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Isolation and Characterization of *Schizosaccharomyces pombe* Mutants Defective in Cell Wall (1-3)β-D-Glucan

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Schizosaccharomyces pombe **thermosensitive mutants requiring the presence of an osmotic stabilizer to survive and grow at a nonpermissive temperature were isolated. The mutants were genetically and biochemically characterized. In all of them, the phenotype segregated in Mendelian fashion as a single gene which coded for a recessive character. Fourteen loci were defined by complementation analysis. Studies of cell wall composition showed a reduction in the amount of cell wall β-glucan in three strains (JCR1, JCR5, and JCR10) when growing at 37°C. Galactomannan was diminished in two others. Strains JCR1 and JCR5, with mutant alleles *cwg1-1* and *cwg2-1*, respectively, were further studied. The *cwg1* locus was mapped on the right arm of chromosome III, 18.06 centimorgans (cM) to the left of the *ade5* marker; *cwg2* was located on the left arm of chromosome I, 34.6 cM away from the *aro5* marker. (1-3)β-D-Glucan synthase activities from *cwg1-1* and *cwg2-1* mutant strains grown at 37°C were diminished, as measured in vitro, compared with the wild-type strain; however, K_m values and activation by GTP were similar to the wild-type values. Mutant synthases behaved like the wild-type enzyme in terms of thermostability. Analyses of round shape, lytic behavior, and low (1-3)β-D-glucan synthase activity in cultures derived from ascospores of the same tetrad showed cosegregation of all these characters. Detergent dissociation of (1-3)β-D-glucan synthase into soluble and particulate fractions and subsequent reconstitution demonstrated that the *cwg1-1* mutant was affected in the particulate fraction of the enzymatic activity while *cwg2-1* was affected in the soluble component. The antifungal agents Papulacandin B and Aculeacin A had similar effects on the enzymatic activities of the wild type and the *cwg2-1* mutant strain, whereas the *cwg1-1* mutant, when growing at 37°C, had a more inhibitor-resistant (1-3)β-D-glucan synthase. It is concluded that the *cwg1*$^+$ and *cwg2*$^+$ genes are related to (1-3)β-D-glucan biosynthesis.**

Fungal cell wall structural polysaccharides are thought to endow cells with shape and mechanical strength and to prevent cell lysis in a hypotonic environment. β-Linked glucan is one of the major structural components of the cell wall in many fungi, including the budding yeast *Saccharomyces cerevisiae* (3) and the fission yeast *Schizosaccharomyces pombe* (17).

Yeast wall β-glucan is a homopolymer of glucose units linked through either (1-3)β- or (1-6)β-D-glycosidic bonds. Nothing is known about the biochemistry of (1-6)β bond formation, but valuable information has been gathered on the biosynthesis of (1-3)β-linked glucose polymers by cell extracts of *S. cerevisiae* (26, 27). These studies showed that the activity of (1-3)β-D-glucan synthase is localized in the inner part of the plasma membrane, is responsible for the synthesis of (1-3)β-D-glucan of the cell wall, and is stimulated in vitro by nucleoside triphosphates, mainly guanosine derivatives (21). Similar findings were extended to *S. pombe* and other fungi (22, 31).

Kang and Cabib (12) dissociated that activity into two proteinaceous components, a membrane-bound fraction, which seemed to contain the catalytic center of the enzyme, and another fraction, solubilized by detergent and NaCl, which bound GTP. They proposed that this second component may modulate the biosynthesis of the fungal cell wall (1-3)β-D-glucan and play a major role in the regulation of fungal cell wall biosynthesis. However, there is as yet no direct evidence about the physiological relevance of this regulatory mechanism. A genetic approach, including isolation and biochemical characterization of mutants affected in β-glucan synthesis, may provide significant information about the role of different cell components in the regulation of cell wall morphogenesis during the cell cycle. In fact, some important studies have been performed with *S. cerevisiae* mutants altered in the biosynthesis of other cell wall polymers such as mannan (4, 15) or chitin (2, 29). Although β-D-glucan is assumed to be essential for yeast cell integrity, very few studies have been done with β-glucan mutants (11, 20, 28), and most of them refer to the (1-6)β-D-glucan from *S. cerevisiae*. A recent study of *KRE* genes, involved in killer factor resistance (1, 18), has provided the first evidence for a pathway of (1-6)-β-D-glucan synthesis and cell wall β-glucan assembly in *S. cerevisiae*.

In this paper, we report the isolation and characterization of some thermosensitive mutants from the fission yeast *S. pombe*. They require osmotic stabilization and are defective in (1-3)β-D-glucan synthase activity when growing at a nonpermissive temperature (37°C).

