Suppression of sorbitol dependence in a strain bearing a mutation in the *SRB1/PSA1/VIG9* gene encoding GDP-mannose pyrophosphorylase by *PDE2* overexpression suggests a role for the Ras/cAMP signal-transduction pathway in the control of yeast cell-wall biogenesis

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Complementation studies and allele replacement in *Saccharomyces cerevisiae* revealed that *PSA1/VIG9*, an essential gene that encodes GDP-mannose pyrophosphorylase, is the wild-type *SRB1* gene. Cloning and sequencing of the *srb1-1* allele showed that it determines a single amino acid change from glycine to aspartic acid at residue 276 (*srb1*^{D276}). Genetic evidence is presented showing that at least one further mutation is required for the sorbitol dependence of *srb1*^{D276}. A previously reported complementing gene, which this study has now identified as *PDE2*, is a multi-copy suppressor of sorbitol dependence and is not, as was previously suggested, the *SRB1* gene. *srb* and *pde2* mutants share a number of phenotypes, including lysis upon hypotonic shock and enhanced transformability. These data are consistent with the idea that the Ras/cAMP pathway might modulate cell-wall construction.

Keywords: *SRB1/PSA1/VIG9/YDL055c*, *PDE2*, cell-wall integrity, GDP-mannose

INTRODUCTION

The cell wall of *Saccharomyces cerevisiae*, a highly complex and dynamic structure, undergoes a number of modifications during different stages of the cell cycle, and also in response to environmental signals. The modifications to the wall must be tightly controlled. Understanding the coordinating factors and mechanisms that underlie cell growth and division is of major scientific and practical interest.

The yeast cell wall consists of almost equal amounts of β -glucans and mannoproteins. The third component, chitin, represents less than 1% of the total mass of the wall, but plays a highly significant role. A great number

of genes have been shown to participate in cell-wall synthesis (see reviews by Klis, 1994; Cid *et al.*, 1995).

Mannan is a complex of proteins modified by the addition of mannose-containing carbohydrate chains linked via N- and O-glycosidic bonds, and in some cases, glycosyl-phosphatidyl-inositol (GPI)-anchors (reviews, Kukuruzinska *et al.*, 1987; Herscovics & Orlean, 1993; Orlean, 1997). The early steps in N-glycosylation, which contributes about 90% of the mannan in the cell wall, are carried out by proteins encoded by essential genes, which indicates that there is a minimal level of N-glycosylation required for cell viability. Most of the remaining mannan is in the form of O-glycosylated proteins (Tanner & Lehle, 1987). Multiple O-glycosylation mutants can exhibit osmotic fragility, thereby indicating the importance of O-glycosylation in maintaining the structural integrity of the cell wall (Gentzsch & Tanner, 1996). The third form of glycosylation, that of addition of GPI anchors to proteins (for reviews see Cross, 1990; Englund, 1993), is essential for cell viability in *S. cerevisiae* (Leidich *et al.*, 1995). All three modes of glycosylation require GDP-

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Abbreviations: GPI, glycosyl-phosphatidyl-inositol; MAP, mitogen-activated protein; PKA, cAMP-dependent protein kinase; STRE, stress-response element.

Table 1. Strains used in this study

Strain	Genotype	Source/reference
<i>S. cerevisiae</i>		
YLG1	<i>MATa his3 leu2 ura3 trp1 ade8 can1</i>	This report
YLG2	<i>MATα his3 leu2 ura3 trp1 ade8 can1</i>	This report
DJ12-9d	<i>MATα his3 leu2 ura3 trp1 ade8 can1</i> <i>pde2::URA3</i>	M. Wigler*
4STLU	<i>MATa srb1-1 srb trp1 leu2 ura3</i>	Stateva <i>et al.</i> (1991)
4SLUP	<i>MATa srb1-1 srb trp1 leu2 ura3 pde2::TRP1</i>	This report
7SLU	<i>MATα srb1-1 srb leu2 ura3</i>	Stateva <i>et al.</i> (1991)
7SUP3 (7SUP6)	<i>MATα leu2 ura3 srb PSA1-LEU2</i>	This report
YPH499	<i>MATa ura3 lys2 ade2 trp1 his3 leu2</i>	Sikorski & Hieter (1989)
BY4741	<i>MATa ura3 leu2 his3 met15</i>	Brachmann <i>et al.</i> (1998)
F7a	<i>MATa ura3 his3 leu2 trp1 ade-mpb1::URA3</i>	This report (spore from CJ19 × SJ21R)
G20b	<i>MATa ura3 lys2 ade2 trp1 his3 leu2 aad4::URA3</i> <i>PSA1-LEU2</i>	This report (based on YPH499)
20hPS1	<i>MATa leu2 ura3 trp1 ade1 his2 psa1::TRP1</i> <i>pURA3/PSA1</i>	Benton <i>et al.</i> (1996)
20hPS1-2d	<i>MATa leu2 ura3 trp1 ade1 his2 psa1::TRP1</i> <i>pLEU2/psa1^{ts}-2d</i>	Benton <i>et al.</i> (1996)
20hPS1-3c	<i>MATa leu2 ura3 trp1 ade1 his2 psa1::TRP1</i> <i>pLEU2/psa1^{ts}-3c</i>	Benton <i>et al.</i> (1996)
BR15-3D	<i>MATa his1 Mal⁻</i>	UMIST collection
DC6	<i>MATα leu2 his4</i>	UMIST collection
CJ19	<i>MATα ura3 ade2 his3 trp1 leu2 met-mpb1::URA3</i>	L. Johnston†
SJ21R	<i>MATa ura3 leu2 ade1</i>	UMIST collection
YPKC1L	<i>MATa pkc1::LEU2 ura3 trp1 leu2</i>	D. Levin‡
DEY293-42c	<i>MATa mpk1::TRP1 ura3 ade2-1 his3-11,15 leu2-3,112 lys2-95 ssd1-02 cgn1-100</i>	P. Sudbery§
<i>E. coli</i>		
JM109	<i>F' traD36 lacI^qΔ(lacZ) M15 proA⁺B⁺/e14⁻(McrA⁻) Δ(lac-proAB) thi gyrA96 (Nal^r) endA1 hsdR17 (r_K⁻ m_K⁺) relA1 supE44 recA1</i>	Yanisch-Perron <i>et al.</i> (1985)
XL1-Blue	<i>[F' proA⁺B⁺ lacI^qΔM15 Tn10 (tet^r)recA1 endA1 gyrA96 (Nal^r) thi-1 hsdR17 (r_K⁻ m_K⁺) supE44 relA1 lac</i>	Bullock <i>et al.</i> (1987)

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mannose, either directly or indirectly. This is produced by the enzyme GDP-mannose pyrophosphorylase which is encoded by the essential gene *PSA1/VIG9* (Benton *et al.*, 1996; Hashimoto *et al.*, 1997). *PSA1* is a cell-cycle-regulated gene with peak expression occurring near START (Benton *et al.*, 1996).

Pkc1p (Levin & Bartlett-Heubusch, 1992; Paravicini *et al.*, 1992) is responsible for the activation of a number of protein-kinase genes whose products function in a typical mitogen-activated protein (MAP) kinase (MAPK) signal-transduction pathway that is significant in maintaining cellular integrity (see Molina *et al.*, 1998

for a review). The MAP kinases under Pkc1p control are the MAPK kinase kinase (MEKK), encoded by *BCK1/SLK1* (Lee & Levin, 1992; Costigan *et al.*, 1992), the MAPK kinase (MEK), encoded by *MKK1* and *MKK2* (Irie *et al.*, 1993) and MAPK, encoded by *MPK1/SLT2* (Lee *et al.*, 1993; Mazzoni *et al.*, 1993). Disruption of *PKC1* results in sorbitol dependency, whilst disruption of the other members of the cascade causes temperature sensitivity that is rescuable by an osmotic stabilizer.

Normal cellular growth and cell-wall synthesis depend on the availability of nutrients. The Ras/cAMP signal transduction pathway has been demonstrated to play a

Table 2. Plasmids used in this study

Plasmid	Details	Source/reference
pUC19	<i>E. coli</i> vector	Yanisch-Perron <i>et al.</i> (1985)
YEpl3	2 μ - <i>LEU2</i> shuttle vector	Broach <i>et al.</i> (1979)
YCp50	CEN-ARS- <i>URA3</i> shuttle vector	Rose <i>et al.</i> (1987)
pRS316	CEN-ARS- <i>URA3</i> shuttle vector	Sikorski & Heiter (1989)
pRS425	2 μ - <i>LEU2</i> shuttle vector	Christianson <i>et al.</i> (1992)
pRS426	2 μ - <i>URA3</i> shuttle vector	Christianson <i>et al.</i> (1992)
pSRB1-2	14.5 kb chromosome IV fragment in YCp50, complements <i>srb1-1</i>	This study
pSRB1-3	pSRB1-2 with <i>SalI</i> fragment removed	This study
pSRB1-4	pSRB1-2 with <i>ClaI</i> fragment removed	This study
pSRB1-5	pSRB1-2 with <i>HindIII</i> fragment removed	This study
pSRB1-9b	<i>ClaI</i> - <i>BamHI</i> fragment of pSRB1-2 in pRS316, contains <i>PSA1</i>	This study
pSRB1-9c	pSRB1-9b with <i>BamHI</i> - <i>EcoRV</i> fragment removed	This study
pSRB1-9d	pSRB1-9c with <i>LEU2</i> in <i>NsiI</i> - <i>NheI</i> sites and is <i>ura3Δ</i>	This study
pSRB1-10c	<i>BamHI</i> - <i>ClaI</i> fragment of pSRB1-2 in pRS426, contains <i>PSA1</i>	This study
<i>psa1</i> ^{ts} -2d	ARS-CEN, <i>LEU2</i> plasmid, temperature-sensitive <i>psa1</i> allele	Benton <i>et al.</i> (1996)
<i>psa1</i> ^{ts} -3c	ARS-CEN, <i>LEU2</i> plasmid, temperature-sensitive <i>psa1</i> allele	Benton <i>et al.</i> (1996)
pSP1	<i>psa1</i> from 7SLU PCR amplified and cloned in pUC19	This study
pSP2	<i>psa1</i> from 7SLU PCR amplified and cloned in pUC19	This study
YEpl95-PKC1	<i>PKC1</i> on a 4.2 kb <i>SphI</i> fragment in a 2 μ - <i>URA3</i> shuttle vector	P. Sudbery*
YEpl352-BCK1-20	<i>BCK1-20</i> on a 2 μ - <i>URA3</i> shuttle vector	D. Levin†
YEpl352-MKK1	<i>MKK1</i> on a 2 μ - <i>URA3</i> shuttle vector	D. Levin†
YEpl352-MPK1	<i>MPK1</i> on a 2 μ - <i>URA3</i> shuttle vector	D. Levin†

