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Isolation and Characterization of *Saccharomyces cerevisiae* Mutants Resistant to Calcofluor White

CÉSAR RONCERO, MARÍA HENAR VALDIVIESO, JUAN CARLOS RIBAS, AND ANGEL DURÁN*

Instituto de Microbiología Bioquímica, Facultad de Biología, Consejo Superior de Investigaciones Científicas and Universidad de Salamanca, Salamanca, Spain

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Calcofluor is a fluorochrome that exhibits antifungal activity and a high affinity for yeast cell wall chitin. We isolated *Saccharomyces cerevisiae* mutants resistant to Calcofluor. The resistance segregated in a Mendelian fashion and behaved as a recessive character in all the mutants analyzed. Five loci were defined by complementation analysis. The abnormally thick septa between mother and daughter cells caused by Calcofluor in wild-type cells were absent in the mutants. The Calcofluor-binding capacity, observed by fluorescence microscopy, in a *S. cerevisiae* wild-type cells during α-factor treatment was also absent in some mutants and reduced in others. Staining of cell walls with wheat germ agglutinin-fluorescein complex indicated that the chitin uniformly distributed over the whole cell wall in vegetative or in α-factor-treated cells was almost absent in three of the mutants and reduced in the two others. Cell wall analysis evidenced a five- to ninefold reduction in the amount of chitin in mutants compared with that in the wild-type strain. The total amounts of cell wall mannan and β-glucan in wild-type and mutant strains were similar; however, the percentage of β-glucan that remained insoluble after alkali extraction was considerably reduced in mutant cells. The susceptibilities of the mutants and the wild-type strains to a cell wall enzymic lytic complex were rather similar. The in vitro levels of chitin synthase 2 detected in all mutants were similar to that in the wild type. The significance of these results is discussed in connection with the mechanism of chitin synthesis and cell wall morphogenesis in *S. cerevisiae*.

In a recent work (21), we indicated that the fluorochrome Calcofluor shows antifungal activity. This compound binds to nascent microfibrils of cellulose or chitin (12, 14). As a consequence of dye interaction, the β-1,4-glucan chains are prevented from cocrystallizing to form microfibrils and their assembly is thus seriously disrupted (4, 11). Because chitin is one of the main structural polysaccharides in fungal cell wall (6, 9, 10, 26), it is concluded that lysis of the fungal cell may be caused by abnormal chitin deposition and subsequent weakening of the wall. On the other hand, Calcofluor enhances the rate of chitin synthesis in vivo (21). The exposure of *Saccharomyces cerevisiae* to Calcofluor induces abnormally thick septa as a result of the massive deposition of anomalous crystallized chitin (21). In an accompanying paper (22), we have shown that basal levels of chitin synthases 1 and 2 (5, 23) increase during Calcofluor treatment. On the basis of these results, we decided to isolate and characterize *S. cerevisiae* mutants resistant to Calcofluor in an attempt to get additional information about the physiological mechanism of chitin synthesis in *S. cerevisiae*.

In this report, we show that the cell walls of Calcofluor-resistant mutants have a reduced level of chitin. Therefore, these mutants must be affected in some gene(s) involved in different steps of yeast chitin synthesis; the more obvious one, the gene for chitin synthase 2 (5, 23), does not seem to be affected.

MATERIALS AND METHODS

Strains and growth conditions. All the strains used in this work are listed in Table 1. Mutants resistant to Calcofluor (Cal^R) were obtained by ethyl methanesulfonate mutagenesis of strain X2180-1A and isolated by plating the survival population in YED-agar (21) plates supplemented with 0.05 to 0.1% Calcofluor. Under these conditions, Calcofluor exerted its antifungal action and only the resistant clones grew. To obtain $Chs1^-$ Cal^R double mutants (strains lacking chitin synthase 1 and resistant to Calcofluor), the *his chs1::URA3* strain was mated to the *cal*^{R} *ura3-52* strain, and the diploid was submitted to sporulation and tetrad analysis. Double mutants were selected from the analyzed tetrads in which the double phenotype Ura^+ Cal^R was present. Growth conditions and genetic crosses were as described previously (25).

