

Research Article

Mutations in Fks1p affect the cell wall content of β -1,3- and β -1,6-glucan in *Saccharomyces cerevisiae*

Gerrit J. P. Dijkgraaf¹, Mitsuhiro Abe², Yoshikazu Ohya² and Howard Bussey^{1*}¹ Department of Biology, McGill University, Montreal, Quebec, Canada H3A 1B1² Department of Integrated Biosciences, Graduate School of Frontier Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan

*Correspondence to:

H. Bussey, Department of Biology, McGill University, 1205 Dr. Penfield Ave., Montreal, Quebec, Canada H3A 1B1.
E-mail: hbusse@po-box.mcgill.ca

Abstract

Fks1p and Fks2p are related proteins thought to be catalytic subunits of the β -1,3-glucan synthase. Analysis of *fks1* Δ mutants showed a partial K1 killer toxin-resistant phenotype and a 30% reduction in alkali-soluble β -1,3-glucan that was accompanied by a modest reduction in β -1,6-glucan. The *gas1* Δ mutant lacking a 1,3- β -glucanoyltransferase displayed a similar reduction in alkali-soluble β -1,3-glucan but did not share the β -1,6-glucan defect, indicating that β -1,6-glucan reduction is not a general phenotype among β -1,3-glucan biosynthetic mutants. Overexpression of *FKS2* suppressed the killer toxin phenotype of *fks1* Δ mutants, implicating Fks2p in the biosynthesis of the residual β -1,6-glucan present in *fks1* Δ cells. In addition, eight out of 12 *fks1*_{ts} *fks2* Δ mutants had altered β -glucan levels at the permissive temperature: the partial killer resistant *FKS1*^{F1258Y N1520D} allele was severely affected in both polymers and displayed a 55% reduction in β -1,6-glucan, while the *in vitro* hyperactive allele *FKS1*^{T605I M761T} increased both β -glucan levels. These β -1,6-glucan phenotypes may be due to altered availability of, and structural changes in, the β -1,3-glucan polymer, which might serve as a β -1,6-glucan acceptor at the cell surface. Alternatively, Fks1p and Fks2p could actively participate in the biosynthesis of both polymers as β -glucan transporters. We analysed Fks1p and Fks2p in β -1,6-glucan deficient mutants and found that they were mislocalized and that the mutants had reduced *in vitro* glucan synthase activity, possibly contributing to the observed β -1,6-glucan defects. Copyright © 2002 John Wiley & Sons, Ltd.

Received: 20 November 2001
Accepted: 24 January 2002**Keywords:** *Saccharomyces cerevisiae*; cell wall; β -1,3-glucan; β -1,6-glucan; Fks1p; Fks2p; Gas1p; *KRE* genes

Introduction

Saccharomyces cerevisiae has an extracellular matrix composed of mannoproteins, β -glucans and chitin. These components are synthesized separately, but are cross-linked to each other at the cell surface in macromolecular complexes that form the cell wall (Lipke and Ovalle, 1998). This essential organelle ensures osmotic stability, determines cell shape, acts as a filter for large molecules and retains enzymatic activities required for nutrient uptake. The cell wall is a dynamic structure and must be remodelled to accommodate different developmental programs such as budding, mating, sporulation and pseudohyphal filamentation (Orlean, 1997).

The β -glucans are glucose polymers that account for half of the cell wall dry weight. Approximately

80% of these β -glucans are β -1,3-linked, have an average length of 1500 residues and contain some β -1,6-linked branching points (Manners *et al.*, 1973). This polymer is made at the cell surface by a β -1,3-glucan synthase activity, which can be dissociated into a membrane-bound fraction and a soluble GTP-binding fraction by high salt and detergent treatment (Kang and Cabib, 1986). *FKS1* and *FKS2* encode a pair of highly homologous proteins predicted to be integral membrane proteins with 16 transmembrane helices (Mazur *et al.*, 1995). Several lines of evidence suggest that Fks1p and Fks2p are components of the β -1,3-glucan synthase complex: (a) both Fks1p and Fks2p co-purify with β -1,3-glucan synthase activity during successive rounds of product entrapment (Inoue *et al.*, 1995); (b) *fks1* Δ mutants have reduced *in vitro*

glucan synthase activity (Douglas *et al.*, 1994); and (c) the glucan synthase activity of yeast extracts can be immunoprecipitated and immunodepleted with Fksp-specific antibodies (Inoue *et al.*, 1995; Mazur *et al.*, 1995). These observations have led to the proposal that Fks1p and Fks2p are redundant catalytic subunits of the β -1,3-glucan synthase, but their precise function remains unknown. Both Fks1p and Fks2p lack clear similarities with other enzymes involved in the formation of glucan polymers, including bacterial curdlan (β -1,3-glucan) synthases (Stasinopoulos *et al.*, 1999). The UDP-glucose binding site R/K-X-G-G found in glycogen synthases of several species (Farkas *et al.*, 1990) is absent in Fks1p and Fks2p, and none of the Fks proteins thus far implicated in glucan synthesis have been shown to bind this sugar nucleotide. Rho1p has been identified as the GTP-binding regulatory subunit of the β -1,3-glucan synthase (Drgonová *et al.*, 1996; Mazur and Baginsky, 1996; Qadota *et al.*, 1996). Membranes of *rho1_{ts}* mutants have thermolabile β -1,3-glucan synthase activity that can be restored by the addition of recombinant Rho1p in a GTP-dependent manner. Furthermore, Rho1p has been shown to co-purify, co-immunoprecipitate and co-localize with Fks1p. Recent work has demonstrated that Rho1p also mediates the de- and repolarization of Fks1p during cell wall stress (Delley and Hall, 1999). This process requires the actin cytoskeleton, the cell wall stress sensor, Wsc1p, and a number of intracellular signalling molecules. Gas1p is attached to the plasma membrane via a GPI-anchor and this 1,3- β -glucanoyltransferase has been implicated in cross-linking the linear β -1,3-glucan chains produced by the glucan synthase (Mouyna *et al.*, 2000). Mutants in *GAS1* have a more spherical morphology and release β -1,3-glucan and β -1,6-glucosylated wall proteins into the medium (Ram *et al.*, 1998). In the absence of Gas1p, compensatory effects such as increased chitin deposition (Popolo *et al.*, 1997) and alternative cross-linking of β -1,6-glucosylated wall proteins to chitin occur (Kapteyn *et al.*, 1997) to retain at least partial cell wall integrity.

The remaining 20% of the β -glucan is β -1,6-linked and highly branched with an average size of 350 glucose residues. This polymer has a glue-like function in the cell wall, interconnecting all major wall components (Kollár *et al.*, 1997). Many genes involved in β -1,6-glucan biosynthesis have been identified through mutations that confer

resistance to the K1 killer toxin, a pore-forming protein that binds to a β -1,6-glucan containing cell surface receptor (Bussey, 1991). These *KRE* (killer toxin resistant) gene products have been localized along the secretory pathway and at the cell surface, but their role in the synthesis of this polymer is unclear. *KRE5* encodes a putative ER protein with significant similarity to UDP-glucose:glycoprotein glucosyltransferase (UGGT) enzymes and loss of Kre5p results in no detectable β -1,6-glucan and extremely slow growth or non-viability (Meaden *et al.*, 1990; Parker *et al.*, 1995). *KRE6* and *SKN1* encode a pair of homologous Golgi proteins with significant similarities to family 16 glycoside hydrolases, and simultaneous loss of both Kre6p and Skn1p also results in a very severe β -1,6-glucan defect and extremely slow growth or lethality (Roemer *et al.*, 1994; Montijn *et al.*, 1999). Other Kre gene products, as well as models for both secretory pathway and cell surface synthesis of β -1,6-glucan, are reviewed in Shahinian and Bussey (2000).

Here, we report that *fks1 Δ* mutants and *fks1_{ts} fks2 Δ* mutants have β -1,6-glucan defects that show no simple correlation with β -1,3-glucan impairment. We also implicate *FKS2* in β -1,6-glucan biosynthesis and demonstrate that the β -1,6-glucan deficient mutants *kre5 Δ* and *kre6 Δ skn1 Δ* have reduced *in vitro* glucan synthase activity and aberrantly localize Fks1p and Fks2p.

Materials and methods

Strains, growth media and procedures

S. cerevisiae strains are listed in Table 1. Standard media and growth conditions were as described (Bussey *et al.*, 1982). Standard procedures were used for genetic crosses, sporulation of diploids and dissection of tetrads (Sherman *et al.*, 1986). Transformation of plasmids was carried out by the one-step transformation procedure for yeast in stationary phase (Chen *et al.*, 1992). The high efficiency method of Gietz *et al.* (1995) was used for transformation of PCR- and restriction digested DNA fragments. Deletion mutants were identified on synthetic minimal media with auxotrophic supplements, except for deletions based on integration of the *kanMX2* cassette, where YEPD containing 200 μ g/ml geneticin (antibiotic G418, GIBCO) was used. Counterselection of yeast strains containing plasmids with the *URA3* selectable

Table I. Yeast strains used in this study

Strain	Genotype	Source/reference
SEY6210 ¹	<i>MATα</i>	S. D. Emr
HAB251-15B ¹	<i>MATα/MATa</i>	4
HAB900 ¹	<i>MATα fks1Δ::GFP-HIS3</i>	5
HAB901 ¹	<i>MATa gas1Δ::kanMX2</i>	This study
HAB902 ¹	<i>MATα fks1Δ::GFP-HIS3 gas1Δ::kanMX2</i>	This study
HAB903 ¹	<i>MATa fks2Δ::kanMX2</i>	This study
HAB904 ¹	<i>MATα/MATa fks1Δ::GFP-HIS3/FKS1 fks2Δ::kanMX2/FKS2</i>	This study
HAB905 ¹	<i>MATα fks1Δ::GFP-HIS3 fks2Δ::kanMX2 + pRS315 FKS1</i>	This study
HAB906 ¹	<i>MATα fks1Δ::GFP-HIS3 fks2Δ::kanMX2 + pRS315 FKS1^{L855R}</i>	This study
HAB907 ¹	<i>MATα fks1Δ::GFP-HIS3 fks2Δ::kanMX2 + pRS315 FKS1^{E1111G}</i>	This study
TA405 ²	<i>MATα/MATa</i>	6
HAB908 ²	<i>MATα</i>	This study
HAB909 ²	<i>MATα fks1Δ::HIS3</i>	This study
HAB910 ²	<i>MATα fks2Δ::kanMX2</i>	This study
HAB911 ²	<i>MATα fks3Δ::LEU2</i>	This study
HAB912 ²	<i>MATα fks1Δ::HIS3 fks3Δ::LEU2</i>	This study
HAB913 ²	<i>MATα fks2Δ::kanMX2 fks3Δ::LEU2</i>	This study
HAB914 ²	<i>MATα ura3Δ::kanMX2</i>	S. Shahinian
HAB915 ²	<i>MATα/MATa fks1Δ::HIS3/FKS1 fks2Δ::kanMX2/FKS2 ura3Δ::kanMX2/ura3Δ::kanMX2</i>	This study
HAB916 ²	<i>MATα fks1Δ::HIS3 fks2Δ::kanMX2 ura3Δ::kanMX2 + pRS316 FKS1</i>	This study
HAB917 ²	<i>MATα fks1Δ::HIS3 fks2Δ::kanMX2 ura3Δ::kanMX2 + pRS315 FKS1</i>	This study
HAB918 ²	<i>MATα fks1Δ::HIS3 fks2Δ::kanMX2 ura3Δ::kanMX2 + pRS315 FKS1-HA</i>	This study
HAB919 ²	<i>MATα fks1Δ::HIS3 fks2Δ::kanMX2 ura3Δ::kanMX2 + pRS315 GFP-FKS1</i>	This study
HAB920 ²	<i>MATα fks1Δ::HIS3 fks2Δ::kanMX2 ura3Δ::kanMX2 + pRS315 FKS2</i>	This study
HAB921 ²	<i>MATα fks1Δ::HIS3 fks2Δ::kanMX2 ura3Δ::kanMX2 + pRS315 FKS2-HA</i>	This study
HAB922 ²	<i>MATα fks1Δ::HIS3 fks2Δ::kanMX2 ura3Δ::kanMX2 + pRS315 GFP-FKS2</i>	This study
YDK5-3B ²	<i>MATa kre5Δ::HIS3</i>	7
HAB923 ²	<i>MATα skn1Δ::kanMX2</i>	H. Li
TR510 ²	<i>MATα kre6Δ::HIS3</i>	6
HAB924 ²	<i>MATα kre6Δ::HIS3 skn1Δ::kanMX2</i>	H. Li
YOC1002 ³	<i>MATα ade3Δ::FKS1;TRP1</i>	Y. Ohya
YOC1072 ³	<i>MATα ade3Δ::FKS1^{T828A};TRP1</i>	Y. Ohya
YOC1074 ³	<i>MATα ade3Δ::FKS1^{V302N};TRP1</i>	Y. Ohya
YOC1076 ³	<i>MATα ade3Δ::FKS1^{T605I M761T};TRP1</i>	Y. Ohya
YOC1078 ³	<i>MATα ade3Δ::FKS1^{A823V D920E};TRP1</i>	Y. Ohya
YOC1080 ³	<i>MATα ade3Δ::FKS1^{I853T A932G E934D F1020Y I1047N};TRP1</i>	Y. Ohya
YOC1082 ³	<i>MATα ade3Δ::FKS1^{F1258Y N1520D};TRP1</i>	Y. Ohya
YOC1084 ³	<i>MATα ade3Δ::FKS1^{E146V Y329N Y335N};TRP1</i>	Y. Ohya
YOC1086 ³	<i>MATα ade3Δ::FKS1^{L872F E907K N982S};TRP1</i>	Y. Ohya
YOC1088 ³	<i>MATα ade3Δ::FKS1^{K877N N899S Q977P};TRP1</i>	Y. Ohya
YOC1090 ³	<i>MATα ade3Δ::FKS1^{I713L I722V};TRP1</i>	Y. Ohya

