

## Chitinase and chitin synthase 1: counterbalancing activities in cell separation of *Saccharomyces cerevisiae*

ENRICO CABIB,\* SANFORD J. SILVERMAN† and J. ANDREW SHAW

Laboratory of Biochemistry and Metabolism, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, USA

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Previous results [E. Cabib, A. Sburlati, B. Bowers & S. J. Silverman (1989) *Journal of Cell Biology* 108, 1665–1672] strongly suggested that the lysis observed in daughter cells of *Saccharomyces cerevisiae* defective in chitin synthase 1 (Chs1) was caused by a chitinase that partially degrades the chitin septum in the process of cell separation. Consequently, it was proposed that in wild-type cells, Chs1 acts as a repair enzyme by replenishing chitin during cytokinesis. The chitinase requirement for lysis has been confirmed in two different ways: (a) demethylallosamidin, a more powerful chitinase inhibitor than the previously used allosamidin, is also a much better protector against lysis and (b) disruption of the chitinase gene in *chs1* cells eliminates lysis. Reintroduction of a normal chitinase gene, by transformation of those cells with a suitable plasmid, restores lysis. The percentage of lysed cells in strains lacking Chs1 was not increased by elevating the chitinase level with high-copy-number plasmids carrying the hydrolase gene. Furthermore, the degree of lysis varied in different *chs1* strains; lysis was abolished in *chs1* mutants containing the *scs1* suppressor. These results indicate that, in addition to chitinase, lysis requires other gene products that may become limiting.

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### Introduction

Two chitin synthases have been found in the yeast *Saccharomyces cerevisiae*, chitin synthase 1 (Chs1) (Kang *et al.*, 1984; Bulawa *et al.*, 1986) and chitin synthase 2 (Chs2) (Sburlati & Cabib, 1986; Orlean, 1987). Recently a third synthase (Chs3) has been detected (Bulawa & Osmond, 1990). Genetic evidence has shown that Chs2 is essential for the formation of the chitin primary septum (Silverman *et al.*, 1988; Shaw *et al.*, 1991). In contrast, null mutations in the structural gene for Chs1 did not affect cell division (Bulawa *et al.*, 1986). A defect was, however, observed in strains carrying such mutations: lysis, mostly restricted to daughter cells, occurred when the strains were grown in a medium that allowed acidification to take place during growth (Bulawa *et al.*, 1986; Cabib *et al.*, 1989).

Normally, cell separation is facilitated by partial digestion of the chitin septum by a chitinase (Correa *et al.*, 1982; Elango *et al.*, 1982; Kuranda & Robbins, 1991). An analysis of the conditions that favour lysis in *chs1*

cells led to the hypothesis that excessive chitinase action may be involved in the lytic process (Cabib *et al.*, 1989), which results in perforation of the daughter cell wall during cytokinesis. This idea was supported by the finding that allosamidin, a specific chitinase inhibitor, decreased lysis (Cabib *et al.*, 1989). Since cells endowed with Chs1 activity do not show lysis even under acidic conditions, it was proposed that Chs1 acts as a repair enzyme, by replenishing part of the chitin lost by chitinase digestion (Cabib *et al.*, 1989). A test of this hypothesis became possible with the recent cloning and disruption of the chitinase structural gene (*CTSI*) by Kuranda & Robbins (1991). The present study provides genetic evidence on Chs1 and chitinase function and also includes additional inhibitor data.

### Methods

**Strains and cell growth.** The strains used in this study are listed in Table 1. Construction of strains was carried out with standard methods of yeast genetics (Sherman *et al.*, 1986). Cells were grown at 30 °C in minimal medium [2% (w/v) glucose, 0.7% Difco yeast nitrogen base] plus nutritional supplements. When succinate, at pH 5.8, was added to the medium, its concentration was 85 mM.

**Plasmid construction and yeast transformation.** pCT21, containing the *CTSI*-2 allele of the chitinase gene in the YEpl352 vector, was

\* Author for correspondence. Tel. (301) 496 1008; fax (301) 496.

† Present address: American Cyanamid, PO Box 400, Princeton, NJ 08543-0400, USA.

Abbreviations: Chs1, Chs2, Chs3, chitin synthase 1, 2, 3.