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major role in nutrient sensing, by regulating the activity of protein kinase A (for reviews see Broach & Deschenes, 1990; Broach, 1991; Thevelein, 1994). An increase in cAMP levels causes activation of the cAMP-dependent protein kinase, PKA (Matsumoto *et al.*, 1982; Toda *et al.*, 1987a, b). This enzyme controls the phosphorylation of a number of different target proteins and, in this way, modulates their activity and function. There have been several reports which implicate a role for the Ras/cAMP pathway in the regulation of a number of cellular processes, such as cell-cycle progression (Baroni *et al.*, 1994; Tokiwa *et al.*, 1994), mating (Arkinstall *et al.*, 1991) and pseudohyphal growth (Gimeno *et al.*, 1992; Pan & Heitman, 1999). The signalling is attenuated by the action of cAMP phosphodiesterase, encoded by *PDE2*, which degrades cAMP (Sass *et al.*, 1986).

The mutation *srb1-1*, which confers hypotonic shock fragility to yeast cells (Venkov *et al.*, 1974; Kozhina *et al.*, 1979), has been an object of investigation. Work on

strains carrying the *srb1-1* mutation found a number of cell-wall changes, including alterations in glucan structure and composition (Blagoeva *et al.*, 1991), and mannan content (Maerkisch *et al.*, 1983). Investigations on the mode of action of actinomycin D (Waltschewa *et al.*, 1976) and rifampicin (Venkov *et al.*, 1975) have been facilitated by the hypersensitivity to antibiotics shown by strains carrying the *srb1-1* mutation.

Initial efforts to identify the *SRB1* gene resulted in the cloning of a complementing gene (Stateva *et al.*, 1991), which was then reported to be the *SRB1* gene. This gene, which we have now identified as *PDE2*, is not the *SRB1* gene but merely a multi-copy suppressor of sorbitol dependence. The wild-type *SRB1* gene has now been isolated and shown to be identical to the previously described gene *PSA1/VIG9* (Benton *et al.*, 1996; Hashimoto *et al.*, 1997) which encodes GDP-mannose pyrophosphorylase. In addition, we present evidence that sorbitol dependency is a synthetic phenotype that

involves at least one further mutant *srb* gene. The finding that *PDE2* can rescue sorbitol dependence implicates the Ras/cAMP pathway in maintenance of cell-wall integrity. We have therefore carried out a number of phenotypic tests to seek further evidence to support this premise.

METHODS

Strains and media. Strains of *Saccharomyces cerevisiae* and *Escherichia coli* used in this study are listed in Table 1, together with relevant references and sources. The *srb* mutations were originally generated in S288C (Venkov *et al.*, 1974).

Yeast media (YEPD, presporulation, sporulation, minimal and selective drop-out media with appropriate combinations of bases and amino acids) were prepared as described by Sherman *et al.* (1986) and Kaiser *et al.* (1994). Bacterial strains were grown in LB and LB + ampicillin prepared as described by Sambrook *et al.* (1989).

Plasmids, DNA preparation and manipulations. Plasmids employed in this study are shown in Table 2 and oligonucleotide primers in Table 3. Bacterial plasmid DNA was isolated using the alkaline lysis method of Birnboim & Doly (1979). All DNA fragments used for subcloning were gel purified by the method of Heery *et al.* (1990). All enzymes (restriction endonucleases, T4 ligase, calf intestinal phos-

phatase, *Taq* DNA polymerase and T4 DNA polymerase) were used according to the recommendations of the manufacturers.

Sequencing was carried out using an Applied Biosystems sequencer; see James *et al.* (1995) for a description of the method employed. The PCR-amplified *srb1-1/psa1* DNA fragment from strain 7SLU was sequenced on both strands, using oligonucleotides *psa1'x'* (Table 3) to identify the mutation, as were the two *psa1^{ts}* alleles. The *psa1^{ts}* plasmids were recovered from strains 20hPS1-2d and 20hPS1-3c by the method of Ward (1990).

Colony PCR was used to check for correct integration of a DNA fragment after transformation into yeast. This was carried out as follows. A 1- or 2-d-old colony was picked and smeared on the inside of a PCR Eppendorf tube, microwaved on full power for 1 min and immediately cooled on ice. The yeast was resuspended in a mixture of all components necessary for PCR (except *Taq*) at the appropriate concentrations (Innis & Gelfand, 1990), 5 U *Taq* DNA polymerase were added and 30 cycles of PCR carried out in a Perkin-Elmer Progene Thermal Cycler with an elongation step of 1 min per kb being used.

Transformation. *E. coli* strains were transformed either by the calcium chloride method as described by Sambrook *et al.* (1989) or electroporation with a Gene Pulser apparatus as recommended by the manufacturer (Bio-Rad). Sorbitol-dependent and *pde2* disruptant yeast strains were transformed

Table 3. Oligonucleotide primers used in this report

Coordinates given for oligonucleotides *psa1a* to *psa1j* relate to GenBank accession no. U19608 (*PSA1*). See Fig. 1 for a schematic representation of the primers used in the replacement of *srb1-1* by a *PSA1-LEU2* cassette.

Name	Sequence (5'–3')	Annealing position
psafor	AGCTCAGAGGTCACATACG	59 bp upstream of <i>HindIII</i> site in GenBank U24437 (nt 538–557)
psarev	CGCGGATCCGCGAATACTA CAGACATTGATAGCC	81 bp downstream of <i>NsiI</i> site in GenBank U19608 (nt 2230–2209), <i>BamHI</i> site in bold
psarev2	CGATTCGCAATCACAAAC	First <i>ClaI</i> site downstream of <i>PSA1</i> , GenBank U19608 (nt 3262–3245)
leuoli	TCGGCTGTGATTTCTTGAC	<i>LEU2</i> ORF, 904 bp downstream of <i>SpeI</i> site, GenBank U03498 (nt 5889–5907)
psaleu2	ACTACACGGAAGTCAATCTCAC	2.61 kb downstream of the first <i>ClaI</i> site downstream of <i>PSA1</i> , GenBank Z74105 (nt 4421–4442)
psa1a	AACAAAAACAGATGAGTG ATATAG	1901–1878
psa1b	GCCAATTTTTTTTGGTTGTC	1498–1480
psa1c	TTTTCAACAAATCTGTCAATC	1249–1229
psa1d	CAGAGAAAGTGATGTTA ACACC	996–975
psa1e	TCACCGATGGTGACATTAG	1609–1591
psa1f	AGCACAAGACAAGCTACAAC	712–731
psa1g	TTTGAAGAAGTACGAAAAGG	950–969
psa1h	TAAATACGGTGTCATTGTCC	1187–1206
psa1i	CAAAGGACTTCTTGTCTGG	1429–1447
psa1j	ACCACTCCTTGGTCAAATC	1660–1678

Fig. 1. Replacement of the *srb1-1* allele by a *PSA1-LEU2* cassette. Arrows show the directions and positions of the oligonucleotide primers. Primer 1 is *psaleu2*, primer 2 is *psarev2*, primer 3 is *leuoli* and primer 4 is *psafor* (Table 3). Primers 2 and 4 were used to amplify the replacement cassette from pSRB1-9d; primers 1 and 3 were used to confirm correct integration of the PCR product. The 3' terminus of *MBP1* is deleted in the gene replacement. C, *Clal*; H, *HindIII*; Nh, *NheI*; Ns, *NsiI*; Sp, *SpeI*; the Nh/Sp join results in a sequence that can not be recut by either enzyme.

by the no-lithium procedure of Philipova (1985) as described by Stateva *et al.* (1991). Other yeast strains were transformed by the method of Hill *et al.* (1991).

Genetic methods. Standard genetic techniques were used (Sherman *et al.*, 1986; Kaiser *et al.*, 1994) and the segregation of markers of interest followed by replica-plating yeast strains onto appropriate selective media. Sorbitol dependence was tested by comparing growth in media with and without 10% (w/v) sorbitol. The mating type of segregants was determined by crossing them with tester strains of both *MATa* (BR15-3D) and *MATα* (DC6) types and selecting for the generation of prototrophic diploids.

Lysis tests. Hypotonic shock lysis tests were performed as described by Stateva *et al.* (1991). Lysis data were normalized as the number of A_{260} units released per OD₆₀₀ unit of pre-lysis cell suspension.

PCR amplification and cloning of *psa1* in a *srb1-1* strain. The *srb1-1/psa1* allele was PCR amplified from strain 7SLU, using oligonucleotide primers *psafor* and *psarev* (Table 3). PCR amplification was carried out as described by Innis & Gelfand (1990). The PCR product was digested with *Bam*HI and *Hind*III to allow cloning into those sites in pUC19. This procedure was carried out twice, completely independently on each occasion.

PSA1 gene replacement. Strains 7SUP3 and 7SUP6 were generated by replacing the *psa1* allele by a *PSA1-LEU2* construct in strain 7SLU. This was made as follows: pSRB1-9b, which was constructed by ligating a *Bam*HI-*Clal* fragment containing *PSA1* into pRS316, was digested with *Bam*HI, the ends filled using T4 DNA polymerase, digested with *Eco*RV and religated. This plasmid, called pSRB1-9c, was constructed in order to remove the *Nsi*I site in the pRS316 vector component.