MATERIALS AND METHODS

Chemicals. Papulacandin B and Aculeacin A were generous gifts from K. Scheibli (Ciba-Geigy, Basel, Switzerland) and K. Mizuno (Toyo Jozo, Tokyo, Japan), respectively. Yeast extract, yeast nitrogen base and agar were from Difco Laboratories, Detroit, Mich. [U-^{14}C]glucose (295 mCi/mmol) and UDP-[U-^{14}C]glucose (240 mCi/mmol) were from Amersham International, Amersham, United Kingdom. Phloxin B, sorbitol, UDP-glucose, and Tergitol Nonidet P-40 were from Sigma Chemical Co., St. Louis, Mo. GTP-γS was from Boehringer GmbH, Mannheim, Germany. Zymolyase-

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TABLE 1. *S. pombe* strains used in this study

Strain	Genotype	Source	Reference
972	h^-	P. Munz	13
975	h^+	P. Munz	13
	h^- *his3-237*	P. Munz	13
	mat2P-B102 lys1-131	R. Egel	6
EG272	h^- *ade6M-216*	R. Egel	9
EG271	h^+ *ade6M-210*	R. Egel	9
EG269	*mat2P-B102 ade6M-210*		
	leu1-32 swi3	R. Egel	9
	h^+ *aro5-110*	NCYC[a]	30
	h^- *ura1-161*	P. Munz	13
	h^- *arg3-10*	P. Munz	13
	h^- *ade4-31*	P. Munz	13
	h^- *leu2-120*	P. Munz	13
B646	h^+ *ura4-294 ade5-36*		
	arg1-230	P. Munz	13
	mat2P-B102 ade6M-210		
	lys1-131 leu1-32 arg1-230	This study	
	h^+ *pap1-8 ade5-36*	J. C. Ribas	24
	h^+ *leu1-32*	P. Munz	13
JCR1A	h^- *cwg1-1*	This study	
JCR1B	h^+ *cwg1-1*	This study	
JCR5A	h^- *cwg2-1*	This study	
JCR5B	h^+ *cwg2-1*	This study	
	h^- *cwg1-1 his3-237*	This study	
	h^- *cwg2-1 his3-237*	This study	
	mat2P-B102 cwg1-1 lys1-131		
	ade6M-216	This study	
	mat2P-B102 cwg2-1 lys1-131		
	ade6M-216	This study	
	h^- *cwg2-1 ade4-31 arg3-10*		
	ura1-161 leu2-120	This study	
	h^- *cwg2-1 aro5-110*	This study	
	h^- *cwg2-1 aro5-110 ura1-161*	This study	

[a] National Collection of Yeast Culture, Norwich, United Kingdom.

100T was from Seikagaku Kogyo Co., Ltd., Tokyo, Japan. Other reagents were of analytical grade.

Strains and growth conditions. *S. pombe* strains used in this work are listed in Table 1. They derive from the heterothallic standard wild-type strains 972 (mating type h^-) and 975 (mating type h^+) originally isolated by Leupold. Yeasts were usually grown on YED medium (1% yeast extract and 1% glucose). The sporulation medium was described by Egel (5). To check the different auxotrophies, minimal medium (1% glucose, 0.7% yeast nitrogen base without amino acids, 0.9 g of KCl per liter, 1 mg of citric acid per liter, 10 μg of biotin per liter, 1 mg of calcium panthotenate per liter, 10 mg of nicotinic acid per liter, and 10 mg of *m*-inositol per liter) supplemented with the necessary requirements was used. Incubations were carried out either at 30 or 37°C. Growth was monitored either by measuring the A_{600} of cultures in a Shimadzu UV-160 spectrophotometer or by counting the number of cells.

Mutagenesis. Cells were mutagenized in YED medium supplemented with 1.2 M sorbitol (7×10^5 cells per ml) with ethylmethane sulfonate (20 μl/ml) for 4 h at 30°C. Surviving cells were incubated in solid medium, YED-sorbitol (1.5% agar), at 24°C. Selection of thermosensitive mutants dependent on osmotic stabilizer was done by replica plating the colonies on YED or YED-sorbitol medium and incubation at 37°C. Lysis was assessed by detection of red colonies in the presence of Phloxin B. An additional criterion included selection by microscopic observation of clones with round or oval cells before lysis at the nonpermissive temperature. That shape is characteristic of *S. pombe* cells growing in the presence of sublethal concentrations of Papulacandin B (32).

Genetic methods. Tetrad analysis was performed by micromanipulation of asci; introduction of convenient auxotrophic markers was carried out as described previously (9, 13). Stable diploids were obtained in crosses with the *mat2P-B102* strain; this mutation blocks the meiotic process at a very early stage so that the diploid stage can be maintained (6, 7). Mapping of the mutations was done as follows: (i) analysis of marker segregation after induced haploidization of stable diploids with *m*-fluoro-DL-phenylalanine (8, 13) (haploids were selected by using *ade6M-216* and *ade6M-210* intragenic complementation markers), (ii) analysis of mitotic recombination induced in diploid strains by ultraviolet light (19), and (iii) analysis of meiotic segregation between the genes under study and the closest available markers. The distances were calculated by using the equation developed by Perkins (23).

