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Homologous Subunits of 1,3-Beta-Glucan Synthase Are Important for Spore Wall Assembly in *Saccharomyces cerevisiae*▽

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During sporulation in *Saccharomyces cerevisiae*, the four haploid nuclei are encapsulated within multilayered spore walls. Glucan, the major constituent of the spore wall, is synthesized by 1,3-β-glucan synthase, which is composed of a putative catalytic subunit encoded by *FKS1* and *FKS2*. Although another homolog, encoded by *FKS3*, was identified by homology searching, its function is unknown. In this report, we show that *FKS2* and *FKS3* are required for spore wall assembly. The ascospores of *fks2* and *fks3* mutants were enveloped by an abnormal spore wall with reduced resistance to diethyl ether, elevated temperatures, and ethanol. However, deletion of the *FKS1* gene did not result in a defective spore wall. The construction of fusion genes that expressed Fks1p and Fks2p under the control of the *FKS2* promoter revealed that asci transformed with *FKS2p*-driven Fks1p and Fks2p were resistant to elevated temperatures, which suggests that the expression of *FKS2* plays an important role in spore wall assembly. The expression of *FKS1p*-driven Fks3p during vegetative growth did not affect 1,3-β-glucan synthase activity in vitro but effectively suppressed the growth defect of the temperature-sensitive *fks1* mutant by stabilizing Rho1p, which is a regulatory subunit of glucan synthase. Based on these results, we propose that *FKS2* encodes the primary 1,3-β-glucan synthase in sporulation and that *FKS3* is required for normal spore wall formation because it affects the upstream regulation of 1,3-β-glucan synthase.

Sporulation in the budding yeast *Saccharomyces cerevisiae* provides a model system for studying the developmental processes of many eukaryotic cells. Sporulation in the **a**/α diploid cells is triggered by carbon starvation and is followed by meiosis and the formation of asci that contain four haploid spores encapsulated within a spore wall (25, 33). The spore wall, which consists of four distinct layers, has been shown to play a central role in protecting the cell from environmental damage. The inner two layers consist of β-glucan and mannan (4), components that are similar to those found in the vegetative cell wall. In contrast, the outer layer consists of chitosan, a polymer of β-1,4-linked glucosamine, and the outermost layer consists of dityrosine, both of which are specific to the spore wall (3, 4, 5, 6, 43). Previous investigations of spore wall formation have focused on the specific components of the spore wall, such as chitosan and dityrosine. The mechanism of assembly of spore walls, including the synthesis of the inner two layers, remains unclear. We speculate that β-glucan is also largely responsible for spore resistance to environmental damage, since glucan is the major constituent of the spore wall (4) and provides rigidity to the cell wall during vegetative growth (36).

In yeast, glucan is constituted predominantly by 1,3-β-glucan, which is synthesized by 1,3-β-glucan synthase (GS), which in turn consists of a catalytic and a regulatory subunit. Two genes for the putative catalytic subunit of GS in budding yeast have been identified: *FKS1*, which synthesizes 1,3-β-glucan, the main structural component of the cell wall (12, 17), and *FKS2*, which was identified by virtue of its cross-hybridization with *FKS1* (31). The regulatory subunit copurifies with glucan synthase and is encoded by *RHO1* (13, 38). Rho1p acts as a molecular switch that monitors and receives upstream signals of cell morphogenesis. A third Fks1p homolog protein, Fks3p (Ymr306wp), was found by homology searching and shares 56% identity with Fks1p and Fks2p (31).

FKS1 and *FKS2* encode a pair of integral membrane proteins with 16 predicted transmembrane domains that share 88% identity. Deletion of *FKS1* leads to a decrease in the level of glucan and an increase in the chitin and mannoprotein levels in the cell wall (27). Deletion of *FKS2* causes no obvious cell wall defect, although the *fks1 fks2* double mutant is nonviable (17, 31), which suggests that in vegetative growth Fks1p and Fks2p are alternative subunits with essentially overlapping functions. However, the role of *FKS3* has not been clarified. The *fks3*-null mutant has no apparent cell wall defects and there are no genetic interactions between *FKS3* and *FKS1* or *FKS2* (11). A synthetic genetic array analysis revealed no synthetic interactions with the *fks3*-null strain (27). In the fission yeast *Schizosaccharomyces pombe*, four genes have been reported for the putative GS catalytic subunits, i.e., $bgs1^+$/$cps1^+$, $bgs2^+$, $bgs3^+$, and $bgs4^+$, the sequences of which share high-level homology with those of *FKS1* and *FKS2* (9, 10, 18, 28, 29, 30). The $bgs1^+$, $bgs3^+$, and $bgs4^+$ genes are essential for vegetative growth and have been shown to be localized to specific sites of cell wall growth (9, 10, 18, 30). The $bgs2^+$ gene is not

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expressed during vegetative growth but is essential for spore wall formation. GS activity is diminished in sporulating *bgs2*Δ diploids (29). Bgs2p localizes to the ascospore periphery and is required for appropriate spore wall maturation (28, 29).

In this study, we analyzed the roles of glucan and glucan synthase in spore wall formation in budding yeast. It has been reported that *fks2* mutants form aberrant spores (31). Recently, Huang et al. (16) have reported that Smk1p, which is a mitogen-activated protein kinase, binds to the 1,3-β-glucan synthase Fks2p and that Fks2p activity is increased in the *smk1* mutant. It has been suggested that Fks2p plays an important role in spore wall assembly through protein kinases. However, nothing is known about the function of *FKS3* in spore wall formation. Our investigation of the roles of two glucan synthase genes and a homologous gene at meiosis and sporulation provides novel information on spore wall assembly.

MATERIALS AND METHODS

Plasmids. The plasmids used in this study are listed in Table 1. For DNA manipulations, standard techniques were used. The 6.0-kb BglII-XhoI fragment that includes *FKS3* (derived from the YEp13 genomic bank) (44) was cloned into the integrating vector pRS305 (42) to generate pYO2728. The 6.0-kb BglII-XhoI fragment that includes *FKS3* was cloned into pBluescript to generate pYO2729. The plasmids pYO2730, pYO2731, and pYO2733 contain the *FKS1*, *FKS2*, and *FKS3* genes, respectively, cloned into the PvuII site of pYO326, and pYO2732 contains a fragment that has the *FKS3* gene cloned into the PvuII site of pYO325. We constructed the *FKS1p-FKS2-HA* fusion (hemagglutinin-tagged *FKS2* gene driven by the *FKS1* promoter) and the *FKS2p-FKS1-HA* fusion, and as controls, we constructed the *FKS1p-FKS1-HA* and *FKS2p-FKS2-HA* fusions as follows. The *FKS1* promoter and the *FKS1*-2×HA (two tandem repeats of hemagglutinin tag) gene fusion were fused at the first ATG codon of the open reading frame (ORF) by using PCR, and the fusion was cloned into the pRS314 plasmid to generate pYO2736. The same procedure was used for the *FKS1* promoter and the *FKS2*-2×HA gene fusion, the *FKS2* promoter and the *FKS1*-2×HA gene fusion, and the *FKS2* promoter and the *FKS2*-2×HA gene fusion to generate pYO2737, pYO2738, and pYO2739, respectively. The fused *FKS1* promoter and *FKS3*-2×HA gene fusion were cloned into pRS316 and pYO326 to generate pYO2740 and pYO2742, respectively. The pYO2764 plasmid is a pRS305 derivative that contains the *FKS3*-2×HA gene fusion, the stop codon of which was replaced with sequences that encode the 2×HA tag.

Strains. *Escherichia coli* strains SCS1 and JM110 were used as plasmid hosts. The yeast strains used in this study are listed as derivatives of the rapidly sporulating strain SK1 (Table 2) (22) and as derivatives of YPH (Table 3) (42). Strains were constructed by using standard genetic crossing, transformation, and other genetic procedures (21). The strains constructed in this study will be deposited in the Yeast Genetic Resource Center Japan (http://yeast.lab.nig.ac.jp/nig/english/index.html or http://bio3.tokyo.jst.go.jp/jst/english/).

The S799 and S800 derivatives of SK1 (Table 2) were constructed by M. Lichten (National Institutes of Health) and provided by K. Ohta (Riken). NKY899, NKY900, NKY486, and NKY487 were provided by N. Kleckner (Harvard University). The *FKS1* genes in YOC4064 and YOC4065 were replaced with the *fks1*::*URA3* allele in plasmid pYO1753, and the *FKS1* genes in YOC4067 and YOC4068 were replaced with the *fks1*::*LEU2* allele in plasmid pYO944. YOC4061, YOC4062, YOC4070, YOC4071, YOC4114, and YOC4115 were constructed using PCR-mediated gene disruptions as previously described (40). Primers were used to amplify the Cg*LEU2* (in plasmid pYO2241) or Cg*URA3* (in plasmid pYO2244) gene of *Candida glabrata*, together with flanking sequences derived from the upstream and downstream regions of the *FKS2* and *FKS3* genes. YOC4070 was crossed with YOC4062, and the resulting diploid was sporulated and subjected to tetrad analysis to generate YOC4078 and YOC4079. The various *FKS1* and *FKS2* expression plasmids were introduced into *fks1 fks2* double-null mutants that harbored the *URA3*-borne *FKS1* plasmid, and this plasmid was subsequently eliminated by treatment with 5′-fluoroorotic acid. In the resultant cells, either the *FKS1* or *FKS2* gene could be expressed in the absence of endogenous Fks1p and Fks2p under the control of the *FKS1* or *FKS2* promoter. Thus, we established the strains YOC4084, YOC4085, YOC4086, and YOC4087.

For the derivatives of YPH (Table 3), the detailed constructions of YOC1001 (*FKS1* Δ*fks2*) and YOC1087 (*fks1-1154* Δ*fks2*) are described elsewhere (41). Briefly, YOC1087 is a temperature-sensitive strain with a deletion of both *FKS1* and *FKS2* and a mutant allele of *fks1* (*fks1-1154*) that is integrated at the *ADE3* locus. YOC1001 is the wild-type control for YOC1087, with *FKS1* and *FKS2* deleted and the wild-type *FKS1* gene integrated at the *ADE3* locus.

TABLE 1. Plasmids used in this study

Name	Description	Reference[a]
pRS305	Yeast integration vector marked with *LEU2*	a
pRS306	Yeast integration vector marked with *URA3*	a
pRS314	Yeast-*E. coli* shuttle vector (CEN) with *TRP1*	a
pRS315	Yeast-*E. coli* shuttle vector (CEN) with *LEU2*	a
pRS316	Yeast-*E. coli* shuttle vector (CEN) with *URA3*	a
pBluescript SK	*E. coli* high-copy-number vector	b
pYO325	Yeast-*E. coli* shuttle vector (2μm) with *LEU2*	c
pYO326	Yeast-*E. coli* shuttle vector (2μm) with *URA3*	c
pYO901	*FKS1p-lacZ* fusion	c
pYO902	*FKS2p-lacZ* fusion	c
pYO946	Δ*fks1*::*LEU2*	c
pYO965	*RHO1* (Q68L) in pRS316	d
pYO991	*FKS1* in pRS316	c
pYO1751	*FKS1* in pRS325	e
pYO1752	*FKS2* in pRS325	e
pYO1753	Δ*fks1*::*URA3*	e
pYO2241	pBS-Cg*LEU2*	f
pYO2244	pBS-Cg*URA3*	f
pYO2367	*ROM2* in pRS316	d
pYO2728	*FKS3* in pRS305	g
pYO2729	*FKS3* in pBluescript SK	g
pYO2730	*FKS1* in pRS326	g
pYO2731	*FKS2* in pRS326	g
pYO2732	*FKS3* in pRS325	g
pYO2733	*FKS3* in pRS326	g
pYO2736	*FKS1p-FKS1-HA* fusion in pRS314	g
pYO2737	*FKS1p-FKS2-HA* fusion in pRS314	g
pYO2738	*FKS2p-FKS1-HA* fusion in pRS314	g
pYO2739	*FKS2p-FKS2-HA* fusion in pRS314	g
pYO2740	*FKS1p-FKS3-HA* fusion in pRS316	g
pYO2742	*FKS1p-FKS3-HA* fusion in pRS326	g
pYO2764	*FKS3-HA* fusion in pRS305	g

[a] a, Sikorski and Hieter (42); b, Stratagene; c, Qadota et al. (38); d, Sekiya-Kawasaki et al. (41); e, Inoue et al. (17); f, Sakumoto et al. (40); g, this study.

