CSD2, *CSD3*, and *CSD4*, Genes Required for Chitin Synthesis in *Saccharomyces cerevisiae*: the *CSD2* Gene Product Is Related to Chitin Synthases and to Developmentally Regulated Proteins in *Rhizobium* Species and *Xenopus laevis*

CHRISTINE E. BULAWA

Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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In *Saccharomyces cerevisiae*, chitin forms the primary division septum and the bud scar in the walls of vegetative cells. Three chitin synthetic activities have been detected. Two of them, chitin synthase I and chitin synthase II, are not required for synthesis of most of the chitin present in vivo. Using a novel screen, I have identified three mutations, designated *csd2*, *csd3*, and *csd4*, that reduce levels of chitin in vivo by as much as 10-fold without causing any obvious perturbation of cell division. The *csd2* and *csd4* mutants lack chitin synthase III activity in vitro, while *csd3* mutants have wild-type levels of this enzyme. In certain genetic backgrounds, these mutations cause temperature-sensitive growth on rich medium; inclusion of salts or sorbitol bypasses this phenotype. Gene disruption experiments show that *CSD2* is nonessential; a small amount of chitin, about 5% of the wild-type level, is detected in the disruptants. DNA sequencing indicates that the *CSD2* protein has limited, but statistically significant, similarity to chitin synthase I and chitin synthase II. Other significant similarities are to two developmental proteins: the *nodC* protein from *Rhizobium* species and the DG42 protein of *Xenopus laevis*. The relationship between the *nodC* and *CSD2* proteins suggests that *nodC* may encode an *N*-acetylglucosaminyltransferase that synthesizes the oligosaccharide backbone of the nodulation factor NodRm-1.

In *Saccharomyces cerevisiae*, chitin plays an important role in cell division. A ring of chitin is made at the base of the emerging bud; following nuclear migration and cytokinesis, this ring is filled in to form a disc or primary septum that separates mother and daughter cells (10).

Three chitin synthases have been detected in cell-free preparations from exponentially growing cells (6). Chitin synthase I and chitin synthase II are zymogens that require partial proteolysis for activity in vitro (10, 42); little or no activity can be detected in untreated membranes (9, 32). In contrast, chitin synthase III does not require proteolysis for activity (6, 32). Because the synthesis of chitin is localized and occurs during a limited period of the cell cycle, it is likely that the activities of these enzymes are regulated temporally and spatially. The regulatory mechanisms used by the cell are not known.

The structural genes for chitin synthase I and chitin synthase II, *CHS1* (8) and *CHS2* (47, 48), respectively, have been cloned and sequenced. Comparison of the deduced amino acid sequences shows that these two proteins are related (47); over the last 750 amino acids, they are about 40% identical. Surprisingly, *chs1::HIS3 chs2::TRP1* double disruptants have no detectable deficiency in the amount of chitin synthesized in vivo (6). In this study, I show that most of the chitin in the cell is made by chitin synthase III and that at least two genes, designated *CSD2* and *CSD4*, are required for this activity.

MATERIALS AND METHODS

Designations. Ts⁻ indicates temperature sensitivity for growth at 37°C; Ts⁺ indicates ability to grow at 37°C.

Strains and plasmids. Strains of *S. cerevisiae* are listed in Table 1. The *S. cerevisiae* YEp24 library was the gift of D.

Botstein; it consists of *Sau3A* genomic DNA fragments inserted into the *Bam*HI site of YEp24. Standard molecular cloning procedures (50) were used. The *S. cerevisiae* *LEU2* gene, which was used as the selectable gene in the disruption experiments, was isolated from the plasmids YEp13 and pJH-L2. The latter is a derivative of pUC18 that has a 2.9-kb *Bgl*II fragment containing *LEU2* inserted into the *Bam*HI site; this plasmid was generously provided by J. Hill (Carnegie-Mellon University). A *leuB* mutant strain of *Escherichia coli* was used to identify recombinant plasmids containing *LEU2*.

Media and growth conditions. The following media (45) were used: YPD (1% yeast extract, 2% peptone, 2% glucose), YPDC (as YPD but containing 100 µg of Calcofluor per ml), 0.5× YPD (0.5% yeast extract, 1% peptone, 1% glucose), 0.5× YPD plus KCl (as 0.5× YPD but containing 0.25 M KCl), SD (0.7% yeast nitrogen base, 2% glucose), and SDC (as SD but containing 0.25 M potassium bipthalate [pH 6.5] and 200 µg of Calcofluor per ml) (38). For solid media, agar was added to 2% except for SDC plates, which contained 1.5% agar. In YPDC medium I used peptone from Difco, having found that peptone from other sources can cause precipitation of the Calcofluor. Roncero et al. (39) used YE medium (1% yeast extract, 1% glucose) for selection of Calcofluor-resistant mutants; I used YPD because certain strains (e.g., PP-1A and derivatives of this strain) were found to grow poorly in the absence of peptone (5).

Colony screen for mutants defective in chitin synthesis in vivo. Strains PP-1A and PP-1B were treated with ethyl methanesulfonate (45) to a level of about 10% survival. A portion of the PP-1B culture was subjected to a suicide selection (see below). To screen for strains defective in chitin synthesis, mutagen-treated cells were spread onto YPD agar to a density of approximately 150 cells per plate

TABLE 1. *S. cerevisiae* strains used^a

Strain	Genotype	Reference or source
PP-1A	<i>MATα ade2-101 chs1-3::URA3 ura3-52 Sup⁻</i>	8
PP-1B	<i>MATα his4 chs1-3::URA3 ura3-52 Sup⁻</i>	8
S-13C	<i>MATα csd1-1 his4 chs1-3::URA3 ura3-52 Sup⁻</i>	This work
2-20A	<i>MATα csd2-1 his4 chs1-3::URA3 ura3-52 Sup⁻</i>	This work
3-2	<i>MATα csd2-2 his4 chs1-3::URA3 ura3-52 Sup⁻</i>	This work
3-4	<i>MATα csd2-3 his4 chs1-3::URA3 ura3-52 Sup⁻</i>	This work
3-5	<i>MATα csd2-4 his4 chs1-3::URA3 ura3-52 Sup⁻</i>	This work
4-7B	<i>MATα csd2-5 ade2-101 chs1-3::URA3 ura3-52 Sup⁻</i>	This work
S-13B	<i>MATα csd3-1 his4 chs1-3::URA3 ura3-52 Sup⁻</i>	This work
4-2	<i>MATα csd3-2 ade2-101 chs1-3::URA3 ura3-52 Sup⁻</i>	This work
3-6	<i>MATα csd3-3 csd4-1 his4-chs1-3::URA3 ura3-52 Sup⁻</i>	This work
PP-2C	<i>MATα his4 ura3-52 Sup⁻</i>	8
XM15-7C	<i>MATα csd4-1 his4 chs1-3::URA3 ura3-52 Sup⁻</i>	This work ^b
XM33-7B	<i>MATα csd2-1 ade2-101 chs1-3::URA3 ura3-52 Sup⁻</i>	This work ^c
XM33-10B	<i>MATα csd2-1 his4 chs1-3::URA3 ura3-52 Sup⁻</i>	This work ^c
XM35-6C	<i>MATα csd3-1 ade2-101 chs1-3::URA3 ura3-52 Sup⁻</i>	Segregant from S-13B × PP-1A
XM36-8D	<i>MATα csd1-1 ade2-101 chs1-3::URA3 ura3-52 Sup⁻</i>	Segregant from S-13C × PP-1A ^d
XM37-2C	<i>MATα csd2-1 ade2-101 ura3-52 Sup⁻</i>	<i>CHS1</i> segregant from XM33-7B × PP-2C
XM37-1D	<i>MATα csd2-1 ade2-101 ura3-52 Sup⁻</i>	<i>CHS1</i> segregant from XM33-7B × PP-2C
XM37-3A	<i>MATα ade2-101 ura3-52 Sup⁻</i>	<i>CSD2 CHS1</i> segregant from XM33-7B × PP-2C
XM43-2C	<i>MATα csd3-1 ade2-101 ura3-52 Sup⁻</i>	<i>CHS1</i> segregant from XM35-6C × PP-2C
XM53-5B	<i>MATα csd4-1 his4 ura3-52 Sup⁻</i>	<i>CHS1</i> segregant from XM15-7C × XM37-3A
DBY1315	<i>MATα leu2-3,112 lys2-801 ura3-52 Sup⁻</i>	D. Botstein
DBY2068	<i>MATα leu2-3,112 his4-619 ura3-52 Sup⁺</i>	D. Botstein
CBY1	<i>MATα chs1::HIS3 leu2-3,112 his3 trp1-Δ1 ura3-52 Gal⁺ Sup⁺</i>	This work ^c
CBY201	<i>MATα chs1::HIS3 csd2-8::LEU2 leu2-3,112 his3 trp1-Δ1 ura3-52 Gal⁺ Sup⁺</i>	<i>csd2-8::LEU2</i> derivative of CBY1 (see text)
XM59-7A	<i>MATα ade2-101 his4 leu2-3,112 ura3-52 Sup⁻</i>	Segregant from XM33-7B × DBY2068
CBY45	<i>MATα csd2-6::LEU2 ade2-101 his4 leu2-3,112 ura3-52 Sup⁻</i>	<i>csd2-6::LEU2</i> derivative of CBY1
PRY288	<i>MATα his4-917 leu2-1 pet9</i>	G. Fink