Labeling and fractionation of cell wall polysaccharides. Exponentially growing cultures of *S. pombe* wild-type or mutant strains incubated at 30 or 37°C in YED-sorbitol medium were supplemented with [U-^{14}C]glucose (2.5 μCi/ml) and incubated for an additional 4 h. Cells were harvested, and unlabeled cells were added to the radioactive samples as carriers. Total glucose incorporation was monitored by measuring the radioactivity in trichloroacetic acid-insoluble material. Mechanical breakage of cells was done as previously described (22). Cell walls were washed with water several times by centrifugation at $1,000 \times g$ for 5 min. Samples of the walls were extracted with 6% NaOH for 60 min at 80°C, followed by neutralization with acetic acid. Precipitation of galactomannan from the supernatant was performed with Fehling reagent with unlabeled mannan as the carrier. Other samples of cell wall suspensions were incubated with Zymolyase-100T (250 μg) in 50 mM citrate–phosphate buffer, pH 5.6, for 36 h at 30°C. A sample without enzyme was included as a control. After incubation, the samples were centrifuged; the pellets, which were washed and counted as described previously (32), were considered the (1-3)α-glucan fraction and the supernatants were considered the β-glucan plus galactomannan fraction.

Other methods. Cell extracts were prepared and (1-3)β-D-glucan synthase activities were assayed and determined as described previously (32). Particulate (1-3)β-D-glucan synthase was dissociated into soluble and membrane fractions as described for *S. cerevisiae* (12) with some modifications. Briefly, particulate enzyme was resuspended in buffer A (50 mM Tris–HCl, pH 7.5, containing 1 mM EDTA and 1 mM 2-mercaptoethanol) with 2% Tergitol Nonidet P-40, 2 M NaCl, and 100 μM GTP-γS. After vigorous vortex mixing, the suspension was centrifuged at $165,000 \times g$ for 1 h. The pellet was subjected again to the same treatment, washed with buffer A, and resuspended in buffer A supplemented with 33% glycerol. This suspension was designated the membrane fraction. The soluble fraction was obtained by pooling the supernatant fluids from the two treatments described above and dialyzing against buffer A. Protein was determined by the Lowry method.

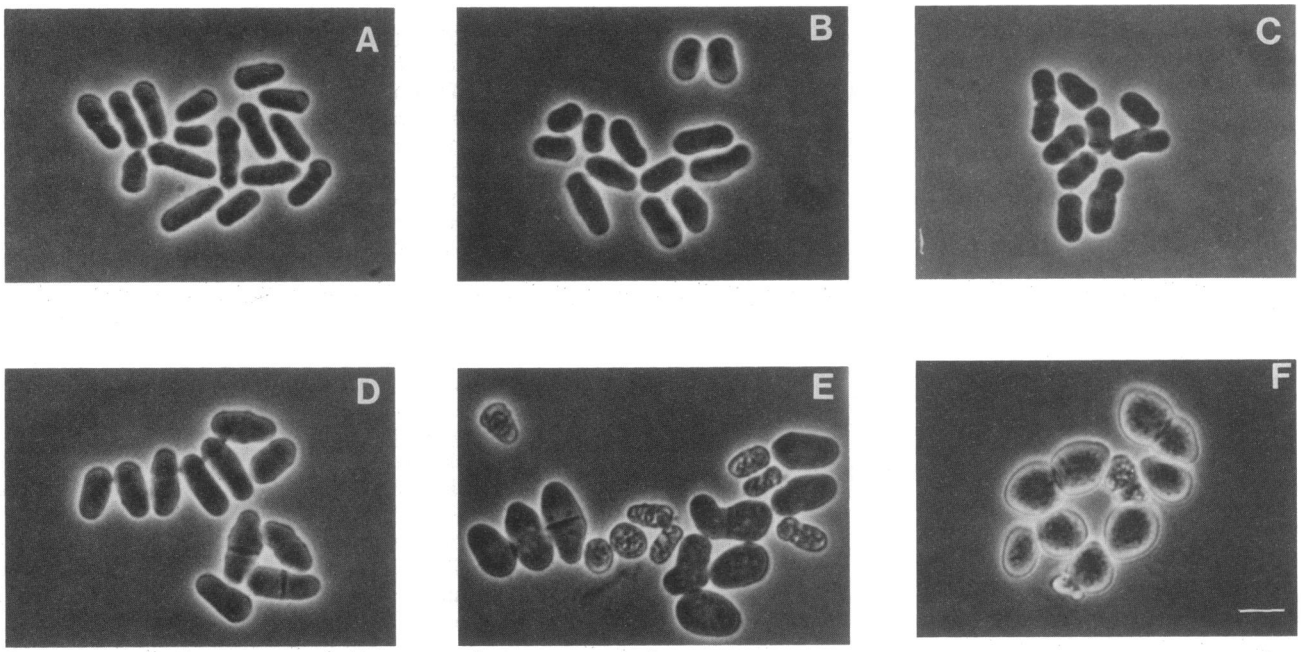

FIG. 1. Phase-contrast micrographs of *S. pombe* cells grown on YED medium at 37°C in the presence (A to C) or absence (D to F) of 1.2 M sorbitol. Wild-type (A and D), JCR1 (B and E), and JCR5 (C and F) strains are shown. Bar, 10 μm.