Media and synchronous sporulation. Yeasts were grown vegetatively in YPD (1% yeast extract, 2% peptone, 2% glucose) and SD (0.67% yeast nitrogen base without amino acids and 2% glucose) supplemented with the appropriate amino acids (21). For solid media, 2% agar was added to each of the above-described media. SPS (presporulation medium: 0.5% yeast extract, 1% polypeptone, 0.17% yeast nitrogen base without ammonium sulfate and amino acids, 0.05 M potassium phthalate, 1% potassium acetate, 0.5% ammonium sulfate [pH 5.0]) and SPM (sporulation medium: 1% potassium acetate) were prepared as previously described (35). Luria-Bertani medium (1% tryptone, 0.5% yeast extract, 1% NaCl) was used for *E. coli*. For synchronous sporulation, cells were grown at 30°C in SPS to a concentration of 1×10^7 to 2×10^7 cells/ml, pelleted, washed in water, and resuspended at the same density in SPM (35). The cells were cultured with vigorous aeration at 30°C and sampled at various times.

Immunoelectron microscopic analysis. Thin sections of the yeast cells were prepared by the freeze-substituted fixation method as described previously (19), except that HPM010 (BAL-TEC AG) or EMCPS (Leica, Solms, Germany) was used for cell freezing and EMAFS (Leica) was used for warming. For 1,3-β-glucan immunolabeling, a mouse monoclonal antibody (MAb) against 1,3-β-glucan (Biosupplies Australia Pty. Ltd., Parkville, Victoria, Australia) and a secondary antibody conjugated with 10-nm gold particles were used. The labeled thin sections were viewed under an electron microscope (model H7600; Hitachi, Tokyo, Japan) at 100 kV.

1,3-β-Glucan and 1,6-β-glucan determinations. Collection of spores by using a Percoll gradient was performed as described previously (4). For cell wall 1,3-β- and 1,6-β-glucan determinations, spores were boiled twice in 50 mM Tris-HCl, pH 7.4, containing 50 mM EDTA, 2% sodium dodecyl sulfate, and 40 mM β-mercaptoethanol and extensively washed with water. The alkali-soluble (AS) and alkali-insoluble (AI) fractions were extracted with 1 N NaOH at 65°C two times for 1 h as described previously (32), except that sodium borohydride (0.5 M) was added to the 1 N NaOH solution. Borohydride was removed after neutralization with acetic acid and evaporation in the presence of methanol.

TABLE 2. Yeast strains used in this study[a]

Strain	Mating type	Genotype	Reference[b]
S799	**a**	*ura3 lys2 ho*::*LYS2 leu2D arg4-bgl cyh2-z*	a
S800	α	*ura3 lys2 ho*::*LYS2 leu2D arg4-bgl cyh2-z*	a
NKY899	**a**	*ura3 lys2 ho*::*hisG leu2*::*hisG ade2*::*LK*	b
NKY900	α	*ura3 lys2 ho*::*hisG leu2*::*hisG ade2*::*LK*	b
NKY487	**a**	*ura3 lys2 ho*::*hisG leu2*::*hisG trp1*::*hisG*	b
NKY486	α	*ura3 lys2 ho*::*hisG leu2*::*hisG trp1*::*hisG*	b
YOC4061	**a**	*ura3 lys2 ho*::*hisG leu2*::*hisG trp1*::*hisG fks3*::Cg*URA3*	c
YOC4062	α	*ura3 lys2 ho*::*hisG leu2*::*hisG ade2*::*LK fks3*::Cg*URA3*	c
YOC4063	**a**/α	YOC4061 × YOC4062	c
YOC4064	**a**	*ura3 lys2 ho*::*hisG leu2*::*hisG trp1*::*hisG fks1*::*URA3*	c
YOC4065	α	*ura3 lys2 ho*::*hisG leu2*::*hisG ade2*::*LK fks1*::*URA3*	c
YOC4066	**a**/α	YOC4064 × YOC4065	c
YOC4067	**a**	*ura3 lys2 ho*::*hisG leu2*::*hisG trp1*::*hisG fks1*::*LEU2*	c
YOC4068	α	*ura3 lys2 ho*::*hisG leu2*::*hisG ade2*::*LK fks1*::*LEU2*	c
YOC4069	**a**/α	YOC4067 × YOC4068	c
YOC4070	**a**	*ura3 lys2 ho*::*hisG leu2*::*hisG trp1*::*hisG fks2*::Cg*LEU2*	c
YOC4071	α	*ura3 lys2 ho*::*hisG leu2*::*hisG ade2*::*LK fks2*::Cg*LEU2*	c
YOC4072	**a**/α	YOC4070 × YOC4071	c
YOC4078	**a**	*ura3 lys2 ho*::*hisG leu2*::*hisG trp1*::*hisG fks2*::Cg*LEU2 fks3*::Cg*URA3*	c
YOC4079	α	*ura3 lys2 ho*::*hisG leu2*::*hisG ade2*::*LK fks2*::Cg*LEU2 fks3*::Cg*URA3*	c
YOC4080	**a**/α	YOC4078 × YOC4079	c
YOC4081	**a**	*ura3 lys2 ho*::*hisG leu2*::*hisG trp1*::*hisG fks1*::*LEU2 fks2*::Cg*LEU2* (pYO950; YCpU-*FKS1*)	c
YOC4082	α	*ura3 lys2 ho*::*hisG leu2*::*hisG trp1*::*hisG ade2*::*LK fks1*::*LEU2 fks2*::Cg*LEU2* (pYO950; YCpU-*FKS1*)	c
YOC4083	**a**/α	YOC4081 × YOC4082 (pYO950; YCpU-*FKS1*)	c
YOC4084	**a**/α	YOC4083 (pYO2736; *FKS1p-FKS1-HA* fusion)	c
YOC4085	**a**/α	YOC4083 (pYO2737; *FKS1p-FKS2-HA* fusion)	c
YOC4086	**a**/α	YOC4083 (pYO2738; *FKS2p-FKS1-HA* fusion)	c
YOC4087	**a**/α	YOC4083 (pYO2739; *FKS2p-FKS2-HA* fusion)	c
YOC4114	**a**	*ura3 lys2 ho*::*hisG leu2*::*hisG trp1*::*hisG fks3*::Cg*URA3 FKS3-HA*::*LEU2*	c
YOC4115	α	*ura3 lys2 ho*::*hisG leu2*::*hisG ade2*::*LK fks3*::Cg*URA3 FKS3-HA*::*LEU2*	c
YOC4116	**a**/α	YOC4114 × YOC4115	c

[a] All strains listed are derivatives of SK1.
[b] a, M. Lichten; b, N. Kleckner; c, this study.

Determination of the total hexose content was performed using the phenol-sulfuric acid procedure with glucose as the standard (14). The amount of 1,3-β- and 1,6-β-glucans in the AI and AS fractions digested by 1,3-β- or 1,6-β-glucanases was quantified by measuring the release of reducing sugars after digestion by the *p*-amino-hydroxybenzoic acid hydrazide method, as described previously (15). For 1,3-β-glucanase digestion, incubation was carried out with recombinant *Thermotoga neapolita* LamA (9 ng/25 to 50 μg total hexoses), purified from *E. coli* bearing a plasmid provided by Vladimir Zverlov (Institute of Molecular Genetics, Moscow, Russia) (45), in sodium phosphate-citrate buffer (50 mM, pH 6.2) for 24 h at 80°C. For 1,6-β-glucanase digestion, incubation was carried out with 0.3 μg of recombinant *Trichoderma harzianum* 1,6-β-glucanase (2) in potassium acetate buffer (50 mM, pH 5.0) for 24 h at 37°C. Values were analyzed by the paired *t* test using JMP5 software.

1,6-β-Glucanase purification. The 1,6-β-glucanase-overproducing *Pichia pastoris* strain GS115/pUR3421-27, kindly provided by Stanley Brul (Unilever Research Laboratories, Vlaardingen, The Netherlands), was grown as described previously (2). The culture supernatant was collected by centrifugation (4,000 × *g* for 5 min), concentrated 20-fold on Amicon-Ultra (Millipore), and prepared for hydrophobic interaction chromatography as described previously (2). Hydrophobic interaction chromatography was carried out on a Phenyl Superose HR 5/5 column (Pharmacia) as described previously (2). Fractions containing 1,6-β-glucanase activity (2) were pooled, dialyzed, and concentrated sixfold on Amicon.

TABLE 3. Yeast strains used in this study[a]

Strain	Mating type	Genotype	Reference[b]
YPH499	**a**	*ade2 his3 leu2 lys2 trp1 ura3*	a
YPH500	α	*ade2 his3 leu2 lys2 trp1 ura3*	a
YOC1001	**a**	*ade2 his3 leu2 lys2 trp1 ura3 fks1*::*HIS3 fks2*::*LYS2 ade3*::*FKS1*::*TRP1*	b
YOC1087	**a**	*ade2 his3 leu2 lys2 trp1 ura3 fks1*::*HIS3 fks2*::*LYS2 ade3*::*fks1-1154*::*TRP1*	b

[a] All strains listed are derivatives of YPH.
[b] a, Sikorski and Hieter (42); b, Sekiya-Kawasaki et al. (41).

Spore viability of asci under conditions of elevated temperatures and in the presence of diethyl ether and ethanol. Assays for measuring the resistance of spores to ether and heat are described elsewhere (5, 26). Sporulated cultures were tested for cell viability after exposure to 50% diethyl ether, a temperature of 55°C, and 25% ethanol for the times indicated in the figures. Cells were sampled at the times indicated in the figures, diluted to between 300 and 500 colonies per plate, and plated onto YPD plates. The cell viability for each mutant was estimated by counting the viable colonies on YPD plates and comparing the numbers to the numbers of colonies at 0 min. The results are expressed as means ± standard deviations.

Quantitative 1,3-β-glucan measurements. The amount of 1,3-β-glucan per cell was measured using aniline blue as described previously (41). The fluorescence of dye-bound 1,3-β-glucan was quantified using a spectrofluorophotometer (RF-5300PC; Shimadzu, Kyoto, Japan) with an excitation wavelength of 400 nm/slit width of 3 nm and an emission wavelength of 460 nm/slit width of 3 nm.