^a Strains that suppress the temperature sensitivity associated with the *csd2*, *csd3*, and *csd4* mutations are designated Sup⁺; those that do not are designated Sup⁻.

^b Segregant from a 0:4 Ts⁺:Ts⁻ tetrad from 3-6 × PP-1A. Complementation analysis showed that the defect in this strain was not allelic to *csd1-1*, *csd2-1*, or *csd3-1*.

^c Segregant from fifth backcross of *csd2-1* to PP-1A.

^d Grows slowly on synthetic medium, a phenotype that appears to be linked to *csd1-1*.

^e *chs1::HIS3* derivative of the haploid 9933-13A (obtained from G. Fink) by transformation with the disrupted gene.

and incubated for 3 days at 26°C. The colonies were replica plated onto filter papers (Schleicher & Schuell no. 589) and grown on the surface of YPD plates for 4 h at 26°C. The replicas were transferred to YPD plates that had been prewarmed to 37°C, incubated for 40 min, and then placed into petri plates containing filters (Schleicher & Schuell no. 589) soaked with 2 ml of labeling medium (10% yeast extract, 20% peptone, 0.05% glucose, 50 μCi of [6-³H]glucosamine per ml [25 Ci/mmol]). After 40 min at 37°C, mannann was extracted from the colonies by transferring the replicas to glass petri plates containing filters (Schleicher & Schuell no. 470) saturated with 0.25 M sodium carbonate (7.5 ml per dish) and autoclaving them for 1 h. The replicas were gently washed with water on a scintered glass funnel and air dried. The alkali-insoluble radioactive material was detected by autoradiography for 4 days, using tritium-sensitive film (Reichert-Jung Ultrofilm; Mager Scientific, Dexter, Mich.), and was identified as chitin on the basis of its sensitivity to partially purified *Serratia marcescens* chitinase.

[³H]glucosamine suicide selection was performed as described previously (21) except that [6-³H]glucosamine (5 mCi/ml, 25 Ci/mmol) was used in place of [2-³H]mannose. After 15 days at -80°C, the survival rate was about 0.3%. The screen described above was used; strains S-13B and S-13C were obtained.

Measurement of chitin in vivo. Cells were harvested and incubated in hot KOH to remove the mannan layer of the cell

wall. The ghosts were washed and treated with purified chitinase. The supernatant was saved and treated with hexosaminidase. The amount of GlcNAc was determined with the Morgan-Elson reaction, a colorimetric assay for *N*-acetyl-2-amino sugars (54). The procedure was performed as described previously (8) except that the hexosaminidase was from *Helix pomatia* (β-glucuronidase).

Chitin synthase III assay. Because chitin synthase I is much more active than the other two chitin synthases, strains with *chs1* null mutations were used for most enzymatic studies. For measurement of chitin synthase III activity, the method of Orlean (32) was used, with slight modifications. All solutions used for the preparation of membranes were chilled to 4°C. One liter of cells grown at 26°C in YPD was harvested at an *A*₆₀₀ of 1, washed with water and buffer A (50 mM Tris HCl [pH 7.5], 1 mM EDTA, 1 mM dithiothreitol), and then resuspended in about 2 ml of buffer A. Glass beads were added to a height of a few millimeters below the meniscus. The samples were mixed on a Vortex Genie for 30 s and chilled on ice for at least 1 min; this procedure was repeated three times. The extract was transferred to a clean tube, the beads were washed four to six times with buffer A, and the walls were removed by centrifugation. The total volume of the tube was adjusted to 20 ml with buffer A, and then the samples were centrifuged at 4°C in a Beckman 50Ti rotor at 35,000 rpm for 45 min. The pellets were resuspended in about 1 ml of buffer B (same as buffer

A except that the dithiothreitol is omitted). Chitin synthase activity was measured by incubating 10 to 20 μ l of membranes with 1 mM UDP-[U- 14 C]*N*-acetylglucosamine (625 cpm/nmol), 40 mM *N*-acetylglucosamine, 50 mM Tris-HCl (pH 7.5), and 5 mM $MgCl_2$ at 25°C. The reaction was stopped with 10% trichloroacetic acid, and the amount of product was quantitated. As noted previously, this enzyme is unstable (6, 32), and the specific activity can vary from preparation to preparation. Therefore, activity is expressed as the percentage of the wild-type control activity.

For proteolytic activation of the *CHS2* gene product, membranes were treated with TPCK-trypsin. After 10 min at 30°C, a twofold excess of soybean trypsin inhibitor was added. These samples were assayed as described above.

Cloning of the *CSD2* gene. The wild-type *CSD2* gene was isolated by complementation of the Ts^- growth of a *csd2-1* mutant. XM37-2C (*csd2-1 ura3-52*) was transformed (45) with a yeast genomic library in YEp24 and was plated on synthetic medium lacking uracil to a density of approximately 500 to 1,000 colonies per plate and incubated at 26°C. The cells were washed from the plate with sterile water. To screen for *CSD2* colonies, approximately 100,000 cells from each transformation plate were spread onto 0.5 $\times$ YPD plates at 37°C. The replating step was necessary because XM37-2C is not Ts^- on synthetic medium (see Results). Plates that had 10 to 100 temperature-resistant colonies were saved. Plasmids were isolated from a few colonies from each plate, passaged through *E. coli*, and retested for the ability to complement XM37-2C. Plasmid pCSD2 was obtained.

Nucleic acid blot hybridization. The chromosome blot was provided by B. Osmond. The conditions for hybridization and washing have been reported elsewhere (6). Southern analysis was performed as described previously (6, 50).

DNA sequencing. Several DNA fragments containing portions of the *CSD2* gene were cloned into M13mp18 and M13mp19. Nested deletions were made by using the exonuclease activity of T4 DNA polymerase (Cyclone I Biosystem; International Biotechnologies, Inc.). The nucleotide sequence of both DNA strands was determined by the method of Sanger et al. (41), using the Sequenase version 2.0 kit (U.S. Biochemical Corp., Cleveland, Ohio). Sequence data were assembled and analyzed by using the programs of Staden (49) and the University of Wisconsin Genetics Computer Group (13).

Nucleotide sequence accession number. The sequence of the *CSD2* locus has been assigned GenBank accession number M73697.

RESULTS

Isolation of mutants defective in chitin synthesis in vivo. It has recently been demonstrated that chitin synthase I and chitin synthase II are not required for most of the chitin synthesis in vivo (6). To identify the genes required for this process, I screened for mutants that are unable to convert exogenous [3 H]glucosamine to cellular chitin. I expected to find two classes of mutants: strains defective in the conversion of [3 H]glucosamine to UDP-*N*-acetyl-[3 H]glucosamine and strains defective in the conversion of UDP-*N*-acetyl-[3 H]glucosamine to chitin. I was primarily interested in the second class; however, because it is not known whether chitin is absolutely required for growth, I designed a screen that would allow the recovery of mutants defective in essential genes and therefore expected to obtain both classes of mutants. Additional characterization of the putative mutants was then used to distinguish between them.