Pulsed-field gel electrophoresis was performed in a Bio-Rad apparatus as suggested by the supplier.

RESULTS

Isolation and characterization of thermosensitive lytic mutants. Mutagenesis with ethylmethane sulfonate was performed on *S. pombe his3-237* h^-. Histidine auxotrophy was used as a marker for Mendelian segregation. Surviving cells (10^4) were tested for thermosensitive lysis in the absence of osmotic stabilizer and for rounded shape before lysis. The morphologies of two representative mutants, JCR1 and JCR5, grown at 37°C in the presence or absence of 1.2 M sorbitol are shown in Fig. 1. Every selected mutant was backcrossed to *S. pombe* 975 h^+ several times. About 50 complete tetrads from each cross were analyzed, and in all cases the osmotic stabilizer dependency at a restrictive temperature segregated in a 2+:2− fashion, indicating that the mutations were monogenic and nuclear. All the diploids obtained by crossing the h^- mutants to *S. pombe mat2P-B102 lys1-131* grew normally at the restrictive temperature, showing that the mutations were recessive. Twenty mutants were selected, and complementation tests between them indicated the existence of 14 different complementation groups. Three mutants belonged to group 2, two belonged to group 6, another two belonged to group 9, and three belonged to group 14. Each one of the other mutants was assigned to a different complementation group. A mutant from each group (JCR1 to JCR14) was chosen to continue the analysis.

The sorbitol dependency of the backcrossed mutants when growing in YED liquid medium with or without 1.2 M sorbitol was tested at permissive and nonpermissive temperatures. Figure 2 shows the growth curves of two mutants, JCR1 and JCR5, and of the wild-type strain 972 h^-. Growth rates at permissive temperatures (ranging between 24 and 30°C) were similar for all the strains in either the absence or the presence of sorbitol (this compound always promoted a lower rate of growth). By contrast, none of the mutants was able to grow for more than three generations on YED at 37°C (Fig. 2B and C). Sorbitol prevented lysis of these mutants and allowed them to grow at 37°C.

In order to ascertain whether the content of (1-3)β-D-glucan or any other of the major cell wall polymers was affected by the mutations, cell wall composition was analyzed, as described in Materials and Methods, in the 14 selected mutants grown in YED medium supplemented with sorbitol at both permissive and restrictive temperatures. *S. pombe* 972 h^- was included as a control in all the experiments. Incorporation of radioactivity into the cell wall polymers was similar in all strains grown at 30°C (data not shown). However, after growth at the restrictive temperature, incorporation of radioactivity into the β-glucan fraction was 40, 69, and 75% of the wild-type value for the JCR1, JCR5, and JCR10 strains, respectively (Table 2). JCR5 also showed a decrease in the incorporation of radioactivity in α-glucan and, as a consequence, in the cell wall as a whole. JCR10 had a lower quantity of galactomannan, whereas JCR1 showed a small increase in the content of galactomannan and α-glucan.

On the basis of these data, it was decided to continue the study of the JCR1 and JCR5 strains, since they had a more marked defect than the others in the amount of cell wall (1-3)β-D-glucan. Therefore, the genes responsible for the phenotype of the JCR1 and JCR5 strains were called *cwg1* (for cell wall glucan) and *cwg2*, respectively (*cwg1-1* and *cwg2-1* are the mutated alleles). Two mutants, JCR2 and JCR13, showed a diminished amount of galactomannan when they were grown at 37°C. These mutants will be analyzed in a separate study.

Chromosomal localization of *cwg1* and *cwg2*. To assign *cwg1* and *cwg2* mutations to a specific chromosome, the method of haploidization with *m*-fluoro-D-phenylalanine was

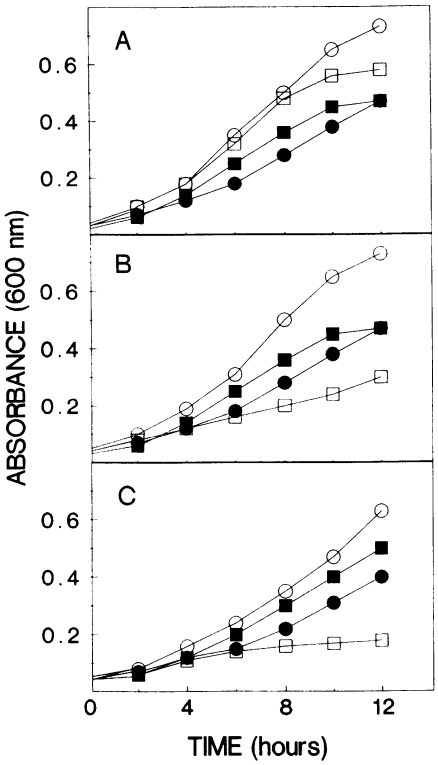

FIG. 2. Growth of *S. pombe* wild-type (A), JCR1 (B), and JCR5 (C) strains in YED medium at 30°C (circles) or 37°C (squares) in the presence (solid symbols) or absence (open symbols) of 1.2 M sorbitol.