Assays of in vitro GS activity. GS was purified after extraction from membranes by successive product entrapments using affinity purification procedures that are based on the affinity of the enzyme for its own product (17). The membrane fraction was prepared as previously described (1). In brief, log-phase cells were resuspended in buffer that contained 1 mM EDTA, 500 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride and lysed with glass beads. The crude lysate was centrifuged at 1,500 × *g* for 5 min to separate the cell debris and unbroken

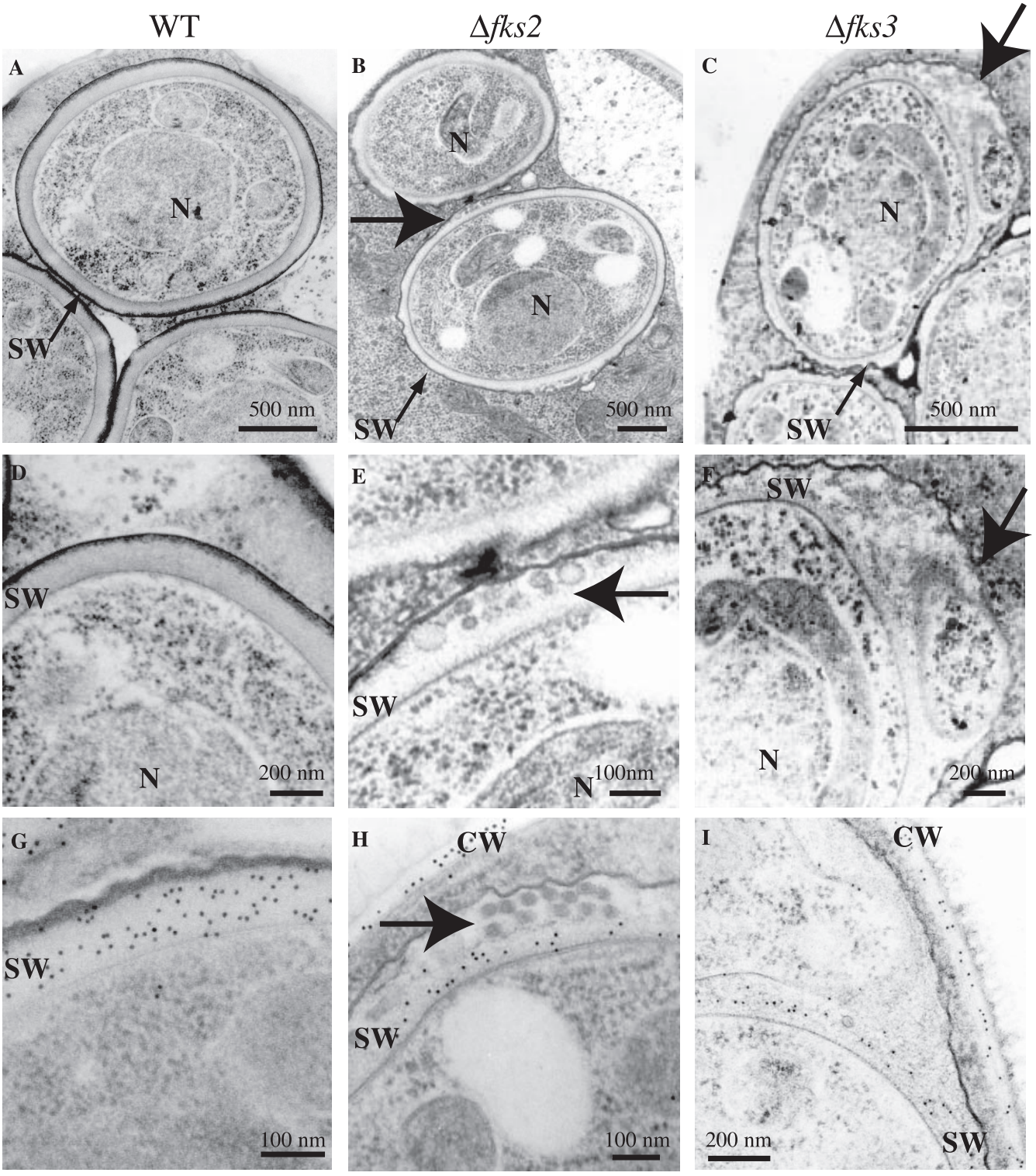


FIG. 1. Electron microscopic analysis of the asci of the *fks2* and *fks3* mutants. Electron microscopic images of the wild-type (WT) (A and D) and *fks2* (B and E) and *fks3* (C and F) mutant cells at a late stage of sporulation are shown. Panel D presents an enlarged image of the spore wall shown in panel A. Panel E shows an enlarged image of the abnormal outer wall (arrow) shown in panel B. Panel F shows an enlarged image of the abnormal outer membrane (arrow) shown in panel C. (G to I) Immunoelectron micrographs of the wild type (G) and *fks2* (H) and *fks3* (I) mutants using an anti-1,3-β-glucan antibody. (J to M) A set of thin serial sections of an *fks2* mutant spore. The abnormal structures are inside the spore. These structures are continuous across the sections. Abnormal structures (arrows), spore walls (SW), nuclei (N), and mother cell walls (CW) are indicated.

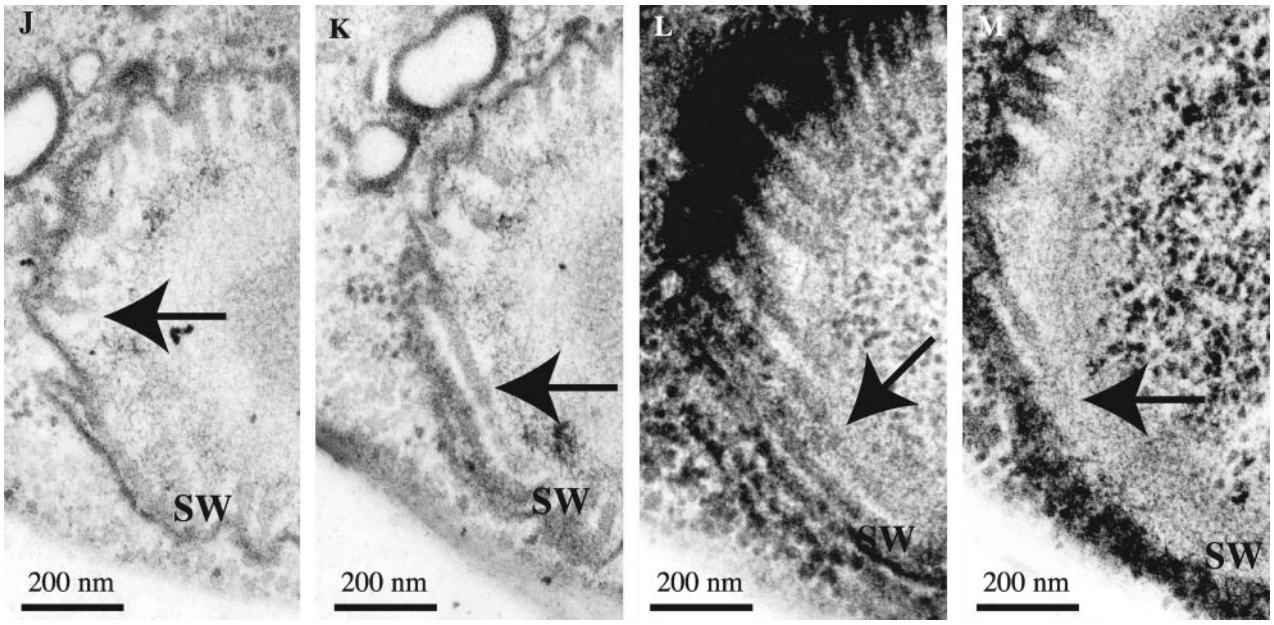


FIG. 1—*Continued.*

cells. After centrifugation at 100,000 × *g* for 30 min, the pellet was suspended in a buffer that contained 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 33% glycerol, and this suspension was used as the membrane fraction. GS activity was measured according to a previously described procedure (17) using an excess of GTPγS.

Western blotting. Protein samples for Western blotting analysis were prepared by disrupting yeast cells with glass beads. The crude lysate was centrifuged at 1,500 × *g* for 5 min to sediment the cell debris and unbroken cells. The supernatant was centrifuged at 100,000 × *g* for 30 min in the model RP70T rotor (Hitachi) with Himac CP 65 (Hitachi). The resultant pellet was suspended and used as the membrane fraction. Equal amounts of protein were loaded and separated on an 8% acrylamide gel. The gels were blotted onto nitrocellulose membranes and probed with mouse MAbs as well as the anti-Fks1p antibody (T2B8) (17) and the anti-HA antibody (11MO; Covance, Princeton, NJ). Signals were detected with the ECL Plus Western blotting analysis system (GE Healthcare, Milwaukee, WI) according to the manufacturer's protocol.

Pull-down assay for active Rho1p. The pull-down assay for active Rho1p was performed as previously described for mammalian RhoA and yeast Rho1p (23; K. Kono, M. Abe, S. Nogami, M. Nishizawa, S. Morishita, D. Pellman, and Y. Ohya, submitted for publication) with some modifications. The region that encodes the Pkc1p Rho1p-binding domain (PRBD) was cloned into pGEX-3X and introduced into *E. coli* strain SCS1. The glutathione-*S*-transferase (GST)-PRBD fusion protein was expressed, purified, and bound to glutathione-Sepharose 4B beads (GE Healthcare). Yeast cells were lysed in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 12 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml tosylsulfonyl phenylalanyl chloromethyl ketone, 25 μg/ml TLCK [*N*α-*p*-tosyl-L-lysine chloromethyl ketone], 25 μg/ml leupeptin, 25 μg/ml pepstatin, 25 μg/ml antipain, 25 μg/ml aprotinin, 25 μg/ml chymostatin, 0.6% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 0.12% cholesteryl hemisuccinate) and incubated with bead-bound GST-PRBD, and proteins bound to the beads were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Bound Rho1p was detected by Western blot analysis using a polyclonal antibody against Rho1p (38).

RESULTS

***FKS2* and *FKS3* are essential for spore wall formation.** To investigate the sporulation defect in mutants with deletions of the *FKS* homologous genes, we constructed *fks1*, *fks2*, and *fks3* deletion strains in the SK1 genetic background (22). These homozygous diploid mutants grew as well as the wild-type cells during vegetative growth and produced viable asci as efficiently as the parental strain upon transfer to sporulation medium (data not shown). To obtain further insight into the nature of sporulation in these mutants, we employed the freeze-substituted fixation method to observe the asci of wild-type cells and *fks1*, *fks2*, and *fks3* mutant diploid cells by electron microscopy (EM). A typical wild-type ascus and spore wall with several layers are shown in Fig. 1A and D. After maturation of the spore wall, most *fks2* mutant spores were enveloped by an abnormal spore wall, which contained abnormal structures (Fig. 1B and E). We observed sets of serial sections with the same structures at the edge of the *fks2* mutant spore (Fig. 1J to M). The abnormal structures were observed on the inside of the spore wall. Careful examination of serial sections revealed that these structures were continuous across the sections rather than being separated structures. Observations of the serial sections of the structures in the middle of the *fks2* mutant spore walls revealed that the small circular structures were also continuous across the sections (data not shown). These morphological observations suggest that the abnormal structures are tubelike. To investigate the distribution of glucan, immunoelectron microscopy (immunoEM) was carried out using an anti-1,3-β-glucan antibody. In a typical wild-type ascus, signals for glucan were detected in all the layers of the mature spore wall (Fig. 1G). However, in the spore walls of the *fks2* mutants, the signals for glucan were detected only in the innermost layers (Fig. 1H). We were unable to stain the tubular structures with the anti-glucan antibody (Fig. 1H). These morphological observations suggest that the *FKS2* genes may perform essential functions related to the formation of the layers of the spore walls and are consistent with previous observations of the *fks2* mutants (16).

Most of the *fks3* mutant spores were enveloped by abnormal spore walls, which were of uneven thickness and in some cases included cytoplasm between the inner and outer layers (Fig. 1C

and F). By using immunoEM, the signals for glucan were found to be delocalized between the inner and outer layers of the spore walls of the *fks3* mutants. Finally, we observed the spore walls of the *fks2 fks3* mutant diploid cells by using EM. Most of the *fks2 fks3* mutant spores were enveloped by abnormal spore walls of uneven thickness similar to the *fks3* mutant spore walls. By using immunoEM, the signals for glucan were found to be delocalized in the spore walls of the *fks2 fks3* mutants, similar to those in the *fks3* mutant spore walls (data not shown). These morphological observations suggest that the *FKS2* and *FKS3* genes perform essential functions in the formation of the layers of the spore wall.

