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# Response to high osmotic conditions and elevated temperature in *Saccharomyces cerevisiae* is controlled by intracellular glycerol and involves coordinate activity of MAP kinase pathways

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In the yeast *Saccharomyces cerevisiae*, response to an increase in external osmolarity is mediated by the HOG (high osmolarity glycerol) MAP kinase pathway. HOG pathway mutant strains display osmosensitive phenotypes. Recently evidence has been obtained that the osmosensitivity of HOG pathway mutants is reduced during growth at elevated temperature (37 °C). A notable exception is the *ste11ssk2ssk22* mutant, which displays hypersensitivity to osmotic stress at 37 °C. This paper reports that overexpression of *FPS1* or *GPD1* (encoding the glycerol transport facilitator and glycerol-3-phosphate dehydrogenase, respectively, and both affecting intracellular glycerol levels) reduces the hypersensitivity to osmotic stress of *ste11ssk2ssk22* at 37 °C. Although in this particular HOG pathway mutant a correlation between suppression of the phenotype and glycerol content could be demonstrated, the absolute level of intracellular glycerol per se does not determine whether a strain is osmosensitive or not. Rather, evidence was obtained that the glycerol level may have an indirect effect, viz. by influencing signalling through the PKC (protein kinase C) MAP kinase pathway, which plays an important role in maintenance of cellular integrity. In order to validate the data obtained with a HOG pathway mutant strain for wild-type yeast cells, MAP kinase signalling under different growth conditions was examined in wild-type strains. PKC pathway signalling, which is manifest at elevated growth temperature by phosphorylation of MAP kinase Mpk1p, is rapidly lost when cells are shifted to high external osmolarity conditions. Expression of *bck1-20* or overexpression of *WSC3* in wild-type cells resulted in restoration of PKC signalling. Both PKC and HOG signalling, cell wall phenotypes and high osmotic stress responses in wild-type cells were found to be influenced by the growth temperature. The data taken together indicate the intricate interdependence of growth temperature, intracellular glycerol, cell wall structure and MAP kinase signalling in the hyperosmotic stress response of yeast.

## INTRODUCTION

Exposure of yeast cells to high osmotic conditions in the surrounding medium results in rapid loss of intracellular water (Hohmann, 1997) followed by a collapse of the cytoskeleton (Chowdhury *et al.*, 1992), intracellular damage and arrest of growth (Gustin *et al.*, 1998; Alexander *et al.*, 2001; Belli *et al.*, 2001; Hohmann, 2002). The aim of the adaptive responses is to regain turgor, reassemble a polarized cytoskeleton, repair cellular damage and resume growth. The response of *Saccharomyces cerevisiae* to a high osmotic challenge is controlled by the HOG (high-osmolarity glycerol) MAP kinase pathway (Gustin *et al.*, 1998). Activation of Pbs2p, the MAPKK of the HOG pathway, occurs through at least two different osmosensing mechanisms (Maeda *et al.*, 1994, 1995). A 'three-component' histidine kinase signalling system links the putative osmosensing protein Sln1p via Ypd1p, Ssk1p and the MAPKKKs Ssk2 and Ssk22p to Pbs2p (Posas *et al.*, 1996; Posas & Saito, 1998). In the other branch of this pathway the most upstream component characterized thus far, viz. Sho1p, functions to link an as-yet-unidentified osmosensor

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Abbreviations: HOG, high-osmolarity glycerol; MAP, mitogen-activated protein; PKC, protein kinase C; SVG, sterile vegetative growth.

to the downstream components Cdc42p, Ste20p, Ste50p and Ste11p (O'Rourke & Herskowitz, 1998; Posas *et al.*, 1998; Raitt *et al.*, 2000; Reiser *et al.*, 2000). In this branch of the pathway Ste11p functions as the MAPKKK that activates Pbs2p. Activated Pbs2p phosphorylates the MAPK Hog1p by dual phosphorylation. Once activated, Hog1p in its turn activates cytoplasmic target proteins such as Rck2p (Bilsland-Marchesan *et al.*, 2000) and, after translocation to the nucleus (Ferrigno *et al.*, 1998; Reiser *et al.*, 1999), regulates osmostress-responsive gene expression (Proft & Serrano, 1999; Rep *et al.*, 1999; Proft *et al.*, 2001). Down-regulation of HOG pathway activity is mediated by dephosphorylation of Hog1p through the phosphatases Ptp2p, Ptp3p and Ptc1p, the latter of which also dephosphorylates Pbs2p (Jacoby *et al.*, 1997; Wurgler-Murphy *et al.*, 1997; Warmka *et al.*, 2001).

Lack of HOG pathway activity results in a dramatic reduction of osmoresistance of yeast cells, indicating the importance of this signalling module in the high osmotic stress response (Brewster *et al.*, 1993). The HOG pathway is also involved in functional control of cell wall architecture, together with the PKC (protein kinase C: Gustin *et al.*, 1998) and SVG (sterile vegetative growth: Lee & Elion, 1999; Cullen *et al.*, 2000) MAP kinase routes. Irrespective of the osmotic conditions, HOG pathway mutants have been shown to be hypersensitive to cell-wall-degrading enzyme cocktails such as Zymolyase (Lai, 1997; Alonso-Monge *et al.*, 2001). On the other hand, HOG pathway mutants display increased resistance to the cell-wall-perturbing agent calcofluor white (Alonso-Monge *et al.*, 1999; Garcia-Rodriguez *et al.*, 2000). In yeast, coordinated activity of the HOG, SVG and PKC pathways, mediating adaptive rearrangements in cell wall composition or architecture, may be crucial to cope with high osmotic stress at elevated temperature (de Nobel *et al.*, 2000; Garcia-Rodriguez *et al.*, 2000; Martin *et al.*, 2000; Alonso-Monge *et al.*, 2001).

We have previously shown that HOG pathway mutants exhibit improved osmoresistance at elevated growth temperature (Siderius *et al.*, 2000). The *ste11ssk2ssk22* strain forms an exception to this rule, since it displays a hyper-osmosensitive phenotype at 37 °C (Alonso-Monge *et al.*, 2001). We have hypothesized that this phenotype may be a consequence of the inability to properly trigger HOG, SVG or PKC pathway signalling, which may cause inadequate adaptation of the cell wall to the respective environmental conditions. In support of this hypothesis, the phenotype of the *ste11ssk2ssk22* strain was shown to be suppressed by overexpression of genes that are known to affect cell wall composition, viz. *LRE1*, *HLR1* and *WSC3* (Lai *et al.*, 1997; Verna *et al.*, 1997; Alonso-Monge *et al.*, 2001). Remarkably, overexpression of *LRE1* and *HLR1* also suppressed the phenotypes of osmosensitivity and sensitivity to cell-wall-degrading enzymes displayed by HOG pathway mutants even at normal growth temperature. Furthermore, *LRE1*, *HLR1* and, to a lesser extent, *WSC3* overexpression also rescued lytic phenotypes associated with PKC pathway mutants. Taken together, these observations suggested that cell wall rearrangements in response to hypo- or hyper-osmotic conditions are controlled by coordinated activity of HOG, PKC and (likely) SVG pathways.

In this paper we describe the characterization of a second class of multicopy suppressors of the *ste11ssk2ssk22* temperature-induced hyper-osmosensitivity. We demonstrate that overexpression of *FPS1* and *GPD1*, which are involved in intracellular glycerol accumulation and synthesis, respectively, allow growth of this HOG pathway mutant under the restrictive conditions (Albertyn *et al.*, 1994; Luyten *et al.*, 1995; Tamás *et al.*, 1999). Furthermore, we describe that these suppressor genes overcome the lack of activation of the PKC pathway at 37 °C.