employed. *lys1-131*, *leu1-32*, and *arg1-230* auxotrophies were used as markers for chromosomes I, II, and III, respectively. Diploid strains with genotypes h^- *cwg1-1 ade6M-216/mat2P-B102 ade6M-210 lys1-131 leu1-32 arg1-230* and h^- *cwg2-1 ade6M-216/mat2P-B102 ade6M-210 lys1-131 leu1-32 arg1-230* were constructed. As a result of cosegregation with the *arg1-230* marker, in 266 haploid colonies analyzed, the *cwg1-1* mutation was assigned to chromosome III. The *cwg2-1* mutation cosegregated with the *lys1-131* marker in 155 haploid colonies analyzed, and therefore it was assigned to chromosome I. A pulsed-field electrophoresis gel of *S. pombe* chromosomes hybridized with a radioactively labeled DNA fragment containing the $cwg2^+$ gene confirmed its location on chromosome I (4a).

More precise locations for the *cwg1* and *cwg2* markers were determined by conventional tetrad analysis after meiotic recombination (Fig. 3). *cwg1* was located 18.1 centimorgans (cM) to the left of *ade5*. In addition, of a total of 89 analyzed from the cross h^- *cwg1-1* × h^+ *pap1-8*, 89 parental ditype tetrads were scored, indicating a strong linkage between *cwg1* and *pap1* (genetic distance, ≤0.56 cM). The *pap1-8* allele accounted for resistance to Papulacandin B (24), an antifungal agent that specifically affects (1,3)β-D-glucan synthesis (22). The *cwg2* marker was assigned to the left arm of chromosome I, between the centromere and the *ura1-161* gene, by mitotic recombination analysis (results not shown). *cwg2* was located 34.6 cM to the right of *aro5* (30), the only available marker in that part of the chromosome. Lack of other markers (10) precluded a more precise location of that gene.

(1-3)β-D-Glucan synthase activity of mutants *cwg1-1* and *cwg2-1*. (1-3)β-D-Glucan synthase activity was determined in vitro after growing *S. pombe* 972 h^- *cwg1-1* and *cwg2-1* strains at permissive and nonpermissive temperatures. The results, shown in Table 3, indicated that (i) the specific activity of the membrane preparation was similar for all the strains when grown at the permissive temperature, whereas it was considerably reduced in *cwg1-1* and *cwg2-1* mutants grown at 37°C; (ii) the activating effect of GTP was approximately 16 times that without GTP for the control and 6 to 7 times that without GTP for the *cwg1-1* and *cwg2-1* mutants; and (iii) the K_ms of the enzymes from mutants grown at 37°C were slightly higher than those of the wild-type enzyme.

Throughout our results, there was cosegregation of the lytic-morphologic defect and the decrease of (1-3)β-D-glucan synthase level, as shown by analysis of tetrads obtained from crosses of both mutants with the h^+ *leu1-32* strain (Table 4). In fact, cosegregation with the in vivo defect in β-glucan synthesis was also observed in a tetrad from a cross

TABLE 2. Incorporation of radioactivity from [^{14}C]glucose into cell wall polysaccharides of *S. pombe* wild-type and thermosensitive lytic strains grown at 37°C

Strain	% Incorporation of [^{14}C]glucose (mean ± SD)[a]			
	Galactomannan	α-Glucan	β-Glucan	Cell wall
Wild type	4.1 ± 0.4 (13.0)	10.6 ± 0.8 (33.8)	16.7 ± 0.6 (53.2)	31.4 ± 0.6
JCR1	4.5 ± 0.1 (16.8)	16.0 ± 0.6 (59.1)	6.6 ± 0.5 (24.1)	27.1 ± 0.3
JCR2	1.3 ± 0.2 (4.2)	11.1 ± 0.7 (36.2)	18.3 ± 1.7 (59.6)	30.7 ± 0.8
JCR3	3.7 ± 0.4 (13.9)	9.5 ± 0.3 (35.9)	13.3 ± 0.4 (50.2)	26.5 ± 1.5
JCR4	3.3 ± 0.4 (11.5)	8.8 ± 0.7 (30.3)	17.0 ± 0.2 (58.2)	29.1 ± 0.4
JCR5	3.3 ± 0.9 (17.4)	7.1 ± 1.4 (37.0)	11.6 ± 1.6 (45.6)	19.2 ± 3.3
JCR6	3.2 ± 0.5 (12.4)	9.4 ± 0.1 (36.6)	13.1 ± 0.6 (51.0)	25.7 ± 2.3
JCR7	3.1 ± 0.5 (10.0)	12.2 ± 2.1 (39.4)	15.4 ± 0.8 (50.6)	30.7 ± 1.3
JCR8	4.3 ± 0.4 (14.6)	10.2 ± 1.0 (34.4)	15.1 ± 1.9 (51.0)	29.6 ± 1.6
JCR9	3.4 ± 0.1 (11.5)	9.8 ± 0.4 (33.4)	16.2 ± 0.7 (55.1)	29.4 ± 1.3
JCR10	2.1 ± 0.3 (7.2)	14.6 ± 0.2 (49.8)	12.6 ± 1.4 (43.0)	29.3 ± 0.9
JCR11	4.8 ± 0.1 (14.6)	12.5 ± 0.1 (38.1)	15.5 ± 1.0 (47.3)	32.8 ± 0.7
JCR12	3.6 ± 0.1 (10.8)	11.4 ± 0.2 (34.6)	18.0 ± 1.1 (54.6)	33.0 ± 1.3
JCR13	1.3 ± 0.1 (3.7)	15.6 ± 0.5 (44.3)	18.3 ± 0.5 (52.0)	35.2 ± 1.0
JCR14	2.7 ± 0.02 (8.4)	14.0 ± 0.3 (43.5)	15.5 ± 0.5 (48.1)	32.2 ± 0.8