Table 1. Continued

Strain	Genotype	Source/reference
MC75	<i>MATα thr5 met</i>	M. W. Clark
MC76	<i>MATα lys1 cry1</i>	M. W. Clark
T158C/S14a	<i>MATα/MATα his4C-864/HIS4 ADE2/ade2-5</i>	8

¹These strains are all derived from the SEY6210 derived diploid HAB251-15B and carry the following additional mutations, which are homozygous in the diploid strains: *leu2-3 112 ura3-52 his3- Δ 200 lys2-801 trp1- Δ 901 suc2- Δ 9*.

²These strains are all derived from the TA405 diploid, and carry the following additional mutations, which are homozygous in the diploid strains: *his3 leu2 can1*.

³These strains are all generated in the same haploid and carry the following additional mutations: *ade2 lys2 his3 trp1 leu2 ura3 fks1 Δ ::HIS3 fks2 Δ ::LYS2*.

⁴Roemer and Bussey (1991).

⁵Ketela et al. (1999).

⁶Roemer et al. (1994).

⁷Meaden et al. (1990).

⁸Brown et al. (1993).

marker was achieved by the addition of 1 g/l 5-fluoroorotic acid (5-FOA, Diagnostic Chemical LTD) to synthetic minimal medium supplemented for auxotrophic requirements. Three *Escherichia coli* strains were used: (a) DH10B, for propagation of plasmids; (b) CJ236 (*dut, ung*), to prepare uracil-containing DNA for site-directed mutagenesis; and (c) BW58 (*dam*), to prepare DNA susceptible to cleavage by restriction endonucleases that are blocked by adenine methylation at GATC sequences. Bacterial strains were grown in 2 \times YT medium and transformed using standard methods (Sambrook et al., 1989).

DNA purification, sequencing and recombinant DNA techniques

Yeast DNA was isolated by the procedure of Hoffman and Winston (1987). Miniprep plasmids were purified from *E. coli* using the boiling method (Sambrook et al., 1989). DNA sequencing was carried out on a 373 DNA Sequencer (Applied Biosystems), using the ABI PRISM[™] dye terminator cycle sequencing ready reaction kit (Perkin Elmer). Restriction endonucleases, calf intestine phosphatase (CAIP), T4 DNA polymerase, T4 DNA ligase, T4 polynucleotide kinase, *TAQ* DNA polymerase, T7 sequenase, Gene 32 protein and Expand[™] high fidelity *TAQ* DNA polymerase were purchased from GIBCO BRL (Gaitersburg, MD), US Biochemicals (Cleveland, OH), Pharmacia LKB Biotechnology (Piscataway, NJ) or Boehringer Mannheim Biochemicals (Indianapolis, IN) and were used according to the manufacturers' instructions.

Oligonucleotide primers and plasmids

The oligonucleotide primers used in this study are listed in Table 2. The plasmids used in this study are listed in Table 3: junctions between plasmid and insert that were non-compatible were rendered blunt with T4 DNA polymerase prior to ligation and detailed procedures for plasmid construction are available upon request. The plasmids YEplac195 *FKS1*, pRS316 *FKS2* and YEplac195 *FKS3* were kindly provided by A. Ram (Fungal Cell Wall Group, University of Amsterdam, The Netherlands). The plasmid pFA6-kanMX2, containing the *kanMX2* cassette used for PCR-based gene disruptions (Wach et al., 1994), was a gift from P. Philippsen (Biozentrum, University of Basel, Switzerland). The plasmid p833 is a pBKS⁻-based vector (Stratagene) with a 717 bp *KpnI-NotI* fragment containing the *F64L S65T* enhanced version of the green fluorescent protein (GFP; Chalfie et al., 1994) and was kindly provided by U. Stochaj (McGill University, Montreal). The plasmids pRS315 and pRS316 are centromeric vectors with a *LEU2* and *URA3* selectable marker (Sikorski and Hieter, 1989), and plasmid pRS425 is a 2 μ -based vector with a *LEU2* selectable marker (Christianson et al., 1992). The *ADH1* promoter of pVT102U (Vernet et al., 1987) was used to create pRS315ADH1.

Gene disruption

Complete gene disruptions were performed in either the SEY6210 derived diploid HAB251-15B or a TA405 derived diploid, followed by sporulation,

Table 2. Oligonucleotide primers used in this study

Name and application	Sequence 5'–3'
Gene disruption ^a <i>gas1-kanMX2-5'</i>	TCA AAG TTA GCA ACC GCT GCT GCT TTT TTT GCT GGC GTC GCA GAT ATC AAG CTT GCC TCG
<i>gas1-kanMX2-3'</i>	GAC ACC AGC GGC AAT GGA TAA GGA AAT GAT GGA GGT AAA GAC GTC GAC ACT GGA TGG CGG
<i>gas1-5'-test</i> <i>fks2-kanMX2-5'</i>	CTA CTA CTG TTG ACA CGG GGG CGG TAT GAT GCA AAT GAG GTG TAC GTA TGT ATA TAT AGC GAT ATC AAG CTT GCC TCG
<i>fks2-kanMX2-3'</i>	CCA GAA AAG ATT AAG ATC AAA TTT GAC GGA GTT GCT CAG GTT GTC GAC ACT GGA TGG CGG
<i>fks2-5'-test</i> <i>kanMX2-int. rev.</i> <i>fks3-LEU2-5'</i>	GGA TTA ATC GAA GCG CTG CAA CAG GCC AGC CAT TAC GGA GTT GAA TAT GGA TTT TAT GAG TCC CAA GTT TTC GCT AAC TGA CCG CAG TTA ACT GTG
<i>fks3-LEU2-3'</i>	TCA AGA ATA TCG TCG TAA AGA CCT TGA AAA GAA TCA GAT GCA TAC CCT ATG AAC ATA TTC
<i>fks3-5'-test</i> <i>LEU2-int. rev.</i> <i>fks1-5'-test</i> <i>HIS3-int. rev.</i>	GTG GCA GTA ACA ACG TCA CGG ATG CAA AGT TAC ATG G CGA TGC GGA AGG ACA AAC GGA AGA TCG AGT GCT CTA
Cloning ^b <i>FKS1pr-NotI-5'</i> <i>FKS1pr-BamHI-3'</i> <i>GFP-BamHI-5'</i> <i>GFP-BamHI-3'</i>	TCC CGG <u>GCG GCC GCA</u> AAC ATC TAC ACA ATT AGC GCG <u>GGA TCC</u> GGT CTG ACC GTT GTA TGA TGA AAG CGC <u>GGA TCC</u> ATG AGT AAA GGA GAA GAA CTT GCG <u>GGA TCC</u> TGC AGC GGC AGC TTT GTA TAG TTC ATC CAT GCC
Site-directed mutagenesis ^b <i>FKS1-BamHI-Nterm</i> <i>FKS2-BamHI-Nterm</i> <i>FKS3-BamHI-Nterm</i> <i>FKS1-NotI-Cterm</i> <i>FKS2-NotI-Cterm</i>	ATA AGG TTG TTG ATC AGT GTT CAT <u>GGA TCC</u> GGT CTG ACC GTT GTA TGA AAG ACT CAA GTT TGG ATC GTT GTA GGA CAT <u>GGA TCC</u> AAC TAT GAC AGT TTA ATA ATT ATT AAA CTT GGG ACT CAT AAA ATC CAT <u>GGA TCC</u> ATT CAA CTC CTT AAC TTT CCT TCT TTC AAG CAA GTA TTG ATT GTA TTA <u>GCG GCC GCT</u> TTT TAT AGT TGA CCA GGT CTT ATA GAT TGT AAA CTA AAA AAA TCA <u>GCG GCC GCT</u> TTT GAT CAT AGA CCA GGT CTT
PCR-based mutagenesis <i>FKS1-GAPIII-5' A</i> <i>FKS1-GAPIII-3' A</i>	TCT CAA GTA TGG AAT GCC TTC TTT ACC AGC GGC CAC
Sequencing <i>FKS1-GAPIII-5' B</i> <i>FKS1-GAPIII-3' B</i> <i>FKS1-NotI-seq 5'</i> <i>FKS1-NotI-seq 3'</i> <i>FKS2-NotI-seq 5'</i> <i>FKS2-NotI-seq 3'</i>	TGG GAA TGT TTT GTT AAG TGG CTC TTC ATC CAA GTA CGT TTG CGT AAG CGT ATG GTC TAG AGG CCG ATA CTG GTG AAA CAA GCA CGC CTA CGT AAA CGT TAC GTT GAT GTT CCC GGA GAG

^aSequences internal to the *kanMX2*, *LEU2* and *HIS3* cassettes are in boldface.

^bSequences that correspond to introduced restriction sites are underlined.

tetrad dissection and mating type determination using tester strains MC75 and MC76 to obtain the disrupted haploid progeny of known mating type. The *fks1Δ::HIS3* disrupted strain HAB909 was prepared via transformation of a 3.1 kb *StuI-XhoI* fragment from plasmid pRS315 *fks1Δ::HIS3* containing the complete *HIS3* gene flanked by a 5' 1250 bp *StuI-MscI* fragment of the *FKS1* locus and

a 3' 625 bp *NsiI-XhoI* fragment of the *FKS1* locus. Strains HAB901 (*gas1Δ*), HAB903 and HAB910 (both *fks2Δ*), and HAB911 (*fks3Δ*) were prepared by a PCR-based disruption method, using the gene disruption primers described in Table 2 and either pFA6-kanMX2 (*gas1Δ* and *fks2Δ*) or pRS315 (*fks3Δ*) as a template. Correct insertion and deletion of the open reading frames was confirmed by