Strains PP-1A and PP-1B (Table 1) were treated with ethyl methanesulfonate, and the survivors were spread onto YPD agar plates and grown at 26°C. The colonies were replica plated onto filter paper, and the plate was saved to allow the recovery of putative mutants. The filter paper replica was shifted to 37°C and pulse-labeled with [3 H]glucosamine, which is incorporated primarily into chitin and mannan. To remove the latter, the replicas were autoclaved in 0.25 M sodium carbonate and washed with water. The radioactive chitin was detected by autoradiography, and the colonies were visualized by staining the filters with Coomassie blue. The autoradiogram was superimposed on the stained filter, and blue colonies that lacked a corresponding gray spot on the autoradiogram were identified as putative mutants.

From about 10,000 mutagen-treated colonies, nine mutants were obtained (Table 1); all are unable to form single colonies at 37°C on YPD plates. Each mutant was backcrossed to the parental strain of opposite mating type and subjected to tetrad analysis. For eight of the mutants, the defect in chitin synthesis segregated 2:2 and cosegregated with Ts^- growth, showing that the mutation of a single nuclear gene causes both phenotypes. For one of the original isolates, designated 3-6, tetrads containing two, three, or four mutant progeny were recovered, suggesting the presence of two unlinked mutations in chitin synthesis in this strain. This was verified by using each of the sisters from a 0:4 Ts^+ : Ts^- tetrad for complementation analyses. Both mutations cause Ts^- growth.

Pairs of the mutants were crossed, and diploids were selected and scored for the ability to grow at 37°C. This analysis revealed four complementation groups, designated *csd1*, *csd2*, *csd3*, and *csd4* (chitin synthesis defective), which were represented by one, five, three, and one allele(s), respectively. The possibility of interallelic complementation was eliminated by crossing representative alleles from each group and performing tetrad analysis. None of the crosses gave a 0:4 Ts^+ : Ts^- segregation pattern (data not shown), demonstrating that these mutations are unlinked.

Analysis of chitin synthesis in vivo in the *csd* mutants. The simplest explanations for the Ts^- growth of the *csd* mutants are that these genes are essential and that the mutant genes encode temperature-sensitive proteins. If so, normal amounts of chitin should be made when the mutants are grown at 26°C, since the mutant protein should be functional at this temperature. An alternative explanation is that the mutant gene products are defective at both 26 and 37°C but are required for growth only at elevated temperatures. In this case, little or no chitin should be present in the mutants regardless of the growth temperature. Measurement of the amount of chitin made in vivo at 26°C (Fig. 1) showed that different explanations apply to different mutants. In the *csd1-1* mutant, no significant reduction in chitin was detected at 26°C, the expected result for a temperature-sensitive mutation. In contrast, a dramatic reduction in chitin content, about 10-fold lower than in the control, was observed for all of the alleles of *csd2* and *csd3*, indicating that the second explanation is more likely for these eight mutants. In the *csd4* mutant, the amount of chitin was reduced by two- to threefold, indicating that the mutant protein has partial activity at 26°C.

The dye Calcofluor binds to bud scars, chitin-rich structures that remain on the mother cell after division. When added to the growth medium, Calcofluor is toxic (37). Roncero et al. (39) isolated Calcofluor-resistant strains and showed that these mutants had ninefold reductions in cellular chitin. I tested the ability of the *csd* mutants to grow in

FIG. 1. Quantitation of the amounts of chitin in cells of the *csd* mutants. Cells were grown overnight in YPD at 26°C, and the amounts of chitin were determined. The amounts of chitin are normalized to the wet weights of the cells.

the presence of Calcofluor at 26°C and obtained the expected results. The *csd1* and *csd4* mutants were sensitive, while the *csd2* and *csd3* mutants were resistant (Table 2).

The *csd* mutants were tested for altered sensitivity to tunicamycin, antimycin, nystatin, EDTA, and glucosamine; no differences from the wild-type strain were detected.

The *csd2* and *csd4* mutants lack chitin synthase III activity. The lack of chitin in the *csd2*, *csd3*, and *csd4* mutants suggested that one or more of these genes may be required for chitin synthase III activity. To test this possibility, one allele from each complementation group was chosen, membranes were prepared, and chitin synthase III activity was measured (Table 2). Membranes from the *csd1* mutant had nearly normal levels of the enzyme, as expected since this mutant synthesizes normal amounts of chitin *in vivo* at 26°C. The membranes from the *csd2* and *csd4* strains had activities almost 10-fold lower than that of the wild-type strain, suggesting that one or both of these genes encode chitin synthase III. Interestingly, the *csd3* mutant, which makes very little chitin *in vivo*, has no reduction in chitin synthase III activity. As a control, all strains were also assayed after preincubation of the membranes with trypsin; this was done in order to measure chitin synthase II activity. In all cases, chitin synthesis could be detected at a rate comparable to that of the wild-type control (data not shown).

The temperature sensitivity of *csd2*, *csd3*, and *csd4* can be suppressed. The obvious strategy for cloning the *csd* genes is the selection of recombinant plasmids that correct the Ts⁻ growth of the mutants. Yeast vectors use *URA3*, *LEU2*, or other amino acid biosynthetic genes as selectable markers

FIG. 2. Growth of various strains at 26 and 37°C in the presence or absence of KCl. Compositions of the media are given in Materials and Methods. The diagram indicates the position and relevant genotype of each strain. Sup⁺ originated from DBY2068. The plates were incubated for either 2 days at 37°C or 3 days at 26°C.

(45), and transformants are grown on synthetic medium (SD). Examination of the growth of the *csd* mutants on SD plates showed that three of the complementation groups, *csd2*, *csd3*, and *csd4*, are not Ts⁻ on this medium. Because SD contains relatively high concentrations of ammonium sulfate, I examined the ability of various solutes to suppress the Ts⁻ phenotype of *csd* mutants. When YPD plates were supplemented with 0.1 M sorbitol, partial restoration of growth was observed. When the concentration of sorbitol was increased to 1 M or when 0.25 M KCl was used, all three mutants grew at 37°C at the same rate as did the CSD⁺ strain. Thus, increasing the osmolarity of the medium suppresses the Ts⁻ growth of the *csd2*, *csd3*, and *csd4* mutants. The *csd1* mutant was Ts⁻ under all of the conditions tested.

By decreasing the osmotic strength of YPD, I observed that the Ts⁻ phenotype scored more cleanly. Therefore, 0.5× YPD plates, which contain half of the usual amounts of yeast extract, peptone, and glucose, were routinely used for testing growth at 37°C. In control experiments, wild-type strains grew at the same rate on YPD and 0.5× YPD at both 26 and 37°C. Figure 2 illustrates the temperature sensitivity of a *csd2* mutant (*csd2* Sup⁻) on 0.5× YPD (compare plates B and C) and the suppression of this phenotype by inclusion

TABLE 2. Characterization of the *csd* mutants

Strain	Relevant genotype	Chitin synthase III sp act ^a (% of wild type)	Growth			
			0.5× YPD ^b		+KCl ^c 37°C	YPDC ^d 26°C
			26°C	37°C		
PP-1A	CSD ⁺ <i>chs1::URA3</i>	100	+	+	+	—
XM36-8D	<i>csd1-1 chs1::URA3</i>	72	+	—	—	—
XM33-7B	<i>csd2-1 chs1::URA3</i>	13	+	—	+	+
XM35-6C	<i>csd3-1 chs1::URA3</i>	113	+	—	+	+
XM15-7C	<i>csd4-1 chs1::URA3</i>	9	+	—	+	—

^a For PP-1A, 1 nmol of *N*-acetylglucosamine was incorporated into acid-insoluble product per h per mg of protein.

^b Solid medium containing 0.5% yeast extract, 1% peptone, 1% glucose, and 2% agar.

^c 0.5× YPD plates supplemented with 0.25 M KCl.

^d YPD plates containing 100 µg of Calcofluor per ml.