Table 1. Strains of *Saccharomyces cerevisiae* used in this study

Strain	Genotype	Source
DBY2068	<i>Mata his4-619 leu2-3,112 ura3-52</i>	D. Botstein*
MKY2068	<i>MATa his4-619 leu2-3,112 ura3-52 cts1::LEU2</i>	M. Kuranda & P. W. Robbins*
ECY18/4B	<i>MATa chs1-23 leu2-2 trp1-1 ura3-52</i>	This work
ECY21-5D	<i>MATa chs1::URA3 leu2-3,112 ura3-52</i>	This work
ECY22-12C	<i>MATa chs1::URA3 cts1::LEU2 his4-619 leu2-3,112 ura3-52</i>	This work
ECY22-12D	<i>MATa chs1::URA3 leu2-3,112 ura3-52</i>	This work
ECY29-4A	<i>MATa chs1-23 cts1::LEU2 leu2 ura3-52 trp1-1</i>	This work
SSY504-6D	<i>MATa chs1::URA3 leu2-3,112 ura3-52</i>	S. J. Silverman
SSY549-19B	<i>MATa chs1-23 his4 leu2-3,112 ura3-52</i>	This work
SSY560-1C	<i>MATa chs1-23 cts1::LEU2 his4 leu2 trp1-1 ura3-52</i>	This work

\* D. Botstein, Stanford University, Stanford, CA, USA; M. Kuranda & P. W. Robbins, Massachusetts Institute of Technology, Cambridge, MA, USA.

generously provided by M. Kuranda and P. W. Robbins (Massachusetts Institute of Technology, Cambridge, MA, USA).

pAS2 was constructed by cleaving pCT21 with *Hind*III and *Bam*HI; the resulting DNA fragment containing the chitinase gene was inserted into YEp351 (provided by A. Myers, Iowa State University, Ames, IA, USA) which had been cleaved with the same two restriction endonucleases.

pEC1 was obtained in a similar fashion, using pAS2 as a source of the chitinase gene and pRS316 (gift of R. Sikorski, Johns Hopkins University, Baltimore, MD, USA), previously cut with *Hind*III and *Bam*HI, as the centromere-containing vector.

pAS1 (Shaw *et al.*, 1991) was derived by inserting the *Xma*I-*Sal*I DNA fragment from pMS1, which contains *CHS1* (Bulawa *et al.*, 1986), into the vector YEp352 (from A. Myers) which had been digested with the same enzymes.

Yeast transformation was carried out using the lithium acetate procedure (Ito *et al.*, 1983).

**Microscopy.** For all observations, exponential phase cultures were used, at a density of  $3-4 \times 10^7$  cells ml<sup>-1</sup>. Refractile (lysed) cells were counted under phase contrast or after staining with trypan blue, as already described (Cabib *et al.*, 1989). Evidence that the phase-refractile cells are lysed in the sense of having lost the permeability barrier has already been presented (Cabib *et al.*, 1989). Sonication was performed on a 1 ml sample with the microtip of a Heat System-Ultrasonics Inc. Model W-385 sonicator with the output control at position 2.5 for 10 s.

**Preparation and analysis of cell walls and intracellular fraction.** Cell walls were prepared essentially as described by Roberts *et al.* (1983) except that a Bead-Beater (Biospec Products) was used to disrupt the cells with glass beads. A washing of the cell walls with 1% (w/v) SDS was carried out before determinations of carbohydrates but omitted when chitinase was measured. The fraction for determination of intracellular chitinase was the 100000g supernatant of the extract obtained by cell disruption with glass beads.

**Enzymic determinations.** Chitinase was measured in intact cells, cell walls or intracellular fraction with 4-methyl-umbelliferyl- $\beta$ -D-N,N',N"-triacylchitotriose, as described by Kuranda & Robbins (1987). *Chs1*

