *pep15-1* marker was monitored as described (16).

Proteinase B-deficient mutants were isolated in ape23 and s126 after ethyl methanesulfonate mutagenesis (16). Treated cells were plated on YEPD at concentrations yielding 30–60 colonies per plate. Incubation was at 23°C for 4 days. Colonies were replica-plated to YEPD. Replicas were incubated at 23°C for 3 days and then transferred to 36°C overnight preceding application of agar overlays containing HPA, a substrate specific for proteinase B (6, 19, 20). The overlaid colonies were incubated an additional 2 days at 36°C. HPA was pulverized by homogenization (VirTis homogenizer) of 200 ml of a slurry (100 mg/ml in 95% ethanol) in a 500-ml flask for 5 min at 40,000 rpm. Aliquots containing 50–100 mg were centrifuged (5 min at 1650 × *g*), the supernatant was discarded, and the pellets were washed with 3 ml of sterile water. Molten agar, $NaDodSO_4$, and cycloheximide were added to the pellets to form the overlay (see legend to Fig. 1).

Preparation of Extracts and Proteinase Activity Assays. Cell-free extracts were prepared as described (16) except that titration with acetic acid was omitted.

Proteinase A and C were assayed as described (16). Proteinase B was assayed according to Juni and Heym (19) with the following modifications: 0.2 ml of various dilutions of untreated crude extract in 0.1 M Tris·HCl buffer at pH 7.6 was added to a reaction mixture containing 20 mg of azocoll, 0.125 ml of 1% Triton X-100 solution, 0.375 ml of 0.1 M Tris·HCl pH 7.6 buffer, and 0.04 ml of 20% $NaDodSO_4$ in 0.1 M Tris·HCl pH 7.6 buffer. The mixture was incubated with shaking at 30 or 37°C as indicated. After 10 or 15 min, depending on the experiment, the reaction was terminated by addition of 1 or 2 ml of cold distilled water. After centrifugation at 1650 × *g* for 5 min, the absorbance at 520 nm was determined. Appropriate dilutions of extract were chosen such that the kinetics of each reaction were linear with time and protein concentration. One unit of proteinase B activity is defined as a change of 1 absorbance unit at 520 nm per minute for the 0.74-ml reaction mixture as assayed at 30 or 37°C. Inactivation of proteinase B was accomplished by incubating samples of crude extract at 50°C for

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Abbreviations: AcPheNap, *N*-acetylphenylalanine β-naphthyl ester; HPA, hide powder azure; $NaDodSO_4$, sodium dodecyl sulfate.

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various intervals, followed by immersion in an ice bath. Treated samples were assayed at 30°C for proteinase B as described above.

Protein concentration was estimated according to Lowry *et al.* (21).

Observations of Fluorescence. Cells grown in YEPD were harvested by centrifugation, washed in 0.01% Calcofluor ST, and then resuspended in 0.01% Calcofluor ST containing 0.5% agar. A drop of the suspension was placed on a slide and covered with a coverglass. The preparations were sealed and the agar was allowed to solidify. Photomicrographs were made with a Zeiss photomicroscope III equipped with a IIIRS epifluorescence condenser. Illumination was provided by a XBO 75-W xenon arc with a Zeiss FITC filter. Tri-X film was developed in Diafine two-stage developer.

RESULTS

When colonies of strains bearing lysis mutations are overlaid with particulate HPA (a colored protein substrate of proteinase B) in the presence of a low concentration of $NaDodSO_4$, the particles of HPA on top of and surrounding the colonies are solubilized. Replica plates bearing clones derived from mutagenized cells were grown to full colony size at 23°C and then tested for ability to solubilize HPA at 36°C after an overnight preincubation at 36°C (to inactivate any temperature-sensitive enzyme). This was done in anticipation that proteinase B activity might be necessary for growth. Of 2400 colonies of ape23 screened, 12 failed to hydrolyze HPA at 36°C. These 12 colonies were recovered from the original master plate and were tested for the ability to cleave HPA at 23, 30, and 37°C and for the ability to grow and divide at the three temperatures. All 12 mutants failed to cleave HPA at any temperature and were able to grow at all temperatures.

