

Chitin Synthase 1, an Auxiliary Enzyme for Chitin Synthesis in *Saccharomyces cerevisiae*

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Abstract. Previously, we showed that chitin synthase 2 (Chs2) is required for septum formation in *Saccharomyces cerevisiae*, whereas chitin synthase 1 (Chs1) does not appear to be an essential enzyme. However, in strains carrying a disrupted *CHS1* gene, frequent lysis of buds is observed. Lysis occurs after nuclear separation and appears to result from damage to the cell wall, as indicated by osmotic stabilization and by a ~50-nm orifice at the center of the birth scar. Lysis occurs at a low pH and is prevented by buffering the medium above pH 5. A likely candidate for the lytic system is a previously described chitinase that is probably involved in cell separation. The chi-

tinase has a very acidic pH optimum and a location in the periplasmic space that exposes it to external pH. Accordingly, allosamidin, a specific chitinase inhibitor, substantially reduced the number of lysed cells. Because the presence of Chs1 in the cell abolishes lysis, it is concluded that damage to the cell wall is caused by excessive chitinase activity at acidic pH, which can normally be repaired through chitin synthesis by Chs1. The latter emerges as an auxiliary or emergency enzyme. Other experiments suggest that both Chs1 and Chs2 collaborate in the repair synthesis of chitin, whereas Chs1 cannot substitute for Chs2 in septum formation.

THE primary septum of the yeast, *Saccharomyces cerevisiae*, consists largely of the polysaccharide chitin (15, 17). The biosynthesis of chitin has been extensively studied, since the septum is both a useful model for morphogenesis and a potential target for antifungal agents (3, 4). Cloning of the structural gene for chitin synthase 1 (Chs1),¹ at that time the only chitin synthase known in yeast, followed by disruption of the gene, produced the unexpected result that Chs1 is not essential for chitin synthesis in vivo (2). Shortly thereafter, a new chitin synthase activity, chitin synthase 2 (Chs2), was detected in yeast (16, 22). Subsequent cloning of *CHS2* and its disruption showed unequivocally that this gene is essential for septum formation and chitin synthesis (24). In the presence of a *CHS2* disruption, Chs1 could not substitute for Chs2 and its function remained obscure.

In previous work (2) it was ascertained that cells carrying a disrupted *CHS1* gene frequently give rise to lysing buds when grown in minimal medium. Starting from this fact, we have accumulated evidence to indicate that Chs1, although not required under optimal conditions, can serve as an auxiliary or repair enzyme in certain limiting situations.

1. *Abbreviations used in this paper:* Chs1, chitin synthase 1; Chs2, chitin synthase 2; DAPI, 4',6-diamidino-2-phenyl-indole; MU(Ch)₃, 4-methylumbelliferyl-β-D-N,N',N''-triacylchitotriose.

Materials and Methods

Reagents and Enzymes

[³H]Chitin was prepared by acetylation of chitosan as described (14). 4-Methylumbelliferyl-β-D-N,N',N''-triacylchitotriose (MU[Ch]₃) was obtained from Calbiochem-Behring Corp. (La Jolla, CA). [2-¹⁴C] Propionic acid (sp. act. 50 mCi/mmol) was from Amersham Corp. (Arlington Heights, IL). Deoxyribonuclease I was purchased from Sigma Chemical Co. (St. Louis, MO). Polyoxin D was kindly provided by Schering-Plough Corp. (Bloomfield, NJ) and by Kaken Chemical Company (Tokyo, Japan). Allosamidin was a generous gift of A. Isogai (The University of Tokyo, Tokyo, Japan) and of P. Somers (Eli Lilly and Company, Indianapolis, IN). The product from Eli Lilly, which was used in most experiments because of the larger amount available, was named A82516, but appears to be identical to allosamidin. Purified yeast chitinase was from an earlier preparation obtained in this laboratory (5).

Strains and Cell Growth

The strains of *S. cerevisiae* used in most experiments were D3A (*MATα, ade2-101, ura3-52, chs1::URA3*), D3B (*MATα, his4, ura3-52, chs1::URA3*), D3C (*MATα, ura3-52*), and D3D (*MATα, ade2-101, his4, ura3-52*), all from the same tetrad. In some experiments, a diploid (*prb1-1122/prb1-1122*, see reference 5) was used to measure chitinase activity in intact cells.

Yeast was grown at 30°C either in YEPD (2% glucose, 2% peptone, 1% yeast extract) or in minimal medium (2% glucose, 0.7% yeast nitrogen base) plus nutritional supplements. When a buffer at pH 5.8 was added to minimal medium, the final concentration was 85 mM for succinate and 100 mM for 2-(*N*-morpholino)ethane sulfonic acid (MES) or phthalate.

Counting of Refractile Cells

Counting of normal and refractile cells was performed with a Universal

Zeiss microscope equipped with a phase contrast condenser. Between 350 and 450 cells were counted in each determination. A bud was counted as a separate cell. In a blind experiment, coded samples containing suspensions of *chs1::URA3* and *CHS1* cells in different proportions were counted by one experienced operator and two operators without previous experience. In all three cases, the percentage of refractile cells showed a linear dependence on the proportion of the *chs1::URA3* strain (results not shown). In this experiment, the range of percentages of refractile cells covered most of the values found in the present study.