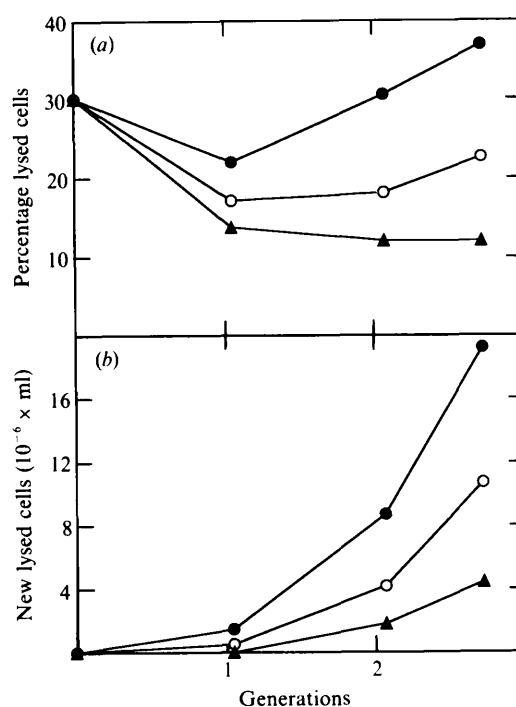


Fig. 1. Effect of different chitinase inhibitors on lysis in *chs1* cells (ECY22-12D). The cells were pre-grown in minimal medium without any added buffer, which explains the high initial percentage of lysed cells. (a) Variations of percentage of lysed cells during growth. The initial decrease in the control is probably due to the increase in pH when the pre-grown culture was diluted in fresh medium. (b) Results presented as increase in new lysed cells per ml. The data were calculated from the total number of cells per ml and the percentage of lysed cells. The initial number of lysed cells per ml was subtracted. ●, No additions; ○, 150 µg allosamidin ml<sup>-1</sup>; ▲, 20 µg demethylallosamidin ml<sup>-1</sup>.

was assayed on cells permeabilized with digitonin as reported by Fernandez *et al.* (1982). *Chs2* and *Chs3* are not detected with this assay if present at wild-type levels.

## Results

### Correlation between inhibition of chitinase and of lysis

Previous results (Cabib *et al.*, 1989) showed that allosamidin, a chitinase inhibitor, partially suppressed lysis of daughter cells in *chs1* strains. More recently, Sakuda *et al.* (1990) reported that demethylallosamidin is a much more potent inhibitor of yeast chitinase. If the effect of allosamidin on lysis were due to its action on chitinase, it would be expected that demethylallosamidin would be a much better protector against lysis. This prediction was confirmed experimentally. At a concentration of 20 µg ml<sup>-1</sup>, demethylallosamidin was about twice as effective in preventing lysis as allosamidin at 150 µg ml<sup>-1</sup> (Fig. 1). At 20 µg ml<sup>-1</sup>, allosamidin would be almost without effect (Cabib *et al.*, 1989). The inhibitors did not significantly affect the growth rate.

Table 2. *Effect of chitinase gene disruption on cell lysis*

For determinations of enzymic activities and of refractile cells, see Methods.

Strain	Relevant genotype	Chitin synthase activity [nmol min <sup>-1</sup> (mg dry wt) <sup>-1</sup> ]	Chitinase activity [pmol min <sup>-1</sup> (mg dry wt) <sup>-1</sup> ]	Refractile cells (%)
DBY2068	<i>CHS1 CTS1</i>	0.17	5.9	1.8
MKY2068	<i>CHS1 cts1::LEU2</i>	0.20	0	1.5
ECY22-12D	<i>chs1::URA3 CTS1</i>	<0.005	8.7	34.0
ECY22-12C	<i>chs1::URA3 cts1::LEU2</i>	<0.005	0	3.3

Table 3. *Restoration of cell lysis by plasmids carrying the chitinase gene*

Strain	Relevant genotype*	Chitinase activity [pmol min <sup>-1</sup> (mg dry wt) <sup>-1</sup> ]	Refractile cells (%)
SSY560-1C	<i>chs1 cts1::LEU2</i>	0	3.5
SSY560-1C[pEC1]	<i>chs1 cts1::LEU2</i> [p <i>CTS1</i> -2(cm)]	13.7	18
SSY560-1C[pCT21]	<i>chs1 cts1::LEU2</i> [p <i>CTS1</i> -2(h-c)]	47	10
ECY29-4A	<i>chs1 cts1::LEU2</i>	0	4.3
ECY29-4A[pEC1]	<i>chs1 cts1::LEU2</i> [p <i>CTS1</i> -2(cm)]	34	17.5
ECY29-4A[pCT21]	<i>chs1 cts1::LEU2</i> [p <i>CTS1</i> -2(h-c)]	91	12

\* cm, Centromere plasmid; h-c, high-copy-number plasmid.

*Chitinase is required for lysis in chs1 cells*

It was reasoned that if chitinase is required for lysis in cells deficient in *Chs1*, introduction of a chitinase gene disruption in such cells should eliminate lysis. Accordingly, cells with a *CHS1* disruption were mated to a strain carrying a chitinase gene disruption, and segregants containing both disruptions were isolated. Because of the chitinase defect (Kuranda & Robbins, 1991), such strains grow in clumps which prevents observation and counting of lysed cells which are refractile under phase contrast (see Cabib *et al.*, 1989). However, refractile cells can be easily detected after light sonication to disrupt the clumps, a treatment that also separates lysed buds from mother cells. Whereas numerous lysed (refractile) cells could be observed in the control *chs1 CTS1* strain, only an occasional bright cell was visible in the strain with the double disruption (results not shown). The results are shown in quantitative form in Table 2, where it can also be seen that activities of *Chs1* and chitinase in the different strains were as expected. Determination of lysed cells by the trypan blue staining method gave similar results (not shown). A small increase of lysed cells in strains containing the double disruption compared to wild-type (3–4% compared to 1.5–2%; see also Table 3) was usually observed. We do not know the reason for this difference; many of the lysed cells in those strains, however, had aberrant forms.