Labeling and fractionation of cell wall polysaccharides. Exponential-phase cultures of *S. cerevisiae* wild-type and mutant strains were supplemented with [U-^{14}C]glucose (6 to 12 μCi/ml). Cells (1×10^7 to 5×10^7) were harvested after 3 generation times. Carrier cells (4×10^9) were added to the radioactive samples and then submitted to mechanical breakage as previously described (20). Cell walls were washed with water several times by centrifugation at 1,000 × *g* for 5 min and then extracted twice with 6% NaOH for 90 min at 80°C. Precipitation of mannan from the alkali extract with Fehling reagent was performed as described previously (2). Alkali-soluble β-glucan and mannan were precipitated together by the addition of 2 volumes of absolute ethanol to the alkali extract. The difference between the counts obtained in the ethanol-precipitated pellet and that produced by Fehling reagent was taken to represent the alkali-soluble β-glucan fraction. The residue remaining after NaOH extraction was washed by centrifugation until the pH was neutral and suspended in 1.2 ml of water. Samples (250 μl) were incubated for 36 to 48 h at 30°C with 60 mU of chitinase (18) or 250 μg of zymolyase 100T (Miles Laboratories, Inc.) in 50 mM phosphate buffer at pH 6.3 in a final volume of 0.4 ml. A sample without enzyme was always included as a control. After incubation, the samples were centrifuged, washed, and counted as described previously (19). The residues remain-

* Corresponding author.

TABLE 1. Strains of *S. cerevisiae* used in this study

Strain	Relevant genotype	Source
X2180-1A	**a**	YGSC[a]
X2180-1B	α	YGSC
CR1	α *cal*^{R}*1 ade1 his3*	This study
CR1312C	α *cal*^{R}*1 ura3*	This study
CR4	α *cal*^{R}*2 ade1 his6*	This study
CR4217A	α *cal*^{R}*2 ura3*	This study
HV23	*cal*^{R}*3*	This study
HV2324D	α *cal*^{R}*3 ura3*	This study
HV25	*cal*^{R}*4*	This study
HV2526A	α *cal*^{R}*4 ura3*	This study
HV26	*cal*^{R}*5*	This study
HV2627B	α *cal*^{R}*5 ura3*	This study
DB3	**a** *his chs1::URA3*	E. Cabib
TD-28	**a** *ura3*	F. Rey
MC193	**a** *ade1*	F. Rey
A3618C	α *his3*	F. Rey
X59/1	**a** *his6*	F. Rey
CR1CHS1	α *cal*^{R}*1 his chs1::URA3*	CR1312C × DB3
CR4CHS1	**a** *cal*^{R}*2 his chs1::URA3*	CR4217A × DB3
HV23CHS1	**a** *cal*^{R}*3 his chs1::URA3*	HV2324D × DB3
HV25CHS1	**a** *cal*^{R}*4 his chs1::URA3*	HV2526A × DB3
HV26CHS1	**a** *cal*^{R}*5 his chs1::URA3*	HV2627A × DB3

[a] YGSC, Yeast Genetic Stock Center.

ing after digestion with chitinase or zymolyase were taken to represent alkali-insoluble β-glucan or chitin fractions, respectively. All determinations were done in duplicate.