Data obtained from genome-wide microarray studies indicate that the amount of *FKS1* mRNA declines to background levels during meiosis, that the level of *FKS2* mRNA increases moderately during meiosis, and that *FKS3* is expressed in a meiosis-specific fashion as a middle gene (7). To verify these data, we prepared total-RNA samples from cells at different stages of sporulation and investigated the presence of *FKS1*, *FKS2*, and *FKS3* mRNAs by Northern analysis. Consistent with the microarray data, the levels of *FKS1* transcripts decreased according to the progression of sporulation whereas the levels of *FKS2* transcripts increased severalfold under the same conditions. The *FKS3* transcripts were strongly induced during the first 2 h after transfer to sporulation medium, with maximal accumulation at 4 h (data not shown). Our results confirm the genome-wide results and indicate that *FKS2* and *FKS3* genes are responsible for the formation of spore walls.

The AI fraction of the ascospore cell wall that represents the skeleton of the cell wall is composed mostly of glucan and chitosan. In contrast, the AS fraction of the ascospore cell wall that is the amorphous cement is composed mainly of mannan and glucan. High ratios of the hexose concentrations in the AI fractions to the total hexose concentrations in the AI and AS fractions may indicate a tight structural organization of the cell wall. Accordingly, Fig. 2A suggests that the ascospore cell walls of the *fks2 fks3* and *fks2* mutants were less structured than the cell walls of the ascospores of the wild type and the *fks1* and *fks3* mutants. Figure 2B shows that the ascospore cell walls of all wild-type and mutant strains contained a significant amount of 1,3-β-glucans. The concentrations of 1,3-β-glucans varied, however, among the different mutants and the AI and AS fractions. The most striking cell wall perturbations were seen in the *fks2 fks3* mutant, where the largest amount of 1,3-β-glucan was released from the AI fraction by the 1,3-β-glucanase. This result was in agreement with EM data and suggested that the cell walls of the ascospores of this mutant were the least organized. In addition, the amounts of 1,3-β-glucan in the AS fractions of the *fks2 fks3* and *fks2* mutants were the smallest. These variations in 1,3-β-glucans were not compensated for by a modification of 1,6-β-glucans since the degradation of the AI and AS fractions by the 1,6-β-glucanase never exceeded 3% of the total hexose concentration in the AI and AS fractions (data not shown).

Sensitivities of the asci of *fks2* and *fks3* mutants to diethyl ether, heat, and ethanol. It has been shown that the asci of several mutants that have defects in spore wall production are more sensitive to certain stresses, including exposure to diethyl ether, heat, and ethanol, than those of the wild type (5, 6, 8, 26). We tested the resistance of the asci of the wild type and

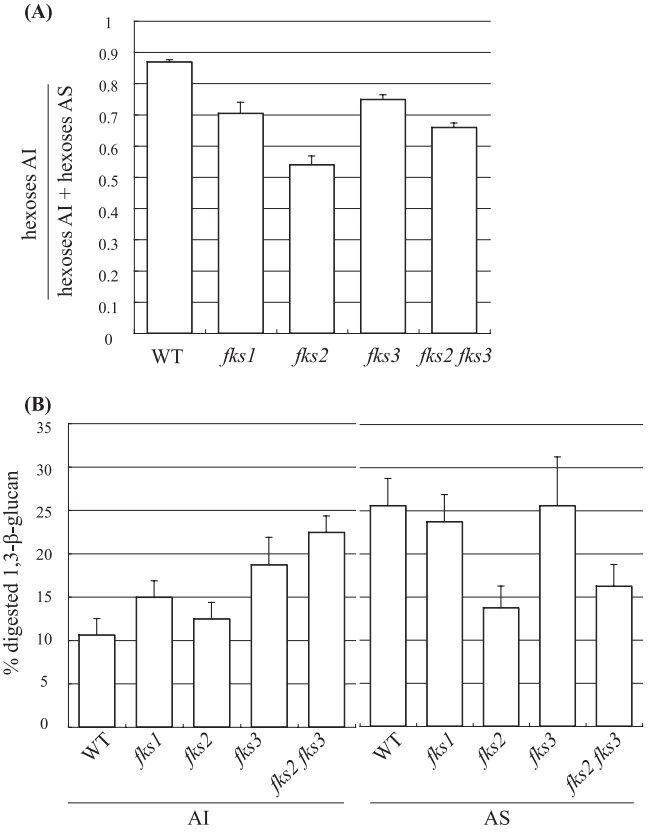


FIG. 2. Hexose and 1,3-β-glucan determination for the ascospore cell wall. The ratios of the hexose concentrations in the AI fractions to the total hexose concentrations in the AI and AS fractions for the different mutants (A) and concentrations (100) of reducing sugar released by the recombinant 1,3-β-glucanase LamA (B) are shown. Data are presented as means ± standard errors of the means of results for four replicates. WT, wild type.

the *fks1*, *fks2*, *fks3*, and *fks2 fks3* mutant diploids to diethyl ether. The asci of the *fks2*, *fks3*, and *fks2 fks3* mutants were 200- to 500-fold more sensitive to a 9-min exposure to diethyl ether than the asci of the wild type (Fig. 3A), although the asci of the *fks1* mutant had a survival rate that was similar to that of the asci of the wild type (Fig. 3A). Next, to confirm the stress-sensitive phenotype, we tested all of the mutants for resistance to heat and ethanol. We examined the thermotolerance of the wild-type and mutant spores after exposure to 55°C for various time periods. As shown in Fig. 3B, the asci of the *fks2*, *fks3*, and *fks2 fks3* mutants were more sensitive than the asci of the wild type to the elevated temperature. Again, the asci of the *fks1* mutants had a survival rate that was similar to that of the asci of the wild type. Finally, we examined the viability of the wild-type and mutant spores after exposure to 25% ethanol for various periods of time. The asci of the *fks2*, *fks3*, and *fks2 fks3* mutants were more sensitive to ethanol than those of the wild type, whereas the asci of the *fks1* mutants had a survival rate that was similar to that of the asci of the wild type (data not shown). The correlation of these phenotypic effects in the *fks2* and *fks3* mutants showing mutational effects on spore wall structure suggests that spore wall formation is linked to spore resistance to stresses, which include exposure

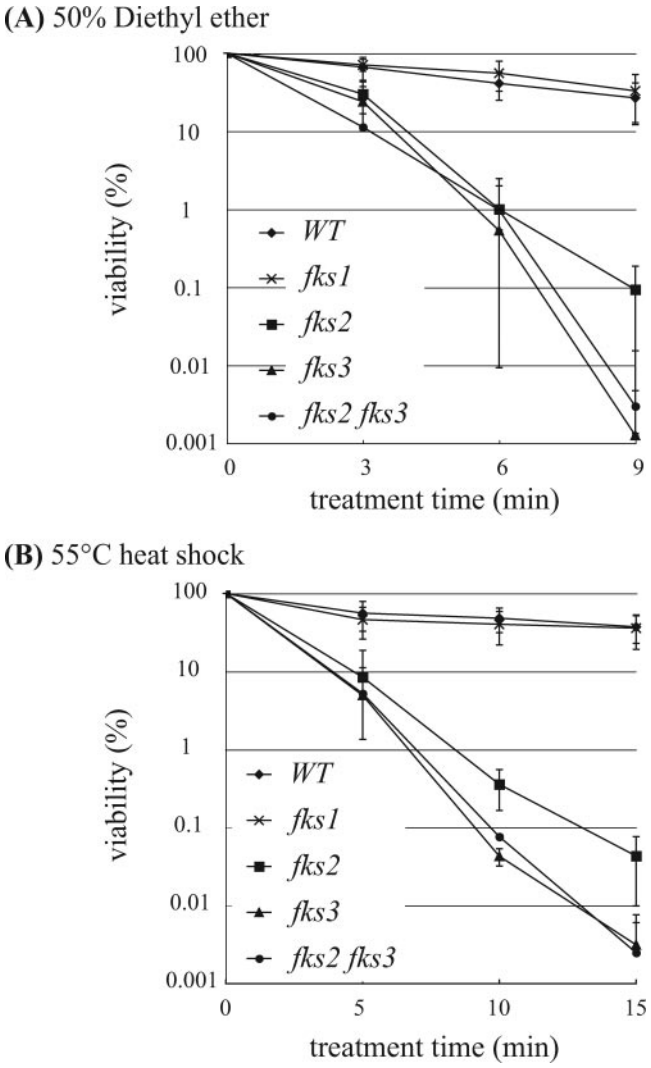


FIG. 3. Spore viability under stress conditions. Sporulated cultures of the wild type (WT) and *fks1*, *fks2*, *fks3*, and *fks2 fks3* mutants were tested for cell viability after exposure to 50% diethyl ether (A) and 55°C (B) for the indicated times. Spore viability was measured as described in Materials and Methods.

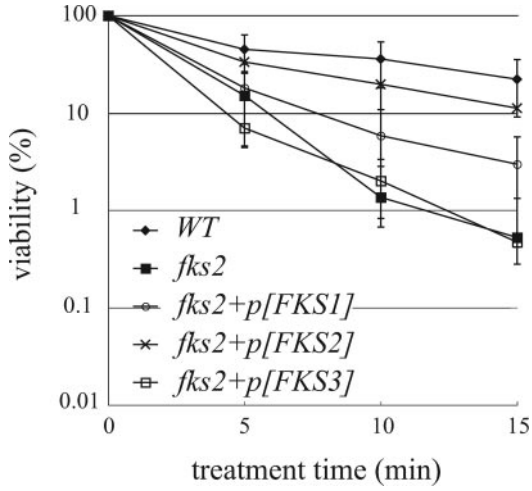


FIG. 4. Spore viability at elevated temperatures for *fks2* mutant asci transformed with plasmids. Sporulated cultures of the wild type (WT), *fks2* mutants, and *fks2* mutants that overexpress the *FKS1*, *FKS2*, or *FKS3* gene were tested for cell viability after exposure to 55°C for the indicated times. Spore viability was measured as described in Materials and Methods.

to diethyl ether, heat shock, and ethanol. In addition, the sensitivities of the asci of the *fks2 fks3* mutants to these stresses (based on survival rates) were the same as those of the *fks3* mutants, which suggests that the *fks2* mutation confers no effect additional to that of the *fks3* mutation.

Expression of *FKS2* under the control of the *FKS2* promoter is important for spore wall assembly. Since Fks1p and Fks2p are highly homologous, they may have overlapping functions in sporulation. Therefore, we investigated whether the overexpression of Fks1p or Fks3p suppressed the spore defect of *fks2* mutants. Multiple copies of the *FKS1* gene partially suppressed the heat-sensitive phenotype of the *fks2* mutant spores (Fig. 4). We postulated that this partial suppression might be due to the difference in the expression levels of Fks1p and Fks2p. Therefore, we constructed plasmids that contained *FKS1p*-driven *FKS1*, *FKS1p*-driven *FKS2*, *FKS2p*-driven *FKS1*, and *FKS2p*-driven *FKS2* (Fig. 5A), and each of these plasmids was introduced into the *fks1 fks2* double-null mutants. All of the strains (YOC4084, YOC4085, YOC4086, and YOC4087) were viable and produced asci of which more than 80% had four spores. Western blotting analysis with an anti-HA antibody revealed that these proteins were present in all the transformed strains (data not shown). We examined the spores of the wild type and the transformed mutants following exposure to 55°C for various periods of time. The asci of the *fks1 fks2* mutant cells transformed with the *FKS1p*-driven Fks1p and Fks2p plasmids showed heat sensitivities similar to those of the *fks2* mutant asci, whereas the asci of the *fks1 fks2* mutant cells transformed with the *FKS2p*-driven Fks1p and Fks2p plasmids showed heat sensitivities similar to those of the wild-type asci (Fig. 5B). These results suggest that the molecular function of Fks2p in spore wall assembly resembles that of Fks1p and that the induction of *FKS2* serves an important role in spore wall assembly. In contrast, multiple copies of the *FKS3* gene did not suppress the heat-sensitive phenotype of the *fks2* mutant spores (Fig. 4). This result and the expression data on the *FKS3* gene expressed at sporulation suggest that Fks3p does not have any function overlapping with that of Fks2p in sporulation.