The notable correlation between glycerol content, MAP kinase signalling and growth temperature prompted us to investigate how temperature affects the osmostress response in wild-type cells. The results presented in this study demonstrate the intricate relationship between the potential to accumulate internal glycerol and the activation of the HOG and PKC pathways.

## METHODS

**Yeast strains and growth conditions.** Yeast strains used in this study are listed in Table 1. To culture cells, standard yeast media and growth conditions were used. YNB medium (0·67 % Yeast Nitrogen Base, 2 % glucose) supplemented with the appropriate amino acids was used whenever selective conditions were required. Osmosensitivity was tested on solid medium: YPD (2% glucose, 2% Bactopeptone, 1% yeast extract) supplemented with various concentrations of sorbitol. Serially diluted (1 : 10, starting $OD_{660}$ 0·1) cell suspensions (5 µl) were spotted to examine growth of yeast strains. Cells were grown at 30 °C and 37 °C as indicated in the figures. The *BCK1* gene was deleted in the KK311 strain using the short flanking homology method (AM001). To recreate a *URA3* deficiency, this marker was replaced by the kanamycin-resistance gene in strain AM002 using the same knock-out strategy (Güldener *et al.*, 1996). The resulting strains were checked using PCR and growth on media containing 5-fluoroorotic acid (5FOA).

**Multicopy suppression screening and DNA manipulation.** Transformants of strain KK311 (*ste11ssk2ssk22*), which display osmoresistance at 37 °C, were identified in the genetic screening procedure described by Alonso-Monge *et al.* (2001). Transformation of the yeast strains with isolated plasmids was performed using the freeze–thaw method (Klebe *et al.*, 1983). Nucleotide sequences were determined by the dideoxynucleotide method using the Thermal Sequenase kit (Amersham).

To verify multicopy suppression of the *ste11ssk2ssk22* hyper-osmosensitive phenotype at 37 °C, this strain was transformed with different plasmids. YEp*lac195*-based plasmids with the *FPS1* wild-type allele and the *fps1-Δ1* allele were kindly provided by Dr M. Tamás (University of Göteborg, Sweden). The plasmid carrying the *GDP1* gene was provided by Professor S. Hohmann (University of Göteborg). The plasmid with the *bck1-20* allele was kindly given by Professor D. Levin (Johns Hopkins University, Baltimore, MD, USA).

**β-1,3-Glucanase sensitivity assays.** Cell cultures were inoculated to an $OD_{600}$ of 0·025 in YPD medium supplemented with different amounts of Zymolyase 100T (Seikagaku, Japan). $OD_{600}$ was measured

**Table 1.** Yeast strains and plamids used in this study

| Strain/plasmid | Genotype | Source/reference |
|---|---|---|
| **Strains** | | |
| TM141 | *MAT***a** *leu2 ura3 trp1 his3* | Alonso-Monge *et al.* (2001) |
| TM233 | *MAT***a** *leu2 ura3 his3 lys2 hog1::TRP1* | |
| TM261 | *MAT***a** *ura3 trp1 his3 pbs2::LEU2* | |
| KK311 | *MAT***a** *leu2 ura3 trp1 his3 ssk2::KAN ssk22::LEU2 ste11::TRP1* | |
| AM001 | *MAT***a** *leu2 ura3 trp1 his3 ssk2::KAN ssk22::LEU2 ste11::TRP1 bck1::URA3* | This study |
| AM002 | *MAT***a** *leu2 ura3 trp1 his3 ssk2::KAN ssk22::LEU2 ste11::TRP1 bck1::KAN* | |
| **Plasmids** | | |
| YEp*lacWSC3* | *URA3* 2μ *WSC3* | Alonso-Monge *et al.* (2001) |
| YEp*lacHLR1* | *URA3* 2μ *HLR1* | This study |
| YEp*lacGPD1* | *URA3* 2μ *GPD1* | Albertyn *et al.* (1994) |
| YEp*lacFPS1* | *URA3* 2μ *FPS1* | Tamás *et al.* (1999) |
| YEp*lacfps1Δ-1* | *URA3* 2μ *fps1-Δ1* | |
| p*RS423GPD1* | *HIS3* 2μ *GPD1* | This study |
| p*RS423FPS1* | *HIS3* 2μ *FPS1* | This study |
| YCp*bck1-20* | *URA3* 2μ *bck1-20* | Lee & Levin (1992) |

after overnight culturing at 30 °C. Zymolyase sensitivity is expressed as percentage of growth compared to growth in YPD without Zymolyase.

**Western blotting and antibody staining.** Total protein samples were isolated as described previously (Davenport *et al.*, 1995; Siderius *et al.*, 1997). Proteins (20 μg total protein) were separated on 10 % polyacrylamide gels and blotted onto nitrocellulose. Dual phosphorylation of Hog1p and Mpk1/Slt2p was examined in a Western analysis using an anti-dually-phosphorylated p38 antibody (New England Biolabs) or anti-phospho-p42/44 MAPK (New England Biolabs) respectively. To detect Hog1p the anti C-terminal Hog1p antibody (Yc20, Santa Cruz Biotechnology) was used. For detection of Mpk1/Slt2p we used a polyclonal antiserum kindly provided by Dr M. Molina (Universidad Complutense de Madrid, Spain) (Martin *et al.*, 2000). Antibody binding was visualized using ECL (Amersham) after binding of a horseradish-peroxidase-conjugated second antibody.

**Glycerol measurements.** Cells grown overnight in selective medium were diluted to $OD_{660}$ 0·3 and grown for 4 h at 24 °C. Then they were subjected to increased osmolarity (sorbitol, to a final concentration of 0·8 M). At time points indicated in the figures, two 10 ml samples were taken (one for glycerol measurement, one for determination of protein concentration). Cells were pelleted, quickly washed with cold YNB and centrifuged again at 4 °C. Pellets were resuspended in 1 ml 50 mM Tris/HCl pH 7·5, boiled for 15 min and centrifuged to remove cellular debris. The supernatant was used for glycerol measurement according to the manufacturers' instructions (glycerol measurement kit, Boehringer Mannheim).

Precultured cells were diluted to $OD_{660}$ 0·1 and grown overnight to $OD_{660}$ 1·5–2 under different conditions. Then cultures were diluted to half of their original density and growth was continued for another 3 h. Samples of 10 ml were taken for intracellular glycerol measurement (see above). For total glycerol measurement 2 ml of culture was boiled and then centrifuged to remove cellular debris. The supernatant was used for glycerol measurements.

## RESULTS

### Hyper-osmosensitivity of the *ste11ssk2ssk22* strain at 37 °C is suppressed by overexpression of genes regulating internal glycerol concentration

As we have previously demonstrated, HOG pathway mutants show a diminished osmosensitivity when they are cultured at elevated temperature. In contrast, the *ste11ssk2ssk22* mutant displays a hyper-osmosensitive phenotype at 37 °C (Siderius *et al.*, 1997; Alonso-Monge *et al.*, 2001). With the aim of further elucidating the cellular functions that determine osmotolerance of yeast cells, the *ste11ssk2ssk22* strain was used in a multicopy suppression screening. One of the plasmids isolated in this way appeared to contain an insert with *FPS1*, *AGP10* and part of *SDH2* (chromosome 12, coordinates 49 712–53 558). The occurrence of the *FPS1* gene was most striking, since *FPS1* encodes the plasma membrane glycerol facilitator protein, which closes upon exposure of cells to high osmotic stress, hence preventing the efflux of intracellular glycerol (Tamás *et al.*, 1999). To examine the possibility that overexpression of *FPS1* caused suppression of the *ste11ssk2ssk22* phenotype, we transformed this strain with a YEp*lac195*-based plasmid carrying the *FPS1* wild-type allele. As can be concluded from the data presented in Fig. 1, overexpression of *FPS1* clearly suppressed the hyper-osmosensitivity of *ste11ssk2ssk22* at 37 °C. This finding suggests that accumulation of intracellular glycerol leads to suppression of the phenotype. To verify this hypothesis, we analysed the suppressive effect of the *fps1-Δ1* allele encoding a truncated protein unable to close upon exposure to increased osmolarity (Tamás *et al.*, 1999). Overexpression of the mutant *FPS1* allele still resulted in some level of suppression, but it was greatly