Nuclear Staining

4',6-Diamidino-2-phenyl-indole (DAPI) was used to stain nuclei. Manipulation, including centrifugation, had to be kept to a minimum to prevent detachment of lysed buds from the mother cell, which occurs very easily. Also, fixation was omitted to distinguish intact cells from lysed cells, which were already permeable to DAPI. Therefore, staining was performed simply by adding 0.1 ml of 1 M phosphate, pH 7.2, and 1 μ l of DAPI at 1 mg/ml to 0.9 ml of culture. After 10 min at room temperature, a drop of the preparation was mounted on a glass slide and observed both under phase contrast and fluorescence. In controls where it was desired to stain all nuclei, cells were permeabilized by adding 0.1 ml of 1 M phosphate, pH 7.2, and 0.1 ml of 10% digitonin to 0.9 ml of culture. After 15 min incubation at 30°C, DAPI was added as above.

Incubations with DNase were performed for 4.5 h at 37°C in the presence of 50 mM Tris chloride, pH 7.5, 10 mM MgCl₂, and 1 mg/ml of the enzyme.

Samples were observed for fluorescence with a Zeiss ICM 405 microscope equipped with a G365 exciting filter, an FT395 beam splitter, and an LP 420 barrier filter.

Electron Microscopy

Cells were harvested, washed with water, and enrobed in agar, followed by fixation with 3% glutaraldehyde in 0.1 M phosphate, pH 7, for 1 h at room temperature. After overnight storage at 5°C, the fixed cells were rinsed twice (15 min each time) in 0.1 M sodium phosphate, pH 6.8, and postfixed in 1% OsO₄ in the same buffer for 30 min at room temperature. Ethanol dehydration was followed by embedding in Epon 812. Thin sections were stained with 1% aqueous uranyl acetate and 1% lead citrate for 5 min each.

Internal pH and Hydrogen Pump Efficiency

For the determination of internal pH, the uptake of [2-¹⁴C] propionic acid in the presence of different buffers was determined according to De La Pena et al. (6) as a function of time, until equilibrium was attained. The buffers used were *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid (pH 7), MES/Tris (pH 6.5), MES (pH 6.0, 5.5), ϵ -aminocaproic acid (pH 5.0, 4.5), and glycine (pH 3.6, 3.0).

For measurement of the ability to excrete protons, the procedure of Serrano (23) was adopted. Logarithmic phase cells (200 mg/ml, wet weight) were incubated in water at room temperature for 2 h under a current of nitrogen before adding 2 M glucose to a final concentration of 0.26 M and following the subsequent change in pH as a function of time.

Preparation and Assay of Chitinase

To obtain chitinase from the periplasmic space, the procedure previously used was followed (8). The extract was concentrated to a final volume equivalent in milliliters to the grams of yeast (wet weight) used in the experiment. The pH was adjusted to 3.0 with 1 M citric acid. The precipitate that formed was removed by centrifugation at 20,000 *g* for 10 min and the supernatant fluid was dialyzed overnight against 25 mM MES, pH 6.3.

Chitinase activity was determined both in intact cells and in extracts with MU(Ch)₃, as described by Kuranda and Robbins (12) or, in extracts only, with [³H]chitin as previously reported (14).

Treatment of Cells with Polyoxin D

To cultures of either D3B (*chs1::URA3*) or D3C (*CHS1*) containing $\sim 1.2 \times 10^7$ cells/ml in minimal medium, polyoxin D was added from a 20 mg/ml aqueous sterile solution to yield a final concentration of antibiotic between 125 and 750 μ g/ml. Cultures were incubated at 30°C until the control without polyoxin went through approximately two generations. Appropriate dilutions were plated on YEPD and the number of survivors was determined by counting the colonies 2 d later.

Results

Lysis of Cells Devoid of *Chs1* Occurs at the End of the Cell Cycle

As reported earlier, when cells of strains carrying a disrupted *CHS1* gene grow in minimal instead of rich medium, numerous buds and sometimes pairs of cells appear refractile when observed by phase contrast microscopy (see Fig. 10 in reference 2 and Fig. 1 A in this article). The refractile cells are actually lysed, since they take up trypan blue (Fig. 1 B). To define this phenomenon more clearly, an attempt was made to determine at which point in the cell cycle lysis took place. Because nuclear separation occurs rather late in the cycle, the presence of nuclei in the refractile buds was ascertained. For this purpose, it was necessary to modify the usual technique for DAPI staining because fixation obliterates the difference between refractile and nonrefractile cells, which thus become indistinguishable after staining. Direct addition of DAPI without fixation stained only refractile cells (Fig. 2, D and E). Phase-dark cells showed no nuclear staining (Fig. 2, A and B) although they manifested a dimmer, punctate fluorescence. Prior permeabilization with digitonin resulted in nuclear staining both in control cells and in those with a disrupted *CHS1* gene (Fig. 2, C and F). The staining of spontaneously lysed cells was more intense and diffuse than that of those permeabilized with digitonin (Fig. 2). This may be due to some nuclear lysis and dispersion of the chromatin in those cells. Incubation of the cells with pancreatic DNase before DAPI staining led to disappearance of most of the fluorescence from refractile cells, leaving only a diffuse, dim background. On the other hand, DNase treatment of normal