TABLE 3. Genetic suppression of the temperature-sensitive growth of the *csd* mutants^a

Cross	No. of tetrads				
	4:0 ^b	3:1	2:2	1:3	0:4
Expt A^c					
XM33-7B (<i>csd2-1</i>) × PP-2C	0	1	9	0	0
XM35-6C (<i>csd3-1</i>) × PP-2C	0	0	9	0	0
XM15-7C (<i>csd4-1</i>) × PP-2C	0	0	10	0	0
Expt B^d					
XM33-10B (<i>csd2-1</i>) × DBY1315	0	1	8	0	0
XM33-7B (<i>csd2-1</i>) × DBY2068	3	3	2	0	0
XM37-1D (<i>csd2-1</i>) × CBY1	3	6	2	0	0
XM43-2C (<i>csd3-1</i>) × CBY1	5	4	2	0	0
XM55-5B (<i>csd4-1</i>) × CBY1	2	3	2	0	0

^a The indicated haploid strains were mated, and diploids were selected, sporulated, micromanipulated, and germinated at 26°C on YPD plates. Segregants were scored for growth at 37°C on 0.5× YPD plates.

^b Ts⁺:Ts⁻.

^c Backcrosses of the *csd* mutants to the parental strain.

^d Outcrosses of the *csd* mutants to various nonparental strains.

of 0.25 M KCl into the medium (compare plates C and D). Similar results were obtained with *csd3* and *csd4* mutants (data not shown).

Suppression of the Ts⁻ growth due to the *csd2*, *csd3*, and *csd4* mutations was also observed during outcrossing. As stated above, Ts⁻ growth segregated 2:2 when each of the *csd* mutants was backcrossed to the parental strain (Table 3, experiment A). When any of these mutants was outcrossed to certain nonparental strains, such as DBY2068 or CBY1, tetrads with zero, one, or two Ts⁻ haploids were recovered (Table 3, experiment B), indicating that these strains harbor genes that allow the *csd2*, *csd3*, and *csd4* mutants to grow at elevated temperatures. Suppression does not restore chitin synthesis, as indicated by the 2:2 segregation of Calcofluor resistance and by chemical measurement of cellular chitin (e.g., CBY201; see below). Strains that harbor suppressor genes are designated Sup⁺, while those lacking suppressor genes are designated Sup⁻. To assess the efficiency of suppression, *csd2* Sup⁺ and *CSD2* strains were streaked onto a 0.5× YPD plate and the growth of single colonies was monitored at 37°C. *csd2* Sup⁺ strains grew at the same rate as the wild-type strain (*CSD2* Sup⁻) (Fig. 2, plate C). Similar results were obtained with *csd3* and *csd4* mutants (data not shown). *CSD2* Sup⁻ strains have no obvious defects in growth. Other nonparental strains do not contain a suppressor gene (Table 3, experiment B, DBY1315).

These data indicate that under certain conditions, the *csd2*, *csd3*, and *csd4* mutants are temperature sensitive for growth. Since all three mutants are chitin deficient, it is possible that this trait causes the Ts⁻ phenotype. Under other conditions, growth and division proceed quite normally with less than 10% of the wild-type level of chitin. The observation that most of the cellular chitin is not essential for growth has been made previously, using the *cal* mutants (39).

***CSD2* is dominant to *csd2-1*.** Before attempting to clone *CSD2* by complementation of *csd2-1*, I tested whether the mutation is recessive to the wild-type allele. Three diploid strains, *csd2/csd2* Sup⁻/Sup⁻, *CSD2/csd2* Sup⁻/Sup⁻, and *CSD2/CSD2* Sup⁻/Sup⁻, were constructed, and their growth on 0.5× YPD was examined. The *csd2/csd2* Sup⁻/Sup⁻ strain did not grow on these plates at 26°C (Fig. 2, plate B), which was unexpected since both of the haploid parents

grow well under these conditions (data not shown). The *CSD2/csd2* Sup⁻/Sup⁻ strain grows as well as the wild-type strain at 26°C (Fig. 2, plate B), but at 37°C, the heterozygote forms small colonies (Fig. 2, plate C). Thus, the wild-type allele is dominant at 26°C; at 37°C, a gene dosage effect is observed. These results indicate that it should be possible to isolate *CSD2* from a high-copy-number yeast genomic library.

In related experiments, I found that Sup⁺ is dominant to Sup⁻ at 26°C (Fig. 2, plate B; compare *csd2/csd2* Sup⁻/Sup⁻ with *csd2/csd2* Sup⁺/Sup⁻). At 37°C, Sup⁺ is less effective (Fig. 2, plate C). All of the diploids grew well on media supplemented with 0.25 M KCl (Fig. 2, plate D). I have also found that spores derived from *csd2/csd2* Sup⁻/Sup⁻ diploids are hypersensitive to zymolyase; lysis of spores was observed even when digestions are performed in buffers containing 1 M sorbitol.

Molecular cloning of *CSD2*. Because *CSD2* and *CSD4* are the putative structural genes for chitin synthase III, both were analyzed in detail. This report describes the cloning, disruption, and sequencing of *CSD2*; a similar analysis of *CSD4* will be reported elsewhere. *CSD2* was isolated from a yeast genomic library in YEp24, a high-copy-number vector, by complementation of the Ts⁻ growth of XM37-2C (see Materials and Methods). One plasmid, designated pCSD2, was obtained, and it simultaneously corrects all of the defects associated with the *csd2* mutation. The restoration of cellular chitin synthesis eliminates the possibility that pCSD2 carries a bypass suppressor, e.g., the gene(s) that confers the Sup⁺ phenotype. Because pCSD2 is a derivative of YEp24, a high-copy-number vector (45), it was possible that the transformants would overproduce chitin synthase III. However, this was not the case; the transformants had wild-type amounts of this enzyme (data not shown).

A partial restriction map of pCSD2 is shown in Fig. 3. Deletion analysis together with subcloning of the DNA insert (Fig. 3) showed that the 3.7-kb *Xho*I-to-*Pvu*II fragment corrects the Ts⁻ growth of XM37-2C (*csd2-1*). Since the size of this fragment correlates well with the size of the mRNA (~4 kb; data not shown), it is likely that this fragment contains most of the *CSD2* gene.

Disruption and chromosomal mapping of *CSD2*. In previous sections, I presented evidence suggesting that the *CSD2* gene product is required for growth only when certain strains and culture conditions are used. To prove this unequivocally, I tested whether *csd2* null mutations confer a conditional-lethal phenotype. The structures of the disrupted genes are shown in Fig. 3. Strains DBY1315 and DBY2068 were mated, and the resulting diploid was transformed with DNA fragments containing the *csd2-6::LEU2* and *csd2-7::LEU2* genes. These two disruptions differ only in the orientation of the *LEU2* gene. Four independent Leu⁺ colonies, three transformed with *csd2-6::LEU2* and one transformed with *csd2-7::LEU2*, were sporulated. Six tetrads from each were micromanipulated and germinated at 26°C on a YPD plate. Twenty-one of twenty-four tetrads produced four viable colonies, indicating that *CSD2* is not essential. Eleven tetrads were analyzed in detail (Table 4). In every case, the ratio of Leu⁺ to Leu⁻ was 2:2 (Table 4, first row), and Calcofluor resistance cosegregated with Leu⁺. As discussed above, DBY2068 has a gene that suppresses the Ts⁻ growth caused by the *csd2-1* mutation. If the disrupted *csd2* gene can be suppressed, then the segregation of Ts⁺:Ts⁻ should be predominantly 3:1. This was in fact observed (Table 4, third row). All Ts⁻ strains were also Leu⁺ and Calcofluor resistant. When KCl was present in the plates, all of the