Table 3. Plasmids used in this study

Name	5'	Insert ^{a,b}	3'
pRS315		None	
pRS315 <i>FKS1</i>	Apal / <i>SphI</i>	7.5 kb <i>FKS1</i>	<i>AflIII</i> / SacI
pRS315 <i>FKS1</i> ^{L855R}	Apal / <i>SphI</i>	7.5 kb <i>FKS1</i> ^{L855R}	<i>AflIII</i> / SacI
pRS315 <i>FKS1</i> ^{E1111G}	Apal / <i>SphI</i>	7.5 kb <i>FKS1</i> ^{E1111G}	<i>AflIII</i> / SacI
pRS315 <i>FKS1</i> -HA	Apal / <i>SphI</i>	7.6 kb <i>FKS1</i> -HA	<i>AflIII</i> / SacI
pRS315 <i>GFP-FKS1</i>	NotI / <i>NotI</i>	7.9 kb <i>GFP-FKS1</i>	<i>PstI</i> / PstI
pRS315 <i>fksl</i> Δ::HIS3	Apal / <i>SphI</i>	3.5 kb <i>fksl</i> Δ::HIS3	<i>AflIII</i> / SacI
pRS315 <i>FKS2</i>	SmaI / <i>EcoRI</i>	7.8 kb <i>FKS2</i>	<i>SacI</i> / SacI
pRS315 <i>FKS2</i> -HA	SmaI / <i>EcoRI</i>	7.9 kb <i>FKS2</i> -HA	<i>SacI</i> / SacI
pRS315 <i>GFP-FKS2</i>	SmaI / <i>EcoRI</i>	8.5 kb <i>GFP-FKS2</i>	<i>SacI</i> / SacI
pRS315 <i>FKS3</i>	HindIII / <i>HindIII</i>	11.0 kb <i>FKS3</i>	<i>Sall</i> / SacI
pRS315 <i>FKS1</i> pr	NotI / <i>NotI</i>	1.2 kb <i>FKS1</i> pr	<i>BamHI</i> / BamHI
pRS315 <i>FKS1</i> pr <i>FKS2</i>	NotI / <i>NotI</i>	7.4 kb <i>FKS1</i> pr <i>FKS2</i>	<i>StuI</i> / SmaI
pRS315 <i>FKS1</i> pr <i>FKS3</i>	NotI / <i>NotI</i>	8.2 kb <i>FKS1</i> pr <i>FKS3</i>	<i>HindIII</i> / HindIII
pRS315 <i>ADH1</i> pr	PvuII / <i>SphI</i>	0.8 kb <i>ADH1</i> pr	<i>SphI</i> / PvuII
pRS315 <i>ADH1</i> pr <i>FKS1</i>	PvuII / <i>SphI</i>	6.8 kb <i>ADH1</i> pr <i>FKS1</i>	<i>SphI</i> / PvuII
pRS315 <i>ADH1</i> pr <i>FKS2</i>	PvuII / <i>SphI</i>	7.8 kb <i>ADH1</i> pr <i>FKS2</i>	<i>SphI</i> / PvuII
pRS315 <i>ADH1</i> pr <i>FKS3</i>	PvuII / <i>SphI</i>	7.8 kb <i>ADH1</i> pr <i>FKS3</i>	<i>SphI</i> / PvuII
pRS316		None	
pRS316 <i>FKS1</i>	KpnI / <i>SphI</i>	7.5 kb <i>FKS1</i>	<i>AflIII</i> / SacI
pRS316 <i>FKS2</i>	EcoRI / <i>EcoRI</i>	9.7 kb <i>FKS2</i>	<i>EcoRI</i> / EcoRI
pRS425		None	
pRS425 <i>FKS1</i>	PstI / <i>PstI</i>	9.7 kb <i>FKS1</i>	<i>PstI</i> / PstI
pRS425 <i>FKS2</i>	SmaI / <i>EcoRI</i>	7.8 kb <i>FKS2</i>	<i>SacI</i> / SacI
pRS425 <i>FKS3</i>	HindIII / <i>HindIII</i>	11.0 kb <i>FKS3</i>	<i>Sall</i> / XhoI
pRS425 <i>FKS1</i> pr	NotI / <i>NotI</i>	1.2 kb <i>FKS1</i> pr	<i>BamHI</i> / BamHI
pRS425 <i>FKS1</i> pr <i>FKS2</i>	NotI / <i>NotI</i>	7.4 kb <i>FKS1</i> pr <i>FKS2</i>	<i>StuI</i> / SmaI
pRS425 <i>FKS1</i> pr <i>FKS3</i>	NotI / <i>NotI</i>	8.2 kb <i>FKS1</i> pr <i>FKS3</i>	<i>HindIII</i> / HindIII
YEplac195 <i>FKS1</i>	PstI / <i>PstI</i>	9.7 kb <i>FKS1</i>	<i>PstI</i> / PstI
YEplac195 <i>FKS3</i>	HindIII / <i>HindIII</i>	11.0 kb <i>FKS3</i>	<i>Sall</i> / SacI
pFA6-kanMX2	EcoRV / <i>EcoRV</i>	1.4 kb <i>kanMX2</i>	<i>Sall</i> / SacI
pVT102U		None	
pSM491	NotI / <i>NotI</i>	0.1 kb 3 × HA	<i>NotI</i> / NotI
p833	KpnI / <i>KpnI</i>	0.7 kb <i>GFP</i> ^{F64L S65T}	<i>NotI</i> / NotI

^aRestriction sites within the plasmid backbone are indicated in bold. The complete insert size is indicated; junctions between insert and plasmid are represented by /.

^bAbbreviations used in the inserts: kb = kilo bases and pr = promoter.

PCR analysis, using 5'-test and 3'-internal reverse oligonucleotides primers (Table 2). Strains carrying multiple disruptions were generated from crosses of the appropriate haploid strains by standard genetic methods.

PCR-based mutagenesis and isolation of *fksl*_{ts} alleles

Random mutagenesis was performed by PCR as described (Jiang and Cyert, 1999), using the oligonucleotide primers *FKS1-GAPIII-5'A* and *FKS1-GAPIII-3'A* (Table 2) and pRS315 *FKS1* as a template. The mutagenic PCR product was

co-transformed with *SacI*-*NdeI* gapped pRS315 *FKS1* into strain HAB916, according to the gap-repair method of Muhlrad *et al.* (1992). Transformants were replica-plated onto 5-FOA plates at 23°C to counterselect for plasmid pRS316 *FKS1*, which originally kept HAB916 alive. Incubation of these transformants at 23°C and 37°C was made possible by a second round of replica plating. We screened for colonies that grew at 23°C but failed to grow at 37°C. Plasmids from these colonies were extracted, amplified in *E. coli* and re-transformed into HAB904. These transformants were sporulated and tetrads were dissected to confirm plasmid-dependent temperature-sensitive growth of *fksl*Δ

fks2 Δ double mutants. HAB906 and HAB907 displayed temperature-sensitive growth, and plasmids recovered from these strains were subjected to DNA sequence analysis using primers *FKSI-GAPIII-5'A* and *B* and *FKSI-GAPIII-3'A* and *B* (Table 2) to identify the responsible mutations.

β -1,6-glucanase purification

The β -1,6-glucanase overproducing *Pichia pastoris* strain GS115-pUR3421-27, kindly provided by Stanley Brul (Unilever Research Laboratories, The Netherlands), was grown as described (Bom *et al.*, 1998). The culture supernatant was collected by centrifugation ($4225 \times g$ for 15 min) and prepared for hydrophobic interaction chromatography (HIC) as described, except that solid ammonium sulphate was added to a final concentration of 0.8 M. HIC was carried out at 4°C on a column (2.5×45 cm) containing a 50 ml Phenyl Sepharose[®] FF (Pharmacia) bed equilibrated with 50 mM potassium acetate, pH 5.0, containing 0.8 M ammonium sulphate. This bed bound all β -1,6- and β -1,3-glucanase activity present in 71 HIC prepared culture supernatant. The column was washed with equilibration buffer and bound protein was eluted with 50 mM potassium acetate, pH 5.0, containing 50% ethylene glycol. Fractions containing β -1,6-glucanase activity were identified, pooled and prepared for anion exchange chromatography (AEC) by dialysis against 10 mM Tris-HCl, pH 8.5, containing 25 mM sodium chloride. AEC was also carried out at 4°C on the same column, containing a 25 ml Q Sepharose[®] FF (Pharmacia) bed equilibrated with dialysis buffer. The column was washed with dialysis buffer and β -1,6-glucanase was obtained by elution with 10 mM Tris-HCl, pH 8.5, containing 80 mM sodium chloride. The β -1,6-glucanase fractions were pooled, concentrated 18-fold (Centriprep YM-10, Amicon Bioseparations), dialysed against 50 mM potassium acetate, pH 5.5, and stored at -80°C. Determination of β -1,6-glucanase activity was performed as described by De la Cruz *et al.* (1995): a unit of activity was defined as the amount of enzyme that can liberate reducing groups from pustulan (β -1,6-glucan) equivalent to 1 μ mol glucose/min at pH 5.5 and 37°C.

Cell wall analyses

β -Glucan analysis were performed on 50 ml stationary-phase cultures grown in YEPD as

described (Dijkgraaf *et al.*, 1996), with some modifications outlined here. The three hot alkali extractions to remove mannoproteins and alkali-soluble glucan were collected and the amount of alkali-soluble hexose was determined by the method of Badin *et al.* (1953). After digestion of the alkali-extracted cell walls, the β -1,3-glucanase resistant material was removed by centrifugation ($15\ 000 \times g$ for 15 min) and washed once with 0.5 ml water and split in two. One half was washed with 0.5 ml 50 mM sodium phosphate buffer, pH 6.3, and resuspended in 125 μ l of the same buffer: a 50 μ l aliquot was used to determine the *N*-acetylglucosamine (GlcNac) content as described (Ketela *et al.*, 1999), and two 10 μ l aliquots were used to determine the total hexose content of the β -1,3-glucanase resistant material. The other half was washed with 0.5 ml 50 mM potassium acetate buffer, pH 5.5, resuspended in 300 μ l of the same buffer containing 20 units β -1,6-glucanase and digested at 37°C for 20 h. The glucanase-resistant material was removed by centrifugation ($15\ 000 \times g$ for 15 min), and the amount of β -1,6-glucanase solubilized hexose was determined. All values were initially calculated as μ g/mg dry weight cell wall, but are reported as μ g/mg total cellular protein, as the latter is less likely to vary between wall mutants than cell wall dry weight. To this extent, we multiplied each calculated value with the dry weight cell wall: total cellular protein ratio. This ratio was established by breaking cells of a 50 ml stationary-phase yeast culture with glass beads (greater than 99% lysis efficiency) in a fixed volume: total cellular protein content was measured via the dye-binding assay of Bradford (1976; Biorad, Mississauga, ON), using bovine serum albumin as a standard, and the cell walls were isolated by centrifugation ($3000 \times g$ for 5 min), washed once with water and aspirated onto a pre-weighed paper filter to determine their dry weight. These ratios are: WT, 1.15 ± 0.02 (SEY6210); *fks1* Δ , 1.40 ± 0.03 (HAB900); *gas1* Δ , 1.45 ± 0.05 (HAB901); *fks1* Δ *gas1* Δ , 1.53 ± 0.04 (HAB902) for Table 4 and Figure 1; and WT, 1.18 ± 0.01 (HAB905); A6, 1.05 ± 0.01 (HAB906); F4, 1.11 ± 0.03 (HAB907); WT, 1.02 ± 0.01 (YOC1002); 1014, 1.09 ± 0.02 (YOC1072); 1082, 1.02 ± 0.01 (YOC1074); 1093, 1.76 ± 0.02 (YOC1076); 1104, 1.01 ± 0.02 (YOC1078); 1114, 1.11 ± 0.02 (YOC1080); 1125, 0.97 ± 0.02 (YOC1082); 1132, 1.07 ± 0.03 (YOC1084); 1144, 1.06 ± 0.03 (YOC1086); 1154, 1.02 ± 0.01 (YOC1088); and 1163, 1.07 ± 0.01 (YOC1090) for Table 6 and Figure 4. Values are the mean of

Table 4. Analyses of alkali-extracted cell walls from wild-type, *fks1*Δ, *gas1*Δ and *fks1*Δ *gas1*Δ mutant cells

	β -1,3-Glucanase-releasable material		β -1,3-Glucanase-resistant pellet			Total alkali-insoluble β -1,6-glucan	Killer zone size (mm)
	β -1,6-Glucan	β -1,3-Glucan	Total hexose	β -1,6-Glucanase-sensitive hexose	GlcNac		
WT	96 ± 5	166 ± 5	6 ± 1	6 ± 1	12 ± 2	102 ± 5	15 ± 1
<i>fks1</i> Δ	71 ± 2	186 ± 4	24 ± 4	16 ± 1	83 ± 5	87 ± 2	9 ± 1
<i>gas1</i> Δ	63 ± 3	210 ± 3	55 ± 6	47 ± 2	66 ± 3	109 ± 4	15 ± 1
<i>fks1</i> Δ <i>gas1</i> Δ	42 ± 2	221 ± 5	69 ± 1	42 ± 1	162 ± 23	85 ± 2	8 ± 1

Values were determined as described in Materials and methods and are given in $\mu\text{g}/\text{mg}$ total cellular protein. All values are the mean of three independent determinations \pm 1 SD.

three independent determinations and the error represents one SD. Isolation and immunodetection of alkali-soluble glucan was performed as