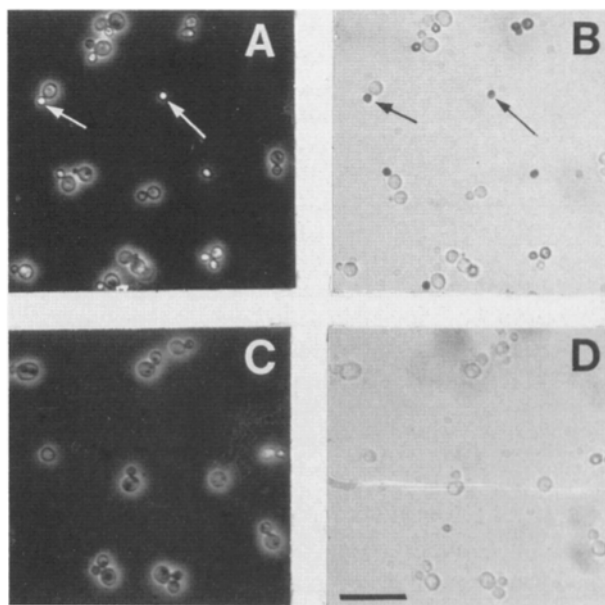


Figure 1. Lysis of buds in strain D3B (*chs1::URA3*) and its correction by succinate. (A and B) Cells grown in minimal medium. (C and D) Cells grown in minimal medium plus succinate. (A and C) Cells observed under phase contrast. (B and D) Cells stained with trypan blue (9) and observed under bright field illumination. Arrows point to some of the cells that are both refractile under phase contrast and stained by trypan blue. Bar, 20 μ m.

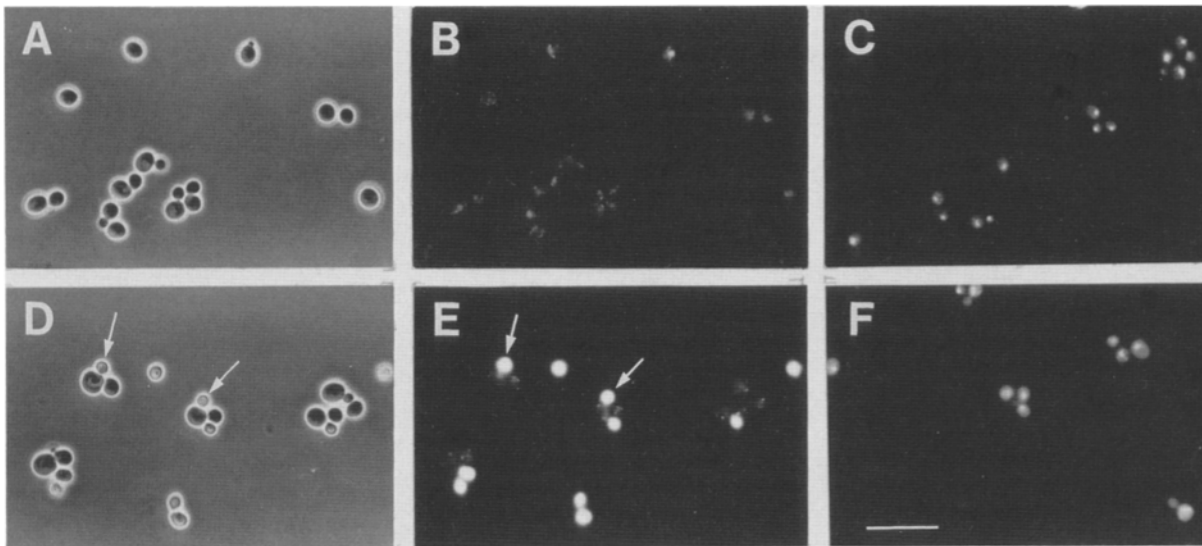


Figure 2. Nuclear staining with DAPI. (A–C) Strain D3C (control); (D–F) strain D3B (*chs1::URA3*). (A, B, D, and E) Intact cells, either under phase contrast (A and D) or fluorescence (B and E). (C and F) Cells permeabilized with digitonin, observed under fluorescence. Arrows in D and E point to some of the refractile cells that are also stained by DAPI. In these cells, the fluorescent staining seems to be uniform over the whole cell. At shorter exposures a smaller area could be identified that was much brighter than the surroundings. At that exposure, however, the unstained cells were no longer visible. Bar, 20 μ m.

cells did not affect subsequent DAPI staining in the digitonin-permeabilized cells (results not shown). From these results, it is concluded that lysis of the cell occurs after nuclear separation.