The *LEU2* gene was cut from YEp13 on a *Spe*I-*Nsi*I fragment and ligated into the *Nhe*I and *Nsi*I sites located downstream of *PSA1* on pSRB1-9c. This plasmid, called pSRB1-9d, was then used as the template for the gene replacement.

The region of pSRB1-9d containing the *PSA1-LEU2* construct was PCR amplified using *psafor* and *psarev2* (Fig. 1). The PCR reaction mixture was digested with *Pvu*I to cut the pSRB1-9d template, thereby reducing the chance of false positives (i.e. colonies growing without leucine because they contain the plasmid, not the integrated construct). The PCR product was then transformed into 7SLU and transformants selected on minimal media containing sorbitol and uracil, but lacking leucine.

After colonies had developed, they were checked for correct integration of the construct. Oligonucleotides *leuoli* and *psaleu2* (Fig. 1), which anneal to the *LEU2* gene and outside the gene-replacement cassette respectively, were used to check for correct insertion of the *PSA1-LEU2* cassette. Correct insertion of the cassette should give a 4.03 kb band, which was the case for 7SUP3 and 7SUP6.

RESULTS

PDE2 is an extragenic suppressor of sorbitol dependence

Stateva *et al.* (1991) isolated a 4.0 kb *Bam*HI fragment (YEp13::*Bam*HI) that complemented the sorbitol dependence of strain 4STLU. However, two pieces of data cast doubt on this plasmid containing the *SRB1* gene and led us to suspect that an extragenic suppressor may have been cloned. First, the cloned fragment was only capable of complementation when present on a multi-copy plasmid. Second, the disruption mutant (which was created using a 1.5 kb *Pst*I subfragment of the cloned gene) displayed a less severe phenotype than the original *srb1-1* mutant. Although lysis occurred when subjected to hypotonic shock, the disruptant strain was capable of growth on media lacking an osmotic stabilizer, the key phenotype for which we have screened. Consequently, we sequenced this clone and identified *PDE2* as the complementing gene. To demonstrate that *PDE2* is a suppressor rather than the *bona fide* *SRB1* gene, genetic analysis was performed on a diploid heterozygous for *srb1-1* and homozygous for the loss of *PDE2*, disrupted by the insertion of *URA3* on one allele and *TRP1* on the other. The results of this genetic analysis (Table 4) show that *PDE2* and *SRB1* are not allelic. From this, we conclude that *PDE2* is merely a multi-copy suppressor of sorbitol dependence, not the *SRB1* gene itself, as previously reported (Stateva *et al.*, 1991). In the light of these results, which demonstrate that the previously reported clone YEp13::*Bam*HI contains the *PDE2* gene, the disruption strains constructed and described previously (Stateva *et al.*, 1991) should now be referred to as *pde2::TRP1* rather than *srb1::TRP1*. The 2:2 segregation pattern of sorbitol-dependent to sorbitol-independent colonies in the tetrad analysis also led us to believe that the *srb1-1* mutation is the only one causing sorbitol dependence in *srb* strains. However, subsequent work revealed the situation to be more complex.

Identification of *PSA1* as the *bona fide* *SRB1* gene

In another attempt to isolate the *SRB1* gene, we transformed a *srb1-1* mutant (7SLU; Table 1) with a YCp50-based genomic library (Rose *et al.*, 1987). Selection was carried out on the basis of growth without an osmotic stabilizer in the medium. Analysis of plasmids recovered through this procedure showed that they contained a 14.5 kb insert. Subcloning and sequence analysis, described by Gardner *et al.* (1996), identified several ORFs in the insert. Different subclones were constructed and transformed into 7SLU to identify the gene complementing sorbitol dependence. The maps of

Table 4. Segregation pattern from the analysis of 48 four-spored tetrads of a cross between strains DJ12-9d and 4SLUP

URA3 was used for disruption of *PDE2* in strain DJ12-9d and *TRP1* was used for disruption of *PDE2* in strain 4SLUP.

Tetrad type	Genotype					
	<i>srb/URA3</i>	<i>srb/TRP1</i>	<i>TRP1/URA3</i>	<i>MAT/URA3</i>	<i>MAT/TRP1</i>	<i>MAT/srb</i>
Parental ditype	4	4	47	11	11	9
Non-parental ditype	7	8	0	8	9	9
Tetratype	37	36	1	29	28	30
Total	48	48	48	48	48	48

Fig. 2. Subcloning of pSRB1-2, which complements sorbitol dependence. Only constructs that contain the entirety of *PSA1* (pSRB1-2, pSRB1-4 and pSRB1-9b) are able to complement sorbitol dependence. The plasmid name is on the right side of the figure. The chromosomal centromere lies to the right of the figure. Arrows show the direction of gene transcription. YCp50 is the vector for each construct, except pSRB1-9b for which it is pRS316. B, *Bam*HI; C, *Cl*aI; E, *Eco*RI; H, *Hind*III; Nh, *Nhe*I; Ns, *Nsi*I; S, *Sa*II; J, *Sau*3A1/*Bam*HI join between insert and vector; *, site not cut by restriction enzyme.

the constructs and the results of the complementation analyses are shown in Fig. 2. They indicate that, of all the genes present on the originally cloned insert, the sequences corresponding to the *PSA1* gene (Benton *et al.*, 1996) alone were capable of functional complementation of sorbitol dependence. The following experiments were carried out in an attempt to show whether *SRB1* and *PSA1* define the same gene.

First we tested the temperature sensitivity (at 37 °C) of strains bearing *psa1*^{ts} mutant alleles, isolated and described by Benton *et al.* (1996), and kindly provided by the authors. We found that these mutants can be complemented by the addition of 1 M sorbitol, a feature which had not been described previously. Therefore, it would appear that if *SRB1* and *PSA1* define the same gene, then *srb1-1* and *psa1*^{ts} mutants should differ in their dependence upon osmotic stabilizers for growth at 30 °C, but share the phenotype of sorbitol dependence at 37 °C. Two of the *psa1*^{ts} alleles (designated *psa1*^{ts}-2d and *psa1*^{ts}-3c) were recovered and transformed into the *srb1-1* mutant, 7SLU. These transformants were then tested for sorbitol dependence at 30 °C and 37 °C. Strain 7SLU, carrying *PSA1* on a single-copy plasmid (pSRB1-9b), and the *psa1*^{ts} mutants in a *psa1*Δ strain (Table 1) were also included for comparison. The *psa1*^{ts} alleles

successfully restored growth in 7SLU without sorbitol at 30 °C, but failed to do so at 37 °C, just as was observed with the *psa1*Δ strains bearing the *psa1*^{ts} mutant alleles. However, when 7SLU was transformed with the wild-type *PSA1* allele, growth without sorbitol was restored at both 30 °C and 37 °C. As the *psa1*^{ts} alleles conferred the same phenotype on both 7SLU and the *psa1* null mutant, this finding is consistent with the hypothesis that *srb1-1* and *psa1*^{ts} are alleles of the same gene.

A DNA fragment carrying the *PSA1* gene with a *LEU2* marker located beside it was used to replace the *psa1* gene in a *srb1-1* strain (see Methods). Strain 7SUP3, generated in this way, was tested for its susceptibility to hypotonic shock stability when grown on complete or minimal media, with or without sorbitol, at 30 °C and 37 °C (Fig. 3). Replacing one allele with another at the same locus is the best strategy to avoid misleading results due to variations in expression or gene dosage. Therefore, the finding that replacement of the mutant allele by a wild-type allele of the *PSA1* gene simultaneously enabled growth to occur without sorbitol on both complete and minimal media at 30 °C and 37 °C demonstrates that *PSA1* is not merely a suppressor of *srb1-1*, but actually represents the wild-type allele of the gene.

Fig. 3. Integration of a *PSA1-LEU2* cassette into a *srb1*^{D276} strain (7SLU) restores the ability to grow without sorbitol. (a) Growth without sorbitol, (b) growth with sorbitol. For both parts, growth was on YEPD agar at 30 °C, with a ten-fold serial dilution from left to right; top row 7SUP6, middle row 7SUP3, bottom row 7SLU.

Identification of the mutations in the *srb1-1* and *psa1*^{ts} alleles of *PSA1*

The *psa1* allele in a *srb1-1* mutant was PCR amplified and cloned (see Methods). Two independently produced clones, called pSP1 and pSP2, were sequenced on both strands of the DNA. The sequence revealed a single nucleotide change from a guanosine in the wild-type *PSA1* at position 1588 (GenBank accession no. U19608) to an adenosine in the allele derived from a *srb1-1* strain. In the amino acid sequence of the protein produced (Fig. 4), this causes a glycine at position 276 to be replaced by an aspartic acid. We sequenced this region of the *PSA1* allele obtained from the gene library (on pSRB1-9b) using oligonucleotide *psa1a* and found that it matched the sequences in the database (GenBank accession nos U19608, U24437 and Z74103), most notably at the point where *srb1-1* differs. The replacement in strain 7SLU of this mutant allele by a wild-type, thereby replacing this one nucleotide, enables growth to occur without sorbitol. This indicates that *PSA1* is the wild-type *SRB1* gene. Having identified the nature of the *srb1-1* mutation, we shall henceforth refer to it as *srb1*^{D276}. Next, we sequenced, using the same oligonucleotides, the *psa1*^{ts} alleles that had been generated by Benton *et al.* (1996) using mutagenic PCR. It is interesting that this revealed the presence of mutations in *psa1*^{ts}-3c close to the site of the *srb1*^{D276} mutation, indicating that this region may be of importance to the protein's function.