Enrichment with Fks3p is not observed during purification of GS in vegetative growth. To determine if Fks3p is incorporated into a component of the GS complex, we monitored the amount of Fks3p during the purification of GS. We constructed a plasmid that expressed HA-tagged Fks3p under the control of the *FKS1* promoter (*FKS1p-FKS3*) (Fig. 6A). Western blot analysis with an anti-HA antibody revealed that this protein was present in the membrane fractions of transformed strains (Fig. 6B). We measured the in vitro GS activities of the membrane fraction and the purified GS fraction of cells that expressed *FKS1p-FKS3*. Endogenous Fks1p was enriched in the partially purified GS fractions (Fig. 6C), and the specific activity of GS increased approximately 200-fold (Fig. 6E). However, *FKS1p*-driven Fks3p-HA was not detected with the anti-HA antibody (Fig. 6D). These results suggest that Fks3p is

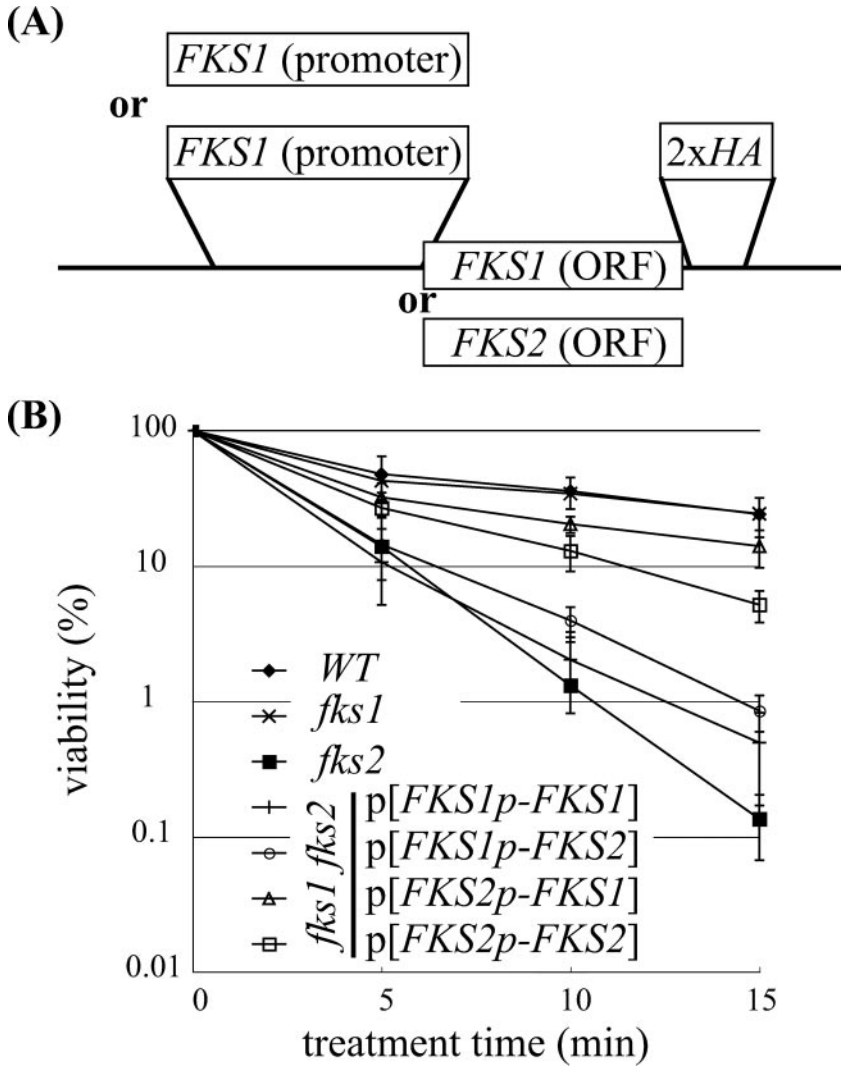


FIG. 5. Effect of the promoter on spore viability. (A) Schematic drawing of the gene constructs. The *HA*-tagged *FKS1* or *FKS2* ORF was fused downstream of the *FKS1* or *FKS2* promoter. (B) Sporulated cultures of the indicated strains were tested for cell viability after exposure to 55°C for the indicated times. Spore viability was measured as described in Materials and Methods. WT, wild type.

lost during the preparation of the purified GS fraction and that Fks3p is not a tightly bound component of the GS complex.

The in vivo glucan synthesis defects are rescued by the expression of Fks3p. To determine the molecular function of Fks3p in 1,3-β-glucan synthase, we examined whether the expression of the *FKS1p-FKS3* allele could suppress the growth defect of the *fks1-1154* Δ*fks2* mutant, a temperature-sensitive mutant of GS (hereinafter referred to as the *fks1-1154* mutant). Recently, Sekiya-Kawasaki et al. (41) have identified seven multicopy suppressors of the *fks1-1154* mutation as positive regulators of GS. Therefore, we examined the growth phenotype of the *fks1-1154* mutant cells, which were able to grow at 25°C but failed to grow at temperatures above 35°C. Figure 7A shows the growth at various temperatures (33 to 37°C). Robust growth was observed for *fks1-1154* mutant cells transformed with multiple copies of *FKS1p-FKS3*, which indicates that the *FKS1p-FKS3* allele expressed from a high-copy-number plasmid is able to suppress the *fks1-1154* mutation.

To examine the possible mechanisms of suppression whereby multiple copies of *FKS1p-FKS3* restore the defect in 1,3-β-glucan synthesis, we tested in vivo glucan synthesis in *fks1-1154* mutant cells that expressed Fks3p under the control of the *FKS1* promoter. First, we stained the mutant cells with aniline blue, which is a fluorescent dye that interacts preferentially with 1,3-β-glucan. As reported previously (41), most of the *fks1-1154* mutant cells appeared to lose their staining signal specifically in the bud at the restrictive temperature (Fig. 7B, panel a). The *fks1-1154* mutant cells that were transformed with multiple copies of *FKS1* and the *FKS1p-FKS3* mutant cells exhibited uniform staining of the cell walls on the cell surface similar to the wild type (Fig. 7B, panels c and e). We quantified the populations of cells that exhibited lower levels of 1,3-β-glucan staining in the bud. Four hours after the shift to 35°C,