Next, we investigated the nature of the lytic process. In general, lysis may result from damage to the cell wall or to the plasma membrane. Only in the former case would an osmotic stabilizer prevent lysis. Inclusion of 1 M sorbitol in the growth medium afforded almost complete protection from lysis (not shown). It was concluded that damage to the cell wall was probably the cause of lysis. Indeed, many of the refractile cells that had separated from their mother cell showed a small appendage at one end at high magnification under phase contrast, suggesting that some material may have seeped out of the cell at that site. The appendage was found in \sim 40% of the single cells after staining with trypan blue, a condition that afforded better visibility of this feature. The actual value is probably much higher because observation of the small irregularity depended on a favorable position of the cell and on continued adherence of the material to the cell surface. To obtain a more detailed view, electron microscopy of thin sections was used to search for images that would correspond to those observed in the light microscope. In favorable sections a small hole (\sim 50 nm) could be seen in the cell wall. The hole was located in the daughter cell, at the center of the birth scar, and membranous material appeared to escape from it (Fig. 3). No such feature was found in sections of wild-type cells, either in this study or in many previous observations. These images suggested that the injury to the cell wall occurs after septa have already been formed and during the process of cell separation.

In several instances, plumes of membranous material seemed to emerge from the center of a bud scar, but in no case could an orifice be associated with them. It should be noted, however, that in some cases both mother and daughter cell were lysed (see Fig. 10 in reference 2) and perforations at the bud scar or elsewhere in the cell are also a possibility.

Effect of pH on Lysis of Cells Lacking Chs1 and Its Possible Meaning

It was mentioned above that appearance of lysed cells in strains carrying a *CHS1* disruption was observed in minimal but not in rich medium (YEPD). This difference is apparently due to the different buffering capacity of the two media. Yeast is known to excrete protons during growth (20). The pH of poorly buffered minimal medium falls to 3.0 or below in late growth. The peptone and yeast extract present in rich medium maintain the pH \sim 5.0. It was found that addition of a buffer to minimal medium, such as succinate at pH 5.8, almost completely prevented cell lysis (Fig 1, C and D) without affecting growth rate. MES was almost equally effective, whereas phthalate, while maintaining constant pH, gave rise to a substantial percentage of refractile cells (see below).

These results correlated lysis with acidification of the medium. It seemed possible that cells with a *CHS1* disruption might be unable to maintain internal pH in the face of an acidic medium: lysis would be a secondary effect of this acidification. Nevertheless, both measurement of internal pH of cells suspended in media of different acidity (Table I) and determination of proton pump efficiency (Table II) failed to disclose significant differences between wild-type and *chs1::URA3* cells. Thus, the effect of pH appeared to be external. In the light of this notion and of the evidence described in the preceding section, it can be seen that the system responsible for the cell lysis must meet several requirements: (a) it should be related to chitin metabolism because an intact *CHS1* gene abolishes lysis; (b) it should be exposed on the outside of the plasma membrane because of its sensitivity to external pH; (c) it should function maximally in a very acidic range of pH, as cell lysis is only observed below pH 5 and increases as the pH of the culture falls to 3; and (d) it should normally be active towards the end of the vegetative cell cycle because lysis occurs at that point (Fig. 3). A system that seems to meet all these requirements is the