<i>psa1p</i>	MKGLILVGGYGTRLRPLTLTVPKPLVEFGNRPMLHQIEALANAGVTDIVLAVNYRPEVM
<i>srb1p</i>	MKGLILVGGYGTRLRPLTLTVPKPLVEFGNRPMLHQIEALANAGVTDIVLAVNYRPEVM
<i>ts2dp</i>	MKGLILVGGYGTRLRPLTLTVPKPLVEFGNRPMLHQIEALANAGVTDIVLAVNYRPEVM
<i>ts3cp</i>	MKGLILVGGYGTRLRPLTLTVPKPLVEFGNRPMLHQIEALANAGVTDIVLAVNYRPEVM
<i>psa1p</i>	VETLKKYEKEYGVNITFSVETEPLGTAGPLKLAEDVLKKNDSPEFVLNSDVICYPFKEL
<i>srb1p</i>	VETLKKYEKEYGVNITFSVETEPLGTAGPLKLAEDVLKKNDSPEFVLNSDVICYPFKEL
<i>ts2dp</i>	VETLKKYEKEYGVNITFSVETEPLGTAGPLKLAEDVLKKNDSPEFVLNSDVICYPFKEL
<i>ts3cp</i>	VETLKKYEKEYGVNITFSVETEPLGTAGPLKLAEDVLKKNDSPEFVLNSDVICYPFKEL
<i>psa1p</i>	ADFHKAHGKGTIVATKVDEPSKYGVIVHDIAIATPNLIDRFVEKPKFVGNINAGLYILN
<i>srb1p</i>	ADFHKAHGKGTIVATKVDEPSKYGVIVHDIAIATPNLIDRFVEKPKFVGNINAGLYILN
<i>ts2dp</i>	ADFHKAHGKGTIVATKVDEPSKYGVIVHDIAIATPNLIDRFVEKPKFVGNINAGLYILN
<i>ts3cp</i>	ADFHKAHGKGTIVATKVDEPSKYGVIVHDIAIATPNLIDRFVEKPKFVGNINAGLYILN
<i>psa1p</i>	PEVIDLIEMKPTSIEKETFFILVEEKQLYSFDLEGFWMDVGQPKDFLSGTVLYLNSLAKR
<i>srb1p</i>	PEVIDLIEMKPTSIEKETFFILVEEKQLYSFDLEGFWMDVGQPKDFLSGTVLYLNSLAKR
<i>ts2dp</i>	PEVIDSIEMKPAIEKETFFILVEEKQLYSFDLEGFWMDVGQPKDFLSGTVLYLNSLAKR
<i>ts3cp</i>	PEVIDLIEMKPTSIEKETFFILVEEKQLYSFDLEGFWMDVGQPKDFLSGTVLYLNSLAKR
<i>psa1p</i>	QPKKLTATGANIVGNALIDPTAKISSTAKIGPDVIGPNVTIGDGVIRTSVVLNCSSTIKN
<i>srb1p</i>	QPKKLTATGANIVGNALIDPTAKISSTAKIGPDVIGPNVTIGDGVIRTSVVLNCSSTIKN
<i>ts2dp</i>	QPKKLTATGANIVGNALIDPTAKISSTAKIGPDVIGPNVTIGDGVIRTSVVLNCSSTIKN
<i>ts3cp</i>	QPKKLTATGANIVGNALIDPTAKISSTAKIGPDVIGPNVTIGDGVIRTSVVLNCSSTIKN
<i>psa1p</i>	HSLVKSTIVGWNSTVGGWCRLEGVTVLGGDVEVKDEIYINGGKVLPHKSISDNVPKEAII
<i>srb1p</i>	HSLVKSTIVGWNSTVGGWCRLEGVTVLGGDVEVKDEIYINGGKVLPHKSISDNVPKEAII
<i>ts2dp</i>	HSLVKSTIVGWNSTVGGWCRLEGVTVLGGDVEVKDEIYINGGKVLPHKSISDNVPKEAII
<i>ts3cp</i>	HSLVKSTIVGWNSTVGGWCRLEGVTVLGGDVEVKDEIYINGGKVLPHKSISDNVPKEAII
<i>psa1p</i>	M
<i>srb1p</i>	M
<i>ts2dp</i>	M
<i>ts3cp</i>	M
	*

Fig. 4. Alignment of the amino acid sequences of the proteins specified by different *PSA1* alleles. The top line is the wild-type sequence, the second is that of the *srb1*^{D276} mutant (the amino acid change is shown in bold), the third and fourth are the *psa1*^{ts} alleles described by Benton *et al.* (1996). * indicates identical amino acids. indicates an amino acid changed to one of similar characteristics and a space indicates a change to an amino acid of different characteristics.

Table 5. Tetrad analysis data from a cross between 7SLU and F7a

Tetrad type	<i>MBP1/MAT</i>	<i>SRB1/MAT</i>	<i>MBP1/SRB1</i>
Parental ditype	9	7	33
Non-parental ditype	7	6	0
Tetratype	41	22	2
Total	57	35	35

However, in *psa1*^{ts}-2d, the mutations are away from this region, suggesting that there are other areas of functional importance in this protein.

Tetrad analysis of a *srb1*^{D276} mutant reveals the presence of at least one further mutant gene

As well as the allele-replacement technique, we also used the more conventional technique of tetrad analysis to see if *PSA1* is the wild-type *SRB1* gene. However, when a

Table 6. Tetrad analysis data from a cross between 7SLU (*srb1-1/psa1 AAD4*) and G20b (*PSA1-LEU2 aad4::URA3*)

AAD4 is located close to the telomere of the left arm of chromosome IV (Delneri *et al.*, 1999), *SRB1/PSA1* is marked by a *LEU2* gene. Of the 52 tetrads analysed, 37 showed 2:2 segregation of sorbitol independence:dependence, 13 were 3:1 and 2 were 4:0, hence only 37 of the *srb1-1/PSA1-LEU2* tetrads could be classified. Only tetrads where both *URA3/ura3* and *LEU2/leu2* segregated in a 2:2 manner were used for this analysis.

Tetrad type	<i>PSA1-LEU2/ AAD4</i>	<i>srb1-1/ PSA1-LEU2</i>
Parental ditype	10	37
Non-parental ditype	11	
Tetratype	31	
Total	52	37

tetrad analysis of a cross between 7SLU and F7a (a wild-type strain) was conducted, only 38.6% of the spores were sorbitol dependent, as 22 of the 57 tetrads yielded more than two sorbitol-dependent colonies (Table 5). Since this is in contrast to our previous tetrad analysis result, in which sorbitol dependence to independence segregated 2:2 (Table 4), the analysis was repeated. This time however, strain 7SLU was crossed to YPH499PS, a wild-type yeast strain with a *LEU2* allele integrated beside the *PSA1* gene so that the segregation of *PSA1* could be easily followed. Table 6 shows that, again, 3:1 and 4:0 segregation of sorbitol independence to dependence was observed. The ability to follow the segregation pattern of *PSA1* allowed us to see that many *srb1*^{D276} allele-bearing colonies were not sorbitol dependent.

Taking this information together, we had to produce a new explanation for what was occurring. For reasons we outline in the Discussion, we believe that there is at least one further mutant *srb* gene in strain 7SLU that plays a role in determining sorbitol dependence.

To support our belief that there is more than one *srb* gene, a tetrad that yielded 3 sorbitol-independent and 1 sorbitol-dependent progeny from a 7SLU × YPH499 diploid was taken and the region of the *PSA1* gene in which the *srb1*^{D276} mutation is found was PCR amplified using primers *psa1a* and *psa1i*. When sequenced, using these same primers, it was found that the *srb1*^{D276} mutant allele segregated 2:2 in these colonies with the wild-type *PSA1* allele. Hence, as well as the sorbitol-dependent colony containing a *srb1*^{D276} allele, one of the sorbitol-independent colonies also contained this allele. This demonstrates that the *srb1*^{D276} gene is not the only one responsible for sorbitol dependence in strain 7SLU. The fact that *srb1*^{D276} does play a role in sorbitol dependence in 7SLU cannot be disputed, given the evidence above (particularly the finding that replacement of the *srb1*^{D276} allele in 7SLU by a wild-type *PSA1* allele restores the ability of the strain to grow without

Table 7. Lysis test results

The *A*₂₆₀ is an indication of the amount of nucleic acid released upon hypotonic shock. Thus higher values indicate greater lysis. Values are the mean of two tests.

Yeast strain (with relevant genotype)	<i>A</i> ₂₆₀
4SLUP (isogenic to 4STLU, <i>srb pde2</i>)	12.0
7SLU (<i>srb</i>)	9.6
7SUP3 (isogenic to 7SLU, <i>srb PSA1</i>)	8.4
4STLU (<i>srb</i>)	7.5
DJ12-9d (<i>pde2</i>)	3.5
F7a (wild-type strain)	1.8
YLG1 (isogenic to DJ12-9d, wild-type)	1.0
YPH499 (wild-type strain)	0.9

sorbitol). To confirm this finding, we repeated this test with a tetrad generated from a cross of BY4741 (an S288C-derived wild-type strain) with 7SLU and obtained an identical result.

The question of the initial tetrad analysis (Table 4), which did give 2:2 segregation of sorbitol-dependent to -independent colonies, also had to be addressed. For reasons explained in the Discussion, we believe the homozygous *pde2* background essentially masks the presence of additional *srb* gene(s) in the tetrad analysis in Table 4. Thus an additional analysis was conducted using a cross between YLG2 and 4STLU, which are isogenic to strains DJ12-9d and 4SLUP, but are wild-type for *PDE2*. Analysis of the tetrads from this cross again revealed 3:1 and 4:0, in addition to 2:2, sorbitol-independent to -dependent segregants, thus supporting our hypothesis [out of 33 tetrads (132 spores), 49 spores (37%) produced sorbitol-dependent colonies, a value consistent with the other tetrad dissections].