The mutants were crossed to an essentially isogenic strain to isolate the mutations free of *pep15-1* (which decreases proteinase levels) and in the opposite mating type. Segregants free of *pep15-1* but unable to cleave HPA were again outcrossed and segregation of the HPA-negative phenotype was followed. In all cases examined, cleavage ability segregated 2:2 in tetrads, as shown for *prb1-2* (proteinase B deficient, see below) in Fig. 1. Because the plate tests are only semiquantitative, the level of proteinase B activity in colonies that solubilize HPA cannot be immediately inferred.

Levels of proteinases A, B and C are shown in Table 1 for the parents and the segregants of tetrads 1 and 2 of Fig. 1. The *prb1-2* mutation eliminated proteinase B activity but had no effect on proteinases A and C. The absence of proteinase B activity segregated 2:2 in meiotic tetrads and cosegregated with the HPA-negative phenotype seen on plates.

Complementation tests for the 12 mutations were performed at 36°C by crossing strains in all pairwise combinations. All strains used were free of *pep15-1*. Three complementation groups were found, based on the ability of the doubly heterozygous diploids to cleave HPA. Ten of the 12 mutations fell into the *prb1* group, 1 fell into *prb2*, and 1, into *pep16* (AcPheNap cleavage ability was also decreased). It can be inferred from the complementation analyses that all 12 mutations are recessive. Diploids homozygous for the 10 *prb1* mutations did not sporulate.

If one is to determine the consequences to the cell of loss of proteinase B activity, it is essential that any mutants being analyzed be deficient only in proteinase B activity and not deficient for this enzyme activity as a consequence of a pleiotropic mutation. Hence, we wished to isolate mutations in the structural gene for proteinase B. Because the *prb1* mutants had enzymatic phenotypes consistent with those we would expect for

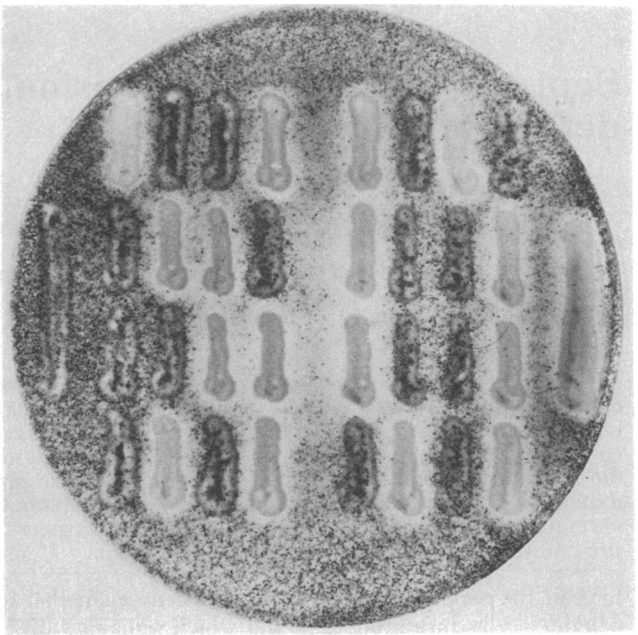

FIG. 1. Meiotic segregants from a *prb1-2*/+ diploid. Approximately 4 ml of 0.5% agar containing 50–100 mg of HPA, 20 mg of $NaDodSO_4$, and 1 mg of cycloheximide was poured over the surface of cells and YEPD agar in the Petri dish. The *prb1-2* mutant is the long stripe at the left; the wild type is at the right. Each horizontal group of four stripes is one tetrad. Stripes of proteinase B-deficient segregants remain covered and lack a clear halo. Tetrad 1, in the upper left, shows a segregation of +−−+; tetrad 2, immediately beneath, is −++−.

structural gene mutants (Table 1), we enlarged our collection of mutants and sought those with thermolabile proteinase B activity. Eighty thousand clones derived from mutagenized cells of s126 were screened for the HPA-negative phenotype. To date 135 mutants in s126 have been assigned to four complementation groups; with 116 in *prb1*, 1 in *prb2*, 17 in *prb3*, and 1 in *prb4* (the last two are new groups). The initial assignment of mutations to the *prb1* complementation group was based on inability of the doubly heterozygous diploids to sporulate because we have previously found that diploids homozygous for

Table 1. Proteinase levels in crude extracts of parents and meiotic segregants of a +/*prb1-2* diploid

