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Proteinase C (Carboxypeptidase Y) Mutant of Yeast

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A mutant of yeast lacking proteinase C (carboxypeptidase Y) activity has been found by using a histochemical stain to screen mutagenized colonies. This defect segregates 2:2 in meiotic tetrads. Cell extracts lacked the esterolytic, amidase, and proteolytic activities associated with proteinase C. The absence of proteinase C does not affect mitotic growth and has no obvious effect on the formation of viable ascospores or meiotic segregation. The mutant grows on peptides known to be cleaved by proteinase C in vitro. This finding is consistent with the idea that other enzymes exist in vivo with overlapping substrate specificities.

The following different types of functions have been found for proteinases; (i) maturation of proteins by removal of the N-terminal methionine residue from newly synthesized proteins (33); (ii) specific modifications of individual proteins resulting in activation or inactivation of a particular enzyme activity (6) or modification of enzyme activity or specificity (30); (iii) selective elimination of defective protein molecules (9); (iv) protein turnover for supplying amino acids and energy in the case of starvation and cell differentiation (sporulation) at the expense of unneeded cellular protein (10, 24); and (v) utilization of peptides as sources of amino acids and nitrogen (in the case of peptidases [29]).

The proteolytic enzymes best characterized biochemically in yeast are the proteinases A and B (12, 13, 22, 31) and proteinase C (also called carboxypeptidase Y) (7, 13, 15, 17, 21). There is very little known concerning their physiological role in the yeast cell. Several in vitro observations were recently reported that gave some indications of the possible in vivo physiological functions of proteinases A and B: studies showed the participation of proteinase A and B in the inactivation of tryptophan synthase (20, 31, 34). Proteinase B was also found to activate chitin synthetase (3, 4, 11). The role of proteinase C is not known. All three proteinases were shown to be inhibited by cellular protein inhibitors in in vitro studies (2, 28, 32). The proteinases activate themselves in an autocatalytic cycle in vitro (32). Nothing is known about the physiological role of the inhibitors.

A convincing analysis of the biological role of these proteinases requires both a biochemical and a genetic approach. Mutant strains, that lack specifically one or more of the proteolytic enzymes, could provide the important link between the in vitro analysis of the proteolytic activity and the biological events catalyzed by that activity. A study of proteinase-deficient mutants could determine which of these enzymes perform vital functions for the cell.

In this paper we report the isolation of a mutant lacking proteinase C. The growth properties of this strain together with the biochemical studies suggest the presence of other peptidases with overlapping specificities.

MATERIALS AND METHODS

Yeast strains. The haploid yeast strains used in this work were strain S288C (α, *mal*$^-$), obtained from R. K. Mortimer, and strain 4275-2A (*a*, *mal*$^-$), our standard mating type *a* strain. All mutants were derived from these strains.

Media and growth conditions. The minimal medium contained 0.7% yeast nitrogen base (Difco) and 2% glucose. The complete medium (YPD) contained 1% yeast extract (Difco), 2% peptone (Difco), and 2% glucose. When these media were used as solid media, they contained 2% agar (Difco). Liquid cultures were grown for 48 h at 30 C into stationary phase and aerated by shaking. The presporulation medium contained 5% dextrose, 1% yeast extract, 3% nutrient broth, and 2% agar. Two methods of sporulation were used. In one procedure the diploids were sporulated on agar plates. This medium contained: 0.98% potassium acetate, 0.1% dextrose, 0.125% yeast extract, and 2% agar. In the alternative procedure diploids were sporulated in liquid medium. After growth for 14 h at 30 C in YPD medium, cells were diluted into 1% potassium acetate so that the cell density was 1×10^7 to 2×10^7 cells/ml.