Hypotonic shock fragility and the Ras/cAMP dependent pathway

PDE2/SRA5, the gene encoding the yeast high-affinity cAMP phosphodiesterase, has been disrupted previously with a modest increase in the concentration of intracellular cAMP being reported (Sass *et al.*, 1986; Wilson & Tatchell, 1988). However, under most conditions, *pde2* mutants have a nearly wild-type phenotype. As it has been demonstrated that there is more than one *srb* gene, where traits associated with the presence of *srb1*^{D276} plus the other *srb* gene(s) are being referred to, we prefer the designation '*srb*' (without a number). Since *PDE2* has been found to be a multi-copy suppressor of sorbitol dependence in 4STLU, it was of interest to see if *pde2* disruptants have similar phenotypes to those conferred by the *srb* genes.

Table 7 gives the results of lysis tests performed with strains of different genotypes. The *srb* and *pde2* mutant strains had similar lysis levels; however, the *srb pde2* double mutant shows a substantially increased lysis ability. Comparison of the isogenic pair 7SLU and

Table 8. Transformation frequencies using the no-lithium method

Results are the mean of two experiments. YEp13 was used throughout, except for strains 7SLU and 7SUP3 where pRS425 was used.

Strain (with relevant genotype)	No. transformants ($\mu\text{g DNA}$) ⁻¹
4STLU (<i>srb</i>)	3.7×10^3
4SLUP (isogenic to 4STLU, <i>srb pde2</i>)	3.6×10^3
7SLU (<i>srb</i>)	6.1×10^2
DJ12-9d (<i>pde2</i>)	3.1×10^2
7SUP3 (isogenic to 7SLU, <i>srb PSA1</i>)	85
YLG1 (isogenic to DJ12-9d, wild-type)	9

7SUP3 (where 7SUP3 carries a wild-type *PSA1* gene) reveals that, although 7SUP3 has a reduced lysis ability, this is still much higher than that of wild-type strains YLG1 and YPH499. This is consistent with the premise that there is more than one *srb* gene. Disruption of *pde2* in strain DJ12-9d shows that it, too, has an increase in lysis ability compared to its isogenic wild-type partner, YLG1. Thus lysis ability can be regarded as a trait shared by *pde2* disruptants and *srb* family genes.

Intact cells carrying *srb* mutations can be transformed by a modification of the procedure described by Ito *et al.* (1983), which does not require the use of lithium ions (Philipova, 1985; Stateva *et al.*, 1991). We transformed several strains using this procedure. The results (Table 8) show that, like *srb* mutants, *pde2* disruptants were capable of transformation without the use of lithium ions. However, the *srb pde2* mutant, 4SLUP, did not show any increased competence for transformation compared to an isogenic strain (4STLU) carrying just the *srb* mutations. Comparison of the isogenic strains 7SLU (*srb1^{D276} srb*) and 7SUP3 (*PSA1 srb*) reveal that replacement of the *srb1^{D276}* allele results in a ten-fold drop in the transformation frequency, however this is still ten times that of a wild-type strain, YLG1. This, too, supports the finding that there is more than one *srb* gene.

Interaction with the *PKC1* pathway

As the *srb* mutations cause phenotypes similar to those caused by mutations in components of the *PKC1* MAP-kinase cascade, we investigated whether it was possible for overexpression of members of the cascade to rescue the sorbitol-dependence phenotype. Transformation of the *srb* strain 7SLU with one of either *PKC1*, *BCK1-20* (a hyper-activated allele of the *BCK1* gene), *MKK1* or *MPK1* on a multi-copy plasmid failed to rescue the sorbitol-dependency phenotype. *PKC1* was also tested to see if it could suppress the temperature sensitivity of the *psa1^{ts}* alleles, but was found to be unable to do so. Transformation of *pkc1* (YPKC1L) and *mpk1* (DEY293-42c) mutant strains with *PSA1* on a multi-copy plasmid

was also incapable of rescuing the sorbitol dependence and temperature-sensitivity phenotypes.

DISCUSSION

PSA1/VIG9, encoding GDP-mannose pyrophosphorylase, is the *bona fide* *SRB1* gene

The *srb* mutants were originally isolated on the basis of their sorbitol-dependent phenotype (Venkov *et al.*, 1974). Additional phenotypes include the ability to lyse upon hypotonic shock, antibiotic hypersensitivity and increased permeability of the cell wall and membrane due to defects in their structure and composition (Maerkisch *et al.*, 1983; Blagoeva *et al.*, 1991; Kopecka *et al.*, 1991). In a previous study (Stateva *et al.*, 1991), a yeast chromosomal gene was isolated that complemented sorbitol dependence in a number of *srb* strains, including 4STLU. We have now identified this gene as *PDE2* and further study has shown it to be a multi-copy suppressor of sorbitol dependence not, as previously reported, the *SRB1* gene. Tetrad analysis clearly demonstrates this point (Table 4). This suppressor activity of *PDE2* and the phenotypes indicative of compromised cell-wall integrity displayed by *pde2* mutants, provide evidence that the Ras/cAMP signal-transduction pathway plays a role in maintaining the structural integrity of the yeast cell wall. Such a function for the Ras/cAMP pathway in both yeasts and filamentous fungi has been suggested previously (Trevillyan & Pall, 1979; Baroni *et al.*, 1994; Tokiwa *et al.*, 1994).

In the attempt to clone the *bona fide* *SRB1* gene described in this paper, we isolated the gene *PSA1/VIG9* (Benton *et al.*, 1996; Hashimoto *et al.*, 1997). This gene complements the *srb1^{D276}* mutation when transformed into yeast on a single-copy plasmid. The temperature sensitivity at 37 °C of *psa1^{ts}* alleles was rescued by sorbitol, and *psa1^{ts}* alleles confer the same phenotype on both *srb1^{D276}* and *psa1Δ* mutants when carried on the same centromeric plasmid. Replacement of *PSA1* in a *srb1^{D276}* strain results in a sorbitol-independent phenotype (Fig. 3). Isolation and sequencing of the *PSA1* gene from a *srb1^{D276}* strain revealed the presence of a single base pair change, resulting in a glycine being replaced by an aspartic acid (Fig. 4) in the gene product. These results allow us to conclude that *PSA1* is the wild-type *SRB1* gene.

SRB1/PSA1/VIG9 has already been described in a number of reports. Benton *et al.* (1996) identified it as a plasmid suppressor of *alg1*. Disruption of this gene is lethal; however, phenotypes associated with mutations in it include vanadate resistance, geneticin hypersensitivity (Hashimoto *et al.*, 1997) and temperature sensitivity (Benton *et al.*, 1996). As we have found the temperature-sensitive phenotype to be rescuable by the addition of sorbitol to the growth media, susceptibility to hypotonic shock can also be added to this list of phenotypes. J. Schultz and G. Sprague (personal communication and GenBank accession no. U24437) isolated

PSA1 as a multicopy suppressor of a *dpm1* mutant. Both *ALG1* (Albright & Robbins, 1990) and *DPM1* (Orlean *et al.*, 1988) are essential genes involved in N-glycosylation (for reviews see Kukuruzinska *et al.*, 1987; Herscovics & Orlean, 1993; Orlean, 1997), and both use GDP-mannose as one of their substrates. *DPM1* has also been found to function in the O-mannosylation and GPI-anchoring pathways (Orlean, 1990).

The process of O-glycosylation has been found to be particularly important to cell-wall integrity. It involves addition of up to five mannose residues, donated either directly or indirectly by GDP-mannose, to a serine or threonine in a protein. Genes in the seven-member *PMT* family are responsible for the addition of the first mannose. Disruption of different members of this family can cause, in some cases, sorbitol dependence and, in others, death (Gentzsch & Tanner, 1996, 1997). Disruption of the addition of the second mannose causes a slow-growth phenotype (Lussier *et al.*, 1997a), as well as resistance to killer toxin and hypersensitivity to calcofluor white; traits associated with cell-wall defects (Lussier *et al.*, 1996, 1997b). N-glycosylation is also thought to play a role in maintaining cellular integrity, as manifested by the osmotic sensitivity of *mn9* disruptants, which are defective in 1,6- α -mannose backbone construction (Yip *et al.*, 1994). N-glycosylation mutants also exhibit a higher wall porosity, resulting in increased sensitivity to antibiotics such as neomycin and geneticin (Shimma *et al.*, 1997). In this last study, *PSA1* was cloned as a suppressor of the neomycin sensitivity of an *alg1* mutant. Increased cell-wall porosity is consistent with our finding that *srb* mutants are transformable without the need for lithium (Table 8).

Sorbitol dependence of *srb1-1* fragile mutants is a synthetic phenotype

Addressing the tetrad-analysis results, we suggest that the reason for the 3:1 and 4:0 segregation pattern in some tetrads is that sorbitol dependence is not dictated by *srb1*^{D276} alone, but requires the presence of at least one further mutant *srb* gene. However, as the total number of colonies that were sorbitol dependent from the two analyses given in Tables 5 and 6 is approximately 40%, i.e. between 25 and 50%, we can conclude that *srb1*^{D276} is linked to the other *srb* gene(s). The 2:2 segregation of sorbitol dependence to independence in our first tetrad analysis (Table 4) is, we suggest, due to it being conducted in a homozygous *pde2* background. The lysis test is a simple gauge of the degree of osmotic fragility of yeast strains. It measures the quantity of nucleic acid released when a strain is subjected to hypotonic shock. The lysis-test data (Table 7) demonstrate that a *pde2* disruptant exhibits increased lysis compared to a wild-type strain. The *srb pde2* strain 4SLUP shows very severe lysis, considerably worse than that of the isogenic equivalent 4STLU, which has a wild-type *PDE2* gene. Thus *pde2* disruption clearly affects cell-wall integrity and this is an additive effect with other mutations. We suggest that the *srb1*^{D276} mutation on its

own does not cause sufficient weakening of the cell wall to cause sorbitol dependence, but that it requires a further gene, which also plays a role in maintaining cell-wall integrity, to be mutated or disrupted. Thus, in the first tetrad analysis (Table 4), *srb1*^{D276} was always with a *pde2* disruptant regardless of how any other *srb* gene segregated. However, the other tetrad analyses were conducted in homozygous *PDE2* backgrounds with, in each case, one of the parents being essentially wild-type in character. Hence, when segregation of the *srb1*^{D276} gene away from the other *srb* gene(s) occurred, there were a number of 3:1 and 4:0 sorbitol-independent to sorbitol-dependent tetrads. In a separate study, a comparison of strain 7SUP3 (*PSA1 srb*) to BY4741 (an S288C derived wild-type strain) on calcofluor white media (0.5 mg l⁻¹ in YEPD agar) and SDS media (0.09% SDS in YEPD agar) found that 7SUP3 was considerably more sensitive than BY4741. As these are standard tests for cell-wall sensitivity (Ram *et al.*, 1994; Lussier *et al.*, 1997c), these findings indicate phenotypes that could be expected of additional mutant *srb* gene(s), and may subsequently be used in cloning experiments.