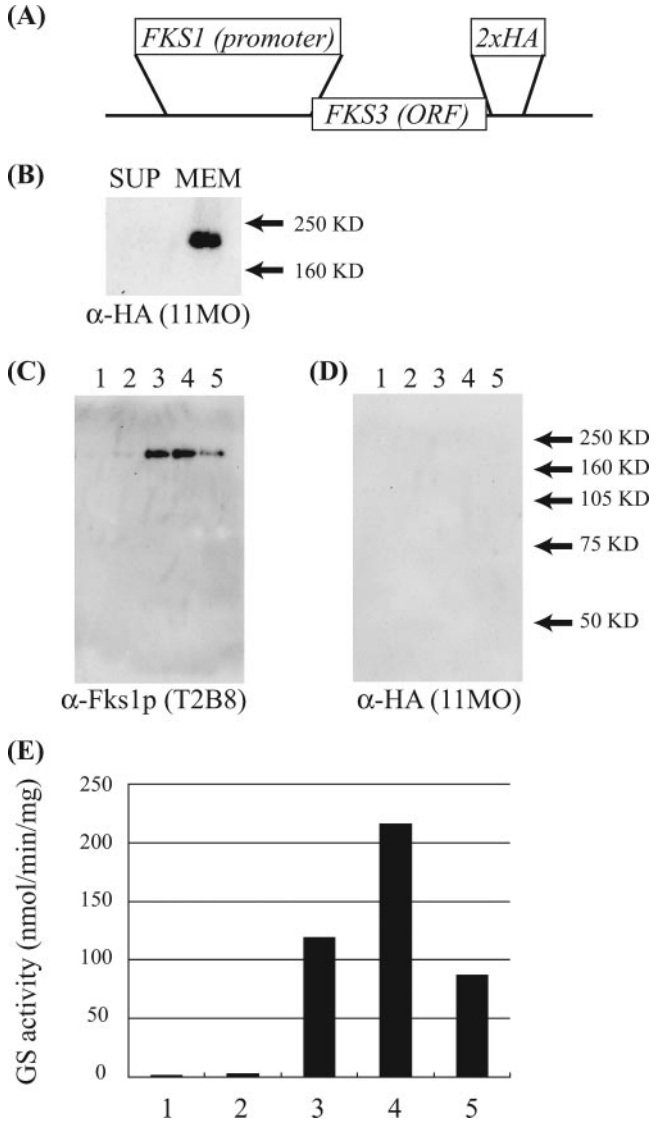


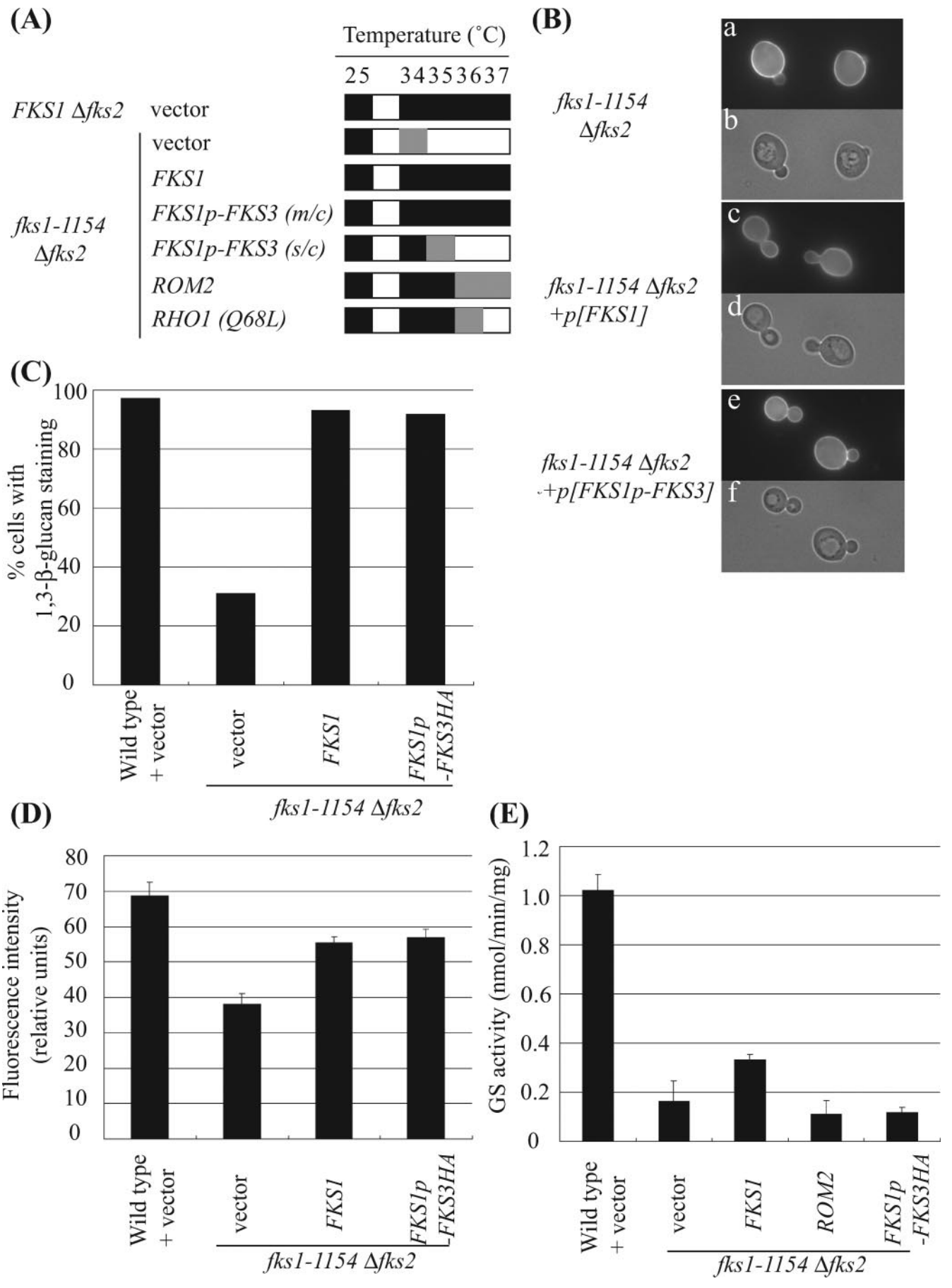
FIG. 6. Purification of GS from cells that express *FKS1* promoter-driven Fks3p during vegetative growth. (A) Schematic drawing of the construct. The *HA*-tagged *FKS3* ORF was fused downstream of the *FKS1* promoter. (B) Protein samples for Western blot analysis of the supernatant (SUP) and membrane (MEM) fractions of yeast lysates. Equal amounts of protein were loaded and separated on an 8% acrylamide gel. A mouse monoclonal antibody against HA (11MO) was used as the primary antibody. (C and D) Western blot analysis with the anti-Fks1p antibody (T2B8) (C) and anti-HA antibody (11MO) (D) of samples taken during purification. (E) GS-specific activity during purification. The purification samples are as follows: lane 1, membrane fraction; lane 2, detergent extract; lane 3, first product entrapment; lane 4, second product entrapment; lane 5, third product entrapment.

more than 70% of the *fks1-1154* mutant cells with vector alone exhibited a loss of glucan staining in the bud. In contrast, the overexpression of *FKS1* and *FKS1p-FKS3* removed the defects in glucan staining in the *fks1-1154* mutant cells (Fig. 7C). Next, we quantified the total amount of 1,3-β-glucan in *fks1-1154* mutant cells with *FKS1* and *FKS1p-FKS3* by using a fluorescence spectrophotometer with aniline blue staining. The *fks1-1154* mutant cells that were transformed with multiple copies of *FKS1p-FKS3* showed increased levels of glucan (Fig. 7D). From these results, we conclude that multiple copies of *FKS1p-FKS3* have a positive effect on the in vivo GS of *fks1-1154* mutant cells.

To examine the positive effect of Fks3p in vitro, we measured the in vitro GS activity of the membrane fraction from the *fks1-1154* mutant cells that overexpressed *FKS1p-FKS3*, which we found to be as low as that from cells with the vector alone (Fig. 7E). This result was similar to that of *ROM2*, a multicopy suppressor of the *fks1-1154* mutation. The overexpression of Rom2p, the GDP-GTP exchange factor of Rho1p (37), increased in vivo GS activity by shifting the equilibrium of Rho1p to the GTP-bound state. Since in vitro GS activity was assayed in the presence of an excess amount of GTPγS, which is a nonhydrolyzable analog of GTP (17), all of the Rho1p was assumed to be in the active state in this in vitro GS assay. These results support the idea that the expression of *FKS1p-FKS3* increases in vivo GS activity by shifting the equilibrium of Rho1p to the GTP-bound state.

Relationship between Fks3p and Rho1p. To examine whether the expression of *FKS1p-FKS3* increases in vivo GS activity through Rho1p activation, *fks1-1154* mutant cells with *FKS1p-FKS3* were transformed with a plasmid that expressed the active form of Rho1p. If the expression of *FKS1p-FKS3* activated GS by converting Rho1p into the active form, no additional effect on GS activity should be observed. We quantified colorimetrically the total amount of 1,3-β-glucan in *fks1-1154* mutant cells with plasmids by using aniline blue staining. No additional effect on GS activity was observed, which suggests that Fks3p activates GS by converting Rho1p into the active form (Fig. 8A). Next, to confirm Rho1p activation by Fks3p in vivo, we measured the amount of the active form of Rho1p. The state of Rho1p was monitored in a pull-down assay using the GST-fused Rho1p-binding domain of Pkc1p, since the Rho1p-binding domain of Pkc1p binds specifically to the active form of Rho1p (34). Our established system specifically pulls down the active form of Rho1p (GTP-fixed Rho1p), while the inactive form (GDP-fixed Rho1p) is not pulled down (K. Kono, M. Abe, S. Nogami, M. Nishizawa, S. Morishita, D. Pellman, and Y. Ohya, submitted for publication). As a control, we performed the pull-down assay for active Rho1p with *fks1-1154* mutant cells and with *fks1-1154* mutant cells transformed with the *ROM2* gene. The active-Rho1p level was higher in the *fks1-1154* mutant cells transformed with the *ROM2* gene than in the untransformed *fks1-1154* mutant cells (Fig. 8B), which is consistent with the previous results. Next, we performed the same pull-down assay using *fks1-1154* mutant cells that overexpressed Fks3p under the control of the *FKS1* promoter. The active-Rho1p level was higher in *fks1-1154* mutant cells that overexpressed Fks3p than in *fks1-1154* mutant cells without Fks3p overexpression (Fig. 8B). These results suggest that the overexpression of *FKS1p-FKS3* has a positive effect on GS activity through the activation of Rho1p in vivo.

Fks1p and Fks2p do not suppress the phenotypes of *fks3* mutants. To examine whether Fks3p has a role distinct from Fks1p and Fks2p at sporulation and whether *FKS1* or *FKS2* acts in the same pathway as *FKS3* at sporulation, we introduced plasmids that overexpressed Fks1p, Fks2p, and Fks3p into *fks3* mutant cells and examined the phenotypes of their


(A)
Temperature (°C)
25 34353637
FKS1 Δfks2
vector
fks1-1154 Δfks2
vector
FKS1
FKS1p-FKS3 (m/c)
FKS1p-FKS3 (s/c)
ROM2
RHO1 (Q68L)
(B)
fks1-1154 Δfks2
fks1-1154 Δfks2 +p[FKS1]
fks1-1154 Δfks2 +p[FKS1p-FKS3]
a
b
c
d
e
f
(C)
% cells with 1,3-β-glucan staining
100
80
60
40
20
0
Wild type + vector
vector
FKS1
FKS1p -FKS3HA
fks1-1154 Δfks2
(D)
Fluorescence intensity (relative units)
80
70
60
50
40
30
20
10
0
Wild type + vector
vector
FKS1
FKS1p -FKS3HA
fks1-1154 Δfks2
(E)
GS activity (nmol/min/mg)
1.2
1.0
0.8
0.6
0.4
0.2
0
Wild type + vector
vector
FKS1
ROM2
FKS1p -FKS3HA
fks1-1154 Δfks2

spores. After exposure to 55°C, the asci of the *fks3* mutant cells transformed with multiple copies of *FKS3* had a survival rate similar to that of the wild-type asci (Fig. 9). However, multiple copies of the *FKS1* or *FKS2* gene did not restore the heat-sensitive phenotype of the *fks3* mutant asci (Fig. 9). Next, we constructed plasmids that contained *FKS3p*-driven *FKS1* and *FKS3p*-driven *FKS2*, both of which were introduced into the *fks3* mutant cells. The asci of the *fks3* mutant cells transformed with *FKS3p*-driven Fks1p and the Fks2p plasmid showed heat sensitivities similar to that of the *fks3* mutant asci, which suggests that these genes do not restore the heat-sensitive phenotype (data not shown). Our findings suggest that Fks3p exerts a distinct effect on GS during spore wall maturation.

DISCUSSION

In budding yeast, the spore wall has been shown to play a central role in protecting the cell from environmental damage. In this paper, we demonstrate that Fks2p, the putative catalytic subunit of the glucan synthase, is required for spore wall maturation, consistent with the results reported by Huang et al. (16), and that Fks3p, which shares homology with the catalytic subunits, is also required for spore wall maturation. In addition, we found that the lack of the *FKS2* gene and the *FKS3* gene causes assembly defects in the 1,3-β-glucan layers and reduces the resistance to certain stresses.

The *fks2* mutant shows abnormal morphology under EM. In addition, the abnormal features (a low ratio of the hexose concentration in the AI fraction to the total hexose concentration in the AI and AS fractions and a small amount of alkali-soluble 1,3-β-glucan) may represent a less organized cell wall in this mutant. In spite of abnormal spore wall morphology, the *fks3* mutant shows a normal ratio of the hexose concentration in the AI fraction to the total hexose concentration in the AI and AS fractions. However, fine structural modifications could exist but could not be investigated with our enzymatic methodology since in contrast to those of the vegetative cells, the ascospore cell wall is very resistant to 1,3-β- and 1,6-β-endoglucanase (70% of the hexoses of the yeast cell wall are degraded by LamA and the 1,6-β-endoglucanase) (data not shown). This result suggests different structural organizations of the polysaccharides of the yeast and ascospore cell walls. The structural 1,3-β-glucan modifications of the *fks2 fks3* mutant and its abnormal morphology show that this mutant results in the additional effect of the absence of the two *FKS2* and *FKS3* genes.

Based on these observations and previous studies, functional differences among glucan synthase subunits and their homologous proteins and their roles in glucan synthesis during the sporulation process are discussed.

Differences among 1,3-β-glucan synthase subunits involved in spore wall assembly. The assembly of the 1,3-β-glucan layers of spores requires the 1,3-β-glucan synthase, which is located in the spore plasma membrane. Two genes in *S. cerevisiae* encode putative catalytic subunits of the synthase. *FKS1* encodes the subunit that is primary during vegetative growth and *FKS2* appears to encode the subunit predominant during sporulation (17, 31). It has been shown that the expression of these genes is controlled differentially. *FKS1* is regulated in the cell cycle and predominates during growth on glucose, whereas *FKS2* is expressed in the absence of glucose, especially during sporulation (31). It has also been shown that Fks1p and Fks2p exhibit different sensitivities to some 1,3-β-glucan synthase inhibitors, such as L-733,560 and aerothricin3 (12, 24). In the present study, we show that Fks1p is functional in spore wall assembly and is able to compensate for the sporulation defect in *fks2* asci when expressed under the control of the *FKS2* promoter. This finding suggests that there are few functional differences between Fks1p and Fks2p and that the regulation of expression of the glucan synthase gene plays an important role in spore wall assembly (Fig. 5B). Although the expression of the *FKS2* gene is important for spore wall formation, the *FKS1* gene may have a role in this process because, even in the *fks2* mutant, the spore wall is formed and contains 1,3-β-glucan (Fig. 1H and 2). A residual amount of Fks1p expressed from its authentic promoter can substitute functionally for Fks2p in spore wall assembly, although it may not be sufficient for complete formation of the normal spore wall. In fission yeast, it has been reported that $bgs1^+$, $bgs3^+$, and $bgs4^+$ gene products under the control of the $bgs2^+$ promoter are unable to compensate for the sporulation defect in *bgs2*Δ (29), which suggests that each *bgs* gene product has a different role in sporulation, in contrast to the case for budding yeast.