Procedure for detecting the absence of esterolytic activity in yeast colonies grown on solid medium. A culture of yeast was treated with ethyl

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methane sulfonate (Eastman Organic Chemicals) for mutagenesis as described by Fink (8) and plated on either solid minimal medium or on minimal medium supplemented with 1% acid casein hydrolysate (Difco). Glass petri dishes were used because the toluene procedure destroys plastic plates. After the mutagenized yeast cells grew into colonies, the plates were placed in a hood at room temperature and the surface of each was covered with 10 ml of toluene. The toluene evaporated completely within about 2 h. Mutants lacking proteinase activity were detected by subjecting the cells to a staining procedure similar to that described by Miller and MacKinnon (29). The knowledge that proteinase C cleaves the synthetic chymotrypsin substrate *N*-acetyl-tyrosine ethylester (7), led to the use of *N*-acetyl-phenylalanine-β-naphthyl-ester (APNE) (Schwarz/Mann) as substrate. After toluene treatment, the cells on the plate were stained for enzymatic activity by a soft agar overlay (about 2-mm thick) containing the proteinase reagents. The overlay was 0.7 g of agar, 0.2 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.4), 2 ml of APNE solution (10 mg/ml in dimethylformamide), 200 mg of Fast Garnet GBC (Sigma Chemical Co.), and 8 ml of dimethylformamide (Sigma Chemical Co.) in 100 ml. The reagents were added to molten 0.7% agar made up in the tris-(hydroxymethyl)aminomethane-hydrochloride buffer. To prevent precipitation of the APNE, it was necessary to add the dimethylformamide before adding the APNE solution. Colonies containing proteinase C showed a dark red color within 1 to 2 h, whereas mutants showed only a slight reddish color within this time.

Isolation of the pro mutant. Colonies which stained weakly with APNE were picked with a sterile inoculating needle and streaked on minimal medium. It was possible to pick directly from the staining plate because the toluenization and staining procedure fail to kill the entire colony. The purified clones were tested again for staining ability, and a single colony, picked from each presumptive mutant, was grown and restreaked. After this purification, the putative mutant was tested again by the staining procedure to make sure that the colony chosen had a phenotype different from wild type. Stains showing a clear-cut mutant phenotype were studied biochemically. The strains were grown and crude extracts were prepared and tested for proteinase C activity as described above. Any strain that appeared defective in this activity was crossed to wild type. Spore progeny showing segregation of the staining ability were again backcrossed to wild type.

Genetic analysis. The procedures for sporulation and tetrad dissection have been described by Hawthorne and Mortimer (14).

Preparation of extracts. Cells were harvested by centrifugation, washed once with distilled water, and resuspended in 0.075 M imidazole chloride buffer (pH 7.4). The ratio of cells to buffer was 1:1 (wt/vol). The cells were broken (3 × 15 s each) with glass beads (two times the weight of cells plus buffer) in a homogenizer (Braun-Melsungen, West Germany). The suspension was centrifuged 40 min at 30,000 × *g* in a Sorvall RC2B centrifuge. In case the crude extract was not clear, it was recentrifuged 1 h at 100,000 × *g* in a Beckman model L2-65B centrifuge.

Gel electrophoresis. Proteins were separated by electrophoresis on acrylamide gel columns according to the procedure of Hedrick and Smith (19) with the following modifications. To 100 ml of the spacer gel buffer 0.1 ml of *N,N,N',N'*-tetramethylethylene diamine (Sigma Chemical Co.) was added. A separating gel of 7.5% acrylamide was prepared according to Davis (5). Sixty microliters of the cell-free extract (approximately 12 mg/ml of protein) was layered on top of the gel. Electrophoresis was allowed to proceed at 1.25 mA/tube until the sample had entered the separating gel and then at 2.5/tube for approximately 2 h. The temperature during electrophoresis was maintained at 8 C.

Gel stain for esterolytic activity. The acrylamide gels were stained for esterolytic activity after electrophoresis using the same substrate (APNE)-dye (Fast Garnet GBC) mixture used for the screening of the proteinase C mutant, except that the agar was omitted. The staining buffer was 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.0).