Our finding of shared phenotypes for *pde2* disruptants and *srb* strains also supports the premise that the Ras/cAMP pathway plays a role in maintaining cell-wall integrity. However, the lysis and transformation data (Tables 7 and 8) also demonstrate the complexity of the relationships involved. Whilst introduction of a wild-type *PSA1* allele into strain 7SLU (to produce strain 7SUP3) rescues sorbitol dependence and greatly reduces transformability using the no-lithium method, lysis ability remains high. Disruption of *PDE2* in strain YLG1 (to produce DJ12-9d) also increases both transformability and lysis ability without causing sorbitol dependence. Whilst a *srb* strain carrying a *pde2* disruption (strain 4SLUP), as would be expected, remains sorbitol dependent, and shows enhanced lysis ability compared to its isogenic partner 4STLU, there is no increase in transformability. Although sorbitol dependence, lysis ability and transformability are all cell-wall-dependent features, different elements of the cell wall are clearly of importance in determining the nature of each trait.

A role for the Ras/cAMP signal-transduction pathway in the control of wall biogenesis

A clear elucidation of the signal pathways involved in cellular integrity in the *srb* strains will require not only the identification of the other *srb* gene(s), but also a greater understanding of the Ras/cAMP and *PKC1* signal cascades, as rescue of the strains may be due to the impact of cAMP depletion on *srb* gene(s) other than *SRB1*, or a wide range of genes and cellular processes leading to the general effect of increased cell-wall rigidity. Our finding that members of the *PKC1* MAP kinase cascade did not suppress sorbitol dependence implies that these genes do not regulate the *srb* family. However, it is known that the *PKC1* MAP kinase cascade regulates the Swi4/6p transcription factor

(Madden *et al.*, 1997), which in turn acts on SCB (Swi4-Swi6-dependent cell-cycle box) boxes (Dirick *et al.*, 1992). Benton *et al.* (1996) reported near-consensus SCB boxes in the promoter region of *PSA1/SRB1*. This would suggest that *PSA1* could be under the control of the *PKC1* MAP kinase. Our failure to find any rescue of the sorbitol dependence of *srb* mutants may indicate that the regulatory distance is too great and the mechanism too complex to allow this to occur.

Our findings that overexpression of *PDE2* rescues a sorbitol-dependent strain, and that disruption of *pde2* causes lysis ability (Table 7) and transformability without lithium (Table 8), traits shared by the *srb* mutant strains which are known to have a fragile cell wall, allow us to conclude that the Ras/cAMP pathway plays a role in maintaining cellular integrity. The role of Ras/cAMP in nutrient sensing (Thevelein, 1994) and phenotypes related to *pde2* disruption, such as heat shock and starvation sensitivity (Sass *et al.*, 1986), have long been known. Links with cell-wall construction have become apparent in recent years. The pseudohyphal development that yeast undergoes in response to nitrogen starvation is regulated in part by the Ras/cAMP pathway. Ras2p is able to signal either to the MAP kinase pheromone-response pathway, causing shmoo formation, or to protein kinase A, causing filamentation (Mosch *et al.*, 1999). In the PKA pathway, it is the three PKA catalytic subunits that can either stimulate (*TPK2*) or inhibit (*TPK1* and *TPK3*) pseudohyphal development (Pan & Heitmann, 1999). Both MAP kinase and cAMP signals converge on the large promoter of *FLO11* (Rupp *et al.*, 1999).

In addition to a role in pseudohyphal formation, a function for the Ras/cAMP pathway in the osmotic stress response is becoming apparent. One known downstream target of the PKA pathway is the stress-response element (STRE) promoter sequence (CCCCT). This site is important in the regulation of stress-related genes since it is a target of the HOG MAP-kinase cascade (Varela *et al.*, 1995; Molina *et al.*, 1998) and the transcription factors Msn2p and Msn4p (Schmitt & McEntee, 1996; Martinez-Pastor *et al.*, 1996) which increase transcription of genes that enable the cell to endure greater stress. In contrast, the PKA pathway is thought to negatively regulate genes bearing an STRE sequence (Varela *et al.*, 1995; Thevelein & de Winder, 1999; Sasaki *et al.*, 2000), thereby reducing the cell's tolerance to stress, but also allowing more rapid growth. The *GRE1* gene, which has an unknown function, was found to be induced by different stresses, including hyperosmotic conditions. As well as being under the control of the HOG pathway, it is also regulated negatively by PKA and positively by the transcription factors Msn2/4p (Garay-Arroyo & Covarrubias, 1999). Norbeck & Blomberg (2000) conducted an extensive analysis of genes that are transcriptionally regulated by PKA in response to hypertonic stress. Low PKA activity and high osmotolerance correlated with increased expression of stress-induced proteins such as Ctl1p (an oxidative-stress resistance protein). The promoters of

all induced PKA-dependent genes had STRE sites. They also found that mutants with constitutively low PKA activity, as would be the case when *PDE2* is overexpressed, caused an expression profile consistent with osmotically stressed cells, thereby making them more tolerant of this stress. Cells in stationary phase show increased resistance to almost all forms of stress (Werner-Washburne *et al.*, 1993). This is consistent with our work; mid-exponential phase cells are used for the lysis test, as those in the stationary phase give lower lysis values (data not shown). Cells undergo an increase in heat-shock tolerance as they enter stationary phase (Parry *et al.*, 1976; Elliott & Fletcher, 1993). As cells growing slowly or in stationary phase have low PKA activity (Thevelein, 1994), it is possible that overexpression of *PDE2* may exert its influence by altering the cells expression profile to one that is more tolerant of stress. Research carried out thus far has linked the low PKA activity induced by osmotic stress to hyperosmotic conditions. In this study, we have found evidence that it also appears to rescue cells in hypotonic stress conditions.

Growth is integrated with division in the G₁ phase of the yeast cell cycle, therefore the synthesis of the cell-wall components should be tightly regulated with the cell cycle during bud enlargement. A cell-division-cycle pathway exists linking *PKC1* to activation of *CDC28* at START (Marini *et al.*, 1996), thus providing a link between glucan synthesis and the cell cycle. *PSA1* has a similar expression profile to that of *CLN2* (Benton *et al.*, 1996). As *CLN2* is negatively regulated by PKA in order to delay START when cells are growing on rich media, thereby allowing a large cell to develop before budding commences (Baroni *et al.*, 1994; Tokiwa *et al.*, 1994), similar regulation of *PSA1* could ensure that its peak expression is timed to coincide with the cell committing to budding.

As well as general effects on the cell, PKA activity may also more directly impact on cell-wall integrity. Mannosylphosphorylation involves the transfer of a mannose 1-phosphate residue from GDP-mannose to N- and O-linked oligosaccharides (Odani *et al.*, 1996; Nakayama *et al.*, 1998). Two genes involved in this process, *MNN6* (Wang *et al.*, 1997) and *MNN4* (Odani *et al.*, 1996), encode a mannosylphosphate transferase and its positive regulator, respectively. Odani *et al.* (1997) found that under conditions of osmotic stress and in late exponential/stationary phase, the level of mannosylphosphorylation increased. It was also found that the process of mannosylphosphorylation was negatively regulated by PKA, with overexpression of the Tpk1p subunit decreasing mannosylphosphorylation and overexpression of Pde1p (Nikawa *et al.*, 1987) increasing it. In a strain carrying a *srb1-1* mutation, it would be expected that GDP-mannose availability would be reduced. As overexpression of *PDE2* could be expected to optimize mannosylphosphorylation of mannoproteins, this could be a factor in increasing cell-wall rigidity of *srb* mutants to allow rescue of sorbitol dependency.