Strain	Relevant genotype	Proteinase A, units/mg	Proteinase B, units/mg	Proteinase C,* units/mg × 10^3
Parents				
(AI-0)-19C	+	1.84	0.305	1.46
(AI-3)-2B	*prb1*	2.13	0.0	1.84
Segregants				
1A	+	2.05	0.348	1.71
1B	*prb1*	2.02	0.0	1.84
1C	*prb1*	1.89	0.0	1.73
1D	+	2.20	0.296	1.65
2A	*prb1*	2.31	0.0	1.79
2B	+	2.10	0.313	1.82
2C	+	1.97	0.367	1.75
2D	*prb1*	2.12	0.0	1.57

Cells were grown for 52 hr to stationary phase in YEPD at 30°C. Assays were carried out at 37°C.

* SDS assay as described (16).

prb1 were unable to sporulate. Eighty-seven mutations were assigned to *prb1* on this basis. However, all complementation tests were repeated by assessing HPA solubilization at 36°C with diploids homozygous for the lysis mutation. These tests confirmed assignment of the initial 87 mutations to *prb1* and added 29 additional mutations to the *prb1* group. Whether the ability of these 29 diploids to sporulate was due to low levels of proteinase B activity or to intracistronic complementation is unknown, although current evidence indicates that proteinase B is monomeric (6).

Colonies of two of the *prb1* mutants isolated in s126, *prb1-29* and *prb1-38*, were able to digest HPA at 23 and 30°C but not at 37°C. As expected, diploids homozygous for these mutations were able to sporulate at 23°C.

We anticipated that diploids heterozygous for *prb1* would show gene dosage if *prb1* were the structural gene for proteinase B, because structural gene loci in eukaryotes commonly show gene dosage. A wild-type diploid and a diploid heterozygous for *prb1-38* and *can1* were constructed. The homoallelic *prb1-38* diploid was selected as a canavanine-resistant mitotic recombinant from the doubly heterozygous diploid (*can1* and *prb1* are tightly linked). That the mitotic recombinant was homozygous for *prb1-38* was verified by *in vitro* assay of proteinase B at 37°C, a temperature at which little or no proteinase B activity is detectable in this temperature-sensitive mutant. The specific activity in the *prb1-38* diploid was about 12% of that for the wild-type diploid (Table 2). One would expect a heterozygous diploid to show 56% of the wild-type specific activity (0.241 unit/mg) if each chromosome were to function independently without regulatory compensation. The observed value, 0.253 unit/mg, was close to the predicted value. When equal amounts of protein from extracts made from wild-type and homozygous *prb1-38* diploids were combined and the proteinase B specific activity of the resulting mixture was measured, it was virtually the same as that measured in the extract of the heterozygote and very close to that predicted. This result eliminated the possibility that the proteinase B deficiency associated with the *prb1-38* mutation was due to an excess of some inhibitory substance.

We extended the dosage analysis to tetraploids. Five tetraploid strains were constructed bearing from zero to four wild-type alleles and from four to zero *prb1-38* alleles. The three tetraploids bearing both alleles (Fig. 2) had the expected specific activities on the basis that each *prb1-38* allele contributed 0.018 unit/mg (0.072/4) and each wild-type allele contributed 0.126 unit/mg (0.504/4). The expected slope of the line for these five tetraploids was 0.108 (0.126 − 0.018); the slope by linear regression was 0.107. Similar data were observed for tetraploids involving *prb1-29*, the other temperature-sensitive allele. The contribution was 0.131 unit/mg per wild-type allele and 0.008 per *prb1-29* allele. The expected slope was 0.123; that obtained by linear regression was 0.128.

Table 2. Activity of proteinase B in crude extracts of wild-type diploids and those heterozygous or homozygous for *prb1-38*

Relevant genotype	Proteinase B, units/mg
+/+	0.431
+/*prb1-38*	0.253
prb1-38/*prb1-38*	0.051
prb1-38/*prb1-38* plus +/+	0.243*

Cells were grown for 72 hr to stationary phase at 23°C in YEPG. Proteinase B was assayed at 30°C.

* A 1:1 (protein/protein) mixture of crude extracts of *prb1-38*/*prb1-38* and +/+ diploids was assayed at 30°C.