Proteinase activity assays. Proteinase A activity was measured according to Saheki and Holzer (31) using hemoglobin as a substrate. The trichloroacetic acid-soluble products released by proteinase A action were measured by using the Folin reagent according to Lowry et al. (25). One unit is defined as 1 μg of tyrosine containing peptides released into the supernatant fluid per min per mg of enzyme at 25 C. Proteinase B activity was measured with azocoll as substrate according to Saheki and Holzer (31). Proteinase C was measured by two methods: (i) its ability to hydrolyze 4% casein at pH 6.0 is described by Doi et al. (7); (ii) its ability to cleave the synthetic substrate *N*-benzoyl-L-tyrosine-*p*-nitroanilide (BTPNA) (Sigma Chemical Co.; Aibara et al. [1]).

Protein determination. Protein was determined by the method of Lowry et al. (25) using insulin as a standard for comparison.

RESULTS

Detection of proteinase C: activity. Mutants with reduced staining activity were screened for reduced proteinase C activity by subjecting crude extracts to a number of biochemical tests. Since proteinase C is the major activity cleaving ester substrates known to be cleaved by chymotrypsin, reduced staining of the colonies is likely to result from lower proteinase C activity. A number of other mutational events could, however, lead to the same phenotype. For example, crude extracts contain an inhibitor of proteinase C (16, 18, 28) which is destroyed by proteinase A and B (16, 28, 32). Since proteinase C activity is dependent upon the activity of proteinases A and B, lowered proteinase C in the petri plate staining proce-

dure might result from mutational alterations in the structure of proteinase A or B. Strains with altered proteinase C inhibitors insensitive to activation by proteinases A and B might also appear as mutants in our procedure. It is also possible that, rather than proteinase C, another proteinase with esterolytic activity is rendered inactive by the mutation. Proteinase B is known to have esterolytic activity.

Specific detection of proteinase C was achieved by subjecting crude extracts of cells, which were grown 48 h into stationary phase, to a battery of three tests: (i) ability to hydrolyze casein, (ii) activity against BTPNA, and (iii) the appearance of electrophoresis gels stained with APNE. In all these tests, the inhibitor of proteinase C posed a potential problem. Proteinase C is reported to be located in the vacuole of the yeast cell (23, 27) and the inhibitor is reported to be in the cytoplasm (23, 27, 28). However, breaking of the yeast cells leads to destruction of the vacuoles resulting in the inhibition of proteinase C. Reactivation of proteinase C activity can be achieved by incubating crude extracts at around pH 5.0 for 20 h at 25 C or at 4 C for a longer period (16, 18, 32), leading to digestion of the inhibitor by proteinase A and B. The reactivation can also be achieved in a shorter period of time by incubation with organic solvents (16, 18).

APNE activity on acrylamide gels. When crude yeast extracts were subjected to electrophoresis and stained, several bands of activity against APNE appeared (Fig. 1A). There were two major activities (band 1 and 2) and one minor activity (band 3). If the crude extract was incubated for 24 h at pH 5.2 and 25 C prior to electrophoresis, bands 1 and 2 were absent, the intensity of band 3 was dramatically increased, and four new minor bands appeared (Fig. 1B). A number of observations suggest that bands 2 and 3 result from proteinase C. (i) When the gels were sliced and the contents were eluted and assayed for activity with casein as substrate, the positions of the activity against casein were in reasonable agreement with the APNE activity (Fig. 1). (ii) In fresh crude extracts (gel A), the activity against casein from