Other methods. Cell extracts were obtained by breaking the cells with a Braun homogenizer as described previously (19). Cell walls were removed by centrifugation at 1,000 × *g* for 5 min. The supernatant fluid was centrifuged at 48,000 × *g* for 40 min, and the pellets were washed with 25 mM Tris hydrochloride (pH 7.5)–5 mM $MgSO_4$ and finally suspended in the same buffer supplemented with 33% glycerol. Assays for chitin synthases 1 and 2 were performed as described previously (21). Cells were stained with wheat germ agglutinin-fluorescein complex (WGA-FITC) (Miles) as follows. Cells were extracted with 6% NaOH for 60 min at room temperature and washed with water until neutral pH. To 10 μl of alkali-extracted cells was added 1 μl of WGA-FITC (2 mg/ml) and 10 μl of 50 mM sodium bicarbonate. After 10 min at room temperature, cell suspensions were diluted to 0.4 ml with sodium bicarbonate, centrifuged, and suspended in the original volume of 50 mM sodium bicarbonate. Fluorescence was usually observed as described previously (21); however, an excitation filter (UV 330-380), a dichroic mirror (DM400), and a barrier filter (420K) were used to examine Calcofluor fluorescence in some experiments. Other methods and sources of materials are described in an accompanying paper (22). Benomyl (methyl 1-[butylcarbamoyl]-2-benzimidazolecarbamate) and polyoxin D were kindly provided by R. J. Howard (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) and E. Cabib (National Institutes of Health, Bethesda, Md.), respectively.

RESULTS

Genetic characterization of Calcofluor-resistant mutants. After three independent mutagenesis, 28 mutants resistant to Calcofluor were selected from 3.7 × 10^6 surviving cells. A total of 21 mutants were unable to conjugate with a wild-type complementary strain. All these clones were self-sporulating, but only three of them produced mature asci; however, the viability of ascospores was almost null, and the few viable clones were also sterile. Therefore, further analysis of these mutants was discontinued. The remaining seven mutants were mated to different strains (Table 1). The heterozygous diploids did not grow on Calcofluor-supplemented plates; tetrad analysis showed that sporulation produced a segregation of 2Cal^S:2Cal^R, indicating that Calcofluor resistance was a single recessive chromosomal mutation in every examined mutant. By complementation analysis, the seven Cal^R mutants fell into five complementation groups, each group defining a different gene (*CAL1* to *CAL5*). Calcofluor resistance cosegregated with resistance to Congo red, another dye that also stained chitin and showed antifungal activity against *S. cerevisiae* (21).

The Mendelian segregation of the Calcofluor resistance defined by mutant CR1 was clearly observable in YED-agar plates at 24°C but not so easily at 37°C, although growth of the mutant in the absence of Calcofluor did not show temperature dependence. It seemed that the susceptibility to Calcofluor of this mutant increased with temperature, and only a small percentage of cells were resistant to the fluorochrome at 37°C. Consequently, at 37°C the colony had a rate of growth slower than that at 24°C and a papillated aspect (results not shown).

Rates of conjugation, measured as percentages of viable diploids from the total number of micromanipulated zygotes, between **a** and α cells from any Cal^R mutant were quite similar to that observed between the corresponding wild-type cells. Fresh growing cultures from HV23 mutant cells were absolutely required for conjugation in that mutant. Sporulation of homozygous diploids from every mutant was severely affected, however.

Fluorescence microscopy. (i) Vegetative cells. Observations of mutant cells were made under a fluorescence microscope with Calcofluor and WGA-FITC as markers. When yeast cells were observed under UV light in the presence of these compounds, fluorescence was detected in regions where chitin was located (7, 17). In intact cells, chitin is covered by the other polysaccharides that form the cell wall and is only accessible to small molecules, such as fluorochrome (13). To be accessible to larger molecules (i.e., WGA-FITC), chitin has to be uncovered by alkali extraction (17). All mutants showed a drastic decrease in Calcofluor-binding capacity. With a blue excitation filter (IF420-485), almost no fluorescence could be seen in CR1 and CR4 mutants compared with definite fluorescence in the wild-type cells (Fig. 1B, D, and F). When cells were observed by using a UV excitation filter (UV 300-380), some fluorescence in septa and over the cell surface was detected in CR1 and CR4 mutant cells, respectively. Under these conditions, wild-type cells were observed as very shiny spots (data not shown). HV25 and HV26 mutants behaved similarly to CR1, and HV23 behaved similarly to CR4 (data not shown). The abnormally thick septa between mother and daughter cells caused by Calcofluor in wild-type cells (21) were absent in mutant cells (Fig. 1). This character cosegregated with Calcofluor resistance in every tetrad analyzed.