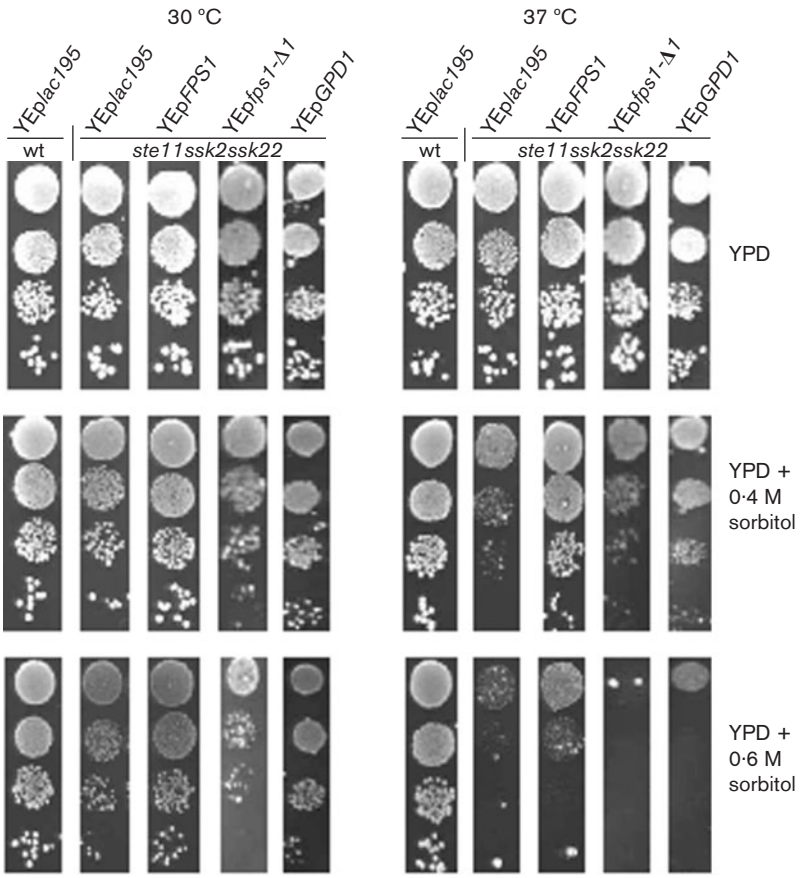


**Fig. 1.** Temperature-dependent hyper-osmosensitivity of the *ste11ssk2ssk22* strain is suppressed by overexpression of *FPS1* or *GPD1*. Serial dilutions of the TM141 wild-type (wt) and *ste11ssk2ssk22* strains transformed with YEp*lac195*, YEp*FPS1*, YEp*fps1-Δ1* and YEp*GPD1* were spotted on YPD plates supplemented with 0·4 and 0·6 M sorbitol. Cells were cultured for 3 days at 30 °C and 37 °C.

diminished as compared to the wild-type allele (Fig. 1). At 30 °C overexpression of *FPS1* did not have a significant effect. In addition, *FPS1* overexpression resulted only in a slightly improved osmoresistance of the *hog1* strain but not of the other HOG pathway mutants, at both 37 °C and 30 °C (not shown).

To further examine the possibility that an increase in intracellular glycerol can suppress the hyper-osmosensitivity of the *ste11ssk2ssk22* strain at 37 °C, we investigated transformants overexpressing *GPD1*, encoding glycerol-3-phosphate dehydrogenase (Albertyn *et al.*, 1994). As is shown in Fig. 1, overexpression of *GDP1* resulted in suppression of the phenotype. In contrast to the suppressor genes described previously, overexpression of *FPS1* and *GPD1* did not increase resistance of HOG pathway mutants to the cell-wall-degrading enzyme cocktail Zymolyase (not shown). This result suggests that the mode of action of the second set of suppressing genes is different from that of the previously reported ones (see Introduction).

## Osmotolerance of *ste11ssk2ssk22* transformants is correlated with increased intracellular glycerol levels

Since the mutant allele *fps1-Δ1*, encoding a constitutive open Fps1p glycerol facilitator, appeared to be less able to suppress the hyper-osmosensitive phenotype of the *ste11ssk2ssk22* strain at 37 °C, we hypothesized that increase in intracellular glycerol levels accounts for the observed suppressive effect of *FPS1* and *GPD1* overexpression. To verify this possibility, intracellular glycerol levels were analysed in the *ste11ssk2ssk22* strain transformed with multicopy plasmids carrying *FPS1* and *GPD1*, and grown at 30 °C, 37 °C or 37 °C combined with increased extracellular osmolarity (Fig. 2). Comparison of the intracellular glycerol levels in the different *ste11ssk2ssk22* transformants under the different conditions indicated that overexpression of *FPS1* resulted in a slight increase in the intracellular glycerol concentration, whereas *GPD1* overexpression led to a significant increase. Culturing the *ste11ssk2ssk22* strain (or transformants) at elevated temperature resulted in an increased glycerol level, as described before for the *hog1* mutant (Siderius *et al.*, 2000). Subjecting the *ste11ssk2ssk22* strain (or transformants) grown at 37 °C to increased osmolarity did not further raise the intracellular glycerol concentration. In conclusion, for the *ste11ssk2ssk22* strain a correlation between glycerol content and osmotolerance at elevated temperature is apparent.

## Levels of intracellular glycerol do not reflect an absolute measure of osmotolerance

In order to test whether intracellular levels of glycerol in general represent a crucial determinant for osmotolerance in yeast, we subsequently compared glycerol content in the wild-type strain (not sensitive under the conditions

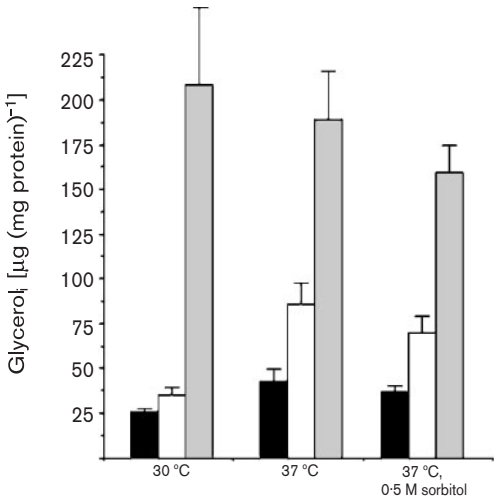


**Fig. 2.** Intracellular glycerol levels in the *ste11ssk2ssk22* strain overexpressing *FPS1* or *GPD1*. Transformants of the *ste11ssk2ssk22* strain with YEp*lac195* (black bars), YEp*FPS1* (white bars) and YEp*GPD1* (grey bars) were cultivated at 30 °C and 37 °C with or without 0·5 M sorbitol, and intracellular glycerol was determined. Means of duplicate measurements are shown ±SD.