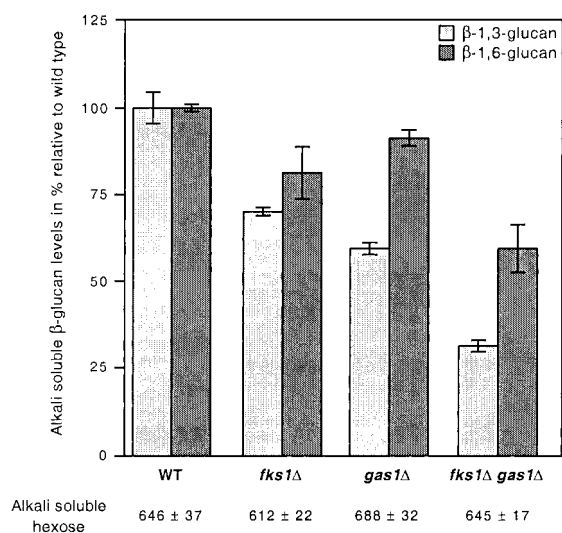


Figure 1. Amount and relative β -glucan content of alkali-soluble hexose fractions from wild-type, *fks1*Δ, *gas1*Δ and *fks1*Δ *gas1*Δ cells. The amount of alkali-soluble hexose was determined for each genotype, as described in Materials and methods. Values are given in $\mu\text{g}/\text{mg}$ of total cellular protein and are the mean of three independent determinations \pm 1 SD. To determine the relative β -glucan content, total cell lysates equivalent to 2 μg protein were extracted in a final volume of 100 μl 3% NaOH for 1 h at 75°C, after which the alkali-insoluble material was removed by centrifugation. One μl of these extracts and of four subsequent two-fold serial dilutions were spotted onto nitrocellulose membranes and subjected to Western analysis using β -1,3- and β -1,6-glucan-specific antibodies. The signals were quantitated by densitometry and are expressed in percentages relative to wild-type. Three independent preparations were analysed per genotype and error bars represent SDs

described (Lussier *et al.*, 1998): β -1,3-glucan was detected with an anti- β -1,3-glucan monoclonal antibody (Biosupplies Australia Pty Ltd, Australia) and β -1,6-glucan was detected with an affinity purified anti- β -1,6-glucan polyclonal antibody (Lussier *et al.*, 1998). Dot-blot were scanned using a UMAX Astra 1220s scanner and signals were quantitated with Adobe® Photoshop® software, using the histogram function. Values represent the mean of three independent preparations \pm 1 SD and are expressed in percentages relative to wild-type. Sensitivity of yeast strains to the K1 killer toxin was determined as described (Brown *et al.*, 1993). Spotting to test Calcofluor white (CFW) sensitivity of yeast strains was performed as described (Ketela *et al.*, 1999).

Glucan synthase assay

Glucan synthase activity was determined as described (Douglas *et al.*, 1994), with some modifications outlined here. Crude cell lysates were subjected to centrifugation at 4°C (1400 \times g for 10 min) and the supernatant was diluted 1:1 with 50 mM sodium phosphate, pH 7.5, containing 66% (vol/vol) glycerol and stored at -80°C . The protein concentration of these samples varied (3.5–5.5 mg/ml) and the specific activity of the 5 mM UDP-D-[6- ^3H]glucose (Amersham) was 5574 dpm/nmol. The assay was initiated by the addition of 10 μl -80°C cell extract, and after a 1 h incubation at 37°C stopped by the addition of 1 ml ice-cold 90% (vol/vol) methanol. Methanol-insoluble material was aspirated onto 25 mm glass fibre filters (Gelman Sciences, MI), washed with 5 ml 90% (vol/vol) methanol and 5 ml 90% (vol/vol) ethanol and quantitated by liquid scintillation counting.

Epitope tagging of Fks1p and Fks2p

Single-stranded, uracil containing DNA of pRS315 *FKS1* and pRS315 *FKS2* was prepared using the M13 helper phage R408 (Stratagene) and *E. coli* strain CJ236 transformed with these plasmids. A *NotI* site was introduced immediately 5' to the stop codons of the *FKS1* and *FKS2* ORFs by the method of Kunkel *et al.* (1987), using the site-directed mutagenesis primers *FKS1-NotI-Cterm* and *FKS2-NotI-Cterm* (Table 2). A 117 bp *NotI-NotI* fragment of pSM491, containing three copies of the HA epitope, was inserted to create pRS315 *FKS1-HA* and pRS315 *FKS2-HA*. These plasmids were analysed by DNA sequencing using the sequencing primers *FKS1-NotI-seq 5'* and *3'* and *FKS2-NotI-seq 5'* and *3'* (Table 2), to confirm correct orientation and in-frame fusion of the triple HA epitope. Functionality of Fks1p-HA and Fks2p-HA was assessed by the ability to rescue the inviability of an *fks1* Δ *fks2* Δ double mutant (yeast strains HAB918 and HAB921) and by glucan synthase activity: Fks1p-HA displayed 94% of the specific activity of Fks1p and Fks2p-HA displayed 85% of the specific activity of Fks2p.

Western immunoblotting

Early log-phase yeast cells grown in synthetic selective media, were broken by vortexing in the presence of glass beads and protease inhibitors (CØmplete[™], Boehringer Mannheim, Germany). Crude cell lysates were centrifuged at 4°C (1400 \times *g* for 10 min) to remove cell walls and unbroken cells. The protein content of the supernatant was determined and the cell lysates were resuspended in sample buffer. Protein samples were separated on 8% SDS-polyacrylamide gels (Laemmli, 1970) and transferred to Hybond-C nitrocellulose membranes (Amersham, Oakville, ON). Membranes were incubated with 1:2500 diluted anti-HA monoclonal primary antibody (HA 11, Babco, Richmond, CA), followed by incubation with 1:2500 diluted horseradish peroxidase-coupled goat anti-mouse secondary antibody (Amersham). Western blots were developed using the enhanced chemiluminescence technique (Amersham).

Localization of Fks1p and Fks2p

A 0.7 kb *GFP*^{F64L S65T} fragment flanked by *Bam*HI sites was obtained by PCR amplification using the

cloning primers *GFP-Bam*HI-5' and 3' (Table 2), the high fidelity *TAQ* DNA polymerase Expand[™] and p833 as a template. A *Bam*HI site was introduced immediately 5' to the *FKS1* and *FKS2* ORFs, using the site-directed mutagenesis primers *FKS1-Bam*HI-*Nterm* and *FKS2-Bam*HI-*Nterm* (Table 2). An in-frame fusion of *FKS2* to *GFP* was obtained by inserting the *Bam*HI-digested PCR fragment into the *Bam*HI site of pRS315 *Bam*HI-*FKS2*, to generate pRS315 *GFP-FKS2*. A 6.1 kb *Bam*HI-*Pst*I fragment of pRS315ADHpr *FKS1* was inserted into the *Bam*HI and *Pst*I sites of pRS315Fks1pr. An in-frame fusion of *FKS1* to *GFP* was obtained by inserting the *Bam*HI-digested PCR fragment into the *Bam*HI site of the latter plasmid, generating pRS315 *GFP-FKS1*. Functionality of the GFP-Fks1p and GFP-Fks2p fusions was assessed by the ability to rescue the inviability of a *fks1* Δ *fks2* Δ double mutant (yeast strains HAB919 and HAB922) and by glucan synthase activity: GFP-Fks1p displayed 92% of the specific activity of Fks1p and GFP-Fks2p displayed 87% of the specific activity of Fks2p. Localization of GFP-Fks1p and GFP-Fks2p was determined by examination of live, early-log-phase yeast cells carrying pRS315 *GFP-FKS1* or pRS315 *GFP-FKS2*. Images were obtained using a Zeiss Axioplan (Germany) fluorescence microscope, equipped with a SPOT digital camera (Diagnostic Instruments Inc., Sterling Heights, MI) using SPOT Advanced software.

Results

The *fks1* Δ mutant has reduced cell wall β -1,3- and β -1,6-glucan levels

To identify additional genes involved in β -1,6-glucan biosynthesis, we screened transposon mutagenized yeast for altered K1 killer toxin sensitivity and identified several *fks1* mutants that were partially killer resistant (data not shown). We verified this phenotype with an *fks1* Δ mutant (Table 4) and asked if the *fks1* Δ mutant had a reduction in cell wall β -1,6-glucan. Previous studies on *fks1* Δ cell walls reported a 40% increase in mannan, a 10-fold increase in chitin and an approximate 50% reduction in β -glucans, but did not distinguish between β -1,3- and β -1,6-glucan (Ram *et al.*, 1995; Dallies *et al.*, 1998). The reduction in β -glucans was assumed to be in β -1,3-glucan, as *fks1* Δ mutants display a strong reduction in *in vitro* β -1,3-glucan

synthase activity (Douglas *et al.*, 1994). We first measured the β -1,3- and β -1,6-glucan content of the alkali-insoluble hexose fraction (Table 4). This fraction represents almost one-third of the total cell wall hexose and contains $\pm 85\%$ of the β -1,6-glucan and $\pm 30\%$ of the β -1,3-glucan. Thus, the majority of β -1,6-glucan is alkali-insoluble, while the majority of β -1,3-glucan is alkali-soluble. The amount of alkali-insoluble, β -1,3-glucanase releasable material in an *fks1* Δ mutant was similar to wild-type, but its composition changed slightly with a 26% reduction in β -1,6-glucan and a small increase in β -1,3-glucan. This reduction was partially due to increased cross-linking of β -1,6-glucan to chitin, as the alkali-insoluble, β -1,3-glucanase resistant pellet of the *fks1* Δ mutant contained 2.5 times as much β -1,6-glucan as the wild-type. Overall, the *fks1* Δ mutant displayed a 15% reduction in alkali-insoluble β -1,6-glucan compared to wild-type. We next analysed the alkali-soluble hexose fraction, to assess whether this modest reduction was due to alternative cross-linking (Figure 1). The alkali-soluble hexose fraction represents 70% of the total cell wall hexose and is composed of both mannans and β -glucans. The amount of alkali-soluble hexose in *fks1* Δ cells did not significantly differ from wild-type (Figure 1). The relative β -1,3- and β -1,6-glucan content of this fraction was determined by immunodetection: the *fks1* Δ mutant displayed a 30% reduction in β -1,3-glucan, which was accompanied by a modest reduction in β -1,6-glucan (Figure 1). Collectively, these analyses show that *fks1* Δ mutants have reduced cell wall β -1,3- and β -1,6-glucan levels. We standardize our hexose measurements against total cellular protein (see Materials and methods) and our data may therefore differ from previous reports on the *fks1* Δ cell wall composition, which were based on relative amounts of monosaccharides liberated from isolated cell walls and did not take into account that the mutant wall might differ significantly in weight from the wild-type wall (Ram *et al.*, 1995; Dallies *et al.*, 1998).

Fks1p is strongly implicated in β -1,3-glucan synthesis and the modest reduction in cell wall β -1,6-glucan of *fks1* Δ mutants may be an indirect consequence of the reduction in alkali-soluble β -1,3-glucan. Mutants in *GAS1* lacking a 1,3- β -glucanosyltransferase required for β -1,3-glucan assembly (Mouyna *et al.*, 2000) have a 50% reduction in alkali-insoluble β -1,6-glucan (Popolo *et al.*, 1997) and secrete β -1,6-glucosylated proteins into the medium (Ram *et al.*, 1998), suggesting that

β -1,6-glucan reduction may be a common phenotype among β -1,3-glucan biosynthetic mutants. To confirm this possibility, we re-examined the β -glucan levels of the *gas1* Δ mutant on a 'per cell' basis: previous studies on the sugar composition of *gas1* Δ cell walls reported a 40% increase in mannan, a five-fold increase in chitin and a 30% reduction in β -glucans (Popolo *et al.*, 1997; Ram *et al.*, 1998). The amount of alkali-insoluble, β -1,3-glucanase releasable material in a *gas1* Δ mutant was comparable to wild-type and had a composition similar to that of the *fks1* Δ mutant, with a 34% reduction in β -1,6-glucan and a 27% increase in β -1,3-glucan (Table 4). In contrast to *fks1* Δ mutants, *gas1* Δ cells are as sensitive to the K1 killer toxin as wild-type cells, suggesting that they have no overall reduction in β -1,6-glucan, but rather cross-link this polymer in a different way. The alkali-insoluble, β -1,3-glucanase resistant pellet of the *gas1* Δ mutant contained nine-fold more hexose than the wild-type pellet and the majority of this hexose could be released by β -1,6-glucanase digestion, indicating that increased cross-linking of β -1,6-glucan to chitin occurs in the absence of Gas1p (Table 4). The *gas1* Δ mutant has no overall reduction in alkali-insoluble β -1,6-glucan, when these two β -1,6-glucan fractions are combined. The amount of alkali-soluble hexose and the relative β -1,6-glucan content of this fraction in *gas1* Δ cells was comparable to wild-type, despite a 40% reduction in the relative β -1,3-glucan content (Figure 1). We conclude from these analyses that *gas1* Δ mutants have no reduction in β -1,6-glucan, but cross-link this polymer in an alternative manner.