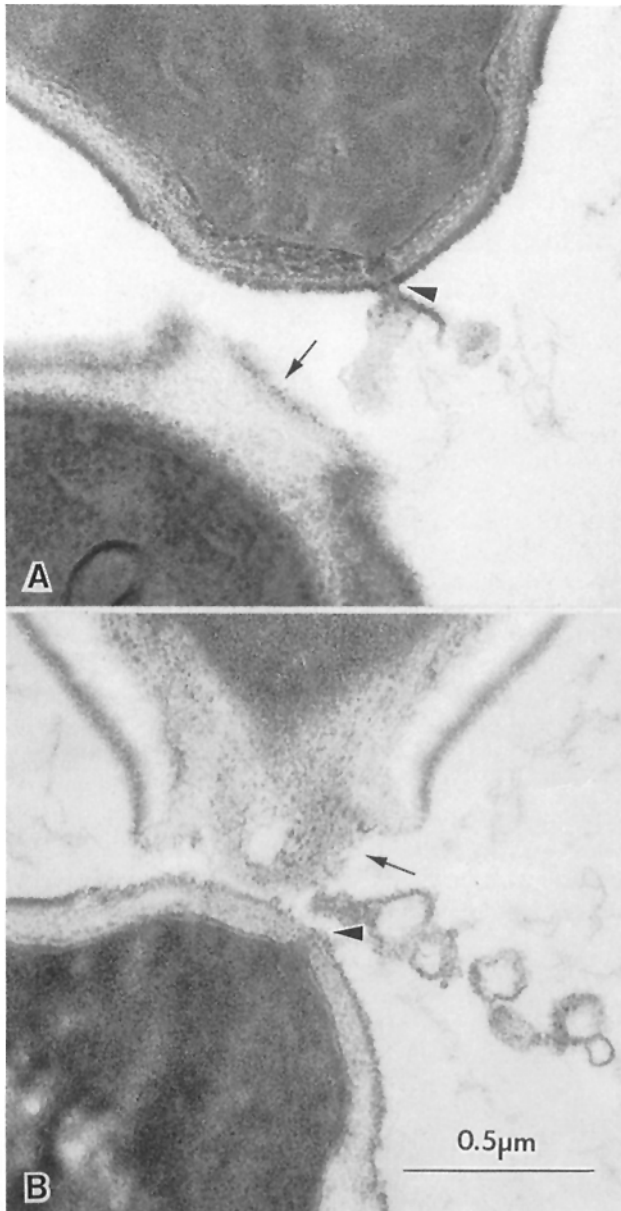


Figure 3. (A and B) Two examples of cell wall lesions in lysed cells of strain D3B (*chsl::URA3*). Arrows point to bud scar on mother cell. Arrowheads point to orifice at the center of the daughter cell birth scar. Some membranous material that can be seen near the orifice probably exited through it.

yeast chitinase previously detected and studied in this laboratory (5, 8). This chitinase is (a) obviously related to chitin metabolism, as a degradative enzyme; (b) exposed on the cell surface, since about half of it was found in the periplasmic space (8); (c) sensitive to pH in the acidic range, with a pH optimum ~ 3.0 (5), where cell lysis reaches a maximum; and (d) probably involved in the last step of the cell cycle, i.e., separation between mother cell and bud, which takes place essentially along the chitin septum (17) and is likely to require some hydrolysis of the polysaccharide.

The hypothesis that chitinase is the causative agent of the lysis provides a straightforward interpretation of the facts: the higher activity of chitinase at low pH would lead to exces-

sive degradation of chitin with damage to the cell wall, especially at the thinner daughter cell side. The mother cell would be protected by the thicker chitin layer (17). It should be kept in mind, however, that this protection is not absolute, since sometimes both mother and daughter cell appear to lyse, as mentioned in the preceding section. Our hypothesis also supplies a simple explanation for the function of Chsl. Because the presence of Chsl prevents lysis, it follows that this enzyme must be able to counteract the effect of the chitinase by synthesizing extra chitin; i.e., it acts as a repair or emergency synthase.

Chitinase Activity in Intact Cells

Some of the points considered in the preceding section required further corroboration. For instance, it was desirable to show experimentally that chitinase at the cell surface was exposed to the surroundings by measuring the activity of the enzyme in intact cells. The insolubility of chitin precluded its use as a substrate, because it cannot penetrate the cell wall. Use was made of a small fluorogenic substrate, 4-methyl-umbelliferyl-triacetylchitotriose. Incubation of this substance with intact cells led to an increase in fluorescence, with a linear dependence on time and amount of cells (Fig. 4). Unexpectedly, the chitinase measured in this fashion yielded a higher pH optimum than previously measured with purified enzyme (Fig. 5). However, this effect may be attributed to the substrate, because a similar curve was obtained with a purified chitinase preparation that showed normal behavior with chitin as substrate (Fig. 5). We conclude that the chitinase is exposed to the environment and normally sensitive to pH. It should be noticed, however, that with the strains mainly used in this study (D3B and D3C), crude preparations from the periplasmic space showed a less steep pH-activity curve than shown in Fig. 5 with chitin as substrate. The activity at pH 5 was $\sim 60\%$ of that at pH 3.

In the course of these experiments it could also be ascertained that growth in the presence of a buffer not only decreased chitinase activity because of the higher pH but also led to a lower total activity of the enzyme as measured at pH 3 (Table III). The combined effect may lower the actual enzymatic activity to about one-third that of control cells. Phthalate was less effective than succinate or MES in reducing the level of chitinase, in agreement with the higher proportion of refractile cells found with this buffer (Table III).

Table I. Internal pH of Cells with a Normal or a Disrupted *CHS1* Gene

External pH	Internal pH	
	D3C (<i>CHS1</i>)	D3B (<i>chsl::URA3</i>)
7.0	7.3	7.2
6.5	7.05	6.9
6	7.0	6.9
5.5	7.0	6.9
5.0	6.25	6.1
4.5	6.0	6.0

For technique, see Materials and Methods. Values for external pH values of 3.6 and 3.0 were also determined but are not included because of poor reproducibility. In each individual experiment, however, almost identical values were obtained for both strains.

Table II. Proton Pump Efficiency of Cells with a Normal or a Disrupted *CHS1* Gene

Strain	Relevant genotype	Initial pH	t ^{1/2} * min	Final pH [‡]
D3A	<i>chs1::URA3</i>	7.3	3.3	3.25
D3B	<i>chs1::URA3</i>	7.1	4.1	3.3
D3C	<i>CHS1</i>	7.3	4.4	3.3
D3D	<i>CHS1</i>	7.25	4.5	3.4

Proton pump efficiency was measured from the drop in pH as a function of time, after addition of glucose to a cell suspension (see Materials and Methods).