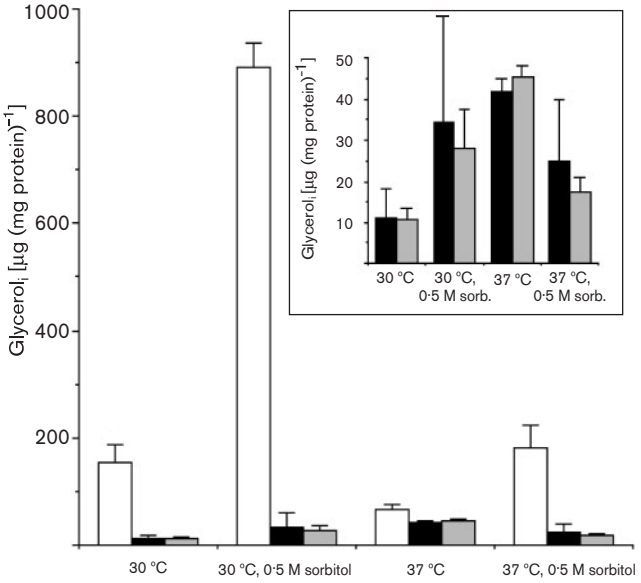


**Fig. 3.** Intracellular glycerol levels in wild-type (white bars), *pbs2* (black bars) and *ste11ssk2ssk22* (grey bars) strains at 30 °C and 37 °C with or without 0·5 M sorbitol. Cells were cultivated at 30 °C and 37 °C with or without sorbitol, and intracellular glycerol was determined. The inset shows data for the *pbs2* and *ste11ssk2ssk22* strains on a different scale. Means of four measurements are shown ±SD.

tested), *pbs2* (osmosensitive at 30 °C, but showing reduced osmosensitivity at 37 °C) and *ste11ssk2ssk22* (hyper-osmosensitive at 37 °C). The size of the cells under the conditions used in this study did not show significant differences (data not shown). As can be concluded from Fig. 3, the level of intracellular glycerol was dramatically reduced in both HOG pathway mutant strains under all conditions. The wild-type strain accumulated glycerol when exposed to increased osmolarity at both 30 °C and 37 °C, but did so to a lesser extent at higher temperature (see below). At 30 °C, the mutants also accumulated glycerol under high osmotic conditions, as has been described before (see inset to Fig. 3). Increased temperature resulted in a higher basal level of intracellular glycerol similarly as we found previously for the *hog1* strain (Siderius *et al.*, 2000). However, despite the fact that both mutant strains do not seem to accumulate glycerol at 37 °C, the *pbs2* strain nevertheless showed improved osmoresistance at 37 °C whereas the *ste11ssk2ssk22* strain is hyper-osmosensitive at elevated temperature. Obviously, the amount of intracellular glycerol is not an absolute measure of the osmotolerance of yeast strains. Probably the effect of glycerol is dependent upon additional cellular properties, for instance the structure of the cell wall. This prompted us to investigate the MAP kinase signalling pathways that control cell wall formation.

## PKC pathway activity at 37 °C under high osmotic conditions is restored by overexpression of *FPS1* and *GPD1*

The hyper-osmosensitivity displayed by the *ste11ssk2ssk22* strain at elevated temperature has previously been postulated to be the result of a lack of rearrangements in the composition or architecture of the cell wall, due to loss of PKC, SVG and HOG pathway signalling (Alonso-Monge *et al.*, 2001). Therefore we tested whether overexpression of *FPS1* or *GPD1* may exert their effect by stimulation of one of the MAP kinase pathways mentioned above. The effect on PKC signalling was assessed by measuring phosphorylation of Mpk1p/Slt2p, the MAP kinase of this pathway. Subjecting yeast cells to high osmotic conditions at 37 °C inhibited the sustained phosphorylation of Mpk1/Slt2p that is characteristic for growth at elevated temperature. Also in the *ste11ssk2ssk22* strain, exposure to increased osmolarity abolished induction of Mpk1/Slt2p phosphorylation at 37 °C (de Nobel *et al.*, 2000; Alonso-Monge *et al.*, 2001; Fig. 4). Interestingly, in the strain overexpressing *FPS1* or *GPD1* the phospho-Mpk1/Slt2p signal appeared to be restored (Fig. 4). As holds for the internal glycerol levels (see above), overexpression of *GPD1* seems to have a larger effect than overexpression of *FPS1*. Note the slight change in the kinetics of Mpk1/Slt2p phosphorylation when *GPD1* is overexpressed.

Hog1p phosphorylation was also examined in the *ste11ssk2ssk22* strain transformed with the YEp*lac*195, YEp*FPS1* and YEp*GPD1* plasmids, but phosphorylation of Hog1p was not detected in any of the transformants at this level of osmotic challenge (not shown).

To examine whether restored activation of the PKC MAP

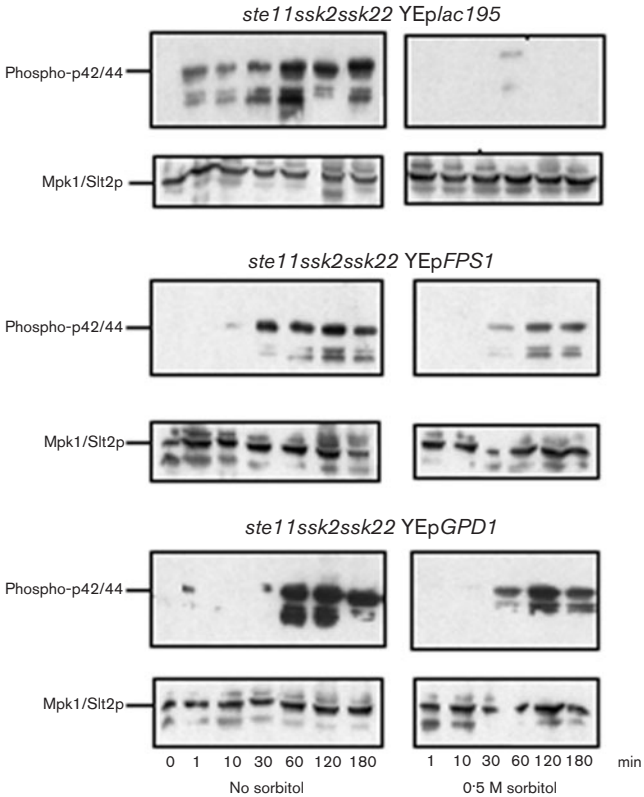


**Fig. 4.** Hyper-osmolarity-induced inhibition of the PKC pathway at elevated temperature is suppressed by overexpression of *FPS1* or *GPD1*. The *ste11ssk2ssk22* strain transformed with YEp*lac195*, YEp*FPS1* or YEp*GPD1* was shifted to 37 °C. The culture was split in two (0 min), and one half was subjected to increased osmolarity (0·5 M sorbitol) for the times indicated. Total protein was isolated, separated by SDS-PAGE and blotted onto nitrocellulose membrane. Phosphorylation of Mpk1/Slt2p was assayed using the anti-phospho-p42/44 antibody. The amount of Mpk1/Slt2p was checked using a polyclonal anti-Mpk1/Slt2p.

kinase pathway may be sufficient for suppression of the hyper-osmosensitive phenotype of the *ste11ssk2ssk22* strain, we tested the effectiveness of expressing the *bck1-20* allele encoding a constitutive active Bck1p, the MAPKKK in the PKC pathway. As is clear from the data presented in Fig. 5(a), expression of the *bck1-20* allele enabled the *ste11ssk2ssk22* strain to grow in the presence of sorbitol at elevated temperature. Again suppression only occurred at relatively low levels of external osmolarity (0·4 M sorbitol; higher concentrations not shown), in contrast with the earlier described suppressor genes *LRE1* and *HLR1* that suppressed at high external sorbitol concentrations (0·8–1 M). If PKC pathway activation, at least downstream of Bck1p, is crucial for suppression of the hyper-osmosensitivity at elevated temperature of the *ste11ssk2ssk22* strain, we argued that additional deletion of *BCK1* in the *ste11ssk2ssk22* background would prevent suppression by overexpression of *FPS1* or *GPD1*. Although