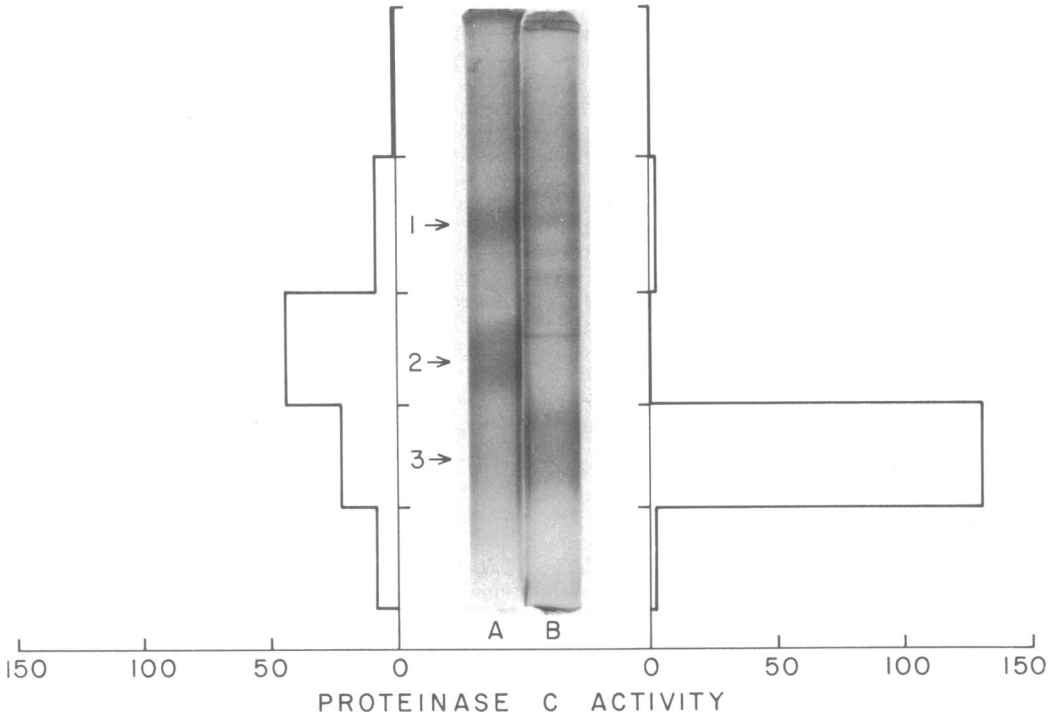

FIG. 1. *Disc electrophoresis of fresh (A) and activated (B) crude extracts of wild-type yeast and subsequent test of proteinase C activity. Gels (length 5.6 cm) were stained with APNE and Fast Garnet GBC. In a parallel experiment, a gel was sliced into five sections (as indicated on the ordinate), which were eluted with 1.2 ml of 0.04 M potassium phosphate buffer (pH 6.0). The hydrolytic activity of each section against casein was measured as the absorbance of trichloroacetic acid-soluble material at 280 nm/min per ml of enzyme.*

the region of band 2 was higher than that in the region of band 3, whereas the hydrolytic activity against casein in activated crude extracts (gel B) was greatly enhanced in the region of band 3 and disappeared in the location of band 2. (iii) Bands 2 and 3 as defined by APNE activity also stained with BTPNA. The color disappeared by diffusion and was difficult to photograph. (iv) Phenylmethylsulfonylfluoride and *p*-chloromercuribenzoate, both potent inhibitors of proteinase C (13, 21), diminished bands 2 and 3 on the gels as well as the hydrolytic activity against casein of the sliced regions of bands 2 and 3 (Table 1). It is known that the binding of the native protein inhibitor is reversible (28). Disappearance of the hydrolytic activity in the location of band 2 and concommitant dramatic increase of this activity in the region of band 3 upon activation of the crude extracts are consistent with the suggestion that the activity of band 2 is due to the proteinase C-inhibitor complex, which dissociates under staining conditions liberating proteinase C, and band 3 is due to proteinase C. This view is also supported by the mode of action of the inhibitors phenylmethylsulfonylfluoride and *p*-chloromercuribenzoate. Although the inhibition of the activity in the location of band 3 was almost complete, inhibition of the activity in location of band 2 was much less (Table 1). This observation could be explained by liberation of proteinase C activity from the native proteinase C-inhibitor complex after incubation with phenylmethylsulfonylfluoride or *p*-chloromercuribenzoate, as was shown in studies using diisopropylfluorophosphate, an inhibitor with the same specificity as phenylmethylsulfonylfluoride (16).