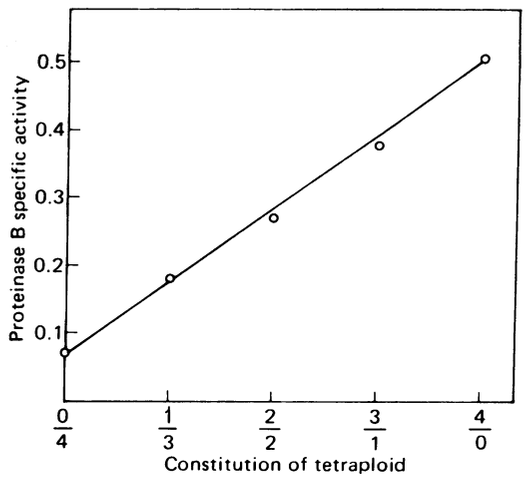

FIG. 2. Specific activities of proteinase B in crude extracts of tetraploid strains bearing various combinations of the wild-type and *prb1-38* alleles (shown as +/*prb1-38*). Cells were grown for 72 hr in YEPG at 23°C. Assays were carried out at 30°C. The specific activities per allele in homozygous wild-type and mutant tetraploids were 0.126 and 0.018 unit/mg, respectively. The *prb1* locus maps just proximal to *can1* (canavanine resistance) on chromosome V (unpublished observations). Two diploids were constructed of genotypes

$$\frac{\alpha\ trp1}{a\ +}\ \frac{prb1}{+}\ \frac{can1}{+}\ \frac{+}{ade6}\ \frac{+}{his5} \quad \text{and} \quad \frac{\alpha\ trp1}{a\ +}\ \frac{+}{ade6}\ \frac{+}{his5}.$$

From these two diploids, six diploids were isolated by mitotic recombination. All six were homozygous for *a* or *α* and for *ade6* or *his5*. Two of the six carried only wild-type alleles for *prb1* and *can1*, two were heterozygous for the two loci, and two were homozygous for *can1* and the *prb1* allele. To ensure that the derivative *can1 prb1* diploids arose by mitotic recombination and not by chromosome loss, each *prb1 can1* diploid was crossed to a diploid homozygous for the wild-type alleles, and the resulting tetraploids were sporulated and dissected. The occurrence of tetrads containing four canavanine-sensitive spore clones that would give canavanine-resistant papillae confirmed euploidy for chromosome V. The six diploids were crossed in appropriate combinations to yield tetraploids with 0, 1, 2, 3, or 4 copies of *prb1-38* or *prb1-29*.

Evidence that mutations in *prb1* result in altered proteinase B molecules was obtained by preincubating samples of crude extracts of tetraploids bearing four wild-type alleles, four *prb1-38* alleles, or three *prb1-38* alleles and one wild-type allele at 50°C for various periods of time and then measuring the specific activity of proteinase B in the treated samples (Fig. 3). The proteinase B activity present in the homozygous *prb1-38* tetraploid was inactivated more quickly than that of the wild-type tetraploid. The proteinase B half-lives, obtained by linear regression, were 3.5 min for the *prb1-38* tetraploid and 8.5 minutes for the wild-type diploid. When extracts of diploids homozygous for *prb1-29* or *prb1-38* were incubated at 50°C, the half-lives of proteinase B were 1 and 4 min, respectively (22). Thus, these two *prb1* alleles resulted in thermolabile proteinase B activities that were distinguishable from the wild-type activity and from each other. In support of the inactivation data described above is the finding that maximal activity for proteinase B is obtained at 41°C in extracts of wild-type diploids and 33°C in extracts of diploids homozygous for *prb1-38*.