* Time required for half the total drop in pH to occur after the addition of glucose.

‡ Value measured after no further change in pH was detected.

The Effect of Allosamidin on Cell Lysis

If chitinase is the causative agent of lysis, addition to the medium of a specific inhibitor of this enzyme should decrease the number of refractile (lysed) cells. Such an inhibitor, allosamidin, recently became available (11, 21). Allosamidin is an analogue of diacetylchitobiose, in which the sugar residue is *N*-acetylallosamine rather than *N*-acetylglucosamine. The disaccharide is joined to an aminocyclitol. Allosamidin does inhibit yeast chitinase both in intact cells and in purified preparations (Fig. 6). With purified chitinase the inhibition was more pronounced when MU(Ch)₃ was used as substrate than with [³H]chitin. Since allosamidin acts as a competitive inhibitor (11), this may be due to the much higher concentration (as *N*-acetylglucosamine residues) of [³H]chitin in the assay. On the other hand, allosamidin was without effect on a crude mixture of yeast β(1-3)-glucanases, when assayed with laminarin as substrate (results not shown).

When added to the growth medium of a *chs1::URA3* strain, allosamidin did significantly reduce the number of refractile cells (Table IV). In the first experiment of Table IV the cells were first allowed to grow in minimal medium with added succinate and then transferred to medium without buffer. Under such circumstances, the appearance of refractile cells was very slow (the pH also decreased slowly) and the effect of allosamidin was most marked. In the second experiment, the starting cells came from unbuffered medium. Refractile cells appeared much more rapidly and the inhibition by allosamidin, although substantial, was weaker. It was also found that the extent of inhibition of lysis by allosamidin is concentration dependent (Fig. 6).

Do *Chs1* and *Chs2* Produce "Equivalent" Chitin?

From the results outlined above and those previously reported (2, 16, 22, 24) the picture that emerges is that of two chitin synthases, one (*Chs2*) essential for the formation of the primary septum, and the other (*Chs1*) able to provide extra chitin in emergency situations. It is not clear, however, whether the chitin made through the agency of *Chs1* has the same distribution in the cell wall and the same role as that synthesized by *Chs2*; the two products may be different. One way of approaching this problem is to ask whether enhancement of *Chs2* activity in vivo, in the absence of *Chs1*, would have the same effect as the presence of *Chs1*, that is, would reduce cell lysis. Certain chitin-binding compounds, Calcofluor white M2R new and Congo red, have been found to in-

crease greatly the chitin content of yeast cell walls in cells with either a normal or a disrupted *CHS1* gene (18, 19). Congo red was added to the growth medium and the formation of refractile cells followed (Calcofluor cannot be used at the acidic pH necessary for the experiment [19]). Considerable inhibition of cell lysis was observed in the presence of Congo red (Table V). It was reasoned that a more pronounced effect might be obtained by using cells with a higher content of *Chs2*, and this was the case. Strain D3B (*chs1::URA3*) when transformed with a plasmid carrying the *CHS2* gene (pSS1; see reference 24) has 50- to 80-fold the *Chs2* activity of wild-type (22). This strain showed fewer lysed cells than the untransformed strain and Congo red inhibited the lysis strikingly (Table V).

It is noteworthy that in the presence of Congo red the absorbance, i.e., total mass, increased identically to that of the control but the number of cells lagged behind. The cell volume should, therefore, be larger, which was confirmed by direct observation. According to data (not shown) from the experiment with strain D3B (*chs1::URA3*) in Table V, the volume of cells grown in the presence of the dye is ~1.4 times that of control cells. These results indicate that, at the concentration used, Congo red has no effect on growth but retards cell division.

Another situation in which *Chs1* and *Chs2* apparently cooperate is suggested by the effect of the chitin synthase inhibitor, polyoxin D, in vivo. As previously mentioned (2), polyoxin D kills cells carrying a disrupted *CHS1* gene more efficiently than it kills wild-type cells (Fig. 7). These results may be interpreted in the following way. In the presence of polyoxin D, less chitin is made but wild-type cells can recruit *Chs1* to increase chitin production and are therefore partially protected. From the results obtained with Congo red, it may be expected that this dye, when added to the growth medium of *chs1::URA3* cells, would also protect against polyoxin. Such an effect was observed, but it was small and its significance is questionable (Fig. 7).

Discussion

The refractile cells observed in the *chs1::URA3* strain growing in minimal medium are lysed in the sense that they have

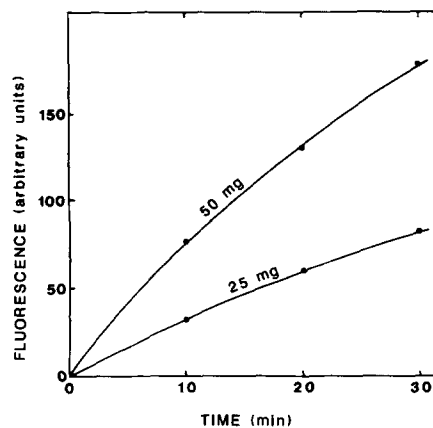


Figure 4. Measurement of chitinase with MU(Ch)₃ in intact diploid cells (*prbl-1122/prbl-1122*). The numbers on the curves refer to the wet weight of cells in each incubation mixture.