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REFERENCES

- Albright, C. F. & Robbins, P. W. (1990). The sequence and transcript heterogeneity of the yeast gene *ALG1*, an essential mannosyltransferase involved in *N*-glycosylation. *J Biol Chem* **265**, 7042–7049.
- Arkininstall, S. J., Papasavvas, S. G. & Payton, M. A. (1991). Yeast α -mating factor receptor-linked G-protein signal transduction suppresses Ras-dependent activity. *FEBS Lett* **284**, 123–128.
- Baroni, M. D., Monti, P. & Alberghina, L. (1994). Repression of growth-regulated G1 cyclin expression by cyclic-AMP in budding yeast. *Nature* **371**, 339–342.
- Benton, B. K., Driscoll-Plump, S., Roos, J., Lennarz, W. & Cross, F. (1996). Overexpression of *Saccharomyces cerevisiae* G₁ cyclins restores the viability of *alg1* *N*-glycosylation mutants. *Curr Genet* **29**, 106–113.
- Birnboim, H. C. & Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res* **7**, 1513–1523.
- Blagoeva, J., Stoev, G. & Venkov, P. V. (1991). Glucan structure in a fragile mutant of *Saccharomyces cerevisiae*. *Yeast* **7**, 455–461.
- Brachmann, C. B., Davies, A., Cost, G. J., Caputo, E., Li, J., Hieter, P. & Boeke, J. D. (1998). Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* **14**, 115–132.
- Broach, J. R. & Deschenes, R. J. (1990). The function of RAS genes in *Saccharomyces cerevisiae*. *Adv Cancer Res* **54**, 79–139.
- Broach, J., Strathern, J. & Hicks, J. (1979). Transformation in yeast: development of a hybrid cloning vector and isolation of the *CAN1* gene. *Gene* **8**, 121–133.
- Broach, J. R. (1991). RAS genes in *Saccharomyces cerevisiae*: signal transduction in search of a pathway. *Trends Genet* **7**, 28–33.
- Bullock, W. O., Fernandez, J. M. & Short, J. M. (1987). XL1-Blue: a high-efficiency plasmid transforming *recA* *Escherichia coli* strain with β -galactosidase selection. *Biotechniques* **5**, 376–378.
- Christianson, T. W., Sikorski, R. S., Dante, M., Shero, J. H. & Hieter, P. (1992). Multifunctional yeast high-copy-number shuttle vectors. *Gene* **110**, 119–122.
- Cid, V. J., Duran, A., Delrey, F., Snyder, M. P., Nombela, C. & Sanchez, M. (1995). Molecular basis of cell integrity and morphogenesis in *Saccharomyces cerevisiae*. *Microbiol Rev* **59**, 345–386.
- Costigan, C., Gehrung, S. & Snyder, M. (1992). A synthetic lethal screen identifies *SLK1*, a novel protein kinase homolog implicated in yeast cell morphogenesis and cell growth. *Mol Cell Biol* **12**, 1162–1178.
- Cross, G. A. M. (1990). Glycolipid anchoring of plasma membrane proteins. *Annu Rev Cell Biol* **6**, 1–39.
- Delneri, D., Gardner, D. C., Bruschi, C. V. & Oliver, S. G. (1999). Disruption of seven hypothetical aryl alcohol dehydrogenase genes from *Saccharomyces cerevisiae* and construction of a multiple knock-out strain. *Yeast* **15**, 1681–1689.
- Dirick, L., Moll, T., Auer, H. & Nasmyth, K. (1992). A central role for SWI6 in modulating cell cycle START-specific transcription in yeast. *Nature* **357**, 508–513.
- Elliott, B. & Fitcher, B. (1993). Stress resistance of yeast cells is largely independent of cell cycle phase. *Yeast* **9**, 33–42.
- Englund, P. T. (1993). The structure and biosynthesis of glycosyl phosphatidylinositol protein anchors. *Annu Rev Biochem* **62**, 121–138.
- Garay-Arroyo, A. & Covarrubias, A. A. (1999). Three genes whose expression is induced by stress in *Saccharomyces cerevisiae*. *Yeast* **15**, 879–892.
- Gardner, D. C. J., Tomlin, G. C., Cele, T., Hamilton, G. A., James, C. M., Stateva, L. I. & Oliver, S. G. (1996). Physical mapping of the centromere-proximal region of chromosome IV-L defines the placement of genes *USO1*, *MBP1*, *PSA1* and *SLC1*. *Yeast* **12**, 411–413.
- Gentzsch, M. & Tanner, W. (1996). The PMT gene family: protein O-glycosylation in *Saccharomyces cerevisiae* is vital. *EMBO J* **15**, 5752–5759.
- Gentzsch, M. & Tanner, W. (1997). Protein O-glycosylation in yeast: protein-specific mannosyltransferases. *Glycobiology* **7**, 481–486.
- Gimeno, C. J., Ljungdahl, P. O., Styles, C. A. & Fink, G. R. (1992). Unipolar cell divisions in the yeast *S. cerevisiae* lead to filamentous growth: regulation by starvation and RAS. *Cell* **68**, 1077–1090.
- Hashimoto, H., Sakakibara, Y., Yamasaki, M. & Yoda, K. (1997). *Saccharomyces cerevisiae* VIG9 encodes GDP-mannose pyrophosphorylase, which is essential for protein glycosylation. *J Biol Chem* **272**, 16308–16314.
- Heery, D. M., Cannon, F. & Powell, R. (1990). A simple method for subcloning DNA fragments from gel slices. *Trends Genet* **6**, 173.
- Herscovics, A. & Orlean, P. (1993). Glycoprotein biosynthesis in yeast. *FASEB J* **7**, 540–550.
- Hill, J., Ian, K. A., Donald, G. & Griffiths, D. E. (1991). DMSO-enhanced whole cell yeast transformation. *Nucleic Acids Res* **19**, 5791.
- Innis, M. A. & Gelfand, D. H. (1990). Optimization of PCR. In *PCR Protocols – A Guide to Methods and Applications*, pp. 3–12. Edited by M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White. San Diego, CA: Academic Press.
- Irie, K., Levin, D. E., Levin, K. S., Bakase, M., Araki, H., Matsumoto, K. & Oshima, Y. (1993). *MKK1* and *MKK2*, which encode *Saccharomyces cerevisiae* mitogen activated protein kinase kinase homologs, function in the pathway mediated by protein kinase C. *Mol Cell Biol* **13**, 3076–3083.
- Ito, H., Fukuda, Y., Murata, K. & Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. *J Bacteriol* **153**, 163–168.
- James, C. M., Indge, K. J. & Oliver, S. G. (1995). DNA sequence analysis of a 35 kb segment from *Saccharomyces cerevisiae* chromosome VII reveals 19 open reading frames including *RAD54*, *ACE1/CUP2*, *PMR1*, *RCK1*, *AMS1* and *CAL1/CDC43*. *Yeast* **11**, 1413–1419.

- Kaiser, C., Michaelis, S. & Mitchell, A. (1994). *Methods in Yeast Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Klis, F. M. (1994). Cell-wall assembly in yeast. *Yeast* **10**, 851–869.
- Kobayashi, O., Suda, H., Ohtani, T. & Sone, H. (1996). Molecular cloning and analysis of the dominant flocculation gene *FLO8* from *Saccharomyces cerevisiae*. *Mol Gen Genet* **251**, 707–715.
- Kopecka, M., Gabriel, M., Necas, O., Svoboda, A. & Venkov, P. V. (1991). Cell surface structures in osmotically fragile mutants of *Saccharomyces cerevisiae*. *J Gen Microbiol* **137**, 1263–1270.
- Kozhina, T., Stateva, L. & Venkov, P. (1979). Genetic analysis of an osmotic sensitive *Saccharomyces cerevisiae* mutant. *Mol Gen Genet* **170**, 351–354.
- Kukuruzinska, M. A., Bergh, M. L. E. & Jackson, B. J. (1987). Protein glycosylation in yeast. *Annu Rev Biochem* **56**, 915–944.
- Lee, K. S. & Levin, D. E. (1992). Dominant mutations in a gene encoding a putative protein kinase (*BCK1*) bypasses the requirement for *Saccharomyces cerevisiae* protein kinase C homolog. *Mol Cell Biol* **12**, 172–182.
- Lee, K., Irie, K., Gotoh, Y., Watanabe, Y., Araki, H., Nishida, E., Matsumoto, K. & Levin, D. (1993). A yeast mitogen activated protein kinase homolog (Mpk1p) mediates signalling by protein kinase C. *Mol Cell Biol* **13**, 3067–3075.
- Leidich, S. D., Kostova, Z., Latek, R. R., Costello, L. C., Drapp, D. A., Gray, W., Fassler, J. S. & Orlean, P. (1995). Temperature-sensitive yeast GPI anchoring mutants *gpi2* and *gpi3* are defective in the synthesis of N-acetylglucosaminyl phosphatidylinositol: cloning of the *GPI2* gene. *J Biol Chem* **270**, 13029–13035.
- Levin, D. E. & Bartlett-Heubusch, E. (1992). Mutants in the *Saccharomyces cerevisiae* *PKC1* gene display a cell cycle-specific osmotic stability defect. *J Cell Biol* **116**, 1221–1229.
- Lussier, M., Sdicu, A. M., Camirand, A. & Bussey, H. (1996). Functional characterization of the *YUR1*, *KTR1*, and *KTR2* genes as members of the yeast *KRE2/MNT1* mannosyltransferase gene family. *J Biol Chem* **271**, 11001–11008.
- Lussier, M., Sdicu, A. M., Bussereau, F., Jacquet, M. & Bussey, H. (1997a). The Ktr1p, Ktr3p, and Kre2p/Mnt1p mannosyltransferases participate in the elaboration of yeast O- and N-linked carbohydrate chains. *J Biol Chem* **272**, 15527–15531.
- Lussier, M., Sdicu, A. M., Winnett, E., Vo, D. H., Sheraton, J., Dusterhoft, A., Storms, R. K. & Bussey, H. (1997b). Completion of the *Saccharomyces cerevisiae* genome sequence allows identification of *KTR5*, *KTR6* and *KTR7* and definition of the nine-membered *KRE2/MNT1* mannosyltransferase gene family in this organism. *Yeast* **13**, 267–274.
- Lussier, M., White, A. M., Sheraton, J. & 17 other authors (1997c). Large scale identification of genes involved in cell surface biosynthesis and architecture in *Saccharomyces cerevisiae*. *Genetics* **147**, 435–450.
- Madden, K., Sheu, Y. J., Baetz, K., Andrews, B. & Snyder, M. (1997). SBF cell cycle regulator as a target of the yeast PKC-MAP kinase pathway. *Science* **275**, 1781–1784.
- Maerkisch, U., Reuter, G., Stateva, L. I. & Venkov, P. (1983). Mannan structure analysis of the fragile *Saccharomyces cerevisiae* mutant VY1160. *Int J Biochem* **15**, 1373–1377.
- Marini, N. J., Meldrum, E., Buehrer, B., Hubberstey, A. V., Stone, D. E., Traynor-Kaplan, A. & Reed, S. I. (1996). A pathway in the yeast cell division cycle linking protein kinase C (Pkc1) to activation of Cdc28 at START. *EMBO J* **15**, 3040–3052.
- Martinez-Pastor, M. T., Marchler, G., Schuller, C., Marchler-Bauer, A., Ruis, H. & Estruch, F. (1996). The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). *EMBO J* **15**, 2227–2235.
- Matsumoto, K., Uno, I., Toh-e, A., Ishikawa, T. & Oshima, Y. (1982). Cyclic AMP may not be involved in catabolite repression in *Saccharomyces cerevisiae*: evidence from mutants capable of utilizing it as an adenosine source. *J Bacteriol* **150**, 277–285.
- Mazzoni, C., Zarzov, P., Rambourg, A. & Mann, C. (1993). The *SLT2/MPK1* MAP kinase homolog is involved in polarised growth in *Saccharomyces cerevisiae*. *J Cell Biol* **123**, 1821–1833.
- Molina, M., Martin, H., Sanchez, M. & Nombela, C. (1998). MAP kinase-mediated signal transduction pathways. In *Yeast Gene Analysis: Methods in Microbiology*, vol. 26, pp. 375–393. Edited by A. J. P. Brown & M. F. Tuite. San Diego, CA: Academic Press.
- Mosch, H. U., Kubler, E., Krappmann, S., Fink, G. R. & Braus, G. H. (1999). Crosstalk between the Ras2p-controlled mitogen-activated protein kinase and cAMP pathways during invasive growth of *Saccharomyces cerevisiae*. *Mol Biol Cell* **10**, 1325–1335.
- Nakayama, K., Feng, Y., Tanaka, A. & Jigami, Y. (1998). The involvement of *mn4* and *mn6* mutations in mannosylphosphorylation of O-linked oligosaccharide in yeast *Saccharomyces cerevisiae*. *Biochim Biophys Acta* **1425**, 255–262.
- Nikawa, J., Sass, P. & Wigler, M. (1987). Cloning and characterization of the low-affinity cyclic AMP phosphodiesterase gene of *Saccharomyces cerevisiae*. *Mol Cell Biol* **7**, 3629–3636.
- Norbeck, J. & Blomberg, A. (2000). The level of cAMP-dependent protein kinase A activity strongly affects osmotolerance and osmo-instigated gene expression changes in *Saccharomyces cerevisiae*. *Yeast* **16**, 121–137.
- Odani, T., Shimma, Y., Tanaka, A. & Jigami, Y. (1996). Cloning and analysis of the *MNN4* gene required for phosphorylation of N-linked oligosaccharides in *Saccharomyces cerevisiae*. *Glycobiology* **6**, 805–810.
- Odani, T., Shimma, Y., Wang, X. H. & Jigami, Y. (1997). Mannosylphosphate transfer to cell wall mannan is regulated by the transcriptional level of the *MNN4* gene in *Saccharomyces cerevisiae*. *FEBS Lett* **420**, 186–190.
- Orlean, P. (1990). Dolichol phosphate mannose synthase is required *in vivo* for glycosyl phosphatidylinositol membrane anchoring, O-mannosylation, and N-glycosylation of protein in *Saccharomyces cerevisiae*. *Mol Cell Biol* **10**, 5796–5805.
- Orlean, P. (1997). Biogenesis of yeast wall and surface components. In *The Molecular Biology of the Yeast Saccharomyces*, vol. 3, pp. 229–362. Edited by J. R. Pringle, J. R. Broach & E. W. Jones. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Orlean, P., Albright, C. & Robbins, P. W. (1988). Cloning and sequencing of the yeast gene for dolichol phosphate mannose synthase, an essential protein. *J Biol Chem* **263**, 17499–17507.
- Pan, X. & Heitman, J. (1999). Cyclic AMP-dependent protein kinase regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. *Mol Cell Biol* **19**, 4874–4887.
- Paravicini, G., Cooper, M., Friedli, L., Smith, D. J., Carpenter, J. L., Klig, L. & Payton, M. A. (1992). The osmotic integrity of the yeast cell requires a functional *PKC1* gene product. *Mol Cell Biol* **12**, 4896–4905.
- Parry, J. M., Davies, P. J. & Evans, W. E. (1976). The effects of ‘cell age’ upon the lethal effects of physical and chemical mutagens in the yeast, *Saccharomyces cerevisiae*. *Mol Gen Genet* **146**, 27–35.
- Philipova, D. (1985). *Transformation of fragile mutants of S. cerevisiae*. PhD thesis, Bulgarian Academy of Science.
- Ram, A. F., Wolters, A., Ten Hoopen, R. & Klis, F. M. (1994). A new