We investigated thermal inactivation of proteinase B activity in the tetraploid bearing one wild-type allele and three *prb1-38* alleles. We reasoned that, if *prb1* were the structural gene for proteinase B, two species of proteinase B, differing in thermolability, should be present in the tetraploid and that the amount of each species should be in proportion to the number of each

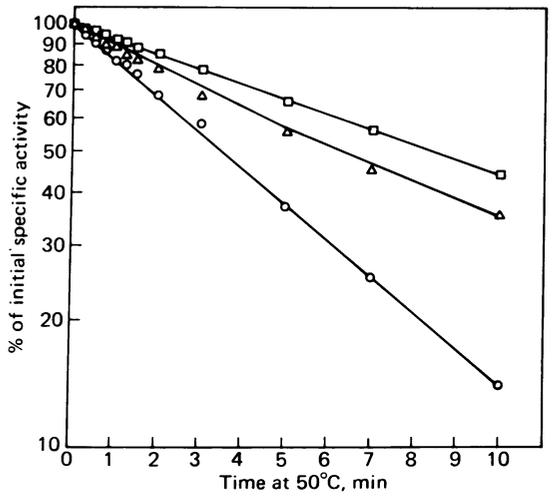

FIG. 3. Kinetics of inactivation of proteinase B in crude extracts of tetraploid strains bearing four wild-type alleles (□), four *prb1-38* alleles (○), or three *prb1-38* and one wild-type allele (△). Cells were grown for 72 hr in YEPG at 23°C. Assays were done at 30°C. Lines are linear regressions for homozygous tetraploids. Δ curve was calculated by using the rates of inactivation of the two different enzymatic species and the number of wild-type and mutant alleles present in the strain.

allele and the input specific activity for that particular allele. The curve for the data for this tetraploid was calculated by using the rates of inactivation of the two species, the number of each allele type, and the contribution per allele (Fig. 3). The data fit quite well to the predicted values. The rate of thermal inactivation of the proteinase B activity present in crude extracts made from the tetraploid bearing one wild-type and three mutant alleles is complex. The rate of inactivation is greatest in the initial portion of the curve but approaches that for the wild-type activity with longer incubation. We infer from this observation that more than one species of proteinase B activity is present in this extract and that the labile species contributes very little to the total activity after about 7 min.

In *S. cerevisiae*, the primary septum that forms between mother and daughter cell, and that is left behind as a bud scar on the mother cell upon cell separation, is composed of chitin (1–4). Ulane and Cabib (6) have postulated that proteinase B is responsible for activation of chitin synthetase zymogen, located in the cell membrane, which then catalyzes synthesis of chitin. If this hypothesis were correct, mutants lacking proteinase B would neither grow nor form septa. All 126 *prb1* mutants we have isolated, including 46 that bear amber or ochre mutations, grow at 23, 30, and 36°C. No obvious differences in growth rates of the mutants were observed. Dominant suppressors of *tyr7-1* and *trp1-1*, known amber mutations, and *lys1-1* and *his5-1*, known ochre mutations, were used in identifying the 46 nonsense mutations (data to be presented elsewhere). The *prb1-9* mutation has been thoroughly investigated. In tetrads derived from a diploid homozygous for *tyr7-1*, *trp1-1*, and *prb1-9* and heterozygous for a dominant amber suppressor, the abilities to synthesize tryptophan and tyrosine and to solubilize HPA cosegregated 2:2. Moreover, when the suppressor-bearing spore clones were crossed to a wild-type strain, meiotic segregants incapable of solubilizing HPA could be recovered.

Several *prb1* mutants have been examined by phase contrast and fluorescence microscopy. All of the mutants, including the one bearing the amber mutation, *prb1-9*, formed septa that stained with Calcofluor ST, a chitin-specific fluorescent stain (23) (Fig. 4).