FIG. 3. Restriction enzyme analysis and disruption of *CSD2*. The thin and heavy lines represent vector and *S. cerevisiae* genomic DNAs, respectively. Strain XM37-2C (*csd2-1*) was transformed with each of the plasmids depicted above; Ura⁺ colonies were tested for growth at 37°C on 0.5× YPD plates. +, Normal growth; −, no growth. (A) Partial restriction map of pCSD2. Restriction sites that occur more than once in the insert are abbreviated as follows: B, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hind*III; P, *Pst*I. The precise position of one of the *Bgl*II sites (B*) is unclear; it is possible that this site lies to the right of the second *Eco*RI site. The extents of the open reading frames of *CSD2* and *FUR4* are indicated by the lengths of the open arrows; the direction of each arrow shows the direction of transcription. (B) Subcloning of pCSD2. Plasmid pCSD2-3 is the 5.4-kb *Cla*I-*Bam*HI fragment of pCSD2 cloned into the same sites of the *CEN*/*ARS* vector pRS316 (46). The restriction sites are abbreviated as in panel A, with the following additions: M, *Mlu*I; Pv, *Pvu*II; poly l, left polylinker (*Kpn*I, *Apa*I, *Xho*I, and *Sal*I); poly r, right polylinker (*Spe*I, *Xba*I, *Not*I, *Sac*I, and *Sac*II). pCSD2-1 was constructed by ligation of the 4.2-kb *Xho*I-*Bam*HI fragment from pCSD2 into the *Sal*I and *Bam*HI sites of YEp24. S/X indicates a hybrid *Sal*I-*Xho*I site. pCSD2-2 is a 3.6-kb *Xho*I-*Pvu*II fragment containing *CSD2* inserted into the *Sal*I and *Sma*I sites of YEp352 (20). Abbreviations: S/X, *Sal*I-*Xho*I hybrid site; Pv/Sm, *Pvu*II/*Sma*I hybrid site; poly l, left polylinker (*Hind*III, *Sph*I, and *Pst*I); poly r, right polylinker (*Kpn*I, *Sac*I, and *Eco*RI). (C) Disruption of *CSD2*. The alleles *csd2-6::LEU2* and *csd2-7::LEU2* (not shown) are insertion disruptions of *CSD2*; they are identical except for the orientation of *LEU2*. Plasmid pCSD2-1 was cut with *Asp* 718 (isoschizomer of *Kpn*I), the ends were filled in with Klenow fragment, and then *Xho*I linkers (pCCTCGAGG) were added. (This step regenerates the *Asp* 718 site.) The DNA was digested with *Xho*I, and a 2.2-kb *Sal*I-*Xho*I fragment containing the *S. cerevisiae* *LEU2* gene (obtained from YEp13; see Materials and Methods) was inserted. Plasmids pCSD2-D1.1 and pCSD2-D1.2 carry *csd2-6::LEU2* and *csd2-7::LEU2*, respectively. To obtain linear DNA fragments for gene disruption experiments, these plasmids were cut with *Bgl*II. The allele *csd2-8::LEU2* was made by replacing the 0.5-kb *Bgl*II fragment of *CSD2* with a 2.9-kb DNA fragment containing *LEU2*. Plasmid pCSD2-2 was digested with *Bgl*II; the 8.5-kb fragment was recovered and ligated to a 2.9-kb *Bsr*YI fragment containing *LEU2* (obtained from pJH-L2; see Materials and Methods) to give pCSD2-D2b. To obtain linear DNA fragments for gene disruption experiments, this plasmid was cut with *Hind*III (one site is in poly l). The abbreviations are the same as in panels A and B, with the following additions: X, *Xho*I; KX/S, a *Kpn*I site adjacent to an *Xho*I-*Sal*I hybrid site (the sequence is GGTACCCTCGAC); XK, adjacent *Xho*I and *Kpn*I sites. Proper integration of the disrupted genes was verified by Southern analysis (data not shown).

TABLE 4. Tetrad analysis of a *CSD2/csd2::LEU2* diploid^a

Medium ^b	Temp (°C)	No. of tetrads				
		4:0 ^c	3:1	2:2	1:3	0:4
SD (−Leu)	26	0	0	11	0	0
YPDC ^d	26	0	0	11	0	0
0.5× YPD ^e	37	3	8	0	0	0
0.5× YPD + KCl	37	11	0	0	0	0

^a Strains DBY1315 and DBY2068 were mated, and the diploid was transformed with DNA fragments containing *csd2-6::LEU2* or *csd2-7::LEU2* (Fig. 3). Leu⁺ transformants were sporulated, micromanipulated, and germinated at 26°C on YPD plates. Eleven tetrads were scored for the ability to grow under the indicated conditions.

^b SD (−Leu) is synthetic medium lacking leucine but supplemented with all other required nutrients. Other media are defined in Materials and Methods.

^c Viable; inviable.

^d Calcofluor resistance was scored after 2 to 3 days at 26°C. With prolonged incubation, it was clear that some Leu[−] colonies, including DBY2068, had a low level of Calcofluor resistance.

^e Strains were scored as viable under these conditions if they formed single colonies after 1 to 2 days at 37°C. The efficiency of suppression was somewhat variable; some strains grew at a faster rate than others, as judged by colony size. Strains scored as inviable did not form single colonies even after several days at 37°C.

progeny grew at 37°C (Table 4, fourth row). Thus, the null allele produces Ts[−] growth in certain strains, and this phenotype is suppressed by high levels of salt or by a suppressor gene present in DBY2068.

A second disruption experiment was done with a deletion disruption (*csd2-8::LEU2*); in this case, the disrupted gene was introduced into the haploids DBY1315, a Sup[−] strain, and CBY1, a Sup⁺ strain. As expected, the Leu⁺, Calcofluor-resistant transformants derived from DBY1315 were Ts[−] for growth, while those derived from CBY1 grew at 37°C.

All five of the *csd2* alleles obtained by ethyl methanesulfonate mutagenesis had low but detectable levels of chitin in vivo. One explanation for this finding is that the mutant *csd2* proteins retain a low level of activity in vivo. Alternatively, the residual chitin may be made by a different chitin synthase. To address this question, I measured the chitin in CBY201, a strain that carries a deletion disruption of *CSD2*. As expected, the amount of chitin is greatly reduced; only 0.042 μg of chitin per mg (wet weight) of cells was detected in CBY201, compared with 1.22 μg of chitin per mg of cells in the isogenic parent CBY1. The small amount of Morgan-Elson (54)-positive material present in CBY201 was characterized further. The visible spectrum (450 to 650 nm) displayed absorbance maxima at 542 nm and 583 nm; identical results were obtained with an authentic *N*-acetylglucosamine standard (data not shown). The simplest explanation for these data is that CBY201 contains a small amount of chitin under these growth conditions; since this strain is *chs1::HIS3*, the residual chitin is not made by chitin synthase I.

CSD2 is located on chromosome II, as determined by chromosome blotting using the 4-kb *Xho*I-to-*Bam*HI fragment as a probe (data not shown). This assignment was confirmed by crossing CBY201 (*csd2-8::LEU2 trp1*) to PRY288 (*pet9*). Tetrad analysis showed that *CSD2* is linked to *pet9* (30), as indicated by the lack of nonparental ditype tetrads (14 parental:0 nonparental:25 tetratype tetrads; map distance of ~30 centimorgans), and to the centromere, as indicated by the segregation of *csd2::LEU2* and *trp1* (15 parental:20 nonparental:4 tetratype; ~5 centimorgans from the centromere). Thus, *CSD2* is located near *CEN2*.

To position *CSD2* more precisely, it was physically mapped by using the prime clone grid filters, a set of 880 lambda clones that contain over three-quarters of the *S. cerevisiae* genome. The 2.3-kb *XhoI-PstI* fragment from *CSD2* hybridized to two clones, 2624 and 6755, that have been shown to carry *FUR4* (36), a gene that has been genetically mapped to the right arm of chromosome II, distal of the *GAL* cluster (53). Examination of the restriction map of *FUR4* (23) indicates that it is carried on pCSD2; its position is shown in Fig. 3. In summary, *CSD2* resides on the right arm of chromosome II, about 1 kb distal of *FUR4* (i.e., *CEN2 GAL10 FUR4 CSD2*). The only gene in this region known to be involved in cell wall synthesis is *MNN2*. From comparison of the restriction maps and transcript sizes (*MNN2* mRNA is 2.2 kb [14], compared with 4 kb for *CSD2* mRNA), it is clear that *MNN2* and *CSD2* are different genes.