[a] Percent incorporation of [^{14}C]glucose = cpm incorporated per fraction × 100/total cpm incorporated. Values are the means and standard deviations calculated from four independent experiments. Values in parentheses are percentages of the corresponding polysaccharides in cell wall composition.

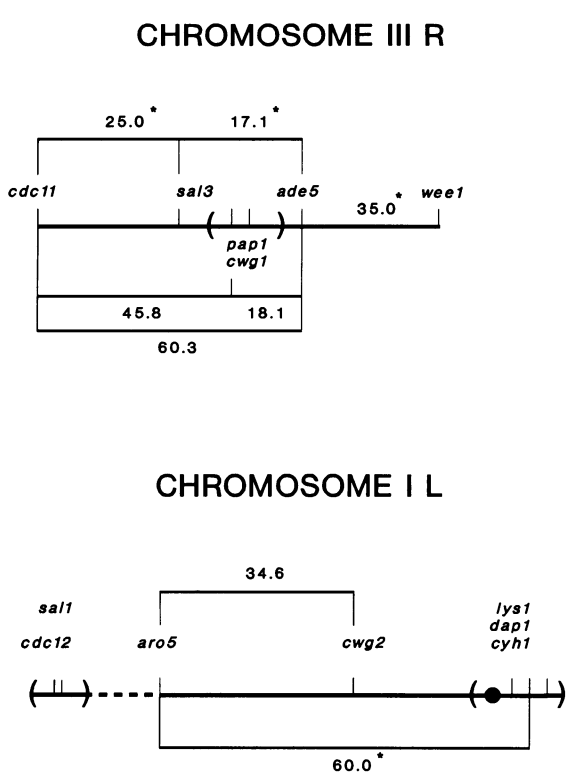

FIG. 3. Genetic localization of *S. pombe cwg1* (A) and *cwg2* (B) genes. A total of 155 tetrads were analyzed to determine the distances between the following pairs of markers: *cwg1-ade5* (104 were parental ditypes [PD], 1 was a nonparental ditype [NPD], and 50 were tetratypes [T]), *cwg1-cdc11* (63 PD, 10 NPD, and 82 T), and *ade5-cdc11* (53 PD, 17 NPD, and 85 T). A total of 103 tetrads were analyzed to determine the distance between *cwg2* and *aro5* (61 PD, 6 NPD, and 36 T). Distances are given in centimorgans. Values marked * are from Gygax and Thuriaux (10).

between another *cwg1* mutant isolated in an independent mutagenesis event (the *cwg1-2* allele) and the wild type (7a).

(1-3)β-D-Glucan synthase activity from wild-type and mutant strains grown at permissive and nonpermissive temperatures was fractionated into two components, soluble and membrane fractions (see Materials and Methods). Reconstitution experiments were performed to determine which fraction was affected by the mutations. As shown in Table 5,

TABLE 3. Characteristics of (1-3)β-D-glucan synthase activity in extracts from *S. pombe* wild-type 972 h^- and h^- *cwg1-1* and h^- *cwg2-1* mutant strains

Growth temp (°C)	Strain genotype	Sp act (mean ± SD)[a]		K_m (mM) (mean ± SD) with GTP
		+ GTP[b]	− GTP	
30	972 h^-	7.3 ± 0.5	0.70 ± 0.10	2.7 ± 0.3
	h^- *cwg1-1*	6.4 ± 0.9	0.85 ± 0.05	2.6 ± 0.2
	h^- *cwg2-1*	9.5 ± 0.4	ND	2.5 ± 0.2
37	972 h^-	5.6 ± 0.8	0.34 ± 0.11	2.7 ± 0.2
	h^- *cwg1-1*	1.4 ± 0.2	0.23 ± 0.08	6.3 ± 0.2
	h^- *cwg2-1*	0.5 ± 0.1	0.07 ± 0.04	4.2 ± 1.0

[a] Specific activity is expressed as milliunits per milligram of protein. Values were calculated from four independent experiments. ND, not determined.
[b] Final concentration in the assay, 150 μM.