A mutant lacking proteinase C activity. Out of about 10^4 plated colonies, 20 colonies, which failed to stain as intensively as wild type with APNE, were grown, extracted, and analyzed for proteinase C activity on acrylamide gels before and after activation. One isolate was found to lack bands 2 and 3, which we had associated with proteinase C. Band 3 was missing even after a period of activation (24 h, 25 C; Fig. 2), which gives a distinctive dark band in gels from wild type. No activity against casein could be obtained from gel slices under conditions, which gave activity in wild type. In addition proteinase C was assayed directly in crude extracts with the use of BTPNA as a substrate. Table 2 shows that strain C29 is completely devoid of proteinase C activity. Furthermore, fresh extracts activated by the organic solvent dimethylformamide (16% vol/vol) or extracts activated by prolonged preincubation at pH 5.2 at 25 C for 48 h did not lead to any activity against BTPNA. Proteinase A and B were present in approximately normal amounts in the extract of the mutant strain. The inhibitor of proteinase C in extracts of mutant C29 was inactivated by treatment which inactivates the inhibitor from wild type. The results shown in Table 3 demonstrate that extracts of mutant C29 have a powerful inhibitor of proteinase C as measured by the BTPNA assay. After incubation, this inhibitor was destroyed and failed to affect the activity of wild-type proteinase C. These results indicate that the absence of proteinase C activity does not result from a failure to inactivate the inhibitor due to some mutation in the inhibitor.

TABLE 1. *Inhibition by phenylmethylsulfonylfluoride (PMSF, 1 mM) and p-chloromercuribenzoate (PCMB, 1 mM) of the proteolytic activity against casein found in band 2 and band 3 after gel electrophoresis of fresh wild-type crude extract*[a]

Inhibitor	% Inhibition	
	Band 2	Band 3
PMSF	60	98
PCMB	25	80

[a] The gel was sliced and eluted as described in the legend to Fig. 1. Then 200-μl samples of the eluate were treated 90 min at 25 C with either PMSF, PCMB, or no added inhibitor and tested.

Segregation pattern of the proteinase C deficiency. Strain C29, deficient in proteinase C, was crossed by wild-type strain 4275-2A, which has normal proteinase C activity. Diploids were sporulated, asci were dissected, and the spore clones were germinated and analyzed.

The ascospore clones were analyzed for their ability to stain with APNE as well as the proteinase C activity of their crude extracts against BTPNA. Twelve tetrads were analyzed and each showed 2:2 segregation of $APNE^+$:$APNE^-$ and $BTPNA^+$:$BTPNA^-$. A typical tetrad is shown in Fig. 3. We have called the mutation which leads to absence of proteinase C in strain C29 *prc1-1*.

Growth behavior. The mutant C29 grew at wild-type growth rates on complete medium as well as on minimal medium at 30 and 37 C. This strain did not grow at a rate significantly different from that of wild type on the dipeptides Gly-Leu, *N*-Cbz-Gly-Leu, or *N*-Cbz-Ala-Leu as sole nitrogen sources, all known to be cleaved by proteinase C in vitro (15, 21).

