

Conditional Osmotic Stress in Yeast

A SYSTEM TO STUDY TRANSPORT THROUGH AQUAGLYCEROPORINS AND OSMOSTRESS SIGNALING*

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The accumulation and transport of solutes are hallmarks of osmoadaptation. In this study we have employed the inability of the *Saccharomyces cerevisiae* *gpd1Δ gpd2Δ* mutant both to produce glycerol and to adapt to high osmolarity to study solute transport through aquaglyceroporins and the control of osmotic stress-induced signaling. High levels of different polyols, including glycerol, inhibited growth of the *gpd1Δ gpd2Δ* mutant. This growth inhibition was suppressed by expression of the hyperactive allele Fps1-Δ1 of the osmogated yeast aquaglyceroporin, Fps1. The degree of suppression correlated with the relative rate of transport of the different polyols tested. Transport studies in secretory vesicles confirmed that Fps1-Δ1 transports polyols at increased rates compared with wild type Fps1. Importantly, wild type Fps1 and Fps1-Δ1 showed similarly low permeability for water. The growth defect on polyols in the *gpd1Δ gpd2Δ* mutant was also suppressed by expression of a heterologous aquaglyceroporin, rat AQP9. We surmised that this suppression was due to polyol influx, causing the cells to passively adapt to the stress. Indeed, when aquaglyceroporin-expressing *gpd1Δ gpd2Δ* mutants were treated with glycerol, xylitol, or sorbitol, the osmosensing HOG pathway was activated, and the period of activation correlated with the apparent rate of polyol uptake. This observation supports the notion that deactivation of the HOG pathway is closely coupled to osmotic adaptation. Taken together, our “conditional” osmotic stress system facilitates studies on aquaglyceroporin function and reveals features of the osmosensing and signaling system.

Osmoregulation is a fundamental biological process that controls cellular water content and turgor pressure. The accumulation of compatible solutes is a well conserved strategy in osmoregulation, although the solute accumulated differs between organisms (1). The yeast *Saccharomyces cerevisiae* employs glycerol, whose production and transmembrane flux are

tightly controlled by osmotic changes (2). In this work we have developed a “conditional osmotic stress” system in which a yeast mutant unable to produce glycerol is stressed by the addition of polyols and allowed to adapt by expression of aquaglyceroporins mediating polyol influx into the cell. We use this experimental set-up to illustrate that (i) different polyols can serve as osmotic stress agents as well as compatible solutes, (ii) to study polyol transport through aquaglyceroporins, and (iii) to probe the feedback mechanisms of the osmosensing/osmosignaling system.

Glycerol is produced in yeast from the glycolytic intermediate dihydroxyacetonephosphate in two steps that are catalyzed by glycerol-3-phosphate dehydrogenase (Gpd) and glycerol-3-phosphatase (Gpp), respectively. Both enzymes exist in two isoforms, Gpd1 and Gpd2 as well as Gpp1 and Gpp2. Deletion of *GPD1* and *GPD2* or *GPP1* and *GPP2* abolishes glycerol production and causes strong osmosensitivity (3–8).

As in other yeasts, active glycerol uptake from the environment has been observed (9) but does not normally contribute to osmoadaptation in *S. cerevisiae* (10). Rather, intracellular glycerol levels are controlled by passive glycerol export, which is mediated by Fps1 (2, 11–13). Upon a hyperosmotic shock the transport capacity of Fps1 is rapidly diminished to ensure that glycerol is maintained inside the cell (2, 11). A specific domain within the N-terminal extension of Fps1 is needed to restrict glycerol transport, and deletion of this domain renders Fps1 hyperactive (12, 14). Yeast cells that express this hyperactive Fps1, Fps1-Δ1, fail to retain glycerol and hence are sensitive to high external osmolarity. Upon a hypo-osmotic shock Fps1 rapidly releases glycerol to prevent excessive cell swelling. Therefore, mutants lacking Fps1 are sensitive to hypo-osmotic shock (12).

Fps1 is a member of the aquaglyceroporin subgroup of MIP channel proteins (15, 16) and hence can mediate passive glycerol flux in both directions. MIP channels, now referred to as aquaporins, occur in all groups of organisms ranging from archaea to humans and play important roles in mediating and controlling water and solute fluxes across cells and tissues (16). Aquaglyceroporins have been shown to transport a range of compounds including polyols, urea, and even metalloids (17–19), and thus the determination of the transport specificity of the many proteins in this ubiquitous family is of considerable importance in the elucidation of their physiological roles.

In yeast, hyperosmotic stress is sensed and signaled by the high osmolarity glycerol (HOG)¹ pathway, an elaborate mito-

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¹ The abbreviations used are: HOG, high osmolarity glycerol; HA, hemagglutinin; MES, 4-morpholineethanesulfonic acid; CF, carboxyfluorescein.

gen-activated protein kinase signal transduction system (2, 20, 21). The activity of the pathway can be monitored by immunological determination of the level of phosphorylated Hog1 mitogen-activated protein kinase or by the mRNA level of target genes such as *GRE2*. Using such markers of HOG pathway activity, it has been demonstrated that a hyperosmotic shock leads to transient activation of the pathway. Negative regulation of the HOG pathway is exerted by protein phosphatases (2, 20, 21). Studies on mutants unable either to produce or retain glycerol or to accumulate glycerol faster than wild type have indicated that deactivation of the HOG pathway correlates with glycerol accumulation and hence successful adaptation (7, 23).² The HOG pathway controls glycerol production at at least two levels. First, Hog1 activates the enzyme phosphofructo-2-kinase, leading to stimulation of glycolytic flux and enhanced glycerol production (24). In addition, Hog1 mediates enhanced expression of the genes *GPD1* and *GPP2* (4, 5, 25) and hence increased capacity to produce glycerol.