TABLE 4. Cosegregation of thermosensitive osmotic lysis and thermosensitive decrease in the level of (1-3)β-D-glucan synthase phenotypes in h^- *cwg1-1* and h^- *cwg2-1* mutants, as determined by tetrad analysis

Genotypic cross	Ascospore genotype	Sp act (mean ± SD) of (1-3)β-D-glucan synthase[a]
h^- *cwg1-1* × h^+ *leu1-32*	h^- *leu1-32 cwg1*$^+$	5.22 ± 0.83
	h^+ *leu1*$^+$ *cwg1-1*	1.50 ± 0.04
	h^+ *leu1-32 cwg1*$^+$	4.71 ± 0.37
	h^- *leu1*$^+$ *cwg1-1*	1.56 ± 0.16
h^- *cwg2-1* × h^+ *leu1-32*	h^+ *leu1-32 cwg2*$^+$	4.66 ± 0.06
	h^- *leu1*$^+$ *cwg2-1*	0.38 ± 0.14
	h^- *leu1*$^+$ *cwg2*$^+$	5.73 ± 1.00
	h^+ *leu1-32 cwg2-1*	1.19 ± 0.48

[a] Specific activity (milliunits per milligram of protein) was measured at 37°C. Values were calculated from four independent experiments.

the *cwg1-1* mutation diminished the activity of the membrane fraction. When the *cwg1-1* soluble fraction was added to the wild-type membrane fraction, most of the synthase activity was recovered; by contrast, when the *cwg1-1* membrane fraction was mixed with the wild-type soluble fraction, the activity was not restored. The opposite situation was found in the *cwg2-1* mutant, where only the soluble component of the enzymatic activity was affected. In all cases, soluble or membrane fractions assayed separately did not show any activity (data not shown).

Thermostability of the different enzymatic preparations obtained from cultures grown at the permissive temperature was tested by preincubating them at 37°C. In every condition tested, the stability of mutant and wild-type enzymatic preparations was similar (data not shown).

In an attempt to further characterize these mutants, we studied the effect of two antifungal agents, Papulacandin B and Aculeacin A, on the enzymatic preparations of both wild-type and mutant strains grown at permissive and nonpermissive temperatures. These two compounds are known inhibitors of (1-3)β-D-glucan synthesis, both in vivo and in vitro (22, 32). (1-3)β-Glucan synthase activity from every

TABLE 5. Reconstitution of (1-3)β-D-glucan synthase activity with soluble and membrane fractions from the *S. pombe* wild type and mutants JCR1 and JCR5 grown at 37°C

Strain and fraction(s)	Sp act (mean ± SD)[a]	% Activity
WT[b]		
Total extract	5.00 ± 0.82	100.0
Soluble + membrane	4.36 ± 0.16	87.2
JCR1		
Total extract	1.80 ± 0.32	36.0
Soluble + membrane	1.18 ± 0.35	23.6
Soluble (WT) + membrane	0.64 ± 0.07	12.8
Soluble + membrane (WT)	3.79 ± 0.54	75.8
JCR5		
Total extract	1.87 ± 0.09	37.4
Soluble + membrane	1.37 ± 0.18	27.4
Soluble (WT) + membrane	3.87 ± 0.35	77.4
Soluble + membrane (WT)	2.09 ± 0.44	41.8

[a] Specific activity is expressed as milliunits per milligram of protein. Values were calculated from four independent experiments.
[b] WT, wild type.

TABLE 6. Effects of antibiotics Papulacandin B and Aculeacin A on (1-3)β-D-glucan synthase from *S. pombe* wild-type 972 h^- and h^- *cwg1-1* and h^- *cwg2-1* mutants grown at 30 and 37°C

Growth temp (°C)	Strain genotype	Sp act (mean ± SD) (% control activity)[a]		
		Control	Papulacandin B[b]	Aculeacin A[b]
37	972 h^-	5.9 ± 0.4	0.7 ± 0.1 (11.9)	2.9 ± 0.2 (49.1)
	h^- *cwg1-1*	1.5 ± 0.2	1.1 ± 0.2 (73.3)	1.2 ± 0.2 (80.0)
	h^- *cwg2-1*	1.4 ± 0.5	0.2 ± 0.06 (14.3)	0.5 ± 0.1 (35.7)
30	972 h^-	7.8 ± 0.6	0.7 ± 0.1 (8.9)	4.0 ± 0.3 (51.3)
	h^- *cwg1-1*	7.7 ± 1.0	1.2 ± 0.4 (15.6)	3.8 ± 0.4 (49.4)
	h^- *cwg2-1*	ND	ND	ND

[a] Specific activity is expressed as milliunits per milligram of protein. Numbers in parentheses represent the percentage of the control activity without antibiotics in the assay. Values were calculated from four independent experiments. ND, not determined.
[b] Final concentration in the assay, 50 μg/ml.

strain grown at 30°C showed the same degree of susceptibility to the antibiotics (Table 6). Surprisingly, the enzyme from the *cwg1-1* mutant cultured at 37°C was more resistant to Papulacandin B and Aculeacin A than the enzyme from the wild type or the *cwg2-1* mutant grown at the same temperature. However, complementation between *pap1-8* (a mutant resistant to Papulacandin B) and *cwg1-1* indicated that they are different mutations (data not shown).