- approach for isolating cell-wall mutants in *Saccharomyces cerevisiae* by screening for hypersensitivity to calcofluor white. *Yeast* **10**, 1019–1030.
- Rose, M. D., Novick, P., Thomas, J. H., Botstein, D. & Fink, G. (1987). A *Saccharomyces cerevisiae* genomic plasmid bank based on a centromere-containing shuttle vector. *Gene* **60**, 237–243.
- Rupp, S., Summers, E., Lo, H. J., Madhani, H. & Fink, G. (1999). MAP kinase and cAMP filamentation signaling pathways converge on the unusually large promoter of the yeast *FLO11* gene. *EMBO J* **18**, 1257–1269.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sasaki, T., Toh-e, A. & Kikuchi, Y. (2000). Extragenic suppressors that rescue defects in the heat stress response of the budding yeast mutant *tom1*. *Mol Gen Genet* **262**, 940–948.
- Sass, P., Field, J., Nikawa, J., Toda, T. & Wigler, M. (1986). Cloning and characterisation of the high-affinity cAMP phosphodiesterase of *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* **83**, 9303–9307.
- Schmitt, A. P. & McEntee, K. (1996). Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* **93**, 5777–5782.
- Sherman, F., Fink, G. R. & Hicks, J. B. (1986). *Methods in Yeast Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Shimma, Y., Nishikawa, A., bin Kassim, B., Eto, A. & Jigami, Y. (1997). A defect in GTP synthesis affects mannose outer chain elongation in *Saccharomyces cerevisiae*. *Mol Gen Genet* **256**, 469–480.
- Sikorski, R. S. & 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.
- Stateva, L. I., Oliver, S. G., Trueman, L. J. & Venkov, P. V. (1991). Cloning and characterisation of a gene which determines osmotic stability in *Saccharomyces cerevisiae*. *Mol Cell Biol* **11**, 4235–4243.
- Tanner, W. & Lehle, L. (1987). Protein glycosylation in yeast. *Biochim Biophys Acta* **906**, 81–99.
- Thevelein, J. M. (1994). Signal transduction in yeast. *Yeast* **10**, 1753–1790.
- Thevelein, J. M. & de Winde, J. H. (1999). Novel sensing mechanisms and targets for the cAMP-protein kinase A pathway in the yeast *Saccharomyces cerevisiae*. *Mol Microbiol* **33**, 904–918.
- Toda, T., Cameron, S., Sass, P. & Wigler, M. (1987a). Three different genes in the yeast *Saccharomyces cerevisiae* encode the catalytic subunits of the cAMP-dependent protein kinase. *Cell* **50**, 277–287.
- Toda, T., Cameron, S., Sass, P., Zoller, M., Scott, J. D., McMullen, B., Hurwitz, M., Krebs, E. G. & Wigler, M. (1987b). Cloning and characterisation of *BCY1*, a locus encoding the regulatory subunit of the cAMP-dependent protein kinase in yeast. *Mol Cell Biol* **7**, 1371–1377.
- Tokiwa, G., Tyers, M., Volpe, T. & Futcher, B. (1994). Inhibition of G_1 cyclin activity by the RAS/cAMP pathway in yeast. *Nature* **371**, 342–345.
- Trevillyan, J. M. & Pall, M. L. (1979). Control of cAMP levels by depolarising agents in fungi. *J Bacteriol* **138**, 397–403.
- Varela, J. C., Praekelt, U. M., Meacock, P. A., Planta, R. J. & Mager, W. H. (1995). The *Saccharomyces cerevisiae* *HSP12* gene is activated by the high-osmolarity glycerol pathway and negatively regulated by protein kinase A. *Mol Cell Biol* **15**, 6232–6245.
- Venkov, P. V., Hadjiolov, A. A., Battaner, E. & Schlessinger, D. (1974). *Saccharomyces cerevisiae* sorbitol dependent fragile mutants. *Biochem Biophys Res Commun* **56**, 559–604.
- Venkov, P. V., Milchev, G. I. & Hadjiolov, A. A. (1975). Rifampicin susceptibility of ribonucleic acid synthesis in a fragile *Saccharomyces cerevisiae* mutant. *Antimicrob Agents Chemother* **8**, 627–632.
- Waltchewa, L. W., Venkov, P. V., Stoyanova, B. B. & Hadjiolov, A. A. (1976). Degradation of ribosomal precursor and poly-adenylic acid-containing ribonucleic acids in *Saccharomyces cerevisiae* caused by actinomycin D. *Arch Biochem Biophys* **176**, 630–637.
- Wang, X. H., Nakayama, K., Shimma, Y., Tanaka, A. & Jigami, Y. (1997). *MNN6*, a member of the *KRE2/MNT1* family, is the gene for mannosylphosphate transfer in *Saccharomyces cerevisiae*. *J Biol Chem* **272**, 18117–18124.
- Ward, A. C. (1990). Single step purification of shuttle vectors from yeast for high frequency back transformation into *Escherichia coli*. *Nucleic Acids Res* **18**, 5319.
- Werner-Washburne, M., Braun, E., Johnston, G. C. & Singer, R. A. (1993). Stationary phase in the yeast *Saccharomyces cerevisiae*. *Microbiol Rev* **57**, 383–401.
- Wilson, R. B. & Tatchell, K. (1988). *SRA5* encodes the low K_m cyclic AMP phosphodiesterase of *Saccharomyces cerevisiae*. *Mol Cell Biol* **8**, 505–510.
- Yanisch-Perron, C., Vieira, J. & Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.
- Yip, C. L., Welch, S. K., Klebl, F., Gilbert, T., Seidel, P., Grant, F. J., O'Hara, P. J. & MacKay, V. L. (1994). Cloning and analysis of the *Saccharomyces cerevisiae* *MNN9* and *MNN1* genes required for complex glycosylation of secreted proteins. *Proc Natl Acad Sci USA* **91**, 2723–2727.

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