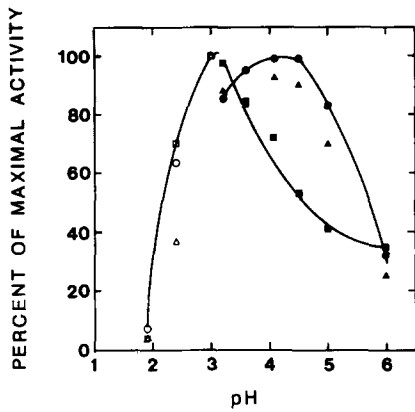


Figure 5. Effect of pH on chitinase measured with either intact cells (MU[Ch]₃ as substrate) or purified enzyme (MU[Ch]₃ or [³H]chitin as substrate) from diploid (*prbl-1122/prbl-1122*). ● and ○, intact cells; ▲ and △, purified chitinase with MU(Ch)₃ as substrate; ■ and □, purified chitinase with [³H]chitin as substrate. Solid symbols, citrate buffer; empty symbols, glycine buffer.

lost the permeability barrier, as shown by the staining with trypan blue and DAPI, by the permeability to DNase, by the lesion observed both with light and electron microscopy and, indirectly, by the protection afforded by sorbitol. However, because of the smallness of the orifice in their cell wall, it is possible that most of their intracellular material is still retained. This situation may explain the unusual appearance in the phase contrast microscope and in the fluorescent microscope with DAPI.

The properties and localization of yeast chitinase match those required of the system responsible for lysis of cells with a disrupted *CHS1* gene. The involvement of chitinase is supported by the correlation between enzymatic activity and lysis of cells grown in the presence of different buffers and confirmed by the inhibition of cell lysis by allosamidin, a specific chitinase inhibitor. The precise mechanism of lysis is not well-understood. The role of chitin at the birth scar on the daughter cell side is unclear. At cell separation most of the septum chitin remains with the mother cell in the bud scar, although the presence of some chitin at the birth scar has been suggested by Beran et al. (1). It is possible that this chitin stabilizes the glucan of the cell wall, as suggested by the results of Mol and Wessels (13) and that its elimination

Table III. Effect of Different Buffers on Production of Refractile Cells and on Chitinase Activity

Buffer	Refractile cells		Chitinase activity	
	%		U*/10 ¹⁰ cells/min	
None	39		137	
Succinate	3		74	
MES	5		89	
Phthalate	14		103	

Cells of strain D3B (*chs1::URA3*) were grown to the same turbidity in minimal medium supplemented with different buffers at pH 5.8, as described in Materials and Methods. The determinations of refractile cells and of chitinase activity were made on the same batch of cells. Chitinase activity was measured at pH 3. * Arbitrary fluorescence units.

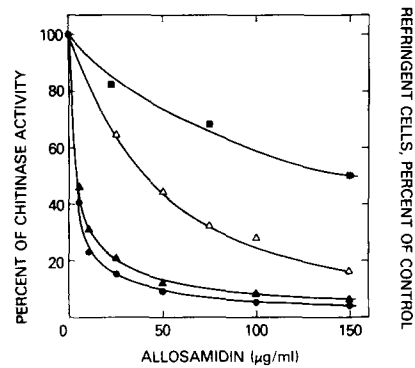


Figure 6. Inhibition of chitinase and of the formation of refractile cells by allosamidin. For the chitinase data, cells and enzyme are from the diploid (*prbl-1122/prbl-1122*). The data on refractile cells were obtained with strain D3B. Chitinase of (●) intact cells and (▲) purified enzyme, both with MU(Ch)₃ as substrate. △, purified enzyme with [³H]chitin as substrate. ■, production of refractile cells. In all cases, the value obtained in the absence of allosamidin is taken as 100.

creates a weak point that cannot resist the turgor pressure of the protoplast. The generation of a small orifice at the center of the birth scar may occur because that is the area of maximal stress or because chitin is a more important component of the wall at that location. It is also possible that other lytic enzymes such as glucanases have a role in the perforation of the cell wall. The complexity of the process is underlined by the finding that some strains appear to contain a gene that suppresses lysis. This suppressor segregated as a single, recessive locus. We could find no correlation between presence of the suppressor and chitinase or chitin synthase activity (Silverman, S. J., A. Sburlati, and E. Cabib, unpublished observations). The participation of other proteins in the lytic process is therefore probable.