In wild-type alkali-extracted cells, WGA-FITC fluorescence was clearly observable around the whole contour of mother cells but not around buds (Fig. 2B). By contrast, CR1 mutants had no fluorescence around the cell contour but only at the level of the septa (Fig. 2D); a similar pattern of fluorescence was observed with HV25 and HV26 mutants (data not shown). A reduced level of WGA-FITC fluorescence around the cell could be observed with CR4 (Fig. 2F) and HV23 (data not shown) mutants, compared with that observed with wild-type cells (Fig. 2B).

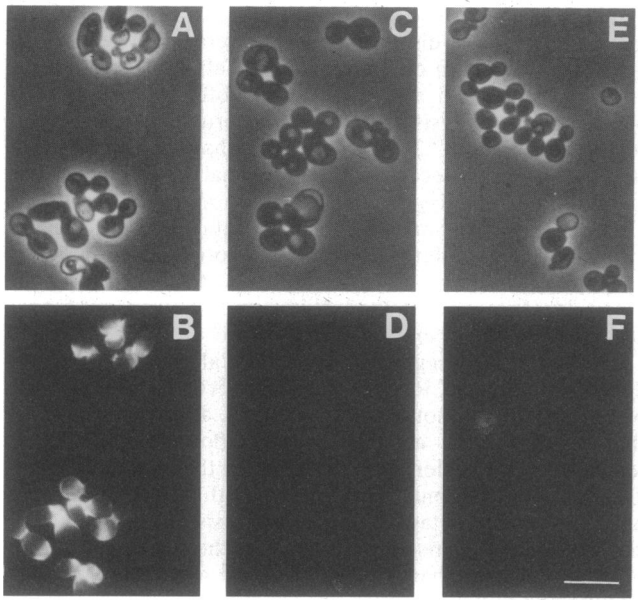

FIG. 1. Fluorescence and phase-contrast micrographs of wild-type and Calcofluor-resistant mutant cells stained with Calcofluor. Cells from wild-type strains (A and B), the *cal*^{R}*1* mutant (C and D), and the *cal*^{R}*2* mutant (E and F) were grown on YED medium in the presence of Calcofluor (50 μg/ml); after 3 h, cells were observed by phase- contrast (A, C, and E) or fluorescence microscopy (B, D, and F). Fluorescence micrographs were taken with the same lenses and exposure times. The bar corresponds to 10 μm.

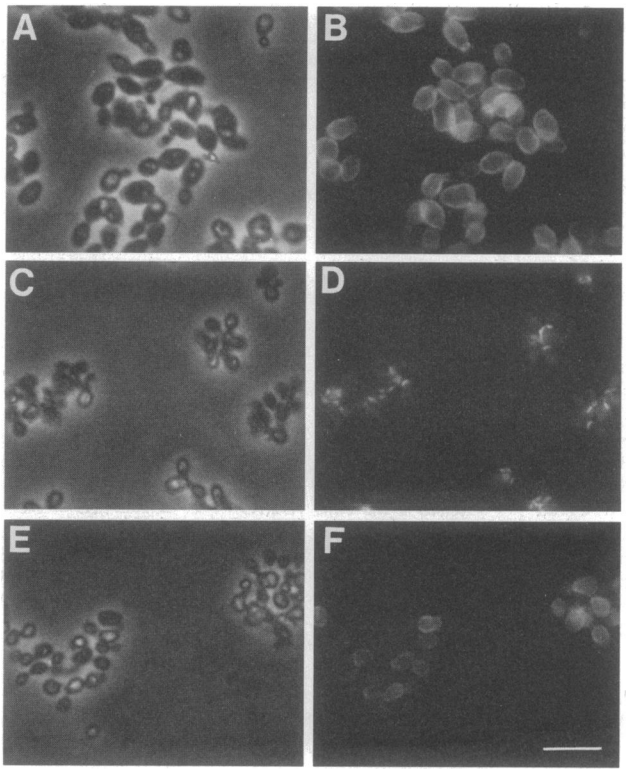

FIG. 2. Fluorescence and phase-contrast micrographs of wild-type and Calcofluor-resistant mutant cells stained with WGA-FITC. Cells grown on YED medium were harvested, extracted with NaOH, and stained as described in Material and Methods. Phase-contrast (A, C, and E) and fluorescence micrographs (B, D, and F) were taken as described in the legend to Fig. 1. A and B, Wild type; C and D, *cal*^{R}*1* mutant; E and F, *cal*^{R}*2* mutant. The bar corresponds to 10 μm.