Some yeast strains contain a suppressor (*scs1*) that eliminates lysis despite the presence of a mutation or disruption of *CHS1* (Cabib *et al.*, 1989; Silverman *et al.*, 1991). It was conceivable that MKY2068, the *cts1::LEU2* parent in the mating that gave rise to ECY22-12C (Table 2), could be such a strain. If this were so, the lack of lysis in ECY22-12C could result from the presence of the suppressor rather than from the double disruption of *CHS1* and *CTS1*. Therefore, DBY2068, which is isogenic with MKY2068 except for the *CTS1*-2 disruption, was examined for the presence of a lysis suppressor. DBY2068 was mated to the lysis-positive strain ECY21-5D (*chs1::URA3*), the resulting diploid was sporulated and tetrads dissected. Samples of colonies from six complete tetrads were allowed to grow in liquid medium and assayed for lysis. If DBY2068 had carried the suppressor, only one out of six tetrads should show a segregation of 2:2 for lysis. However, all six tetrads examined segregated 2:2. Furthermore, all lysing strains were able to grow in the absence of uracil, i.e. they contained the *CHS1* disruption. The latter result was also found in four additional incomplete tetrads, in which only three of the four spores had germinated. It may be concluded that DBY2068, and in consequence MKY2068, did not contain the lysis suppressor.

To confirm the role of chitinase in the lysis, strains devoid of *Chs1* and chitinase were transformed with plasmids carrying a chitinase gene. Strain ECY22-12C

Table 4. Lysis in cells with different chitinase levels

Strain	Relevant genotype*	Chitinase activity† [pmol min <sup>-1</sup> (mg dry wt) <sup>-1</sup> ]	Refractile cells (%)
SSY549-19B	<i>chs1 CTS1</i>	4.7 (7)	16
SSY549-19B[pAS2]	<i>chs1 CTS1</i> [p <i>CTS1</i> -2(h-c)]	27 (61)	14
SSY549-19B[pAS2][pAS1]	<i>chs1 CTS1</i> [p <i>CTS1</i> -2(h-c)][p <i>CHS1</i> (h-c)]	27	4
ECY18/4B	<i>chs1 CTS1</i>	5 (13)	21
ECY18/4B[pEC1]	<i>chs1 CTS1</i> [p <i>CTS1</i> -2(cm)]	(30)	17
ECY18/4B[pAS2]	<i>chs1 CTS1</i> [p <i>CTS1</i> -2(h-c)]	38	25
ECY18/4B[pCT21]	<i>chs1 CTS1</i> [p <i>CTS1</i> -2(h-c)]	38	18
SSY504-6D	<i>chs1::URA3 CTS1</i>	7.7	6
SSY504-6D[pAS2]	<i>chs1::URA3 CTS1</i> [p <i>CTS1</i> -2(h-c)]	48	14
ECY21-5D	<i>chs1::URA3 CTS1</i>	7	17
ECY21-5D[pAS2]	<i>chs1::URA3 CTS1</i> [p <i>CTS1</i> -2(h-c)]	45	22

\* h-c, High-copy-number plasmid; cm, centromere plasmid.

† For chitinase activity, results presented in this Table were obtained with cells grown in medium buffered with succinate, except for the values in parentheses, which come from cells grown in unbuffered medium.

could not be used in this experiment because it lacked a suitable marker for transformation. Therefore, strains with the *chs1 ura3 cts1::LEU2* genotype were constructed by crossing MKY2068 with *chs1 ura3* strains. These strains, like MKY2068, grew in clumps (not shown) and gave rise to very few lysing cells. Transformation with the centromere plasmid pEC1 that carries the chitinase gene, resulted in the appearance of chitinase activity, together with a large increase in lysis (Table 3). These values are not as high as that for strain ECY22-12D (Table 2). However, it should be kept in mind that the *chs1* parents of the strains in Table 3 showed about 20% lysis, close to that determined with the centromere plasmids. As will be shown below, the level of lysis changes from strain to strain. In addition to the strains in Table 3, a third strain, ECY29-12C, yielded similar results (not shown). Surprisingly, transformation with a high-copy-number plasmid containing the *CTS1*-2 gene (pCT21, Table 3) gave rise to less lysis than occurred in strains transformed with a centromere plasmid, although the level of chitinase was higher in the cells with the high-copy-number plasmid. This effect will be considered in the next section.