If chitinase causes the lysis, its prevention by the presence of *Chs1* has a simple explanation: by synthesizing additional chitin, *Chs1* can make up for excessive loss of the polysaccharide caused by hydrolysis. An emergency mechanism of

Table IV. Effect of Allosamidin on Appearance of Refractile Cells

Incubation time	Percent refractile cells	
	Control	Allosamidin (0.15 mg/ml)
Experiment 1		
3.0 hours	4.0	1.0
6.0 hours	10.0	1.0
7.5 hours	18.0	3.5
Experiment 2		
6.0 hours	33.0	15.0
8.0 hours	33.0	24.0

Allosamidin was added as a 15 mg/ml solution in dimethylsulfoxide to a culture of strain D3B (*chs1::URA3*) in minimal medium. The same amount of dimethylsulfoxide was added to control cells. In Experiment 1 the cells had been previously grown in minimal medium with succinate; in Experiment 2 in minimal medium only. The approximate cell concentration was 0.9×10^7 cell/ml at the beginning and 1.4×10^8 cells/ml at the end of Experiment 1. For Experiment 2 the corresponding values were 0.57×10^7 and 6.6×10^7 .

Table V. Effect of Congo Red on Production of Refractile Cells

Strain	Incubation time	Percent refractile cells	
		Control	Congo red (20 $\mu\text{g/ml}$)
	<i>h</i>		
D3B (<i>chs1::URA3</i>)	0.0	6.0	6.0
	4.0	28.0	16.0
	6.0	39.0	27.0
<i>chs1::URA3(pSS1)</i>	0.0	5.0	5.0
	2.0	12.0	7.0
	3.5	15.0	5.0
	5.0	18.5	6.0

Conditions as in Experiment 2 of Table IV, except for the addition of Congo red in place of allosamidin. In the experiment with strain D3B (*top*) the initial number of cells was $1 \times 10^7/\text{ml}$ in both cultures. The final number was 7.8×10^7 for the control and 5.5×10^7 for the Congo red culture. In the experiment with the strain bearing a *CHS2*-containing plasmid (*bottom*) the number of cells was not monitored but the absorbance at 660 nm increased about sevenfold in both cultures.

this sort may also be activated when Chs2 is inhibited by polyoxin D. It follows that the cell must have some system to sense that more chitin is needed. The recruitment of Chs1 probably occurs by activation of the enzyme, most of which is in the zymogen form (7). One might expect to find a higher basal activity of Chs1 (measured without adding trypsin, see reference 7), when the yeast is allowed to grow at a more acidic pH. Indeed, a two- to threefold increase in basal activity was observed (from 2 to 5% of the total activity measured after trypsin treatment). This result may be significant if the additional synthase is located at the birth scar, but there is no means available to check this point. Further progress in this area will require a better understanding of the mechanism by which both Chs1 and Chs2 zymogens are activated *in vivo*.

It seems odd that an auxiliary or repair enzyme such as Chs1 should be the major isozyme in the cell, as suggested by *in vitro* determination of activity (22). It is questionable, however, whether the assays reflect accurately the *in vivo* activity, both because of the arbitrary conditions used and because the zymogen was usually activated with trypsin which of course is not the physiological activator. As a note of interest, we recently observed a threefold increase in the activity of Chs2 when the substrate, UDPGlcNAc, was included during trypsin treatment (Cabib, E., unpublished observations). Even if Chs1 were more abundant than Chs2 in the cell, this would not be an unprecedented situation. In the well-known case of the DNA-polymerases, it has been calculated that 400 molecules of DNA polymerase I, the repair isozyme, and only 10 molecules of DNA polymerase III, the synthetic isozyme, are present per cell of *Escherichia coli* (10).

Finally, why does the fungal cell need specific enzymes for wall repair? Certain events in the cell cycle require partial hydrolysis of the wall, either to soften it (budding in yeast, branch formation in filamentous fungi) or to separate cells. This is dangerous behavior because as we have seen, even a small hole in the cell wall can result in cell death. It is not surprising, therefore, that the cell should have special mech-

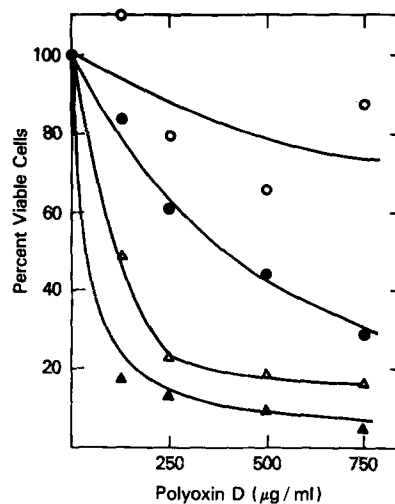


Figure 7. Killing effect of polyoxin D on control or *chs1::URA3* cells, in the absence or presence of Congo red. ● and ○, D3C (*CHS1*) strain; ▲ and △, D3B (*chs1::URA3*) strain. Solid symbols are without and empty symbols with, 20 $\mu\text{g/ml}$ Congo red. For details see Materials and Methods.

anisms and enzymes to deal with such situations. The repair or emergency enzymes may be very important for survival under certain conditions and further research may reveal the existence of other systems of this type.

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