(ii) α-factor treated cells. Polar elongation, due to α-factor treatment of a *S. cerevisiae* cells (shmoo production), is a developmental process in which chitin synthesis has been also implicated (24). Log-phase cells from wild-type strains and Calcofluor-resistant mutants were submitted to α-factor treatment as described previously (22), and the levels of Calcofluor or WGA-FITC fluorescence were observed. Wild-type cells showed fluorescence due to Calcofluor on the area of pheromone-stimulated growth (24; Fig. 3B), whereas no fluorescence at all was observed in CR1 (Fig. 3D), HV25, and HV26 (data not shown) mutants under the same conditions. Concomitant with what occurred in vegetative cells, CR4 and HV23 mutants showed reduced levels of fluorescence, not enhanced by Calcofluor, compared with that observed with wild-type cells.

The fluorescence due to WGA-FITC appeared uniformly distributed around the surface of the alkali-extracted shmoos from the wild-type strain and, again, was completely absent in those from CR1, HV25, and HV26 mutants and reduced in those from CR4 and HV23 mutants (results not shown).

Cell wall fractionation. It was important to ascertain whether the reduced level of fluorescence in mutant cells could be specifically related to a reduced level of chitin in cell walls, as described for another system (7). The incorporation of radioactivity into the chitin fraction from CR1 or CR4 mutants grown without Calcofluor was about 11 or 15%, respectively, of the control value (Table 2). Rates of chitin synthesis in HV25 and HV26 mutants were similar to that in the CR1 mutant (0.12 ± 0.01 and 0.11 ± 0.01, respectively), whereas the rate in HV23 mutant was higher (0.20 ± 0.04). Incorporation of radioactivity in mannan and β-glucan fractions of CR1 and CR4 mutant cell walls was about the same as that of the control. However, the proportion of radioac-

TABLE 2. Incorporation of radioactivity from [^{14}C]glucose into cell wall polysaccharides of *S. cerevisiae* wild-type and Calcofluor-resistant strains[a]

Polysaccharide fraction	Incorporation of [^{14}C]glucose (%) by[b]:		
	Wild type	CR1 mutant	CR4 mutant
Mannan	16.5 ± 1.7 (46.9)[c]	16.3 ± 1.3 (50.3)	13.2 ± 1.2 (45.0)
β-glucan			
Alkali soluble	11.5 ± 2.7 (32.7)	15.0 ± 0.8 (46.3)	15.0 ± 1.5 (51.2)
Alkali insoluble	6.1 ± 1.0 (17.3)	1.0 ± 0.03 (3.1)	0.94 ± 0.15 (3.2)
Chitin	1.1 ± 0.1 (3.1)	0.11 ± 0.02 (0.34)	0.14 ± 0.02 (0.48)

[a] For experimental conditions, see text. The relevant genotypes are as follows: wild type, *cal*^{S}; CR1 mutant, *cal*^{R}*1*; and CR4 mutant, *cal*^{R}*2*.
[b] Percent incorporation of [^{14}C]glucose = (counts per minute incorporated per fraction/total counts per minute incorporated) × 100.
[c] Values in parentheses are percentages of the corresponding polysaccharides in the cell wall composition.