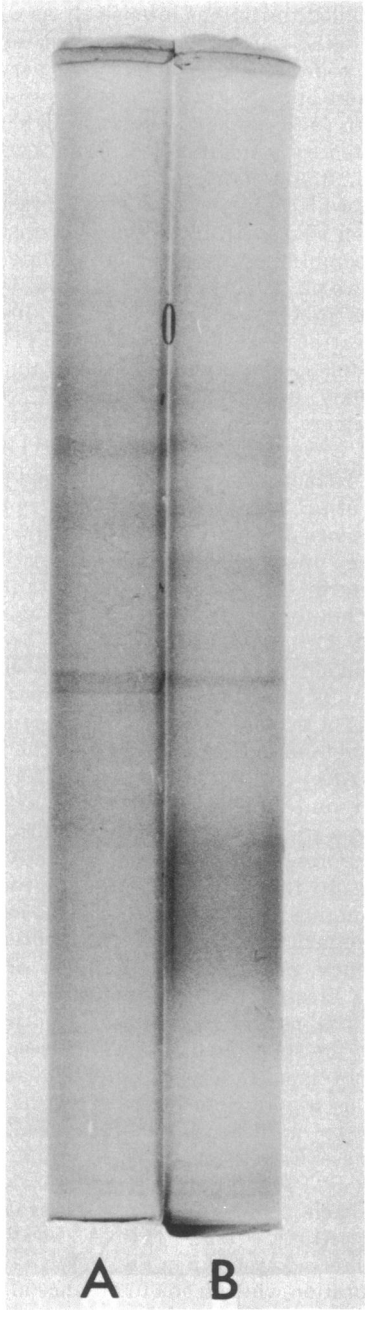

FIG. 2. *Disc gel electrophoresis of activated mutant C29 (A) and wild-type S288C (B) crude extracts followed by activity staining of proteinase C.*

Sporulation. A diploid heterozygous for *prc* (*prc*/+) was sporulated and tetrads were dissected to obtain ascospore clones. These were used as parents in testing the effect of the *prc* gene product on sporulation frequency. Seven *prc* ascospores were mated in different pair-wise combinations to produce six diploids homozygous for *prc* (*prc*/*prc*), and seven wild-type ascospores were mated in a similar manner to produce six diploids homozygous for the wild-type gene (+/+). The frequency of sporulated cells obtained from the mutant (*prc*/*prc*) diploids was 39%, whereas the frequency from the wild-type (+/+) diploids was 44%.

TABLE 2. *Activity pattern of proteinases A, B, and C in crude extracts of the wild-type (S288C) and the mutant strain (C29) after 24 h of activation at 25 C and pH 5.2*

Activity measured	Sp act[a]	
	S288C	C29
Proteinase A	22×10^{-3}	18.5×10^{-3}
Proteinase B	83×10^{-3}	62×10^{-3}
Proteinase C	25×10^{-3}	0

[a] Proteinase A activity was measured against hemoglobin, proteinase B activity against azocoll, and proteinase C activity against BTPNA.

TABLE 3. *Proteinase C[a] activity against BTPNA after addition of fresh and activated crude extracts of the mutant strain C29 (prc1-1)*

Addition	Proteinase C activity ([absorbance at 410 nm]/min per ml)
None	0.390
Fresh mutant crude extract (2.8 mg)	0
Activated mutant crude extract (2.3 mg)	0.355

[a] Every reaction mix contains activated proteinase C from wild type.

DISCUSSION

The results presented in this paper show that a mutation in the *prc* gene leads to the absence of proteinase C activity. There are a number of possible explanations for this phenotype. The *prc* mutation could be a (i) mutation in the structural gene, coding for the proteinase C, (ii) mutation affecting the regulation of the proteinase C, or (iii) mutation affecting the inhibitor-proteinase C relationship. One form of explanation (iii) is that *prc1* is a mutation affecting the inhibitor so that it irreversibly inactivates proteinase C. We have shown that the inhibitor in the *prc1* strain is accessible to digestion by proteinase A and B, and wild-type proteinase C, when added to these digested extracts, has normal activity. These results make possibility (iii) unlikely. Our experiments cannot distinguish between possibilities (i) and (ii). If we had