DISCUSSION

Inhibition of (1-3)β-D-glucan biosynthesis in *S. pombe* by Papulacandin B results first in a morphologic alteration consisting of the swelling of growing cells and later in a lytic effect which can be avoided by the presence of an osmotic stabilizer (32). On the basis of these observations, we decided to obtain *S. pombe* thermosensitive lytic mutants which had spherical shapes before lysis and required osmotic stabilization for growth at a restrictive temperature. It was expected that some of these mutants would be altered in cell wall composition and at least some of them would be affected in (1-3)β-D-glucan synthesis. This approach was tried previously in *S. cerevisiae* (33); however, having a spherical shape before lysis was not adopted as a condition in the selection protocol. The mutants obtained in that study seemed to be affected in the membrane rather than in the cell wall (14).

Fractionation of cell wall polysaccharides showed that in most mutants there was not a clear correlation between the defect in cell wall composition and the lytic-morphologic defect. We have not precisely analyzed the nature of the linkages of the β-glucan fractions from the mutant strains; therefore, we cannot rule out the possibility that small changes in the length of the (1-3)β-linked glucose chains or small differences in the degree of (1-6)β crosslinking possibly affect the secondary and tertiary conformation of the β-glucan and, ultimately, the nature of the network structure in the cell wall and the shape of the cell. In fact, some authors (11, 28) favor the hypothesis that the number of (1-6)β-glycosidic linkages is a key element in controlling the hydrodynamic properties and morphology of the yeast cell wall. Indeed, very recently (1, 18) Bussey and coworkers clearly showed the role of (1-6)β-D-glucan in cell morphology and cell growth in *S. cerevisiae*.

We found two mutants, JCR2 and JCR13, with diminished galactomannan contents. A defect in the mannoprotein fraction has already been related to an increase in wall porosity (34) or to spherical shape and irregularities in the cell wall surface (25). There is some circumstantial evidence of the existence of linkages between glucan and mannoprotein in *S. cerevisiae*; perhaps this kind of mutant may have an altered β-glucan which cannot link some morphogenic relevant mannoproteins to the cell wall.

We also found three mutants with reduced β-glucan content when grown at a nonpermissive temperature. Two of them, JCR1 and JCR5, were further studied. The mutations affected two different genes, *cwg1* and *cwg2*, located in two different chromosomes. *cwg1* mapped very close to *pap1* (defined by a Papulacandin B-resistant locus). Complementation between the *pap1* mutation and *cwg1-1* showed that they are different mutations (data not shown); however, the possibility of intragenic complementation cannot be ruled out.

cwg1 and *cwg2* mutants showed lower in vitro levels of (1-3)β-D-glucan synthase activity than the wild-type strain, as measured after culturing them at 37°C in YED medium supplemented with 1.2 M sorbitol. These lower activity levels always cosegregated with the lytic-morphologic defect. Therefore, it is probable that all these phenotypic features are pleiotropic effects of a single gene mutation. Definitive evidence will come from the cloning of the homologous wild-type gene. Very recently, a putative kinase gene important for growth polarity in *S. pombe* was described (16). Since the morphologic phenotype of *cwg* mutant cells is quite similar to that exhibited by cells carrying a *kin1*$^+$ disruption, complementation studies between these strains may provide some additional information about *S. pombe* cell wall growth.

Kinetic properties and in vitro thermosensitivity of the synthases from both mutants were similar to those of the enzyme from the wild-type strain. These data indicate that the mutated genes may be different from the structural gene for (1-3)β-D-glucan synthase, although they are directly related to its activity. However, it is also possible that only certain allelic mutations would reflect in parallel an in vitro and in vivo thermosensible defect. Since the enzyme has not been purified, we could not determine whether the decrease in the activity is due to a decrease in the amount of synthesized enzyme or whether there is a modification in some regulatory component. The study of new alleles from the *cwg1* locus may shed some additional light on this question.

Fractionation and reconstitution experiments of (1-3)β-D-glucan synthase activity showed that the *cwg1-1* mutation alters the activity of the membrane fraction, which is supposed to contain the catalytic component of the enzyme (12). In contrast, the *cwg2-1* mutation affects the soluble fraction, where the regulatory component of the enzyme has been

assigned (12). Cloning and functional study of the corresponding wild-type alleles is in progress and will help to assess the physiological significance of the corresponding products in *S. pombe* cell wall biosynthesis and assembly.

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