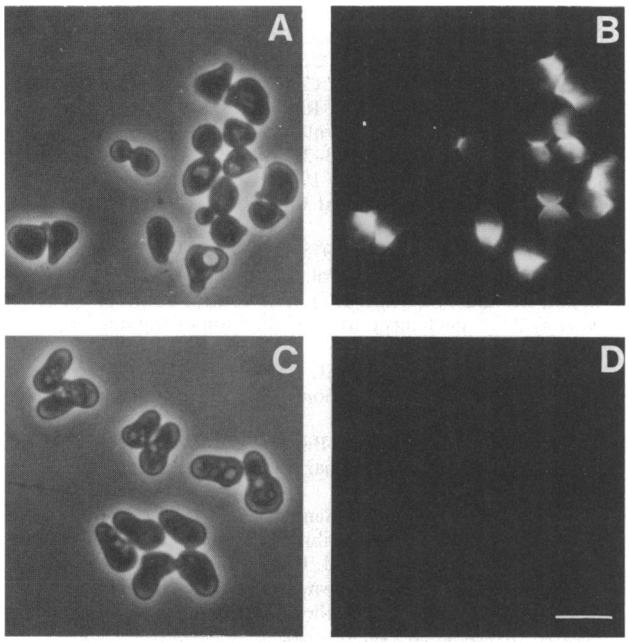

FIG. 3. Fluorescence and phase-contrast micrographs of wild-type (A and B) and *cal*^{R}*1* mutant (C and D) cells treated with α-factor in the presence of Calcofluor. Cells were incubated in YED medium supplemented with α-factor (5 μM), and after 90 min, Calcofluor (50 μg/ml) was added to the cultures. At 2 h later, cells were observed by phase-contrast (A and C) or fluorescence (B and D) microscopy as described in the legend to Fig. 1. The bar corresponds to 10 μM.

tivity in the alkali-insoluble β-glucan fraction in these mutants was considerably smaller (sixfold) than that in wild-type cells (Table 2).

The differences between the cell wall compositions of wild-type and mutant cells could imply differences in cell wall structure; however, the susceptibilities to zymolyase 100T (a cell wall enzymic lytic complex) of wild-type and CR1 and CR4 mutant strains were rather similar (data not shown).

Chitin synthase levels. Since chitin content was qualitatively and quantitatively altered in Calcofluor-resistant mutants, it seemed reasonable to analyze the activities of chitin synthases derived from these mutants. The specific activity levels of chitin synthase 1 from log-phase cultures of all mutants were similar to those from wild-type strain (results not shown). Furthermore, other properties, such as optimal pH, K_m, *N*-acetylglucosamine activation, inhibition by polyoxin, and solubilization by the detergent digitonin, of wild-type and CR1 and CR4 mutant synthases were also identical (results not shown).

Recent results showed that chitin synthase 1 is not required for chitin synthesis in vivo in *S. cerevisiae* (5); therefore, double mutants resistant to Calcofluor and lacking chitin synthase 1 activity (*chs1*::*URA3*) were obtained as described in Materials and Methods and used to determine chitin synthase 2 activity. The Calcofluor resistance phenotype remained unchanged in the double mutants. The differences between the specific activity values for chitin synthase 2 from Cal^S and Cal^R cell extracts (Table 3) were not as significant as expected from the differences in chitin content. Perhaps the basal activity level (expressed in the absence of trypsin activation) in the CR1 mutant was smaller than that in the wild-type strain (Table 3). Furthermore, the increase in chitin synthase 2 basal level measured in Cal^S permeabilized cells after Calcofluor treatment (22) was not observed in any of the mutants, as expected from strains that did not respond to Calcofluor.

DISCUSSION

Calcofluor is a fluorochrome that specifically labels cell wall chitin in *S. cerevisiae* (7, 17); it also shows antifungal activity (21) and enhances the rate of chitin synthesis in several fungal systems (21). On the basis of these observations, we decided to obtain *S. cerevisiae* mutants resistant to Calcofluor, the rationale being that the process of chitin synthesis in such mutants might be affected somehow.