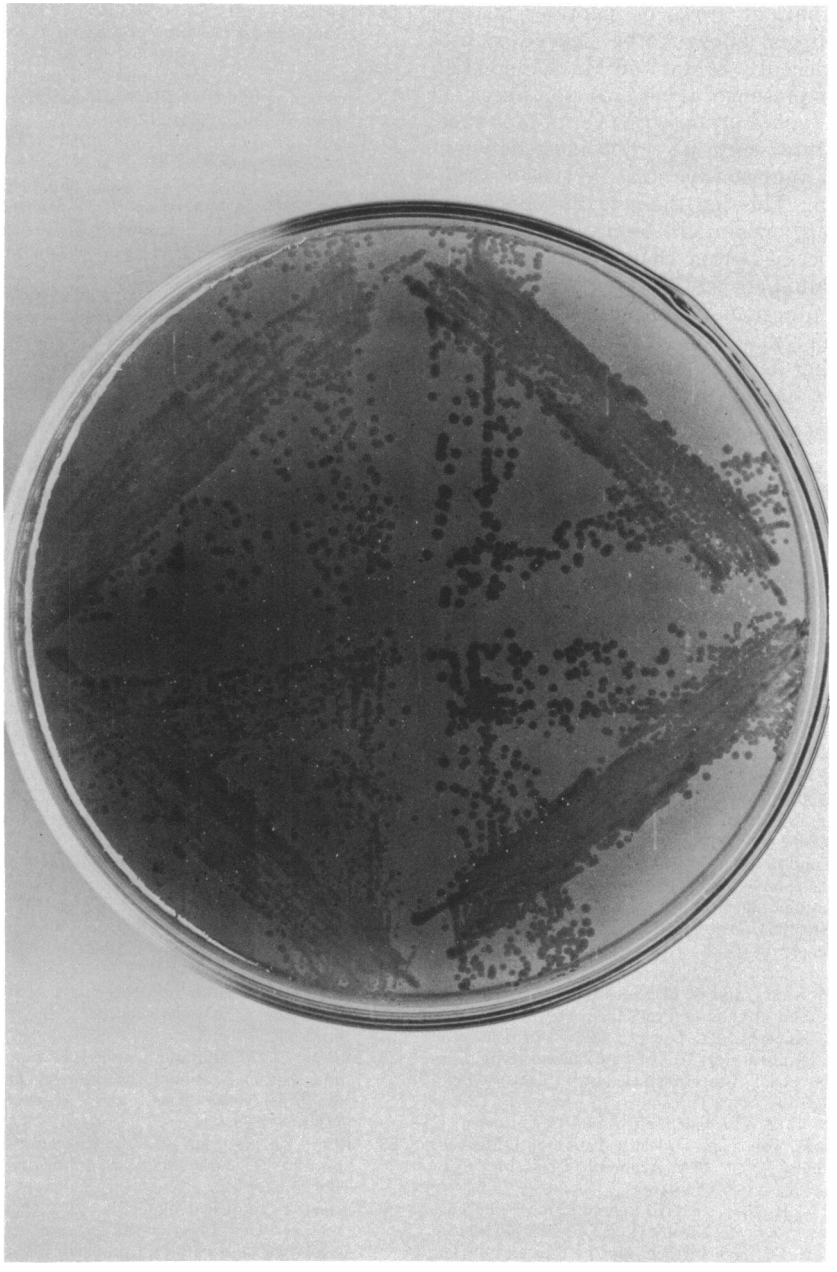

FIG. 3. *Segregation (2:2) of staining ability for proteinase C from a spore tetrad derived from the cross: mutant C29 × wild-type 4275-2A.*

a temperature-sensitive *prc1* mutant with a temperature-sensitive proteinase C, we would have stronger evidence that *prc1* were the structural gene for proteinase C. The proteinase C deficiency did not seem to affect the behavior of the *prc1* mutant during the vegetative cell cycle or the meiotic cycle. Nevertheless, proteinase C could still have a vital role in the life of the cell, but in its absence other proteinases with overlapping specificities might be able to compensate for the deficiency. Several aspects of our studies suggest the existence of proteinases with overlapping specificities. The *prc1* mutant is not totally devoid of activity against APNE. The mutant colonies stain faintly in the petri plate test. Moreover, gel electrophoresis showed several APNE activities distinct from proteinase C remain in the *prc1* mutant. In addition

the *prc1-1* mutant grows on peptides (as sole nitrogen sources) known to be cleaved by proteinase C. Recently Miller and MacKinnon (29) showed the presence of several peptidases in *Salmonella typhimurium* with overlapping specificities. Only multiply peptidase-deficient strains grew more slowly than wild type on minimal medium. This led them to the conclusion that these enzymes might be involved in intracellular processes other than hydrolysis of exogenously supplied peptides. If there are peptidases with functions overlapping those of proteinase C in yeast, strains carrying mutations in these other activities would be necessary to demonstrate the biological role of proteinase C.

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