Several lines of evidence indicate that the cloned gene is *CSD2* rather than a multicopy suppressor. First, the null mutations and *csd2-1* produce identical phenotypes, namely, a 10-fold decrease in cellular chitin, loss of chitin synthase III activity, and temperature-sensitive growth in *Sup*⁻ strains. Second, *csd2-1* is complemented by pCSD2-3 (Fig. 3), a *CEN/ARS* plasmid that carries *CSD2*. Third, the *csd2::LEU2* mutations do not complement *csd2-1* in diploids. Fourth, *csd2-1* genetically maps to the same position as the disrupted alleles. Results obtained from two crosses show that *csd2-1* is centromere linked (XM37-1D [*csd2-1*] × CBY1 [*trp1*]; 4 parental:4 nonparental:3 tetratype tetrads) and linked to *pet9* (XM37-2C [*csd2-1*] × PRY288 [*pet9*]; 14 parental:0 nonparental:17 tetratype tetrads). Fifth, *csd2-1* and *csd2-6::LEU2* are tightly linked. Strains XM37-1D and CBY45 were mated, but because the resulting diploid (*csd2-1/csd2-6::LEU2 Sup*⁻/*Sup*⁻) produced spores that lysed when digested with zymolyase (see above), tetrad dissection was not possible. To circumvent this problem, XM37-1D was transformed with the high-copy-number plasmid pCSD2 and then mated to CBY45. Three independent diploids were sporulated; asci were dissected from each, and the spores were germinated on YPD plates. Thirty-one tetrads were first cured of pCSD2 by growth on agar plates containing 5-fluoroorotic acid and were then subjected to tetrad analysis. In 29 tetrads, all four sisters were both Calcofluor resistant and *Ts*⁻. In the remaining two tetrads, Calcofluor resistance segregated 3:1; the Calcofluor-sensitive strains were *Ts*⁺.

Determination of the nucleotide sequence of *CSD2*. Various DNA fragments from the insert of pCSD2 were cloned into M13mp18 and M13mp19, nested deletions were made (for details, see Materials and Methods), and the nucleotide sequence was determined. The complete nucleotide sequence of *CSD2*, as well as the sequence of 5' and 3' flanking regions, is shown in Fig. 4. There is a single long open reading frame of 3,495 nucleotides which encodes a protein with a molecular weight of 131,568. A consensus TATA box is found at -100 bp. Two putative transcription initiation sites (17), TC(G/A)A, are located between the TATA box and the first ATG; RRYRR does not occur in this region. Because chitin synthesis is induced by mating factors (43), I looked for the pheromone-responsive element, TGAAACA (25), and found two matches, one at -365 bp and one in reverse orientation at -176 bp. In the 3' flanking region, the eukaryotic mRNA processing signal, AAUAAA (2), occurs twice (3,602 and 3,606 bp). The transcription termination signal TTTTATA (19) is not found, nor is the tripartite sequence of Zaret and Sherman (55). Several yeast mRNAs end with the sequence CAA(TG)CTTTG (2); a related se-

quence, CTATCTTTA, occurs downstream of the *CSD2* gene at 3,619 bp.

A FASTA (35) sequence similarity search of the TRANSGEN protein data base, using the deduced amino acid sequence of *CSD2*, revealed similarity to the *CHS1* (initn = 222) and *CHS2* (initn = 209) proteins, suggesting that *CSD2* encodes the catalytic portion of chitin synthase III. The other significant similarities were to the *nodC* proteins from several *Rhizobium* species (initn = 280 for *R. meliloti*) and to the DG42 protein of *Xenopus laevis* (initn = 173).

DIAGON comparisons between the *CSD2* protein and the *CHS2* (47), *nodC* (22), and DG42 (40) proteins are shown in Fig. 5. A portion of the *CSD2* protein spanning amino acids 827 to 999 (region 1 in Fig. 4) is similar to regions in each of the other three proteins (boxed areas marked 1 in Fig. 5A to C). Analysis of the optimal amino acid alignment (Fig. 6A) of these regions shows that there is 28% identity between *CHS2* and *CSD2* (12.4 standard deviations [SD] above random), 30% identity between *CSD2* and *nodC* (7.2 SD above random), and 25% identity between *CSD2* and DG42 (9.1 SD above random). Thus, these similarities are statistically significant and may indicate common ancestry and/or function.

Similarity to a second region of the *CSD2* protein, amino acids 1004 to 1117 (region 2 in Fig. 4), is found in the *CHS2* and *nodC* proteins (boxed areas marked 2 in Fig. 5A and B) but not in the DG42 protein. The only statistically significant relationship among these regions is a short stretch of 34% identity between *CSD2* and *CHS2* (Fig. 6B). A more subtle relationship between these regions was found by comparing their hydrophobicity plots (not shown); in every case, at least one putative membrane-spanning domain was detected. Interestingly, amino acids 408 to 566 of the DG42 protein are predominantly nonpolar. Therefore, all four proteins consist of a common domain (region 1) that is followed by a hydrophobic domain (region 2). The interval between the two domains is short, ranging from 22 to 31 amino acids. The possible biological significance of these observations is discussed below.

During the preparation of this report, I searched GenBank version 69.0 and found one very high-scoring match to *CSD2*, the *S. cerevisiae* *CAL1* protein (51). It should be noted that the designation *cal* has been used twice; it was first given to a mutation causing calcium-dependent growth (15, 31). Subsequently, Roncero et al. (39) assigned *cal* to mutations that confer resistance to Calcofluor. *CSD2* is identical to *CAL1* of Roncero et al. (39) on the basis of several criteria: first, the sequences are 99% identical over a span of 3,826 nucleotides (discrepancies are discussed below); second, *cal1* mutants, like *csd2* mutants, have a 10-fold reduction in cellular chitin and lack chitin synthase III (51); and finally, *CAL1* is located on chromosome II (51).

There are a few discrepancies between the nucleotide sequences of *CAL1* (see reference 51, Fig. 2) and *CSD2* (Fig. 4). First, *CAL1* lacks the adenine residue at position +167 of *CSD2*; consequently, the open reading frame of *CAL1* is 198 bp shorter than that of *CSD2*. I have sequenced this region of *CSD2* several times in both directions; all of the gels support the assignment of an adenine residue at +167. Second, the first nine nucleotides of *CAL1* (-277 to -269), which do not match the corresponding nucleotides of *CSD2* (-81 to -73), are probably incorrect since they are identical to a portion of the polylinker of pUC19 (+421 to +429). Third, the adenine residue at +3489 of *CSD2* is a cytosine residue in *CAL1* (+3291); therefore, the antepenultimate amino acid residue is leucine in the *CSD2* protein and phenylalanine in the

[illegible]

FIG. 4. Complete nucleotide sequence and predicted amino acid sequence of *CSD2*. Putative regulatory elements in the nucleotide sequence are indicated as follows: promoter, boxed; pheromone-responsive element (one mismatch allowed), underline; polyadenylation, two overlapping underlines; and transcription termination, bold underline. The indicated features of the amino acid sequence are region 1 (underline of short dashes) and region 2 (underline of long dashes).

FIG. 5. Pairwise comparisons of the *CSD2* protein with the *CHS2* gene product (47) of *S. cerevisiae* (A), the *nodC* gene product (22) of *R. meliloti* (B), and the DG42 protein (40) of *X. laevis* (C) and comparison of the *nodC* protein with the DG42 protein (D). The Staden program DIAGON was used with a window of 51 and a proportional score of 535.

CAL1 protein. There are two mismatches that do not change the deduced amino acid sequence; they occur at +2817 and +3646 of *CSD2*.

DISCUSSION

I have developed a novel screen for the isolation of mutants defective in chitin synthesis. Although chitin-deficient mutants have been isolated by selecting resistance to Calcofluor (39), the screen described in this study allows the isolation of mutants in essential genes such as those required for UDP-*N*-acetylglucosamine synthesis. Preliminary characterization of the four complementation groups obtained thus far suggests that one, *CSD1*, is an essential gene (see below). Three genes, *CSD2*, *CSD3*, and *CSD4*, are necessary for chitin synthesis in vivo. Mutations in *CSD2* and *CSD4*, but not *CSD3*, abolish chitin synthase III activity. Thus, this enzyme, in contrast to the *CHS* gene products,

synthesizes a substantial fraction of the cellular chitin. The properties of the *csd* mutants are summarized in Table 5.