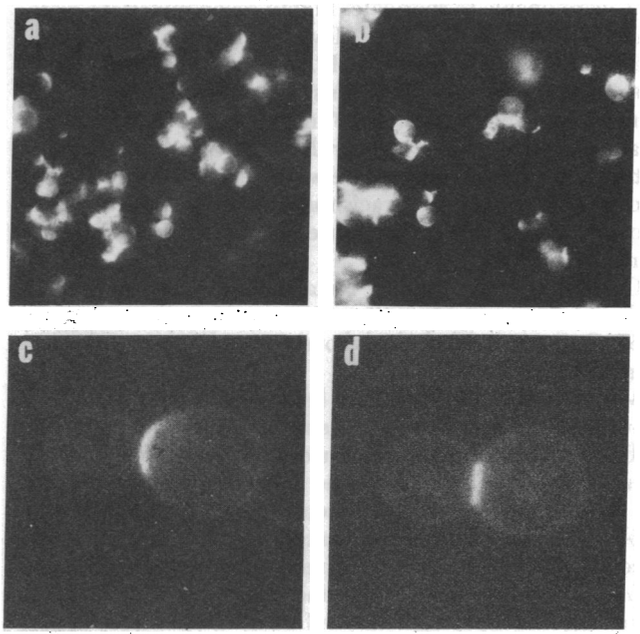

FIG. 4. Fluorescence micrographs showing septum formation in logarithmic phase cells of the wild-type (*a* and *c*) and *prb1-9* (*b* and *d*) strains as in (23). Calcofluor ST binds to chitin in septa and bud scars and fluoresces brightly when so bound. (Calcofluor ST; *a* and *b*, ×556; *c* and *d*, ×3476.)

DISCUSSION

If the phenotypes of mutants that lack a particular enzyme are to be used to infer the physiological function of that enzyme, it is necessary to ensure that the mutations present in those mutants lie in the structural gene for that enzyme. This is especially important if one is to separate pleiotropic effects attending loss of the enzyme activity from pleiotropic effects arising because more than one enzyme activity has been lost as a consequence of the mutation.

We infer that *prb1* is the structural gene for proteinase B because (*i*) two *prb1* alleles, *prb1-29* and *prb1-38*, cause thermolabile proteinase B activities that are distinguishable from one another and from the activity of the wild type and (*ii*) specific activities of proteinase B in extracts of various diploids and tetraploids show a dosage effect—i.e., the specific activity of proteinase B in extracts is proportional to the number and type of alleles present in the diploid or tetraploid.

An alternative explanation would have *prb1* encode a posttranslational modification enzyme. We consider this explanation unlikely. Because most of the *prb1* mutants lack proteinase B activity, one would have to postulate that modification is essential for activity. One could only account for the thermolabile species present in *prb1-29* and *prb1-38* by postulating that proteinase B has been mismodified in the two mutants and that the mismodifications are different in the two mutants.

One expects structural gene mutations to show dosage effects, whereas mutations affecting posttranslational modification enzymes should not show dosage effects on the modified enzyme. This is what has been found for mutations affecting α-mannosidase-1 of *Dictyostelium*. The *modA* mutations, which affect modification of α-mannosidase-1, do not show a dosage effect (24), whereas mutations in the α-mannosidase-1 structural gene, *manA*, do show a dosage effect (25).

In addition, one would not expect posttranslational modification to be the limiting factor in production of proteinase B activity. If one were to account for finding thermolabile proteinase B activity in the proportion detected in the tetraploid containing one wild-type and three *prb1-38* alleles, one would

have to postulate that the modification enzyme is limiting for production of proteinase B activity.

The hypothesis put forth by Ulane and Cabib (6), that proteinase B, present in vacuoles, was responsible for activation of chitin synthetase zymogen by controlled fusion of proteinase B-containing vesicles to the plasma membrane, rested on the observations that chitin synthetase was present in zymogen form in plasma membranes and that the zymogen could be activated, *in vitro*, by proteinase B as well as trypsin. Our results suggest that proteinase B is not essential for activation of chitin synthetase zymogen to allow formation of the primary septum because all 126 *prb1* mutants can grow at all temperatures tested. All of the mutants examined, including the one bearing the amber mutation *prb1-9*, form septa that stain with Calcofluor ST, a chitin-specific stain. Our results do not exclude the possibility that proteinase B might participate in the activation or that, in the absence of proteinase B activity, an auxiliary mechanism might function. The hypothesis implicating proteinase B in the activation was based on *in vitro* experiments. No evidence on the nature of the *in vivo* activation, be it proteolytic or allosteric in nature, is currently available.

Of the 126 mutations we have isolated at the *prb1* locus, 46 are amber or ochre mutations (unpublished observations). This frequency is comparable with frequencies found for other structural gene loci (26–28). Hence, it seems unlikely that the mutations we have isolated at *prb1* are a selected group that result in production of proteinase B molecules that have lost activity toward HPA but retain the ability to activate chitin synthetase zymogen. Until we can isolate deletions for *prb1* or construct a fine structure map for the locus, this question will remain unsolved.

Our finding that most diploids homozygous for *prb1* mutations cannot sporulate provides strong evidence that proteinase B activity is required for sporulation.

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