Function of *FKS3* during spore wall assembly. In this study, we investigated the molecular functions of Fks3p. We found that the overexpression of *FKS1p-FKS3* effectively suppressed the growth defect of the *fks1-1154* mutant (Fig. 7A), which suggests that *FKS1p-FKS3* has a positive effect on in vivo GS of *fks1-1154* cells. However, the overexpression of *FKS1p-FKS3* did not increase in vitro GS activity (Fig. 6 and 7E). Therefore, we postulate that Fks3p has distinct functions for GS and may

FIG. 7. Rescue of growth defect and reduced in vivo GS activation in the *fks1-1154* mutant through expression of the *FKS3-HA* gene under the control of the *FKS1* promoter. (A) Growth of the transformants at various temperatures. YOC1087 (*fks1-1154*) was transformed with a control vector (pRS316), a multicopy vector that expresses the *FKS1* gene, a multicopy (m/c) vector that expresses the *FKS3* gene under the control of the *FKS1* promoter, a single-copy (s/c) vector that expresses the *FKS3* gene under the control of the *FKS1* promoter, a single-copy vector that expresses the *ROM2* gene, or a single-copy vector that expresses the *RHO1* (Q68L) mutant gene. Growth on YPD was scored at the indicated temperatures. The black, gray, and white regions indicate growth equivalent to that of wild-type cells, slower growth, and no growth, respectively. (B) Aniline blue staining of yeast cells. Log-phase cells were shifted from 25°C to 35°C, cultured for 4 h, and stained with aniline blue (a, c, and e). (C) Quantification of cells with normal aniline blue staining. Cells with a small bud or a tiny projection were observed, and the percentages of cells with aniline blue staining in the bud were calculated. (D) Colorimetric quantification of the total amount of 1,3-β-glucan in *fks1-1154* mutant cells that carry the *FKS1p-FKS3-HA* plasmids. The amount of 1,3-β-glucan per cell was measured with aniline blue staining and is expressed as the relative fluorescence intensity. (E) Glucan synthase activity assayed in the membrane fraction from the *fks1-1154* mutant transformed with high-copy-number plasmids carrying the indicated genes. The *fks1-1154* mutant cells transformed with plasmids were shifted from 25°C to 34°C for 2 h and then assayed for GS activity.

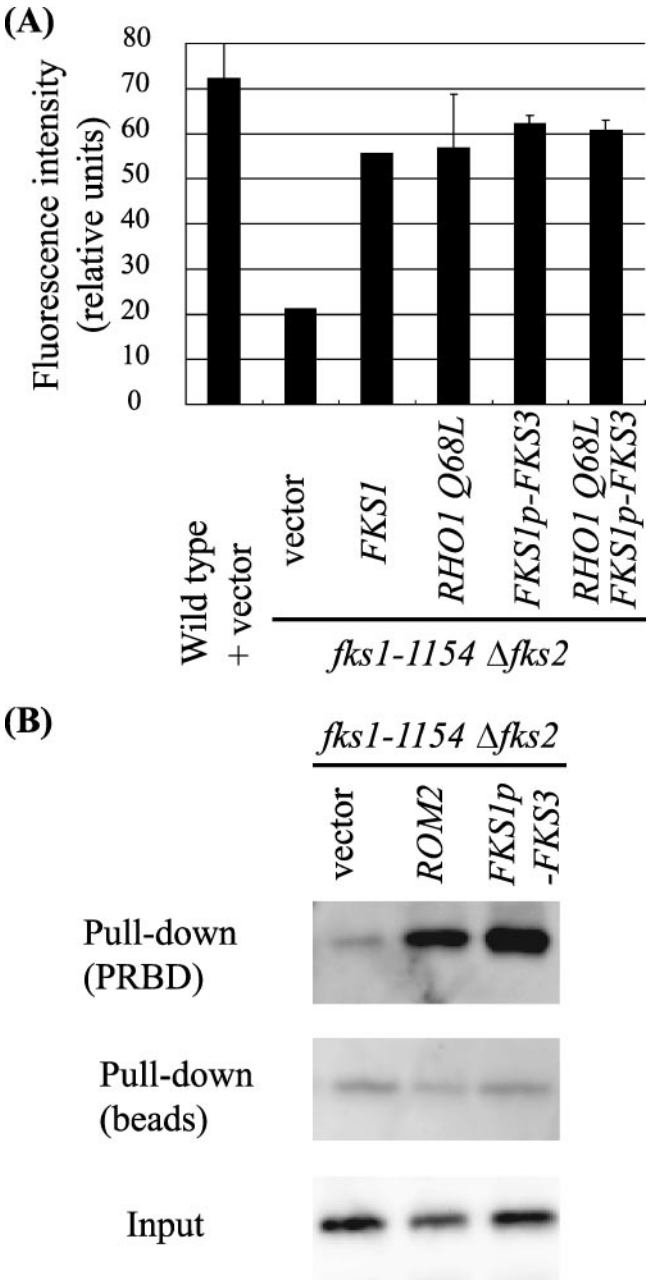


FIG. 8. Expression of *FKS1p-FKS3* activates Rho1p in vivo. (A) Quantification of the total amount of 1,3-β-glucan in *fks1-1154* mutant cells that carry the *RHO1* (Q68L) and *FKS1p-FKS3-HA* plasmids. The amount of 1,3-β-glucan per cell was measured with aniline blue staining and is expressed as the relative fluorescence intensity. (B) Pull-down assay of active Rho1p. Yeast cells were transformed with the control vector (pRS316), a vector that expresses the *ROM2* gene, and a vector that expresses the *FKS3* gene under the control of the *FKS1* promoter. Log-phase, growing yeast were cultured at 25°C in SD lacking uracil and subjected to the pull-down assay.

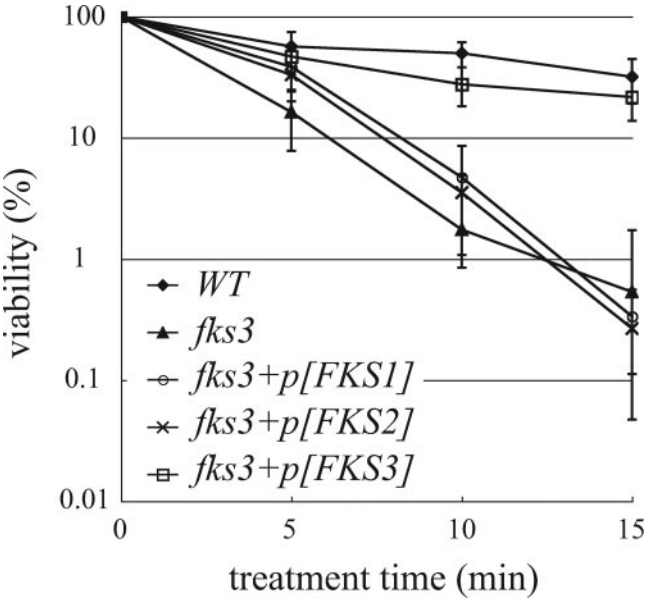


FIG. 9. Spore viability of *fks3* mutant asci transformed with plasmids after treatment at the elevated temperature. Sporulated cultures of the wild type (WT), the *fks3* mutant, and the *fks3* mutants carrying a multicopy plasmid that expresses the *FKS1*, *FKS2*, or *FKS3* gene were tested for cell viability after exposure to 55°C for the indicated periods of time. Spore viability was measured as described in Materials and Methods.

regulate GS through the activation of a GS effector. We found that the active-Rho1p level increased in *fks1-1154* mutant cells transformed with *FKS1p-FKS3* relative to that in *fks1-1154* mutant cells (Fig. 8), which suggests that the *fks1-1154* mutant has a weak glucan synthase activity that can be stimulated by the activation of Rho1p by Fks3p and that the overexpression of *FKS1p-FKS3* acts positively on GS through the activation of Rho1p in vivo. If Fks3p activates Rho1p during spore wall assembly, the active form of Rho1p (*RHO1* [Q68L]) should complement the spore wall defect of the *fks3* mutant. However, the asci of the *fks3* mutant cells transformed with the *RHO1* (Q68L) plasmid showed heat sensitivities similar to those of the *fks3* mutant asci, which indicates that these genes do not restore the heat-sensitive phenotype (data not shown). In addition, the spore wall defects in the *fks3* mutant were not restored when both *FKS1* and *FKS2* were introduced via an expression plasmid (Fig. 9), which suggests that the relationship between Rho1p and Fks1/2p at sporulation is different from that at vegetative growth. Our results support the idea that Fks3p has distinct functions for GS and also regulates GS during spore wall assembly. We speculate that Fks3p interacts with accessory components of GS that modify GS activity and increase GS activity during glucan layer assembly. However, it is unclear whether Rho1p is the only target of Fks3p. It has recently been reported that in fission yeast, the Rho5p GTPase, which is highly homologous to Rho1p, is required for spore wall formation. The asci of the *rho5*Δ strain are less resistant to heat than the asci of the wild-type (39). It is possible that a similar regulation mechanism mediated by Rho1p or other homologous proteins operates during spore wall assembly in budding yeast. In addition, protein kinases Smk1p and Sps1p play roles in the trafficking of both Fks2p and the Chs3p chitin synthase to the spore plasma membrane (16, 20). Both Fks2p and Chs3p localize to the prospore membrane and spore wall (20). We have determined by microscopic observation that Fks3p also localizes to the prospore membrane and spore wall

(data not shown). Fks3p may be required for the trafficking, localization, and activation of Fks2p through interactions with accessory components of GS.

Spore wall resistance is dependent upon glucan assembly. The low organization of the cell wall of the *fks2*, *fks3*, and *fks2 fks3* mutants as shown by EM, ImmunoEM analysis using the anti-1,3-β-glucan antibody, and chemical cell wall analysis (Fig. 1 and 2) is the direct cause of the reduced resistance of the ascospores of these mutants to diethyl ether, elevated temperatures, and ethanol. Therefore, the assembly of the glucan layer and subsequent assembly of the spore wall may be essential for spore wall resistance to environmental damage. In summary, glucan is a key assembly component of the spore wall, and its synthesis and assembly are regulated by a subunit of glucan synthase, Fks2p, and its regulatory homolog Fks3p. Glucan layer assembly is essential for spore wall formation and confers resistance to environmental damage.