#### Factors limiting lysis in *Chs1*-defective strains

As shown in the preceding section, elimination of chitinase activity abolished lysis of strains defective in *Chs1*. It seemed likely that an increase in chitinase activity should bring about, reciprocally, an increase in lysis. This expectation was not borne out by the experimental evidence. When strains with a mutation or disruption of the *CHS1* gene were transformed with

high-copy-number plasmids carrying the chitinase gene, the percentage of lysed buds did not change significantly. In four different strains transformed with the plasmid, the chitinase activity increased substantially. However, the proportion of lysed cells did not show a clear trend, being sometimes a little higher and sometimes a little lower than the control (Table 4). In addition, two other strains yielded comparable results (not shown). As mentioned in the preceding section, a lack of correlation between chitinase activity and the extent of lysis was also noticed in the experiments of Table 3. Simultaneous transformation of cells with two high-copy-number plasmids, one containing *CTS1*-2 and the other *CHS1*, led to sharply reduced lysis, as expected (Table 4, transformants of strain SSY549-19B). It may also be observed that the percentage of lysed cells in untransformed *Chs1*-deficient cells varied over a wide range (Table 2, strain ECY22-12D, and strains in Table 4). As mentioned above, and elsewhere (Cabib *et al.*, 1989; Silverman *et al.*, 1991), lysis is totally absent when the *chs1* cells contain suppressor *scs1*.

In the experiments of Table 3 and 4, chitinase was assayed with intact cells. The assay measures only the chitinase associated with the cell walls, about one-half of the total cell-associated activity (Elango *et al.*, 1982). This portion of the enzyme appears to be strongly bound to the cell wall: isolated cell walls yielded 75–80% of the activity found with intact cells (results not shown). Measurement of intracellular chitinase activity after cell disruption with glass beads showed about the same ratio between cells with and without a chitinase plasmid as was found with intact cells (results not shown). Thus, there is no change in the compartmentation of the enzyme when its activity is elevated by increasing the

number of copies of the structural gene. The chitin content of cell walls, relative to total sugar as measured with anthrone, was not changed by transformation of either wild-type or *chs1* cells with high-copy-number plasmids containing the chitinase gene (results not shown).

Strains transformed with plasmids carrying the chitinase gene showed some aggregation and a variable increase in the size of individual cells (results not shown). These results, that indicate some effect of high levels of chitinase on cell division and separation, remain unexplained. Perhaps these effects are related somehow to the paradoxical decrease in lysis observed in some cases when chitinase expression was increased.

## Discussion

The hypothesis that chitinase activity is an essential requirement for lysis of cells devoid of Chs1 has now been unambiguously confirmed. Demethylallosamidin, a more powerful chitinase inhibitor than allosamidin, was also a much better protector against lysis. Because of the similarity between the structures of the two compounds, this practically eliminates the possibility that the effect of allosamidin on lysis could have been unspecific. More importantly, introduction of a chitinase disruption in *chs1* cells practically abolished lysis. This effect was not due to the presence of a lysis suppressor because the strains used in constructing the double disruption did not contain such a suppressor. Furthermore, reintroduction of a chitinase gene on a plasmid restored the lysis. These results clearly show that at least one function of Chs1 is to replenish chitin lost through chitinase action during the process of cell separation. It remains to be ascertained how the cell senses the need for chitin repair and how it regulates, presumably by zymogen activation (Cabib, 1987), the activity of the repair synthase.

In contrast with the effect of chitinase elimination, the overproduction of chitinase by transformation with suitable plasmids did not result in the expected increase in lysing daughter cells. It was also observed that the extent of lysis in untransformed Chs1-deficient cells varied widely from strain to strain. An extreme case of this situation is the total disappearance of lysis in *chs1* cells containing suppressor *scs1* (Cabib *et al.*, 1989; Silverman *et al.*, 1991). Since the suppressor is recessive (Silverman *et al.*, 1991), it represents loss of a function. This indicates that at least another gene product, in addition to chitinase, is required for lysis (Silverman *et al.*, 1991). Thus, either the *SCS1* product or another protein necessary for lysis, such as a wall-digesting enzyme, may be partially deficient in different strains

and limit the extent of lysis even when chitinase is overexpressed.

From our observations, it is clear that the opposing activities of both chitinase and Chs1 are required in the delicately balanced process of cell separation. At the same time, the participation in this process of other factors and controls, as yet not well identified, is indicated.

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