What are the functions of the *CSD* genes? Which of the genes encode chitin synthase III? The latter question is problematic for two reasons. First, there are two genes, *CSD2* and *CSD4*, that are required for chitin synthase III activity, and therefore two putative structural genes. Second, it is not known whether chitin synthase III is a single polypeptide or a complex of different subunits. The *CSD2* protein has limited, but statistically significant, similarity to the *CHS1* and *CHS2* gene products; this finding suggests that *CSD2* encodes chitin synthase III or a catalytic subunit of a chitin synthase III enzyme complex. In contrast, the deduced amino acid sequence of the *CSD4* protein shows no significant similarity to chitin synthases (7), suggesting that it is regulatory rather than catalytic. If chitin synthase III is multimeric, *CSD4* could be a subunit of the active enzyme

FIG. 6. (A) Alignment of approximately 180 amino acids from the *CHS2* (47), *CSD2*, *nodC* (22), and DG42 (40) proteins. The regions shown are amino acids 432 to 608 of the *CHS2* protein, amino acids 827 to 999 of the *CSD2* protein, amino acids 132 to 284 of the *nodC* protein, and amino acids 232 to 385 of the DG42 protein. Statistical analyses (see Results) were done with the University of Wisconsin Genetics Computer Group program BESTFIT. The marks above the sequences indicate the number of identical amino acids: ., two; *, three; and *, four. (B) Alignment of 26 amino acids from the *CSD2* and *CHS2* proteins. The regions displayed are a portion (amino acids 1090 to 1115) of region 2 of the *CSD2* protein (see Fig. 4 and Results) and amino acids 779 to 804 of *CHS2*. Identities are in boldface type and are marked above with asterisks.

TABLE 5. Summary of the functions of the *CHS1*, *CHS2*, *CSD2*, *CSD3*, and *CSD4* genes

Gene	Essential	Characteristics of mutants				Comments (reference)
		Chitin synthase activity ^a			Chitin ^b in vivo	
		I	II	III		
<i>CHS1</i>	No	–	+	+	>90	Structural gene for chitin synthase I (8). Proposed role in cell wall repair (11). Mutants form refractile buds in acidic medium (11).
<i>CHS2</i>	No	+	–	+	>90–200 ^c	Structural gene for chitin synthase II (48). Mutants have defects in septation (6, 44, 48) and nuclear migration (6, 44). Proposed role in synthesis of septal chitin (44).
<i>CSD2</i>	No	+	+	–	<10	Catalytic component of chitin synthase III ^d (51). Required for chitin synthesis during growth ^d (39), mating (32, 39), and sporulation (4, 34, 51). Related to the <i>nodC</i> and DG42 proteins. ^d Allelic to <i>dit101</i> (4, 34), <i>cal1</i> , ^d and <i>cal4</i> (39, 51). Mutants are temperature sensitive for growth under certain conditions. ^d
<i>CSD3</i>	ND ^e	+	+	+	<10	Mutants are temperature sensitive for growth under certain conditions. ^d
<i>CSD4</i>	No	+	+	–	<10 ^f	Possible activator of chitin synthase III. ^d No homology to chitin synthases (7). Mutants are temperature sensitive for growth under certain conditions. ^d

^a Because the number of different chitin synthases has only recently been established (6), the designations for the enzymes have been inconsistent. Chitin synthase I (or chitin synthase 1) requires proteolysis for activity (8–10, 32). It is stimulated by digitonin and strongly inhibited by Co²⁺ (32, 42). Chitin synthase II (or chitin synthase 2) requires proteolysis for activity (9, 48). It is not inhibited by Co²⁺ (32, 42). Chitin synthase III (chitin synthase II in the report of Orlean [32]) is detectable without protease treatment (6, 32). Because the specific activity of chitin synthase I is 10- to 20-fold higher than that of chitin synthase II, the latter is detectable only in extracts from *chs1* mutants.

^b Expressed as percentage of the wild-type value as determined by the procedure described in Materials and Methods.

^c Values vary with the growth medium (6).

^d Reported in this study.

^e ND, not determined.

^f Value for *csd4::LEU2* strains (7).

complex. Alternatively, if the *CSD2* protein is the sole component of chitin synthase III, the *CSD4* gene product may be required for transcription or translation of *CSD2* or for posttranslational modification of the *CSD2* protein. Regarding the latter possibility, it is known that many chitin synthases are stimulated in vitro by partial proteolysis (discussed further below), and therefore *CSD4* could encode an activating protease. While this idea cannot be excluded, the *CSD4* protein shows no obvious similarity to any protease in the TRANSGEN data base and has none of the motifs that characterize the different families of proteases, as judged by analysis with the program PROSITE. Furthermore, chitin synthase III is not stimulated by trypsin in vitro (6, 32).

The *csd3* mutants lack chitin in vivo, yet chitin synthase III activity is unaffected in vitro. At first glance, these results suggest that the *CSD3* protein might be involved in the biosynthesis of the substrate, UDP-*N*-acetylglucosamine. This is unlikely, however, when the functions of UDP-*N*-acetylglucosamine and the properties of the *csd3* mutants are considered. UDP-*N*-acetylglucosamine is required for protein glycosylation, an essential biosynthetic pathway (21, 26). Despite the fact that the mutant *csd3* proteins are clearly inactive in vivo, as evidenced by the lack of cellular chitin, the *csd3* mutants grow well. Thus, *CSD3* is probably a nonessential gene and therefore is unlikely to encode an enzyme in UDP-*N*-acetylglucosamine biosynthesis. An intriguing possibility is that *CSD3* plays a role in the spatial regulation of chitin synthesis, such as proper localization of chitin synthase III. It is worth noting that microvesicles that contain chitin synthases, called chitosomes, have been described and have been proposed to play a role in the transport of these enzymes (27). All three chitin synthases in

S. cerevisiae appear to lack a conventional amino-terminal signal sequence. Thus, the notion that these proteins have a specific transport system, independent of the secretory pathway, deserves some consideration.

Preliminary characterization of the *csd1* mutant shows that it is phenotypically distinguishable from mutants in the other three *CSD* complementation groups. First, it is temperature sensitive for chitin synthesis in vivo. (The *csd2*, *csd3*, and *csd4* mutants are defective at both 26 and 37°C.) Second, the temperature-sensitive growth of the *csd1* mutant cannot be suppressed. (The Ts[−] phenotype associated with the *csd2*, *csd3*, and *csd4* mutations is conditional; see below.) These observations suggest that *csd1-1* encodes a temperature-sensitive mutant protein, and therefore it is likely that *CSD1* is an essential gene. Regarding the function of *CSD1*, many possibilities exist. To test whether *CSD1* is a component of chitin synthase III, membranes from *csd1-1* and *CSD1* strains were assayed at 37°C. Unfortunately, the wild-type enzyme is inactive in vitro at this temperature, and no conclusions can be drawn from these studies. Alternatively, *CSD1* may be required for the conversion of glucosamine to UDP-*N*-acetylglucosamine. Since at least 10 enzymes are required for this process (6 enzymes for conversion of pantothenate to acetyl coenzyme A and 4 enzymes for conversion of glucosamine to UDP-*N*-acetylglucosamine), it is quite possible that *CSD1* is not specifically involved in chitin synthesis.

The growth defects associated with the *csd2*, *csd3*, and *csd4* mutations are influenced by environmental factors and suppressor genes. The most severe phenotype is failure to grow at 37°C; this defect is suppressed by increasing the osmolarity of the media or by suppressor genes that have been detected in certain strains. It is interesting that the *chs1*

and *chs2* mutations also exhibit suppressible phenotypes. Cabib et al. (11) observed abnormal, refractile buds when *chs1::URA3* mutants were grown in acidic medium; this defect is corrected by buffering the medium, and it is less evident in certain strains. The viability of *chs2* spores can range from 0 to 90%, depending on the strains and germination media that are used (6). These mutants septate poorly in YPD medium, forming large clumps of cells, yet in synthetic medium or in rich medium with glycerol as the carbon source, this phenotype is much less severe (6). Thus, compensatory genes and growth conditions have been detected for all five of these mutations. The media effects may be due to the induction of specific proteins or may reflect a general remodeling or stabilization of the cell wall.

Many yeast mutants are osmotic remedial (18). For some missense mutations, it has been shown that high osmolarity stabilizes the mutant polypeptide, giving partial restoration of function both in vitro and in vivo (24). This is clearly not the explanation for the osmotic remedial behavior of the *csd2* mutants for two reasons. First, certain *csd2* deletion disruption strains are osmotic remedial. Second, when the *csd2-1* mutant is grown in high-salt medium, no restoration of cellular chitin synthesis is detected (5). Thus, a more likely explanation is that high osmolarity stabilizes a weakened, chitin-deficient cell wall.