To further investigate the effect of impaired β -1,3-glucan assembly on β -1,6-glucan levels, we analysed the *fks1* Δ *gas1* Δ mutant. This double mutant was no more killer toxin resistant than the *fks1* Δ mutant, even though the alkali-insoluble, β -1,3-glucanase releasable material contained 56% less β -1,6-glucan and 33% more β -1,3-glucan than wild-type (Table 4). The β -1,3-glucanase-resistant pellet had a 12-fold increase in hexose over the wild-type, but the amount of hexose that could be released by β -1,6-glucanase digestion was comparable to the amount in *gas1* Δ mutants (Table 4). Thus, *gas1* Δ *fks1* Δ and *fks1* Δ mutants appear to have a similar reduction in alkali-insoluble β -1,6-glucan. The massive increase in chitin did not reduce the amount of alkali-soluble hexose, although the 70% reduction in β -1,3-glucan and the 40% reduction in β -1,6-glucan suggest that this wall fraction has a much higher mannan content

than the wild-type fraction (Figure 1). Our findings with *gas1* Δ mutants indicate that β -1,3-glucan assembly defects need not lead to reduced β -1,6-glucan levels. Therefore, the modest β -1,6-glucan defect of *fks1* Δ mutants could be Fks1p-specific, rather than an indirect consequence of impaired β -1,3-glucan assembly.

Overexpression of Fks2p can partially restore β -1,6-glucan levels in *fks1* Δ mutants

Two proteins encoded in the yeast genome are related to Fks1p: Fks2p with 88% identity and Fks3p with 55% identity. Cells lacking both *FKS1* and *FKS2* are synthetically lethal indicating that Fks1p and Fks2p have overlapping functions in β -glucan synthesis (Mazur *et al.*, 1995), while *FKS3* has not been implicated in glucan synthesis. The partial β -1,6-glucan defect of *fks1* Δ mutants suggested the possible involvement of Fks2p and Fks3p in this process. We sought genetic interactions between *FKS1*, *FKS2* and *FKS3* by testing for Calcofluor white (CFW) phenotypes (Figure 2). CFW binds to nascent chains of chitin and prevents their assembly into microfibrils. Mutants with weakened cell walls, including *fks1* mutants, are hypersensitive to this drug (Ram *et al.*, 1995). The *fks1* Δ mutant was CFW hypersensitive as expected, while the *fks2* Δ , *fks3* Δ and *fks2* Δ *fks3* Δ mutants were as sensitive as the wild-type and the *fks1* Δ *fks3* Δ mutant was no more sensitive to CFW than the *fks1* Δ mutant. In killer toxin sensitivity tests, only the *fks1* Δ mutant had a phenotype (Table 4), which was not enhanced by the absence of Fks3p (data not shown). To test for possible carbon source-dependent transcriptional regulation of *FKS3* expression as observed for *FKS2* (Mazur *et al.*,

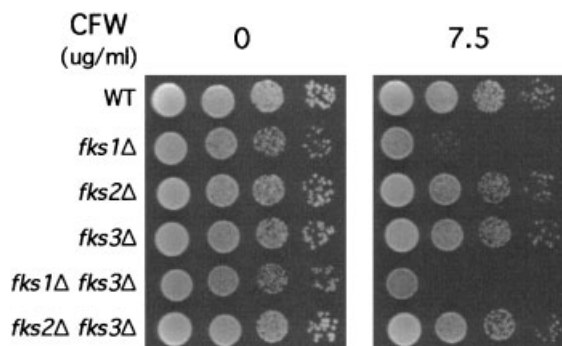


Figure 2. Sensitivity of *fks1*, *fks2* and *fks3* mutants to Calcofluor white. Mid-log phase cells were diluted to OD₆₀₀=0.5 and 5 μ l of this suspension and three subsequent 10-fold serial dilutions were spotted onto YEPD agar \pm 7.5 μ g/ml CFW. Growth was scored after 2 days at 30°C

1995), these analyses were repeated on media containing either galactose or glycerol as a carbon source, with similar results.

We asked if overproduction of Fks2p or Fks3p could rescue the killer toxin phenotype of *fks1* Δ cells. *FKS1* transcripts are cell cycle-regulated during normal vegetative growth, while *FKS2* transcripts are upregulated under stressful conditions (Ram *et al.*, 1995; Mazur *et al.*, 1995; Zhao *et al.*, 1998). We therefore introduced *FKS1*, *FKS2* and *FKS3* in both low- and high-copy-number plasmids behind one of a range of different promoters to reduce effects due to transcriptional regulation. These plasmids were introduced in wild-type and *fks1* Δ cells, and the transformants were tested for killer toxin sensitivity (Table 5). Low-copy-number plasmids containing *FKS2* rescued the killer toxin phenotype weakly (cf. *fks1* Δ +*FKS2* to *fks1* Δ +vector only), with stronger effects seen using

Table 5. KI killer toxin sensitivity of wild-type and *fks1* Δ mutant cells expressing *FKS1*, *FKS2* and *FKS3* behind endogenous (END) and heterologous (*FKS1* or *ADH1*) promoters from both low- and high-copy-number vectors

		cen-END pr	cen-FKS1 pr	cen-ADH1 pr	2 μ -END pr	2 μ -FKS1 pr
WT	+vector only	18 \pm 0.0	19 \pm 0.5	18 \pm 0.3	19 \pm 0.5	17 \pm 0.8
WT	+ <i>FKS1</i>	19 \pm 0.8	19 \pm 0.3	19 \pm 0.5	19 \pm 0.6	18 \pm 0.0
<i>fks1</i> Δ	+vector only	11 \pm 0.3	12 \pm 0.3	11 \pm 0.3	10 \pm 0.3	11 \pm 0.3
<i>fks1</i> Δ	+ <i>FKS1</i>	16 \pm 0.3	18 \pm 0.3	16 \pm 0.3	17 \pm 0.3	16 \pm 0.0
<i>fks1</i> Δ	+ <i>FKS2</i>	13 \pm 0.3	14 \pm 0.3	13 \pm 0.3	16 \pm 0.6	15 \pm 0.8
<i>fks1</i> Δ	+ <i>FKS3</i>	10 \pm 0.3	12 \pm 0.6	10 \pm 0.3	11 \pm 0.0	10 \pm 0.6

Seeded plate assays for KI killer toxin sensitivity were performed as described by Brown *et al.* (1993) and are expressed in mm. Values are the mean of three independent determinations \pm 1 SD.

high-copy-number *FKS2* plasmids. The *FKS3* plasmids did not restore the killer toxin or CFW phenotypes of *fks1Δ* cells, irrespective of plasmid copy-number or promoter type, and they also failed to restore the inviability of *fks1Δ fks2Δ* double mutants (data not shown). The ability of *FKS2* to suppress the partial killer resistant phenotype of *fks1Δ* cells in a dose-dependent manner suggests that Fks2p can affect β -1,6-glucan levels in a manner similar to Fks1p, and that Fks2p may be required for the residual β -1,6-glucan in *fks1Δ* mutants.

The effect of *fks1_{ts}* alleles on β -1,3- and β -1,6-glucan levels

Fks1p has been implicated in β -1,3-glucan synthesis as a putative catalytic sub-unit of the β -1,3-glucan synthase (Douglas *et al.*, 1994; Inoue *et al.*, 1995). If Fks1p is directly involved in β -1,3-glucan synthesis and the β -1,6-glucan phenotype of *fks1Δ* mutants is indirect, we reasoned that reduced β -1,6-glucan levels should correlate with β -1,3-glucan impairment. To test this idea, we examined the β -1,3- and

β -1,6-glucan levels of yeast with temperature-sensitive *fks1* alleles. These conditional alleles were introduced into an *fks1Δ fks2Δ* double mutant background, so that β -glucan synthesis was dependent on them. Ten *fks1_{ts}* alleles were isolated and characterized in work to be published elsewhere (Abe *et al.*, 2002), we identified two additional alleles, and all 12 alleles are shown in Table 6. Biochemical and phenotypic analyses of these mutant alleles allowed the identification of four domains within Fks1p, which are depicted in Figure 3 (Abe *et al.*, 2002). To establish wall-related phenotypes for these conditional *fks1* mutants at a permissive temperature, we analysed their CFW and killer toxin sensitivity. All strains were CFW-hypersensitive to various extents, indicating that the alleles are only partially functional at the permissive temperature (data not shown). Killer toxin analysis indicated that *fks1-1125* was more resistant and *fks1-1014* was more sensitive than wild-type, while the other alleles showed only minor alterations in killer toxin sensitivity (Table 6).

We next measured the amount of alkali-soluble

Table 6. The effect of point mutations in Fks1p on alkali-insoluble β -glucan levels

Group ^a	<i>fks1_{ts}</i> Allele	Point mutation(s)	Alkali-insoluble glucan (%) ^b		Killer zone size (mm) ^c
			β -1,6-Glucan	β -1,3-Glucan	
	WT	–	100 ± 2	100 ± 5	17 ± 0.3/18 ± 0.3*
A	1125	F1258Y N1520D	45 ± 2	75 ± 4	10 ± 0.8
	1114	I853T A932G E934D F1020Y I1047N	65 ± 2	99 ± 4	15 ± 0.3
	F4	E1111G	53 ± 1	64 ± 5	15 ± 0.8*
	A6	L855R	48 ± 3	72 ± 3	17 ± 0.3*
	1154	K877N A899S Q977P	67 ± 1	71 ± 2	16 ± 0.6
	1144	L872F E907K N982S	68 ± 3	80 ± 1	16 ± 0.3
	1104	A823V D920E	57 ± 7	85 ± 3	15 ± 0.5
	B	1163	I713L I722V	95 ± 2	82 ± 1
1082		V302N	88 ± 3	93 ± 5	15 ± 0.3
1014		T828A	114 ± 2	80 ± 3	19 ± 0.3
1132		E146V Y329N Y335N	85 ± 2	91 ± 4	17 ± 0.0
C	1093	T605I M761T	126 ± 3	134 ± 7	18 ± 0.5

^aThe conditional *fks1* mutants were grouped according to their cell wall β -glucan content: Group A mutants have a lower β -glucan content, Group B mutants have no gross alteration in their β -glucan content and the Group C mutant has a higher β -glucan content.

^bThe amount of alkali-insoluble, β -1,3-glucanase releasable glucan was determined as described in Materials and methods and expressed as percentages relative to wild-type. HAB905 is the isogenic wild-type for F4 and A6 and contained 132 ± 4 μ g β -1,6-glucan and 252 ± 4 μ g β -1,3-glucan/mg total cellular protein. YOC1002 is the isogenic wild-type for all other *fks1* alleles and contained 118 ± 2 μ g β -1,6-glucan and 248 ± 13 μ g β -1,3-glucan/mg total cellular protein.

^cKiller zone sizes of *fks1_{ts}* alleles F4, A6 and their isogenic wild-type are marked with an asterisk to indicate a difference in strain background. All values are the mean of three independent determinations ± 1 SD.

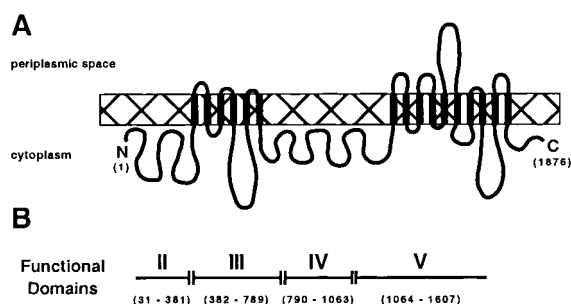


Figure 3. Functional domains of Fks1p. (A) Schematic representation of Fks1p. The solid black line represents the polypeptide chain. Transmembrane domains are shown as solid black bars and the plasma membrane as a long rectangular filled with a cross pattern (adapted from Douglas *et al.* (1994)). (B) Functional domains within Fks1p. The numbers of amino acid residues flanking each domain are indicated in brackets. Domain II has been implicated in the *in vivo* activation of the glucan synthase complex. Domain III has been implicated in localizing glucan synthesis at the site of polarized growth. Domain IV contains the putative catalytic site. Domain V is important for localizing the glucan synthase at the cell surface. Amino acid regions 1–30 and 1608–1876 have not been mutagenized; see Abe *et al.* (2002) for more details

hexose and examined the relative β -1,3- and β -1,6-glucan content of this wall fraction for each mutant strain grown at 25°C (Figure 4). The *fks1-1163*, *fks1-1082*, *fks1-1014* and *fks1-1132* strains had amounts of alkali-soluble hexose that were comparable to wild-type and they did not display strong alterations in their β -1,3- or β -1,6-glucan content. The *fks1-1093* strain had a 70% increase in the amount of alkali-soluble hexose, that was accompanied by a 50% increase in both β -glucan polymers. Strains *fks1-1104*, *fks1-1114*, *fks1-1144* and *fks1-1154* had no significant decrease in alkali-soluble hexose, despite severe reductions in both β -1,3- and β -1,6-glucan. Strains *fks1-F4*, *fks1-A6* and *fks1-1125* had an approximate 15% reduction in alkali-soluble hexose and also showed a diminished β -glucan content. The partial killer resistant *fks1-1125* strain was most severely affected with 75% less β -1,3- and 87% less β -1,6-glucan. Thus, the majority of conditional *fks1* mutants had a reduced alkali-soluble β -1,3- and β -1,6-glucan content at the permissive temperature.