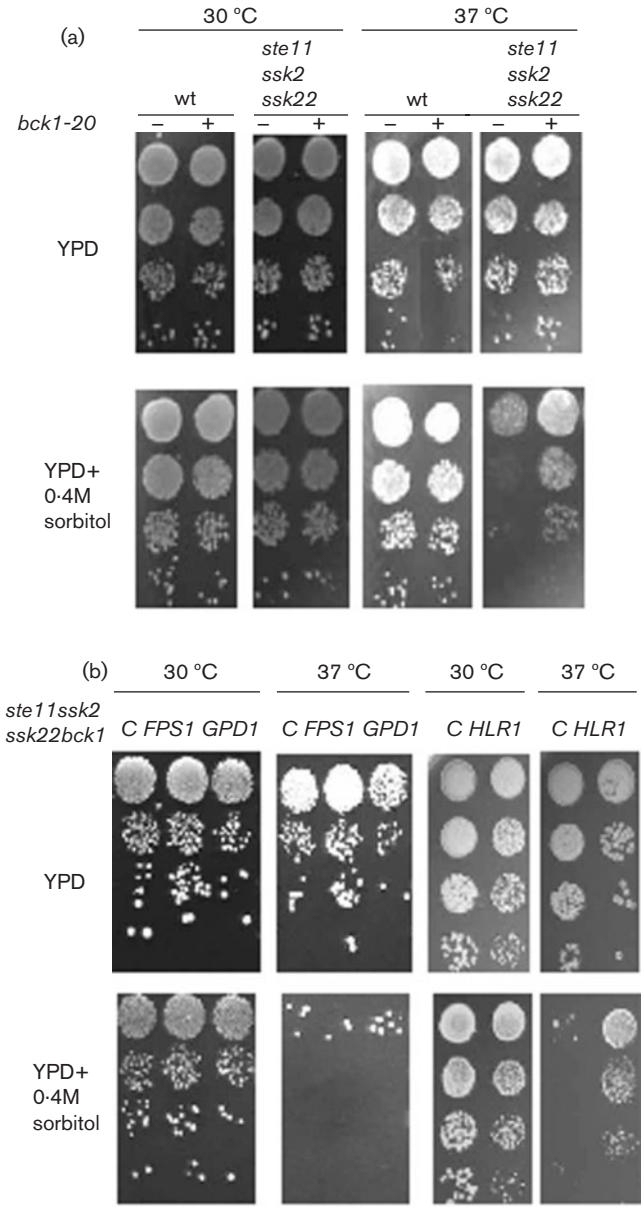


**Fig. 5.** Temperature-dependent hyper-osmosensitivity of the *ste11ssk2ssk22* strain is suppressed by overexpression of *FPS1* or *GPD1* in a Bck1p-dependent manner. (a) Temperature-dependent hyper-osmosensitivity of the *ste11ssk2ssk22* strain is suppressed by expression of the constitutive active *bck1-20* allele. Serial dilutions of the *ste11ssk2ssk22* and wild-type strains transformed with Ycp50 (vector) and Ycp*bck1-20* plasmids were spotted on YPD plates with or without 0·4 M sorbitol. Cells were cultured for 3 days at 30 °C and 37 °C. (b) Suppression of hyper-osmosensitivity of the *ste11ssk2ssk22* strain depends on the presence of Bck1p. Serial dilutions of the *ste11ssk2ssk22bck1* (AM001) strain transformed with p*RS423* (control, C), p*RS423FPS1* or p*RS423GPD1* were spotted on YPD plates with or without 0·4 M sorbitol. The same analysis was performed using the *ste11ssk2ssk22bck1* (AM002) strain transformed with YEp*lac195* and YEp*lacHLR1*. Cells were cultured for 3 days at 30 °C and 37 °C.

the quadruple *ste11ssk2ssk22bck1* strain lacks all MAPKKKs described in *S. cerevisiae*, it is viable on YPD medium at 30 °C and 37 °C (Fig. 5b). This strain also displayed the hyper-osmosensitivity at 37 °C but was viable at moderately high osmolarity conditions (0·4 M sorbitol) at 30 °C. In agreement with our hypothesis, overexpression of *FPS1* or *GPD1* did not suppress the hyper-osmosensitive phenotype of *ste11ssk2ssk22bck1* at elevated temperature. To investigate if this is a specific feature of these suppressors and not due to the result of the quadruple deletion, we tested whether overexpression of *HLR1* could suppress the hyper-osmosensitivity of the *ste11ssk2ssk22bck1* strain. As is clear from Fig. 5(b), this was indeed the case. Again this indicates that the molecular basis of suppression of the hyper-osmosensitivity of the *ste11ssk2ssk22* strain at elevated temperature by overexpression of *GPD1* and *FPS1* is distinct from that by *HLR1* overexpression.

To verify the role of PKC pathway activation in the suppressive effect by the *bck1-20* allele, we examined phosphorylation of Mpk1/Slt2p in strains expressing the activated allele (Lee & Levin, 1992). As can be concluded from Fig. 6, expression of the *bck1-20* allele in both wild-type and *ste11ssk2ssk22* strains resulted in the increase of the level of phospho-Mpk1/Slt2p, even in the presence of 0·5 M sorbitol. Overexpression of *WSC3* also resulted in suppression of the hyper-osmosensitivity of the *ste11ssk2ssk22* strain at elevated temperature. Previously, we did not detect any stimulatory effect of *WSC3* overexpression on the phosphorylation of Mpk1/Slt2p at elevated temperature and increased osmolarity (0·8 M sorbitol) (Alonso-Monge *et al.*, 2001). As it was shown in this work that stimulation of the PKC pathway activity only resulted in suppressive effects at lower osmolarities, we tested whether overexpression of *WSC3* could result in increased phosphorylation of Mpk1/Slt2p in the presence of 0·5 M sorbitol at elevated temperature. Indeed the effect of overexpression of *WSC3* appeared to be only manifest at lower osmolarities, similar to the results obtained with *GPD1*, *FPS1* and *bck1-20*. As can be concluded from Fig. 6, this correlates with increased phosphorylation of Mpk1/Slt2p in the wild-type strain and the *ste11ssk2ssk22* mutant.

## In *S. cerevisiae* the level of glycerol accumulation depends on the growth temperature

As can be concluded from the data presented above, compensatory activation of the PKC MAP kinase pathway may suppress osmosensitivity of the *ste11ssk2ssk22* mutant at elevated temperature and this is correlated with increased levels of glycerol. These data prompted us to study the situation in wild-type yeast cells, for a wild-type strain also displays loss of temperature-induced activation of the PKC pathway activity after transfer to increased osmolarity (see de Nobel *et al.*, 2000; Alonso-Monge *et al.*, 2001; Fig. 6). It turned out that overexpression in the wild-type strain of *GPD1* and – to a lesser extent – *FPS1* or *WSC3*, or expression of *bck1-20*, led to an increase in the level of phosphorylated Mpk1/Slt2p at 37 °C in the presence of 0·5 M sorbitol (Fig. 6).