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REFERENCES

1. **Abe, M., I. Nishida, M. Minemura, H. Qadota, Y. Seyama, T. Watanabe, and Y. Ohya.** 2001. Yeast 1,3-β-glucan synthase activity is inhibited by phytosphingosine localized to the endoplasmic reticulum. J. Biol. Chem. **276:** 26923–26930.
2. **Bom, I. J., S. K. Dielbandhoesing, K. N. Harvey, S. J. C. M. Oomes, F. M. Klis, and S. Brul.** 1998. A new tool for studying the molecular architecture of the fungal cell wall: one-step purification *Trichoderma* β-(1-6)-glucanase expressed in *Pichia pastoris*. Biochim. Biophys. Acta **1425:**419–424.
3. **Briza, P., G. Winkler, H. Kalchhauser, and M. Breitenbach.** 1986. Dityrosine is a prominent component of the yeast ascospore wall. A proof of its structure. J. Biol. Chem. **261:**4288–4294.
4. **Briza, P., A. Ellinger, G. Winkler, and M. Breitenbach.** 1988. Chemical composition of the yeast ascospore wall. The second outer layer consists of chitosan. J. Biol. Chem. **263:**11569–11574.
5. **Briza, P., M. Breitenbach, A. Ellinger, and J. Segall.** 1990. Isolation of two developmentally regulated genes involved in spore wall maturation in *Saccharomyces cerevisiae*. Genes Dev. **4:**1775–1789.
6. **Christodoulidou, A., P. Briza, A. Ellinger, and V. Bouriotis.** 1999. Yeast ascospore wall assembly requires two chitin deacetylase isozymes. FEBS Lett. **460:**275–279.
7. **Chu, S., J. DeRisi, M. Eisen, J. Mulholland, D. Botstein, P. O. Brown, and I. Herskowitz,.** 1998. The transcriptional program of sporulation in budding yeast. Science **282:**699–705.
8. **Coluccio, A., E. Bogengruber, M. N. Conrad, M. E. Dresser, P. Briza, and A. M. Neiman.** 2004. Morphogenetic pathway of spore wall assembly in *Saccharomyces cerevisiae*. Eukaryot. Cell **3:**1464–1475.
9. **Cortes, J. C., J. Ishiguro, A. Duran, and J. C. Ribas.** 2002. Localization of the (1,3)β-D-glucan synthase catalytic subunit homologue Bgs1p/Cps1p from fission yeast suggests that it is involved in septation, polarized growth, mating, spore wall formation and spore germination. J. Cell Sci. **115:**4081–4096.
10. **Cortes, J. C., E. Carnero, J. Ishiguro, Y. Sanchez, A. Duran, and J. C. Ribas.** 2005. The novel fission yeast (1,3)beta-D-glucan synthase catalytic subunit Bgs4p is essential during both cytokinesis and polarized growth. J. Cell Sci. **118:**157–174.
11. **Dijkgraaf, G. J., M. Abe, Y. Ohya, and H. Bussey.** 2002. Mutations in Fks1p affect the cell wall content of beta-1,3- and beta-1,6-glucan in *Saccharomyces cerevisiae*. Yeast **19:**671–690.
12. **Douglas, C. M., F. Foor, J. A. Marrinan, N. Morin, J. B. Nielsen, A. M. Dahl, P. Mazur, W. Baginsky, W. Li, M. El-Sherbeini, J. A. Clemas, S. M. Mandala, B. R. Frommer, and M. B. Kurtz.** 1994. The *Saccharomyces cerevisiae FKS1* (*ETG1*) gene encodes an integral membrane protein which is a subunit of 1,3-β-D-glucan synthase. Proc. Natl. Acad. Sci. USA **91:**12907–12911.
13. **Drgonova, J., T. Drgon, K. Tanaka, R. Kollar, G. C. Chen, R. A. Ford, C. S. M. Chan, Y. Takai, and E. Cabib.** 1996. Rho1p, a yeast protein at the interface between cell polarization and morphogenesis. Science **272:**277–279.
14. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric methods for determination of sugars and related substances. Anal. Chem. **28:**350–356.
15. **Fontaine, T., R. Hartland, A. Beauvais, M. Diaquin, and J. P. Latgé.** 1997. Purification and characterization of an endo-1,3-β-glucanase from *Aspergillus fumigatus*. Eur. J. Biochem. **243:**315–321.
16. **Huang, L. S., H. K. Doherty, and I. Herskowitz.** 2005. The Smk1p MAP kinase negatively regulates Gsc2p, a 1,3-β-glucan synthase, during spore wall morphogenesis in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **102:** 12431–12436.
17. **Inoue, S. B., N. Takewaki, T. Takasuka, T. Mio, M. Adachi, Y. Fujii, C. Miyamoto, M. Arisawa, Y. Furuichi, and T. Watanabe.** 1995. Characterization and gene cloning of 1,3-β-D-glucan synthase from *Saccharomyces cerevisiae*. Eur. J. Biochem. **231:**845–854.
18. **Ishiguro, J., A. Saitou, A. Duran, and J. C. Ribas.** 1997. *cps1+*, a *Schizosaccharomyces pombe* gene homolog of *Saccharomyces cerevisiae FKS* genes whose mutation confers hypersensitivity to cyclosporin A and papulacandin B. J. Bacteriol. **179:**7653–7662.
19. **Ishihara, S., A. Hirata, M. Minemura, S. Nogami, and Y. Ohya.** 2001. A mutation in *SPC42*, which encodes a component of the spindle pole body, results in production of two-spored asci in *Saccharomyces cerevisiae*. Mol. Genet. Genomics **265:**585–595.
20. **Iwamoto, M. A., S. R. Fairclough, S. A. Rudge, and J. Engebrecht.** 2005. *Saccharomyces cerevisiae* Sps1p regulates trafficking of enzymes required for spore wall synthesis. Eukaryot. Cell **4:**536–544.
21. **Kaiser, C., S. Michaelis, and A. Mitchell.** 1994. Methods in yeast genetics: a laboratory course manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
22. **Kane, S. M., and R. Roth.** 1974. Carbohydrate metabolism during ascospore development in yeast. J. Bacteriol. **118:**8–14.
23. **Kimura, K., T. Tsuji, Y. Takada, T. Miki, and S. Narumiya.** 2000. Accumulation of GTP-bound RhoA during cytokinesis and a critical role of *ECT2* in this accumulation. J. Biol. Chem. **275:**17233–17236.
24. **Kondoh, O., T. Takasuka, M. Arisawa, Y. Aoki, and T. Watanabe.** 2002. Differential sensitivity between Fks1p and Fks2p against a novel beta-1,3-glucan synthase inhibitor, aerothricin3. J. Biol. Chem. **277:**41744–41749.
25. **Kupiec, M., B. Byers, R. E. Esposito, and A. P. Mitchell.** 1997. Meiosis and sporulation in *Saccharomyces cerevisiae*, p. 889–1036. *In* J. R. Pringle, J. R. Broach, and E. W. Jones (ed.), The molecular and cellular biology of the yeast *Saccharomyces*: cell cycle and cell biology. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
26. **Law, D. T., and J. Segall.** 1988. The *SPS100* gene of *Saccharomyces cerevisiae* is activated late in the sporulation process and contributes to spore wall maturation. Mol. Cell. Biol. **8:**912–922.
27. **Lesage, G., A. M. Sdicu, P. Menard, J. Shapiro, S. Hussein, and H. Bussey.** 2004. Analysis of β-1,3-glucan assembly in *Saccharomyces cerevisiae* using a synthetic interaction network and altered sensitivity to caspofungin. Genetics **167:**35–49.
28. **Liu, J., X. Tang, H. Wang, and M. Balasubramanian.** 2000. Bgs2p, a 1,3-β-glucan synthase subunit, is essential for maturation of ascospore wall in Schizosaccharomyces pombe. FEBS Lett. **478:**105–108.
29. **Martin, V., J. C. Ribas, E. Carnero, A. Duran, and Y. Sanchez.** 2000. *bgs2+*, a sporulation-specific glucan synthase homologue is required for proper ascospore wall maturation in fission yeast. Mol. Microbiol. **38:**308–321.
30. **Martin, V., B. Garcia, E. Carnero, A. Duran, and Y. Sanchez.** 2003. Bgs3p, a putative 1,3-beta-glucan synthase subunit, is required for cell wall assembly in *Schizosaccharomyces pombe*. Eukaryot. Cell **2:**159–169.
31. **Mazur, P., N. Morin, W. Baginsky, M. El-Sherbeini, J. A. Clemas, J. B. Nielsen, and F. Foor.** 1995. Differential expression and function of two homologous subunits of yeast 1,3-β-D-glucan synthase. Mol. Cell. Biol. **15:** 5671–5681.
32. **Mouyna, I., R. P. Hartland, T. Fontaine, M. Diaquin, C. Simenel, M. Delepierre, B. Henrissat, and J. P. Latgé.** 1998. A β(1-3) glucanosyltransferase isolated from the cell wall of *Aspergillus fumigatus* is a homologue of the yeast Bgl2p. Microbiology **144:**3171–3180.
33. **Neiman, A. M.** 2005. Ascospore formation in the yeast *Saccharomyces cerevisiae*. Microbiol. Mol. Biol. Rev. **69:**565–584.
34. **Nonaka, H., K. Tanaka, H. Hirano, T. Fujiwara, H. Kohno, M. Umikawa, A. Mino, and Y. Takai.** 1995. A downstream target of *RHO1* small GTP-binding protein is *PKC1*, a homolog of protein kinase C, which leads to activation of the MAP kinase cascade in Saccharomyces cerevisiae. EMBO J. **14:**5931–5938.
35. **Ohta, K., A. Nicolas, M. Furuse, A. Nabetani, H. Ogawa, and T. Shibata.** 1998. Mutations in the *MRE11*, *RAD50*, *XRS2*, and *MRE2* genes alter chromatin configuration at meiotic DNA double-stranded break sites in premeiotic and meiotic cells. Proc. Natl. Acad. Sci. USA **95:**646–651.
36. **Orlean, P.** 1997. Biogenesis of yeast wall and surface components, p. 229–362. *In* J. R. Pringle, J. R. Broach, and E. W. Jones (ed.), The molecular and cellular biology of the yeast *Saccharomyces*: cell cycle and cell biology. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
37. **Ozaki, K., K. Tanaka, H. Imamura, T. Hihara, T. Kameyama, H. Nonaka, H. Hirano, Y. Matsuura, and Y. Takai.** 1996. Rom1p and Rom2p are GDP/

GTP exchange proteins (GEPs) for the Rho1p small GTP binding protein in *Saccharomyces cerevisiae*. EMBO J. **15:**2196–2207.

38. **Qadota, H., C. P. Python, S. B. Inoue, M. Arisawa, Y. Anraku, Y. Zheng, T. Watanabe, D. E. Levin, and Y. Ohya.** 1996. Identification of yeast Rho1p GTPase as a regulatory subunit of 1,3-β-glucan synthase. Science **272:**279–281.
39. **Rincon, S. A., B. Santos, and P. Perez.** 2006. Fission yeast Rho5p GTPase is a functional paralogue of Rho1p that plays a role in survival of spores and stationary-phase cells. Eukaryot. Cell **5:**435–446.
40. **Sakumoto, N., Y. Mukai, K. Uchida, T. Kouchi, J. Kuwajima, Y. Nakagawa, S. Sugioka, E. Yamamoto, T. Furuyama, H. Mizubuchi, N. Ohsugi, T. Sakuno, K. Kikuchi, I. Matsuoka, N. Ogawa, Y. Kaneko, and S. Harashima.** 1999. A series of protein phosphatase gene disruptants in *Saccharomyces cerevisiae*. Yeast **15:**1669–1679.
41. **Sekiya-Kawasaki, M., M. Abe, A. Saka, D. Watanabe, K. Kono, M. Minemura-Asakawa, S. Ishihara, T. Watanabe, and Y. Ohya.** 2002. Dissection of upstream regulatory components of the Rho1p effector, 1,3-beta-glucan synthase, in *Saccharomyces cerevisiae*. Genetics **162:**663–667.
42. **Sikorski, R. S., and P. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:**19–27.
43. **Valdivieso, M. H., P. C. Mol, J. A. Shaw, E. Cabib, and A. Duran.** 1991. *CAL1*, a gene required for activity of chitin synthase 3 in *Saccharomyces cerevisiae*. J. Cell Biol. **114:**101–109.
44. **Yoshihisa, T., and Y. Anraku.** 1989. Nucleotide sequence of *AMS1*, the structural gene of vacuolar a-mannosidase of *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. **163:**908–915.
45. **Zverlov, V. V., I. Y. Volkov, T. V. Velikodvorskaya, and W. H. Schwarz.** 1997. Highly thermostable endo-1,3-beta-glucanase (laminarinase) LamA from Thermotoga neapolitana: nucleotide sequence of the gene and characterization of the recombinant gene product. Microbiology **143:**1701–1708.