The alkali-insoluble β -glucan levels of these strains grown at 25°C were consistent with our analyses of the alkali-soluble wall fraction, and

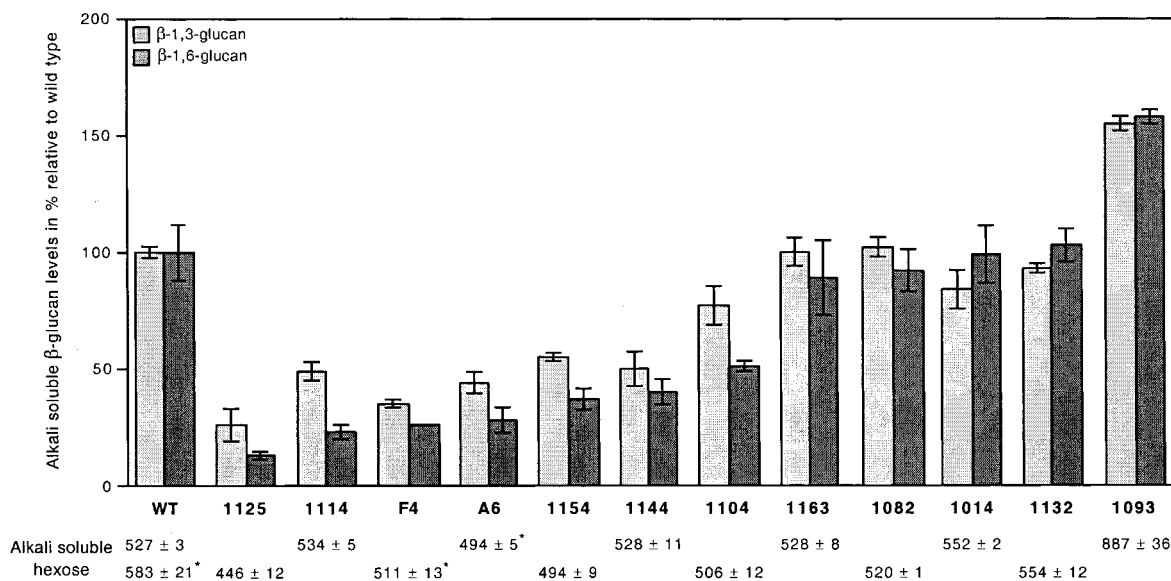


Figure 4. Amount and relative β -glucan content of alkali-soluble hexose fractions from *fks1* Δ *fks2* Δ cells with a wild-type or temperature-sensitive *FKS1* allele. The amount of alkali-soluble hexose was determined for each strain, as described in Materials and methods. Values are given in $\mu\text{g}/\text{mg}$ total cellular protein and are the mean of three independent determinations \pm 1 SD. The values of *fks1*_{ts} alleles F4, A6 and their isogenic wild-type (HAB905) are marked with an asterisk to indicate a difference in strain background. The relative β -glucan content was determined as outlined in the legend of Figure 1 and error bars represent SDs

allowed us to place the *fks1_{ts}* mutants into three groups based on their cell wall β -glucan content (Table 6). Group B consists of mutants without gross alterations in their cell wall β -glucan content. These *fks1* alleles carry mutations within domains II, III and IV of Fks1p, and one is of particular interest. The *fks1-1014* mutant has a killer-hypersensitive phenotype and a slight increase in alkali-insoluble β -1,6-glucan relative to the wild-type control, suggesting that this mutant may be hyperactive for β -1,6-glucan synthesis despite reduced β -1,3-glucan synthesis. The Group C *fks1-1093* mutant appeared hyperactive for the synthesis of both polymers, as this mutant has more alkali-insoluble β -1,3- and β -1,6-glucan. However, the 73% increase in cell wall dry weight of this strain cannot be fully attributed to these increases in β -glucan, suggesting that other structural wall changes occur as well in this mutant. Group A consists of mutants with a reduced β -glucan content and all carry mutations within the central cytoplasmic loop of Fks1p. These analyses demonstrate that mutations within *FKS1* have a range of effects on β -1,3- and β -1,6-glucan levels.

The β -1,6-glucan deficient mutants *kre5 Δ* and *kre6 Δ skn1 Δ* have reduced *in vitro* glucan synthase activity

Our data are consistent with a requirement for Fks1p and Fks2p for β -1,6-glucan biosynthesis. To assess whether the integrity of these proteins was impaired in β -1,6-glucan deficient mutants, we analysed some of their properties in *kre* mutants. An *in vitro* glucan synthase assay was developed and optimized to test for Fks1p activity (Shematek et al., 1980; Douglas et al., 1994). Analysis of the reaction product of this assay indicated that it consisted predominantly of β -1,3-glucan. In light of our results, we tested whether small amounts of β -1,6-glucan were made in this assay. We found that the *in vitro* reaction product was not susceptible to β -1,6-glucanase digestion and did not display immunoreactivity towards our β -1,6-glucan specific antiserum, indicating that *in vitro*-synthesized glucan contains no detectable β -1,6-glucan (data not shown). The *kre6 Δ* mutant is known to have reduced *in vitro* glucan synthase activity (Roemer and Bussey, 1991); we tested if other severe β -1,6-glucan deficient mutants were also affected and included the *fks1 Δ* mutant as a control (Figure 5). The 80% reduction in *in vitro* glucan synthase

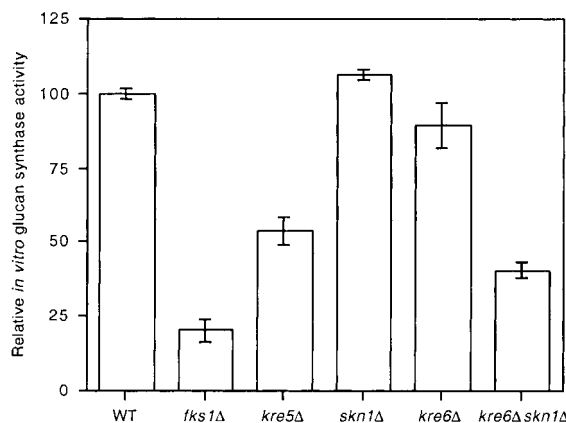


Figure 5. Relative *in vitro* glucan synthase activity of β -1,6-glucan-deficient mutants. Wild-type and mutant cell extracts were assayed for *in vitro* glucan synthase activity, as described in Materials and methods. The glucan synthase activity of wild-type extracts was set at 100% and was equivalent to 11.40 ± 0.27 nmol incorporated glucose/min/mg of protein. The average glucan synthase activity of three different cell extract preparations is shown. Error bars indicate a SD

activity of the *fks1 Δ* mutant is consistent with previous observations (Douglas et al., 1994), but the *kre6 Δ* mutant displayed only a marginal reduction in our hands and loss of the *KRE6* functional homologue Skn1p had no effect. However, the *kre6 Δ skn1 Δ* double mutant showed a 59% reduction in *in vitro* glucan synthase activity and the *kre5 Δ* mutant was also impaired with a 46% reduction. These findings indicate that loss of secretory pathway *KRE* gene products not only affect β -1,6-glucan biosynthesis *in vivo*, but also reduce *in vitro* β -1,3-glucan synthase activity.

One of many possibilities to account for the altered *in vitro* glucan synthase activity is that these mutants have reduced levels of Fks1p and Fks2p. To test this, we generated functional HA-tagged versions of Fks1p and Fks2p that were introduced into the wild-type and *kre* mutant strain backgrounds. Cell lysates obtained from them were analysed for their relative levels of Fks1p and Fks2p (Figure 6). The *kre* mutant cell extracts had comparable Fks1p levels, but contained significantly less Fks1p than the wild-type and *skn1 Δ* cell extracts (Figure 6A). In contrast, both the *kre5 Δ* mutant and the *kre6 Δ skn1 Δ* double mutant contained higher levels of Fks2p (Figure 6B), consistent with their reported elevation of *FKS2*

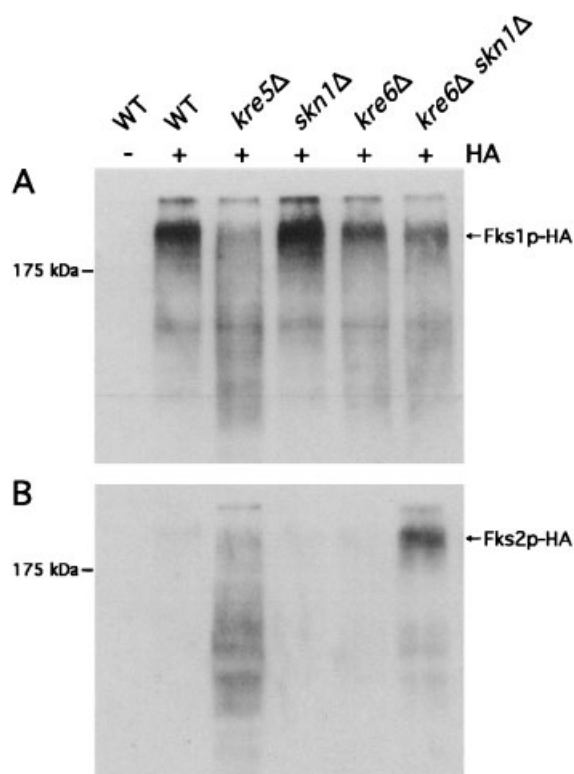


Figure 6. Fks1p and Fks2p levels in β -1,6-glucan deficient mutants. Cell extract samples (8 μ g protein) prepared from the indicated strains transformed with low-copy-number plasmids containing epitope-tagged versions of either Fks1p (A) or Fks2p (B) were immunoblotted with an anti-HA monoclonal antibody, as described in Materials and methods. The first lane of each blot contains a sample from wild-type cells expressing untagged Fks1p (A) or untagged Fks2p (B), to demonstrate the absence of antibody cross-reactivity with endogenous yeast proteins. The position of a 175 kDa standard is indicated on both blots

mRNA levels (Kapteyn *et al.*, 1999b). Thus, it seems unlikely that the altered *in vitro* glucan synthase activity of *kre5*Δ and *kre6*Δ *skn1*Δ mutants is due to an overall reduction in Fks1p and Fks2p levels, although both proteins did appear to be proteolytically degraded in the *kre5*Δ mutant, which may likely affect their activity. However, we did not observe any genetic interaction between *kre5*Δ and *fks2*Δ mutants; *kre5*Δ *fks2*Δ double mutants are viable, grow as slowly as *kre5*Δ mutants and have the same *in vitro* glucan synthase activity (data not shown). Another factor that may influence the *in vitro* glucan synthase activity is the localization of these proteins, which we examine below.

Aberrant localization of Fks1p in β -1,6-glucan deficient mutants

The localization of Fks1p and Fks2p may affect cell wall β -1,6-glucan levels in *kre* mutants, e.g. the *fks1-1125* mutant displayed the strongest reduction in cell wall β -1,6-glucan and is affected in both the *in vitro* activity and localization of the glucan synthase (Abe *et al.*, 2002). To localize these proteins in live cells, we generated functional N-terminal GFP fusions of Fks1p and Fks2p. Fks1p and Rho1p normally co-localize to the site of polarized growth (Qadota *et al.*, 1996) but cell wall stress can depolarize this complex, resulting in a uniform localization at the cell periphery (Delley and Hall, 1999). Our GFP-Fks1p fusion localized to the sites of polarized growth in both wild-type and *skn1*Δ cells (Figure 7A and data not shown). The fluorescent signal of this construct was found to be brighter and slightly more discrete in *fks1*Δ *fks2*Δ double mutant cells lacking endogenous Fks proteins (data not shown). A dramatically different localization pattern of GFP-Fks1p occurred in β -1,6-glucan deficient mutants. The *kre6*Δ cells showed bright patches that appeared to be at the cell periphery, as judged by adjusting the focus of the microscope (Figure 7C). This suggests that the Fks1p containing glucan synthase may be depolarized in this wall mutant. The *kre5*Δ cells had strongly reduced cell surface levels of Fks1p, with the GFP-Fks1p signal present in intracellular vesicular structures (Figure 7E). The *kre6*Δ *skn1*Δ double mutant formed enlarged cells with multiple buds, indicating that this mutant has pleiotropic problems with the establishment and maintenance of cell polarity (Figure 7G). We never observed GFP-Fks1p in the septal region of this mutant, where it is required for the deposition of cell wall material so that cytokinesis can occur. Furthermore, the GFP-Fks1p fusion appeared to be uniformly present at the mother cell surface.