To further ascertain the impact of these results, we wanted to establish whether a correlation exists between intracellular glycerol and activation of the PKC pathway at 37 °C under osmotic stress. Therefore, we measured intracellular glycerol and total glycerol levels in the wild-type strain grown in the presence of 0·5 M sorbitol or 0·8 M sorbitol at 24 °C or 37 °C (Fig. 7a, b). Subjecting the TM141 wild-type strain to increased osmolarity at 24 °C resulted in an accumulation of intracellular glycerol that was proportional

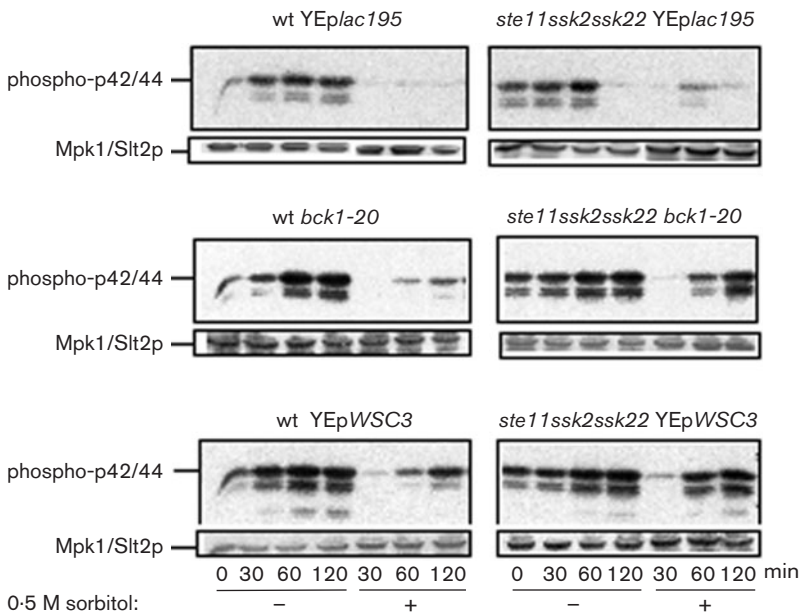


**Fig. 6.** Hyper-osmolarity-induced inhibition of the PKC pathway at elevated temperature is suppressed by overexpression of *WSC3* or expression of *bck1-20*. The wild-type and *ste11ssk2ssk22* strains transformed with YEp*lac195*, YEp*WSC3* and *Ycpbck1-20* were shifted to 37 °C. The cultures were split in two (0 min), and one half was subjected to increased osmolarity (0·5 M sorbitol) for the times indicated. Total protein was isolated, separated by SDS-PAGE and blotted onto nitrocellulose membrane. Phosphorylation of Mpk1/Slt2p was assayed using the anti-phospho-p42/44 antibody. The amount of Mpk1/Slt2p was checked using a polyclonal anti-Mpk1/Slt2p.

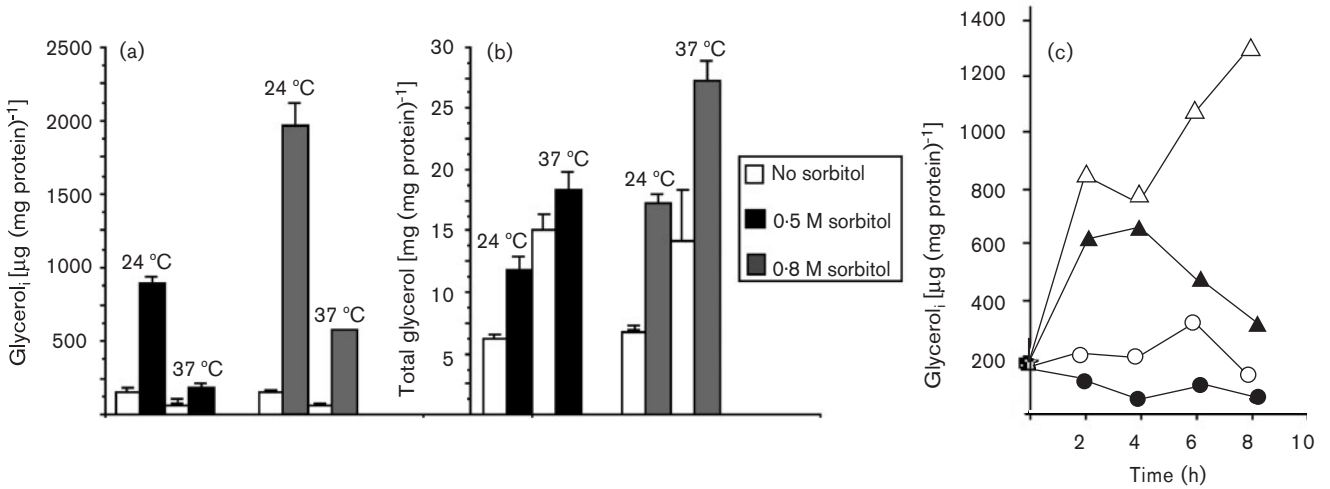


**Fig. 7.** (a, b) Intracellular glycerol accumulation (a) and total production of glycerol (b) by *S. cerevisiae* wild-type strain TM141 subjected to osmotic challenges at different temperatures. TM141 cells cultured at 24 °C and 37 °C were subjected to high osmotic shock (0·5 M or 0·8 M sorbitol). Means of four measurements are shown ±SD. (c) Intracellular accumulation of glycerol by wild-type strain subjected to osmotic challenges at different temperatures. Cells cultured at 24 °C (open symbols) and 37 °C (filled symbols) were subjected to high osmotic shock (0·8 M sorbitol) and intracellular glycerol levels were determined (triangles). Circles represent glycerol levels in control cells (not subjected to osmotic shock).

to the osmolarity of the surrounding medium. In contrast to the W303IA wild-type strain (Siderius *et al.*, 2000), the TM141 wild-type strain displayed a slightly lower basal level of intracellular glycerol at 37 °C. To our surprise, at 37 °C this wild-type strain accumulated much less glycerol upon an osmotic challenge (Fig. 7a). The size of cells grown at 24 °C or 37 °C did not differ significantly (not shown). Therefore, the different level of accumulated glycerol after an osmotic challenge might reflect a different way of sensing and/or response to increased osmolarity at different temperatures. Indeed the actual kinetics of the high-osmolarity-triggered Hog1p activation displayed a marked difference in kinetics of the response at 24 °C as compared to 37 °C. However, the cells did synthesize increased amounts of glycerol at 37 °C, even under non-stress conditions, as can be concluded from the total glycerol measurements (Fig. 7b).

Following the accumulation of intracellular glycerol with time after exposure to increased osmolarity confirmed the temperature-dependent differences described above. Cells (wild-type) grown at 24 °C and 37 °C were subjected to osmotic shock and intracellular levels of glycerol were determined. As is shown in Fig. 7(c), the accumulation of glycerol in cells challenged with high osmolarity at 37 °C displayed a more transient course, never reaching the values that were found in cells cultured at 24 °C. No apparent difference in growth rate of the osmo-adapted cells could be detected at either temperature (not shown) and therefore the differences in glycerol accumulation apparently do not reflect a different extent of osmosensitivity. This set of data clearly demonstrates that growth temperature has a major influence on the accumulation of glycerol in yeast challenged with hyperosmotic stress.