Several lines of evidence complementing each other indicate that Calcofluor-resistant mutants are affected in chitin synthesis. (i) None of these mutants formed the abnormally thick septa between mother and daughter cells caused by the addition of Calcofluor to growing cultures of the wild-type strain. We therefore conclude that the oversynthesis of chitin detected in vivo and the concomitant activation of chitin synthases 1 and 2 detected in vitro as consequences of Calcofluor action (22) do not take place in Calcofluor-resistant mutants. (ii) Fluorescence due to the accumulation of Calcofluor-binding material in pheromone-treated wild-type cells was considerably reduced in CR4 and HV23 mutants and absolutely absent in the others. (iii) Uniform staining by WGA-FITC of the wild-type cell surface due to the small amount of chitin distributed over the whole yeast cell wall (17) was also missing in three mutants and clearly reduced in CR4 and HV23 mutants; a parallel result was observed in a cells after α-factor treatment. (iv) The chitin content of cell walls from mutants was 11 to 20% of the value from the wild-type.

Some additional considerations may also be inferred. (i) Growth rates of Cal^R mutants were similar to that of the wild-type strain; therefore *S. cerevisiae* can grow normally

TABLE 3. Activities of Chitin synthase 2 extracts from *S. cerevisiae* wild-type and Calcofluor-resistant strains

Strain	Sp act[a]		Ratio (+T/−T)
	−T	+T	
DB3 (*chs1*::*URA3*)	0.113 ± 0.02	0.365 ± 0.05	3.23
CR1CHS1 (*cal*^{R}*1 chs1*::*URA3*)	0.079 ± 0.02	0.341 ± 0.04	4.32
CR4CHS1 (*cal*^{R}*2 chs1*::*URA3*)	0.153 ± 0.05	0.490 ± 0.1	3.20
HV23CHS1 (*cal*^{R}*3 chs1*::*URA3*)	0.115 ± 0.02	0.375 ± 0.04	3.26
HV25CHS1 (*cal*^{R}*4 chs1*::*URA3*)	0.137 ± 0.02	0.501 ± 0.01	3.65
HV26CHS1 (*cal*^{R}*5 chs1*::*URA3*)	0.123 ± 0.03	0.327 ± 0.08	2.66

[a] Extracts were prepared and assayed in the absence (−T) or presence (+T) of trypsin as described elsewhere (21). Activities are expressed as milliunits per milligram of protein.

with a cell wall chitin content 10 times smaller than usual. (ii) If chitin synthesis was needed for yeast mating or shmoo formation or both, it is clear that a considerable reduction in chitin content would not be lethal. Indeed, conjugation between α and a strains from the cited mutants was almost not affected. (iii) There is no biochemical evidence for the presence of chitin in yeast spores. However, if chitin was essential for sporulation, it would make sense that diploids homozygous for Calcofluor resistance did not sporulate, as was the case. (iv) The reduction of chitin in the cell wall of the CR1 and CR4 mutants was accompanied by a considerable decrease in the alkali-insoluble β-glucan fraction. This result could be considered circumstantial evidence in favor of the existence of a β-glucan-chitin complex proposed by several authors (3, 15–17).

Chitin synthase 2 levels from wild-type and mutant strains are very similar. It follows then that the structural gene for chitin synthase 2, the presumptive participant in septum formation in *S. cerevisiae* (23), is probably not affected in Calcofluor-resistant mutants and that some other factors, not yet characterized, have to be implicated in yeast chitin synthesis.

How is chitin synthesis impaired in these mutants? That chitin synthesis is regulated by activation of a zymogenic enzyme at a specific time and place is the only hypothesis put forward currently (23). Activation may occur by fusion of activating-factor vesicles with the synthase located at the site of septum formation (8). Microtubules have been involved in the polarization of growth and localization of cell wall deposition in yeast (1). Alteration of the transport of activating-factor vesicles by benomyl may be crucial for chitin synthesis, and indeed, septum formation in the CR1 mutant was drastically inhibited by benomyl (C. Roncero, unpublished results). However, the physiological activation mechanism remains unknown and may involve steps probably defined by the Calcofluor resistance loci cited in this work.

In the past, genetic approaches have solved doubts about the physiological role of an activating factor for chitin synthase 1 (27) or of chitin synthase 1 itself (5) in chitin synthesis in vivo; therefore it seems reasonable to think that additional efforts along the line presented in this report may shed some light on the process of chitin synthesis in yeast.

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