The localization of Fks2p has not been studied, but the expression of *FKS2* is known to be up-regulated during stress (Mazur *et al.*, 1995; Zhao *et al.*, 1998). Our GFP-Fks2p fusion localized to the site of polarized growth in wild-type and *skn1*Δ mutant cells, but the signal was weak, consistent with low-level expression of Fks2p during vegetative growth (Figure 7B and data not shown). GFP-Fks2p localized in a manner that was indistinguishable from GFP-Fks1p in *fks1*Δ *fks2*Δ double mutants, demonstrating the redundant

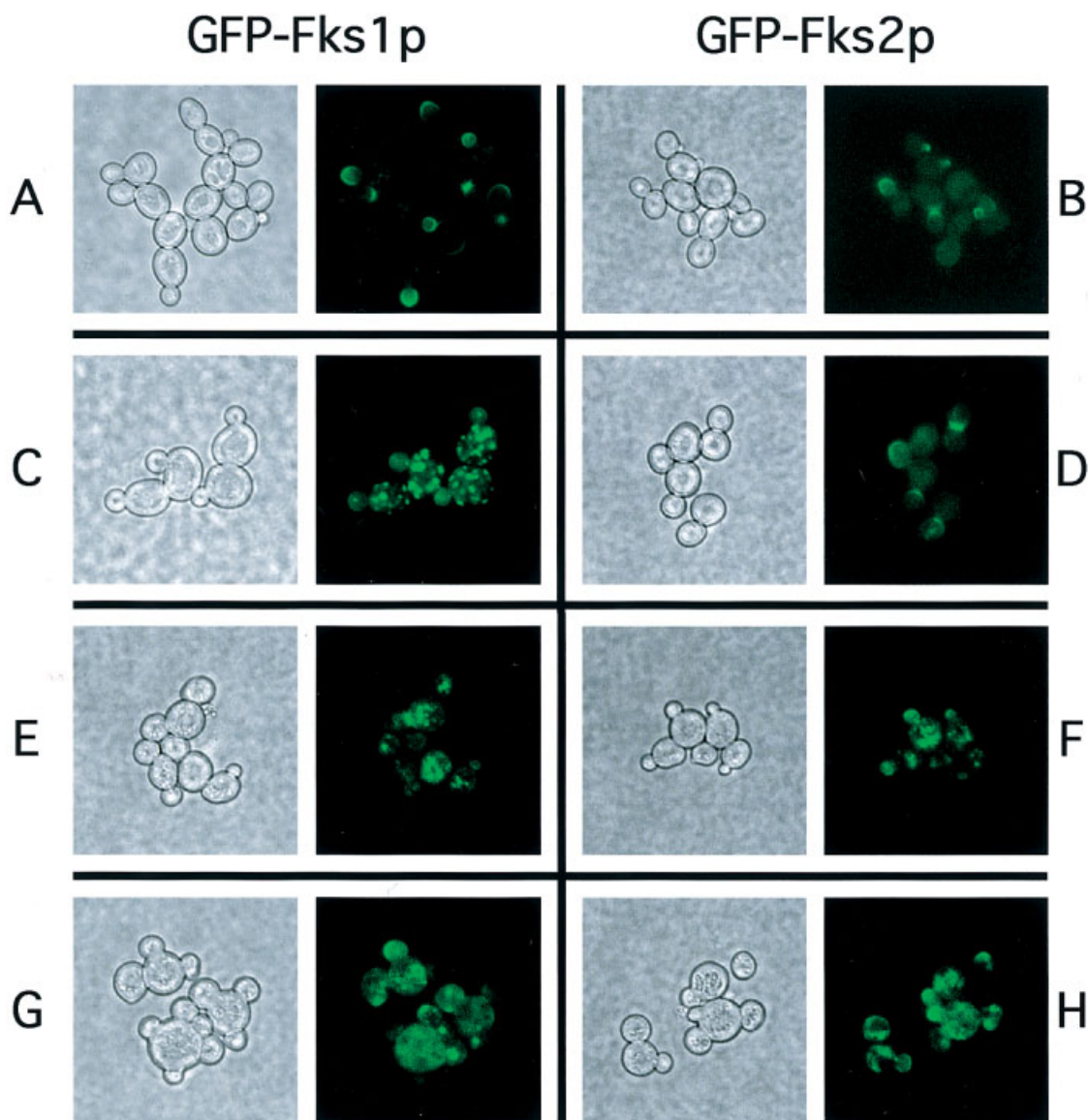


Figure 7. Localization of GFP-Fks1p and GFP-Fks2p in β -1,6-glucan-deficient mutants. Wild-type (A, B), *kre6* Δ (C, D), *kre5* Δ (E, F) and *kre6* Δ *skn1* Δ (G, H) yeast strains were transformed with low-copy-number plasmids containing a N-terminal GFP fusion of either Fks1p or Fks2p. The localization of these fusion proteins was determined in live yeast cells by fluorescence microscopy, as described in Materials and methods

nature of these two proteins (data not shown). The signal obtained from *kre6* Δ cells was slightly stronger and also showed Fks2p localizing at sites of polarized growth (Figure 7D). This implies that Fks2p can localize to sites of polarized growth under conditions when Fks1p is depolarized. The *kre5* Δ mutant cells accumulate the GFP-Fks2p fusion in some intracellular compartment, although significant levels of Fks2p can now also be observed

in the growing bud (Figure 7F). The *kre6* Δ *skn1* Δ mutant cells showed virtually the same localization pattern of GFP-Fks2p as with GFP-Fks1p (cf. Figure 7H–G). All β -1,6-glucan deficient mutants mislocalized Fks1p to various extents, and this aberrant localization pattern in combination with reduced *in vitro* glucan synthase activity may in part account for the β -1,6-glucan defects observed in these *kre* mutant backgrounds.

Discussion

Our results show a requirement of Fks1p and Fks2p for normal β -1,6-glucan levels. Null alleles of *FKS1* are partially killer toxin-resistant and have a small reduction in alkali-insoluble β -1,6-glucan. Overexpression of *FKS2* suppressed the killer-resistant phenotype of *fks1* Δ mutants, implicating Fks2p in the biosynthesis of the residual β -1,6-glucan in *fks1* Δ cells. We could not assess the β -1,6-glucan levels of an *fks1* Δ *fks2* Δ double mutant due to synthetic lethality, but reductions in β -1,6-glucan up to 55% were found in *fks1_{ts}* *fks2* Δ cells. These *fks1_{ts}* alleles are an informative set, as they affected both β -1,3- and β -1,6-glucan levels: most reduced the levels of both polymers, but the *fks1-1093* mutant is hyperactive *in vitro* (Abe *et al.*, 2002) and increased the *in vivo* amounts of both β -glucans. Our results are consistent with a recent study showing that the glucan synthase inhibitor caspofungin can reduce the levels of both β -1,3- and β -1,6-glucans in *Cryptococcus neoformans* (Feldmesser *et al.*, 2000). Nevertheless, the involvement of Fks1p and Fks2p in β -1,6-glucan biogenesis was unexpected. Sugar analysis following acid hydrolysis of isolated cell walls showed that *fks1* Δ mutants have a lower cell wall glucose content (Ram *et al.*, 1995; Dallies *et al.*, 1998), but did not distinguish whether one or both β -glucan types were reduced. The only previous *fks1* alleles tested for β -1,6-glucan defects showed wild-type killer toxin sensitivity (Ram *et al.*, 1995), as did many of our alleles.

The *in vitro* glucan synthase activity was severely affected in *kre5* Δ and *kre6* Δ *skn1* Δ cells and both mutants aberrantly localized Fks1p and Fks2p. Although it is unclear what caused the reduced activity and aberrant localization of Fks1p and Fks2p, both phenotypes are likely to contribute to the severe β -1,6-glucan defects observed in these backgrounds. The *fks1-1125* mutant is also affected in activity and localization of Fks1p (Abe *et al.*, 2002), and displayed the strongest β -1,6-glucan reduction of all *fks1_{ts}* *fks2* Δ mutants analysed (Figure 4; Table 6). Several scenarios can lead to reduced *in vitro* glucan synthase activity and mislocalization of Fks1p, including impaired secretory pathway trafficking (Lee *et al.*, 1999). We did not observe genetic interactions between *kre5* Δ and *fks2* Δ mutants and there appeared to be no overall reduction in Fks1p and Fks2p levels in the *kre5* Δ and *kre6* Δ *skn1* Δ backgrounds. We speculate that these mutants may have a problem with the

regulatory subunit of the glucan synthase, especially since incorrect regulation of Rho1p can affect both the *in vitro* activity and localization of the glucan synthase (Delley and Hall, 1999). However, further experimentation is required to test this conjecture. The aberrant localization of Fks1p and normal localization of Fks2p in *kre6* Δ mutants suggests that Fks2p may localize to sites of polarized growth under conditions when Fks1p is depolarized.

What is the basis of the β -1,6-glucan defect in *fks1* mutants? A role for Fks1p in β -1,3-glucan synthesis was previously established *in vitro* (Douglas *et al.*, 1994; Inoue *et al.*, 1995) and was also observed in this work (Figure 5). In addition, our cell wall analyses indicate that *fks1* Δ and *fks1_{ts}* *fks2* Δ mutants have β -1,3-glucan defects *in vivo* (Figures 1 and 4; Table 6). These findings raise the possibility that the β -1,6-glucan phenotype of *fks1* mutants is an indirect consequence of β -1,3-glucan impairment. Since β -1,3- and β -1,6-glucan are cross-linked to each other in the cell wall, it is possible that the β -1,3-glucan polymer serves as an acceptor for β -1,6-glucan chains and the synthesis of β -1,6-glucan may depend on the availability and/or structure of this acceptor. The reduced *in vitro* glucan synthase activity of *fks1* Δ mutants suggests that this polymer is made at a much slower rate *in vivo* and may therefore be present in limiting amounts, a possibility supported by our finding of the lower alkali-soluble β -1,3-glucan content. Consistent with this, six out of 12 *fks1_{ts}* *fks2* Δ mutants analysed here displayed a greater than 50% reduction in *in vitro* glucan synthase activity and all were severely affected in both β -glucan polymers (Abe *et al.*, 2002; G. Dijkgraaf, unpublished observation). Furthermore, the β -1,3-glucan synthesized by Fks2p or the *fks1_{ts}* alleles may be different in structure and have altered efficiency as a β -1,6-glucan acceptor. For example, impaired substrate binding by the glucan synthase complex is likely to affect polymer length. The *fks1-1125* mutant suffers the greatest reduction in β -1,6-glucan and displays an almost five-fold higher K_m than wild-type at the permissive temperature, although it remains to be established whether this mutant produces shorter β -1,3-glucan chains *in vivo* (Abe *et al.*, 2002). An acceptor role for β -1,3-glucan is not readily compatible with our current understanding of cross-linking events at the cell surface, as β -1,6-glucan chains are thought to be first attached to a remnant of the glycosylphosphatidylinositol (GPI) anchor of wall proteins and these β -1,6-glucosylated mannoproteins are

subsequently cross-linked to the β -1,3-glucan/chitin network (reviewed in Kapteyn *et al.*, 1999a). However, failure to cross-link with β -1,3-glucan could, for example, impair β -1,6-glucan stability. It is clear from our observations that the relationship between Fks1p and these two polymers is more complex than β -1,3-glucan impairment reducing β -1,6-glucan levels. The *in vitro* hyperactive *fks1-1093* mutant has a higher cell wall content of both β -1,3- and β -1,6-glucan *in vivo* and our findings with the *gas1* Δ mutant indicate that a reduction in β -1,3-glucan need not lead to a lower β -1,6-glucan content. Therefore, an alternative explanation for the β -1,6-glucan defect of *fks1* Δ and *fks1_{ts}fks2* Δ mutants may be that Fks1p and Fks2p contribute directly to the synthesis of both β -glucan polymers. The reaction mechanism of β -1,3-glucan synthesis has not been elucidated and existing evidence cannot distinguish between a glucan synthase and a glucan transport role for Fks1p and Fks2p. The lack of clear similarities with other glucan synthesizing enzymes, and a predicted membrane topology that is reminiscent of bacterial and eukaryotic transport proteins (Douglas *et al.*, 1994), suggest the possibility that these proteins may be glucan synthase components involved in transport of β -1,3- and β -1,6-glucan across the plasma membrane. It seems less likely that Fks1p and Fks2p are catalytic subunits of a dual-specificity glucan synthase, since there is no precedent for one enzyme with two processive β -glucosyltransferase activities synthesizing two differently linked glucan polymers. Development of partial *in vitro* assays dissecting the activities of glucan synthase complexes are required to further analyse how Fks1p and Fks2p are involved in glucan synthesis.