## High-osmolarity-induced signalling depends on the growth temperature and may be regulated at the level of inactivation of MAP kinases

To further investigate the temperature dependency of the osmotic stress response, we examined the activation of both PKC and HOG pathways at different temperatures and osmolarities. Wild-type cells cultured at 24 °C and 37 °C were subjected to increased osmolarity and the phosphorylation of Hog1p and Mpk1/Slt2p was followed with time. Increased osmolarity (0·8 M sorbitol) at 24 °C and 30 °C (not shown) resulted in a transient phosphorylation of Hog1p (Fig. 8a). At 37 °C phosphorylation of Hog1p was lost more rapidly and only the 1 min sample showed a phospho-Hog1p signal. This result confirms the observation made previously by our group (Alonso-Monge *et al.*, 2001). However, in those studies cells were subjected to a temperature shift and increased osmolarity simultaneously, and here we show the effect in cells pre-cultured at 37 °C and subjected to a sudden osmotic challenge. This finding indicates that the changed kinetics of Hog1p phosphorylation are an effect of growth at elevated temperature rather than the consequence of exposure to combined stresses. Furthermore we included an additional time point (5 min) to demonstrate that Hog1p phosphorylation at 37 °C is very rapidly lost after a high osmotic challenge. Interestingly, dephosphorylation of Mpk1/Slt2p also occurs very rapidly when cells are subjected to osmotic shock at 37 °C (Fig. 8a): no phospho-Mpk1/Slt2p is detectable at 24 °C but cells

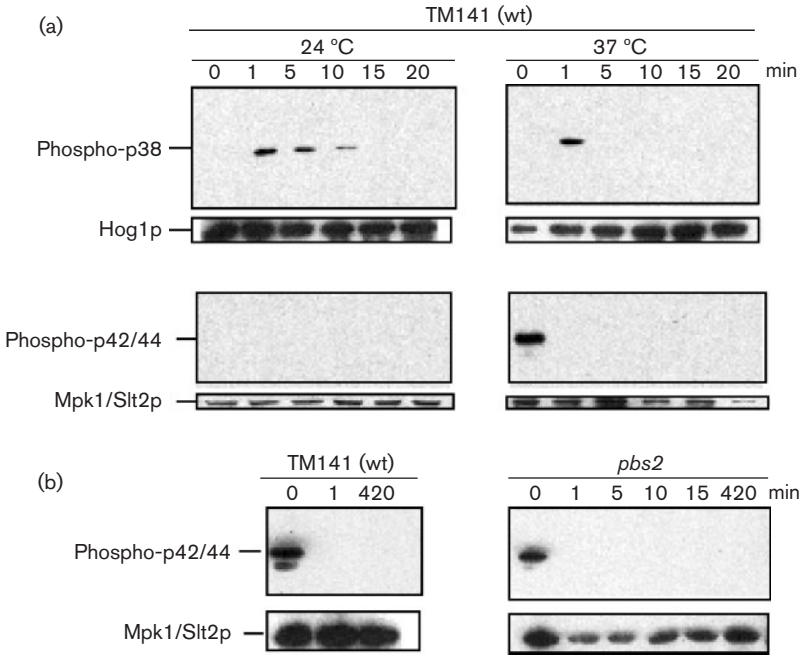


**Fig. 8.** Hyper-osmolarity-induced inhibition of the PKC pathway at elevated temperature is independent of Hog1p activation. (a) Wild-type cells (TM141) cultured at 24 °C and 37 °C were subjected to increased osmolarity (0·8 M sorbitol) and phosphorylation of Mpk1/Slt2p and Hog1p was examined. Dually-phosphorylated forms of Mpk1/Slt2p or Hog1p were detected using anti-phospho-p42/44 and anti-phospho-p38 respectively. Protein loading was checked using a polyclonal anti-Mpk1/Slt2p or anti-Hog1p. (b) Wild-type and *pbs2* strains were grown at 37 °C and subjected to increased osmolarity (0·8 M sorbitol), and phosphorylation of Mpk1/Slt2p was assayed using the anti-phospho-p42/44 antibody. The amount of Mpk1/Slt2p was checked using a polyclonal anti-Mpk1/Slt2p.

cultured at 37 °C showed the characteristic phospho-Mpk1/Sltp2 which disappeared within 1 min after a challenge with high osmolarity.

To examine if the loss of Mpk1/Slt2p phosphorylation after osmotic shock may be due to activation of the HOG pathway we examined Hog1p and Mpk1/Slt2p phosphorylation in a *pbs2* deletion strain. As is shown in Fig. 8(b), loss of Mpk1/Slt2p phosphorylation in the *pbs2* strain was comparable to that in the wild-type, whereas Hog1p was not phosphorylated in this strain (not shown). We therefore concluded that the rapid loss of Mpk1/Slt2p phosphorylation is not due to the activation of Hog1p. As both Hog1p and Mpk1/Slt2p rapidly lost their phosphorylated state in the experiments described here, we postulate that at 37 °C MAP kinase phosphatase activity may be enhanced to shorten the time of MAP kinase activation.

## Temperature determines the severity of cell wall phenotypes associated with HOG pathway mutations

As was shown earlier, HOG pathway mutants display a dramatic sensitivity to the cell-wall-degrading enzyme cocktail Zymolyase (Alonso-Monge *et al.*, 2001). Although the molecular details of cell wall adaptations under high osmotic stress exposure are still unknown, we investigated whether the observed differences in the kinetics of osmo-adaptive signalling at different growth temperatures are also reflected in the cell wall phenotypes of HOG pathway mutants. We analysed the sensitivity of HOG pathway mutants to Zymolyase at 30 °C and 37 °C. As can be concluded from Fig. 9, this phenotype displays a temperature dependency. The *hog1* and *pbs2* pathway mutants are less sensitive to cell-wall-degrading enzymes at 37 °C, whereas the *sho1ssk2ssk22* and *ste11ssk2ssk22* strains, which are less osmosensitive, are slightly sensitive to Zymolyase at 37 °C (not shown). Again this suggests that differences in cellular physiology occur at different temperatures. Furthermore, this finding is another indication that cell wall adaptations correlate with changes in sensitivity to high osmotic stress, which is clearly dependent on the growth temperature.

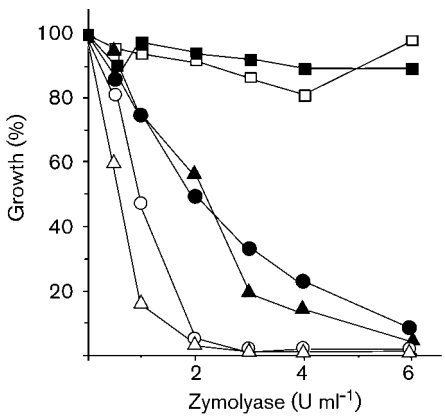


**Fig. 9.** Hypersensitivity of HOG pathway mutants to the $\beta$-1,3-glucanase-containing enzyme cocktail Zymolyase is temperature-dependent. Wild-type and *hog1* and *pbs2* mutant strains were grown overnight at 30 °C (open symbols) and 37 °C (filled symbols) in the presence of different amounts (0·25–6 U ml$^{-1}$) of Zymolyase as indicated, starting with an $OD_{600}$ of 0·025. Growth of the wild-type (squares), *hog1* (triangles) and *pbs2* (circles) is depicted as a percentage of growth in YPD supplemented with Zymolyase as compared to growth in YPD without addition. Results of a representative experiment are shown.

## DISCUSSION

The temperature-dependent hyper-osmosensitivity of the *ste11ssk2ssk22* strain has been explained to be a consequence of the inability to activate the HOG, PKC and SVG MAP kinase pathways (Alonso-Monge *et al.*, 2001). Most likely, lack of signalling through these pathways prevents the cell from properly adapting its cell wall architecture to the demands of the combined condition of increased temperature and high osmolarity. The first reported set of high-copy suppressors of this phenotype indeed encompassed genes that previously were shown to affect cell wall composition of *S. cerevisiae*. Here we show that overexpression of genes implicated in the accumulation of glycerol under high osmotic conditions, viz. *GPD1* and *FPS1*, also suppressed the hyper-osmosensitivity of the *ste11ssk2ssk22* strain at elevated temperature. The mechanism of suppression by multicopy *FPS1* and *GPD1* is distinct from that by *LRE1* and *HLR1*, as the latter, apart from temperature-induced hyper-osmosensitivity of the *ste11ssk2ssk22* strain, also suppressed the Zymolyase sensitivity of the *hog1* and *pbs2* strains, while the former did not.