A small amount of chitin, ~5% of the wild-type level, is detected in *csd2::LEU2 chs1::HIS3* mutants. The simplest explanation is that it is synthesized by chitin synthase II. Alternatively, *S. cerevisiae* may possess a fourth, as yet unidentified chitin synthase. It is curious that loss of chitin synthase III, which makes most of the chitin in the cell, causes no obvious defects in cell division. Conversely, loss of chitin synthase II, which apparently makes a very small percentage of the total chitin, is very pleiotropic, causing defects in septation (4, 44, 48) and nuclear migration (6, 44). There are several possible explanations for the latter observation. First, a portion of the chitin may be undetectable by the assay used in this study so that the contribution by chitin synthase II is actually higher. Second, different synthases might make different pools of chitin that serve specific functions. Third, chitin synthase II may be a bifunctional protein. Although the carboxy terminus is related to other chitin synthases, the first 230 amino acids of chitin synthase II are unique (47). This region may define a domain that plays a role in cell division that is independent of chitin synthetic activity.

Because chitin is a major component of the division septum, it has been suggested that the synthesis of this polysaccharide must be regulated temporally and spatially (10). Chitin synthases from many different fungi are stimulated by trypsin in vitro, and proteolytic conversion of the zymogens to active enzymes has been the predominant, if not the exclusive, model for regulation (10). It should be noted, however, that proteolytic activation in vitro is not necessarily meaningful in vivo. For example, pyruvate oxidase of *E. coli* can be activated by removing a small peptide from the C terminus of the protein by limited digestion with chymotrypsin. Grabau and Cronan (16) constructed a mutant gene that produces a truncated protein similar to the one produced by chymotrypsin cleavage. Although fully active in vitro, the truncated protein has no oxidase activity in vivo, demonstrating that proteolysis of the enzyme is not physiologically relevant. In the cell, oxidase activity is regulated by binding of membrane lipids. It is clear that additional evidence is required in order to assess the role of proteolysis in the regulation of chitin synthase activity.

Previous studies have shown that chitin synthesis is stimulated by mating factors (43). While some progress has been made in understanding this process, no clear picture has yet emerged. Glutamine:fructose 6-phosphate amidotransferase, an enzyme required for UDP-*N*-acetylglucosamine formation, is induced (52), suggesting that substrate availability plays a role in regulation (33, 52). Several pheromone-responsive elements are present in the 5' noncoding region of the amidotransferase gene (52). These elements also occur upstream of *CHS1* (25), and Appeltauer and Achstetter (1) have demonstrated that they are required for induction of chitin synthase I. Surprisingly, there is no detectable reduction in chitin synthesis in *MATa chs1::URA3* mutants treated with α -factor (32). In contrast, *CSD2* is required for synthesis of most of the chitin during mating (32, 39), but no elevation in the specific activity of chitin synthase III has been observed in pheromone-treated cells (32). I found only two possible pheromone-responsive elements in the 500-bp region upstream of the putative *CSD2* promoter (Fig. 4); one of them lacks the highly conserved adenine residue at position five of the TGAAACA sequence (25). Clearly, additional studies are required to establish how chitin synthase III is regulated.

Chitosan, a deacetylated form of chitin, is a major component of the spore wall in *S. cerevisiae* (4). The biosynthesis of chitosan has not been studied in *S. cerevisiae*, but a chitin deacetylase has been identified in *Mucor rouxii* (12), suggesting that chitin is the immediate precursor of chitosan. Preliminary studies by Breitenbach et al. (3) show that *csd2/csd2* diploids produce spores that lack the chitosan and dihydroxy layers of the spore wall. Several mutants deficient in spore wall dihydroxy have been reported (4); one of them, *dit101*, is allelic to *csd2*, as judged by several criteria. First, *csd2* and *dit101* mutants produce identical phenotypes during both sporulation and vegetative growth (3). Like the *csd2* mutants, *dit101* mutants are resistant to Calcofluor and contain very little chitin, as measured by chemical assays (3, 5). Second, pCSD2 complements *dit101* (3). Third, *csd2* and *dit101* fail to complement in diploids (3, 34).

A surprising number of genes are specifically involved in chitin synthesis. In addition to the *CHS* and *CSD* genes, four complementation groups, designated *CAL*, have been reported among mutants resistant to Calcofluor (39). (Although Roncero et al. [39] originally reported five complementation groups, Valdivieso et al. [51] have found that *cal4* is allelic to *cal1*.) As discussed above, *CSD2*, *CAL1*, and *DIT101* are the same gene. We have tested the ability of the cloned *CSD2*, *CSD3*, and *CSD4* genes, carried on *CEN/ARS* plasmids, to complement the *cal2*, *cal3*, and *cal5* mutants, as judged by the restoration of Calcofluor sensitivity. CR4-8D (*MATa cal2 leu2 ura3*) was complemented by *CSD4*, while HV2324D (*MATa cal3 ura3*) was not complemented by any of the *CSD* genes. We found that HV26 (*MATa cal5 ura3*) was complemented by *CSD2* (= *CAL1*), suggesting that *cal5* is allelic to *csd2* (= *cal1*) rather than belonging to a separate complementation group as previously reported (39, 51). Our preliminary analysis suggests a total of four complementation groups: *csd2* = *dit101* = *cal1* = *cal5*, *csd3*, *csd4* = *cal2*, and *cal3*. Clearly, additional genetic studies are required to unequivocally establish the number of different genes.

There are two discrepancies between the original characterization of the *cal1* mutants (39) and my analysis of the *csd2* mutants. First, no enzymatic defect was detected in the *cal* mutants, either with or without preincubation of the membranes with trypsin. Reexamination of the *cal1* mutant has eliminated this discrepancy; Valdivieso et al. (51) have

recently demonstrated a defect in chitin synthase III in these strains. Second, the *cal1* mutant is not Ts⁻. It is likely that the absence of this phenotype is a function of the parental strain and that some, if not all, of the alleles of *cal1* can cause Ts⁻ growth in the appropriate strains (e.g., PP-1A).

My analysis of the *CSD2* protein identifies two proteins, the *nodC* protein of *Rhizobium* species (22, 29) and the DG42 protein of *X. laevis* (40), that may be distant, and unanticipated, relatives of chitin synthases. Both play important roles in development. The *nodC* protein is required for nodulation in all *Rhizobium* species (29); the DG42 protein is expressed transiently during gastrulation, and it displays a distinct pattern of expression within the developing embryo (40). The relationship of these proteins to chitin synthases may be an important clue to their as yet unknown biochemical functions. In the case of the *nodC* protein, previous studies have established that *Rhizobium* species produce extracellular factors that trigger nodule formation in the roots of leguminous plants (29). Recently, Lerouge et al. (28) purified a nodulation factor, termed NodRm-1, from cultures of *R. meliloti*. Structural analyses showed that NodRm-1 is an *N*-acyl-tri-*N*-acetyl- β -1,4-D-glucosamine tetrasaccharide bearing a sulfate group on the reducing sugar moiety. The structural similarity of this molecule to chitin (poly-*N*-acetyl- β -1,4-D-glucosamine), together with the similarity between the *nodC* and *CSD2* gene products, suggests that *nodC* encodes an *N*-acetylglucosaminyltransferase that synthesizes the oligosaccharide backbone of NodRm-1. Consistent with this hypothesis is the observation that *nodC* mutants do not synthesize NodRm-1 (28). To address the question of whether the similarity shown in Fig. 6A is functionally meaningful, I replaced this region of *CSD2* with the analogous region of *nodC*. The *CSD2-nodC* hybrid protein did not correct the Ts⁻ growth or the chitin deficiency of the *csd2* mutant. A similar experiment, which used a *CSD2-CHS2* hybrid protein, also failed. Because there are trivial explanations for the lack of complementation, such as instability of the chimeric protein, these results cannot be interpreted, and additional experiments are required to test this hypothesis.

Almost nothing is known of the biochemistry of the DG42 protein, and any discussion of its function is purely speculative. Nonetheless, the similarity between the *nodC* and DG42 proteins is noteworthy (FASTA initn = 342, 26% identical over 520 amino acids, 14.3 SD above random; Fig. 5D), and it seems likely that the elucidation of the function of the *nodC* protein will have significant implications for an understanding of the DG42 protein. If the hypothesis that *nodC* encodes an *N*-acetylglucosaminyltransferase is correct, the possibility that carbohydrates serve as signals during embryogenesis deserves consideration.

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