Acknowledgements

We thank M. Snyder, T. Roemer and N. Fortin for hospitality and help in screening transposon mutants; S. Shahinian for help with β -1,6-glucanase purification; M. Pardo for the *fks3* Δ mutant; S. Véronneau and P. Ménard for sequencing; M. Whiteway and T. Ketela for comments on the manuscript, and the Bussey laboratory for helpful discussions. Supported by an NSERC operating grant. GJPD was a Max Stern Fellow.

References

- Abe M, Minemura M, Utsugi T, *et al.* 2002. Multiple functional domains of yeast 1,3- β -glucan synthase revealed by mutational analysis (manuscript in preparation).
- Badin J, Jackson C, Schubert M. 1953. Improved method for determination of plasma polysaccharides with tryptophan. *Proc Soc Exp Biol Med* **84**: 228–291.
- Bom IJ, Dielbandhosing KS, Harvey KN, *et al.* 1998. A new tool for studying the molecular architecture of the fungal cell wall: one-step purification of recombinant trichoderma β -(1–6)-glucanase expressed in *Pichia pastoris*. *Biochim Biophys Acta* **1425**: 419–424.
- Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* **72**: 248–254.
- Brown JL, Kossaczka Z, Jiang B, Bussey H. 1993. A mutational analysis of killer toxin resistance in *Saccharomyces cerevisiae* identifies new genes involved in cell wall (1 \rightarrow 6)- β -glucan synthesis. *Genetics* **133**: 837–849.
- Bussey H. 1991. K1 killer toxin, a pore forming protein from yeast. *Mol Microbiol* **5**: 2339–2343.
- Bussey H, Sacks W, Galley D, Saville D. 1982. Yeast killer plasmid mutations affecting toxin secretion and activity and toxin immunity function. *Mol Cell Biol* **2**: 346–354.
- Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. 1994. Green fluorescent protein as a marker for gene expression. *Science* **263**: 802–805.
- Chen D-C, Yang B-C, Kuo T-T. 1992. One-step transformation of yeast in stationary phase. *Curr Genet* **21**: 83–84.
- Christianson TW, Sikorski RS, Dante M, Shero JH, Hieter P. 1992. Multifunctional yeast high-copy-number shuttle vectors. *Gene* **110**: 119–122.
- Dallies N, François J, Paquet V. 1998. A new method for quantitative determination of polysaccharides in the yeast cell wall. Application to the cell wall defective mutants of *Saccharomyces cerevisiae*. *Yeast* **14**: 1297–1306.
- De la Cruz J, Pintor-Toro JA, Benítez T, Llobell A. 1995. Purification and characterization of an endo- β -1,6-glucanase from *Trichoderma harzianum* that is related to its mycoparasitism. *J Bacteriol* **177**: 1864–1871.
- Delley P-A, Hall MN. 1999. Cell wall stress depolarizes cell growth via hyperactivation of RHO1. *J Cell Biol* **147**: 163–174.
- Dijkgraaf GJP, Brown JL, Bussey H. 1996. The *KNH1* gene of *Saccharomyces cerevisiae* is a functional homologue of *KRE9*. *Yeast* **12**: 683–692.
- Douglas CM, Foor F, Marrinan JA, *et al.* 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.
- Drgonová J, Drgon T, Tanaka K, *et al.* 1996. Rho1p, a yeast protein at the interface between cell polarization and morphogenesis. *Science* **272**: 277–279.
- Farkas I, Hardy TA, Depaoli-Roach AA, Roach P. 1990. Isolation of the *GSY1* gene encoding yeast glycogen synthase and evidence for the existence of a second gene. *J Biol Chem* **265**: 20879–20886.
- Feldmesser M, Kress Y, Mednick A, Casadevall A. 2000. The effect of the echinocandin analogue caspofungin on cell wall glucan synthesis by *Cryptococcus neoformans*. *J Infect Dis* **182**: 1791–1795.
- Gietz RD, Schiestl RH, Willems AR, Woods RA. 1995. Studies of the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. *Yeast* **11**: 355–360.

- Hoffman CS, Winston F. 1987. A 10 minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. *Gene* **57**: 267–272.
- Inoue SB, Takewaki N, Takasuka T, et al. 1995. Characterization and gene cloning of 1,3- β -D-glucan synthase from *Saccharomyces cerevisiae*. *Eur J Biochem* **321**: 845–854.
- Jiang B, Cyert MS. 1999. Identification of a novel region critical for calcineurin function *in vivo* and *in vitro*. *J Biol Chem* **274**: 18543–18551.
- Kang MS, Cabib E. 1986. Regulation of fungal cell wall growth: a guanine nucleotide-binding, proteinaceous component required for activity of (1,3)- β -D-glucan synthase. *Proc Natl Acad Sci U S A* **83**: 5808–5812.
- Kapteyn JC, Ram AFJ, A, Groos EM, et al. 1997. Altered extent of cross-linking of β 1,6-glucosylated mannoproteins to chitin in *Saccharomyces cerevisiae* mutants with reduced cell wall β 1,3-glucan content. *J Bacteriol* **179**: 6279–6284.
- Kapteyn JC, Van Den Ende H, Klis FM. 1999a. The contribution of cell wall proteins to the organization of the yeast cell wall. *Biochim Biophys Acta* **1426**: 373–383.
- Kapteyn JC, Van Egmond P, Sievi E, et al. 1999b. The contribution of the O-glycosylated protein Pir2p/Hsp150 to the construction of the yeast cell wall in wild-type cells and β -1,6-glucan-deficient mutants. *Mol Microbiol* **31**: 1835–1844.
- Ketela T, Green R, Bussey H. 1999. *Saccharomyces cerevisiae* Mid2p is a potential cell wall stress sensor and upstream activator of the PKC1-MPK1 cell integrity pathway. *J Bacteriol* **181**: 3330–3340.
- Kollár R, Reinhold BB, Petráková E, et al. 1997. Architecture of the yeast cell wall: β (1 \rightarrow 6)-glucan interconnects mannoprotein, β (1 \rightarrow 3)-glucan and chitin. *J Biol Chem* **272**: 17762–17775.
- Kunkel TA, Roberts JD, Zakour RA. 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol* **154**: 367–382.
- Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.
- Lee D-W, Ahn G-W, Kang H-G, Park H-M. 1999. Identification of a gene, *SOO1*, which complements osmo-sensitivity and defect in *in vitro* β 1,3-glucan synthase activity in *Saccharomyces cerevisiae*. *Biochem Biophys Acta* **1450**: 145–154.
- Lipke PN, Ovalle R. 1998. Cell wall architecture in yeast: new structure and new challenges. *J Bacteriol* **180**: 3735–3740.
- Lussier M, Sdicu A-M, Shahinian S, Bussey H. 1998. The *Candida albicans* *KRE9* gene is required for cell wall β -1,6-glucan synthesis and is essential for growth on glucose. *Proc Natl Acad Sci U S A* **95**: 9825–9830.
- Manners DJ, Masson AJ, Patterson JC. 1973. The structure of a β -(1 \rightarrow 3)-D-glucan from yeast cell walls. *Biochem J* **135**: 19–30.
- Mazur P, Morin N, Baginsky W, et al. 1995. Differential expression and function of two homologous subunits of yeast 1,3- β -D-glucan synthase. *Mol Cell Biol* **15**: 5671–5681.
- Mazur P, Baginsky W. 1996. *In vitro* activity of 1,3- β -D-glucan synthase requires the GTP-binding protein Rho1. *J Biol Chem* **271**: 14604–14609.
- Meaden P, Hill K, Wagner J, Slipetz D, Sommer SS, Bussey H. 1990. The yeast *KRE5* gene encodes a probable endoplasmic reticulum protein required for (1 \rightarrow 6)- β -D-glucan synthesis and normal cell growth. *Mol Cell Biol* **10**: 3013–3019.
- Montijn RC, Vink E, Müller WH, et al. 1999. Localization of synthesis of β 1,6-glucan in *Saccharomyces cerevisiae*. *J Bacteriol* **181**: 7414–7420.
- Mouyna I, Fontaine T, Vai M, et al. 2000. Glycosylphosphatidylinositol-anchored glucanoyltransferases play an active role in the biosynthesis of the fungal cell wall. *J Biol Chem* **275**: 14882–14889.
- Muhlrad D, Hunter R, Parker R. 1992. A rapid method for localized mutagenesis of yeast genes. *Yeast* **8**: 79–82.
- Orlean P. 1997. Biogenesis of yeast wall and surface components. In *The Molecular and Cellular Biology of the Yeast Saccharomyces*, vol 3, *Cell Cycle and Cell Biology*, Pringle JR, Broach JR, Jones EW (eds). Cold Spring Harbor Laboratory Press: New York; 229–362.
- Parker CG, Fessler LI, Nelson RE, Fessler JH. 1995. *Drosophila* UDP-glucose:glycoprotein glucosyltransferase: sequence and characterization of an enzyme that distinguishes between denatured and native proteins. *EMBO J* **14**: 1294–1303.
- Popolo L, Gilardelli D, Bonfante P, Vai M. 1997. Increase in chitin as an essential response to defects in assembly of cell wall polymers in the *gsp1 Δ* mutant of *Saccharomyces cerevisiae*. *J Bacteriol* **179**: 463–469.
- Qadota H, Python CP, Inoue SB, et al. 1996. Identification of yeast Rho1p GTP-ase as a regulatory subunit of 1,3- β -glucan synthase. *Science* **272**: 279–281.
- Ram AFJ, Brekelmans SSC, Oehlen LJWM, Klis FM. 1995. Identification of two cell cycle regulated genes affecting the β -1,3-glucan content of cell walls in *Saccharomyces cerevisiae*. *FEBS Lett* **358**: 165–170.
- Ram AFJ, Kapteyn JC, Montijn RC, et al. 1998. Loss of the plasma membrane-bound protein Gas1p in *Saccharomyces cerevisiae* results in the release of β 1,3-glucan into the medium and induces a compensation mechanism to ensure cell wall integrity. *J Bacteriol* **180**: 1428–1424.
- Roemer T, Bussey H. 1991. Yeast β -glucan synthesis: *KRE6* encodes a predicted type II membrane protein required for glucan synthesis *in vivo* and for glucan synthase activity *in vitro*. *Proc Natl Acad Sci U S A* **88**: 11295–11299.
- Roemer T, Paravicini G, Payton MA, Bussey H. 1994. Characterization of the yeast (1 \rightarrow 6)- β -glucan biosynthetic components, Kre6p and Skn1p, and genetic interactions between the PKC1 pathway and extracellular matrix assembly. *J Cell Biol* **127**: 567–579.
- Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press: New York.
- Shahinian S, Dijkgraaf GJP, Sdicu A-M, et al. 1998. Involvement of N-glycosyl chain glucosylation and processing in the biosynthesis of cell wall β -1,6-glucan of *Saccharomyces cerevisiae*. *Genetics* **149**: 843–856.
- Shahinian S, Bussey H. 2000. β -1,6-Glucan synthesis in *Saccharomyces cerevisiae*. *Mol Microbiol* **35**: 477–489.
- Shematek EM, Braatz JA, Cabib E. 1980. Biosynthesis of the yeast cell wall. I. Preparation and properties of β -(1 \rightarrow 3)glucan synthetase. *J Biol Chem* **255**: 888–894.
- Sherman F, Fink GR, Hicks JB. 1986. *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press: New York.
- Sikorski RS, Hieter P. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**: 19–27.
- Stasinopoulos SJ, Fisher PR, Stone BA, Stanisch VA. 1999. Detection of two loci involved in (1 \rightarrow 3)- β -glucan (curdlan)

- biosynthesis by *Agrobacterium* sp. ATCC31749, and comparative sequence analysis of the putative curdlan synthase gene. *Glycobiology* **9**: 31–41.
- Vernet T, Dignard D, Thomas DY. 1987. A family of yeast expression vectors containing the phage f1 intergenic region. *Gene* **52**: 225–233.
- Wach A, Brachat A, Pöhlmann R, Philippsen P. 1994. New heterologous modules for classical PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* **10**: 1793–1808.
- Zhao C, Sung Jung U, Garrett-Engle P, et al. 1998. Temperature-induced expression of yeast *FKS2* is under the dual control of protein kinase C and calcineurin. *Mol Cell Biol* **18**: 1013–1022.