A connection between accumulation of intracellular glycerol and cell-wall-damaging treatments has been described previously (Garcia-Rodriguez *et al.*, 2000). Furthermore, our group has postulated a 'protective function' for glycerol besides its role as osmolyte in high osmotic stress conditions. This may explain why glycerol is accumulated when cells are shifted to 37 °C and it may explain the effect of glycerol on osmoresistance of HOG pathway mutants (Siderius *et al.*, 2000).

Here we show that intracellular glycerol may play a role in triggering the activity of the PKC MAP kinase pathway in a concentration-dependent manner. Although the increase in intracellular glycerol level by overexpression of *FPS1* has been reported to be twofold (Luyten *et al.*, 1995), in our experiments the increase was more modest. Overexpression of *GPD1*, on the other hand, resulted in a dramatic increase as reported before (Albertyn *et al.*, 1994), which was paralleled by a strong stimulation of Mpk1/Slt2p phosphorylation. These experiments not only verified that the absence of PKC pathway signalling could (partly) cause the hyper-osmosensitive phenotype of the *ste11ssk2ssk22* strain, but also indicated that the lack of PKC pathway signalling under high osmotic conditions may be brought about by loss of turgor. Increasing the intracellular glycerol concentration by overexpression of *FPS1* or *GPD1* did not restore the Mpk1/Slt2p phosphorylation kinetics to those observed in cells only experiencing increased temperature. Overexpression of these genes resulted in transient Mpk1/Slt2p phosphorylation under conditions of high external osmolarity at elevated temperature whereas a shift to increased temperature resulted in a sustained phosphorylation of Mpk1/Slt2p. The suppressive action of *GPD1* and *FPS1* only occurred at lower osmolarity, indicating that increase of intracellular glycerol can not fully rescue the phenotype of the *ste11ssk2ssk22* strain. The mechanism of suppression of the hyper-osmosensitivity of the *ste11ssk2ssk22* strain by overexpression of *WSC3* may be similar to that by *FPS1* or *GPD1* overexpression and by expressing a constitutive active *bck1-20* allele. Also *WSC3* overexpression at lower osmolarities turned out to result in enhanced Mpk1/Slt2p phosphorylation. Together with the observed lack of suppression of the Zymolyase- and osmo-sensitivities of the HOG pathway mutants, the above-mentioned genes seem to share their phenotypic effects. Although the activation of the PKC pathway thus seems to link increased intracellular glycerol content to restored growth of the *ste11ssk2ssk22* strain at elevated temperature and increased osmolarity, it does not fully account for all phenotypes observed with the HOG pathway mutants.

Although the TM141 wild-type strain displayed increased glycerol production at 37 °C, it notably did not accumulate glycerol, unlike the W303IA wild-type strain, which does increase its intracellular glycerol level at 37 °C (Siderius *et al.*, 2000). Interestingly, this difference in ability to accumulate glycerol correlates with differences in cell wall phenotypes between these strains, the W303IA strain being more sensitive to calcofluor white and to Zymolyase than the TM141 strain (I. Wojda, R. Alonso-Monge & M. Siderius, unpublished observations). For instance, Garcia Rodrigues *et al.* (2000) showed that calcofluor white treatment induced accumulation of glycerol. Comparing the molecular differences in cell wall composition between the wild-type yeast strains used in this study may provide essential information on the cell wall components mediating resistance and sensitivity to compounds that influence cellular integrity.

In this study we have shown that *S. cerevisiae* cells sense and respond to high osmolarity in a manner that is influenced by other environmental factors, in particular temperature. MAP kinase signalling pathways involved in osmoregulation display a temperature dependency in the kinetics of their responses. *S. cerevisiae* wild-type cells do not display any growth defects or osmosensitivity at 37 °C. Yet both HOG and PKC pathways are activated more transiently at 37 °C than in cells cultured at lower growth temperature (30 °C or 24 °C). Because exposure to a certain level of osmotic stress at different temperatures does not trigger the responsive signalling pathways to the same extent and it does not dramatically affect the size of cells, we postulate that yeast cells experience a different level of osmotic stress at different temperatures. Interestingly, we have observed a similar effect on HOG pathway activation after osmotic challenge when cells are cultured at different pH (E. Real & M. Siderius, unpublished observations), and again pH differences were proposed to have a significant effect on the composition of the cell wall of yeast (Kapteyn *et al.*, 2001).

Altered osmosensing may be the result of the integrated output of different 'sensors' of yeast. Net osmosensing is the result of the contributions to sensing at many different levels. The plasma membrane has been proposed to be the

most likely site of sensing differences in osmolarity, but integrating pressure from cell wall to plasma membrane, to cytoskeleton or, for example, from within the vacuole, may be alternative ways to trigger both PKC and HOG pathway signalling. A consequence of multiple levels of osmosensing would be that multiple osmosensors are present in the yeast cell. Indeed this has been shown for the PKC pathway, which has many putative upstream sensors that could have complementary functions (Gray *et al.*, 1997; Verna *et al.*, 1997; Ketela *et al.*, 1999). Upstream of the HOG pathway the picture as to how many osmosensing proteins exist is still unclear. Sln1p is a candidate osmosensor that may sense increased osmolarity at the plasma membrane (Tao *et al.*, 1999). Sho1p, however, rather functions to recruit the downstream osmosignalling proteins to an as-yet-unidentified osmosensor (Raitt *et al.*, 2000). Recently O'Rourke *et al.* (2002) have characterized a new putative osmosensor which may be connected to the cell wall: Msb2p. So, the number and nature of proteins involved in sensing and/or responding to increased osmolarity remains to be determined. The phenotypes described in this study suggest, however, that part of the sensing of increased osmolarity may derive from cell-wall-associated proteins that could explain the role of intracellular glycerol in the interplay between HOG and PKC pathway signalling.

The observed rapid loss of Mpk1/Slt2p phosphorylation when cells cultured at 37 °C are subjected to increased osmolarity, together with the rapid loss of phosphorylated Hog1p after osmotic challenge at 37 °C, suggests a prominent role for the corresponding phosphatases in regulation of MAP kinase signalling at elevated temperature. Hog1p and Mpk1/Slt2p share several phosphatases, viz. Ptp2p, Ptp3p and Ptc1p (Jacoby *et al.*, 1997; Wurgler-Murphy *et al.*, 1997; Warmka *et al.*, 2001). Wurgler-Murphy *et al.* (1997) suggested that activated Hog1p may regulate Mpk1/Slt2p activity via induced expression of *PTP2* and *PTP3* (Jacoby *et al.*, 1997; Wurgler-Murphy *et al.*, 1997). However the presumed phosphatase activity described in the present paper is not in agreement with this hypothesis, since it does not seem to be regulated via activation of Hog1p. Expression of *PTC1* also appears to be transiently upregulated by osmotic stress (Gasch *et al.*, 2000), but whether this enzyme is the candidate phosphatase regulating the rapid dephosphorylation of Mpk1/Slt2p and Hog1p under the conditions tested here remains to be investigated.

In this paper we have shown that *S. cerevisiae* experiences changes in environmental osmolarity in a way that depends on additional environmental conditions affecting sensing and induction of adaptive responses. Eventually this results in a different resistance to stress. Further studies will elucidate the components involved in the modulation of osmosensing and signalling which can be of practical importance in biotechnological applications aimed at improving the viability of yeast in complex, potentially stressful environments.

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