In this work we make use of the osmosensitive *gpd1Δ gpd2Δ* mutant as well as the hyperactive Fps1-Δ1. Expression of Fps1-Δ1, or rat AQP9, a mammalian aquaglyceroporin, suppresses the growth defect of the *gpd1Δ gpd2Δ* mutant in the presence of high concentrations of polyols that can be transported by Fps1-Δ1 or AQP9. This has allowed us to establish an experimental "conditional osmotic stress" system to study polyol transport and osmosignaling.

MATERIALS AND METHODS

Strains and Plasmids—The yeast strains used in this study are W303-1A (*MATa leu2-3/11 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2 mal0*) (26) plus its isogenic mutants YSH 642 (*gpd1Δ::TRP1 gpd2Δ::URA3*) (6) and YMT2 (*fps1Δ::HIS3*) (14). Solute transport studies utilized secretory vesicles prepared from strain SY1 (*MATa ura3-52 leu2-3,112 his4-619 sec6-4*) (27) containing a *URA3*-marked vector (pCu426) (28) to drive the copper-inducible, high level expression of hemagglutinin (HA) epitope-tagged *FPS1* or *FPS1-Δ1*. YEpmc-*FPS1* is a 2μ *LEU2* plasmid expressing a *c-myc* epitope-tagged Fps1, and YEpmc-*FPS1-Δ1* mediates expression of a truncated, hyperactive Fps1-Δ1, which lacks amino acids 12–231 (12). The rat AQP9 gene (kindly provided by S. Nielsen) was amplified by PCR and inserted between the *EcoRI* and *SmaI* sites of the pYX242 vector (multi-copy 2μ vector, constitutive *TPI1* promoter, HA tag). The construct was confirmed by sequencing.

Growth Conditions—Yeast cells were grown in 2% peptone, 1% yeast extract, 2% glucose (YPD). Selection and growth of transformants was performed in synthetic medium (YNB, 2% glucose) (29). For growth assays cells were pregrown for 2 days on YNB plates and resuspended in YNB to $A_{600\text{ nm}} = 0.4$, and 5 μl of a 10-fold dilution series were spotted onto agar plates supplemented with osmotica as indicated. Growth was monitored for 2–7 days in 30 °C.

For growth curves the cells were pregrown in YNB for 24 h. The cells were adjusted to $A_{600\text{ nm}} = 0.15$ in 350 μl of YNB supplemented with compounds as indicated and transferred to Bioscreen plates (30). The cells were grown at 30 °C with agitation for 60 s every second minute. The $A_{600\text{ nm}}$ values were automatically recorded at 20-min intervals.

Polyol Transport in Whole Yeast Cells—The cells were harvested by centrifugation in mid-exponential phase, washed, and suspended in ice-cold MES buffer (10 mM MES, pH 6.0) to 60 mg/ml. 30 μl of unlabeled polyol solution was mixed with 0.5 μCi of [¹⁴C]glycerol, [³H]xylytol, or [³H]sorbitol respectively in MES buffer and added to 20 μl of cell suspension to give a final polyol concentration of 100 mM. The reactions were stopped at 15, 30, and 60 s by transferring the cells to ice-cold water and collecting them on filters. Radioactivity was monitored in a scintillation counter. The samples for dry weight were collected on filters and dried at 80 °C. The initial uptake rates were determined by the slope. For each value the average value obtained with transformants carrying the corresponding empty plasmid was subtracted. Presented is the average of at least four individual experiments.

Membrane Preparation and Western Blot Analysis of rAQP9—Transformed cells were harvested in mid-exponential phase, washed (10 mM

Tris-HCl, pH 7.5, 0.5 M sucrose, 2.5 mM EDTA), and resuspended in homogenization buffer (50 mM Tris-HCl, pH 7.5, 0.3 M sucrose, 5 mM EDTA, 1 mM EGTA, 5 mg/ml bovine serum albumin, 2 mM dithiothreitol, protease inhibitor mixture (Roche Applied Science)). The cells were disrupted in a Fast-prep (BIO101) and centrifuged at $10,000 \times g$ for 10 min, and the supernatant was centrifuged at $100,000 \times g$ for 90 min. The membrane pellet was resuspended (10 mM Tris-HCl, pH 7.0, 1 mM EGTA, 1 mM dithiothreitol, 20% (v/v) glycerol, protease inhibitor mixture), 50 μg of protein were separated by SDS-PAGE and blotted (Hybond-ECL; Amersham Biosciences). The membranes were blocked in phosphate-buffered saline Tween 20, 5% milk and probed for 1.5 h with 1:2,000 diluted anti-HA mouse monoclonal antibody (Roche Applied Science), washed, and incubated for 1 h with secondary antibody (horseradish peroxidase-conjugated anti-mouse IgG (Promega), diluted 1:2,500) in phosphate-buffered saline Tween 20, 5% milk. The membranes were incubated with Lumi-Light for detection.

Western Blot Analysis of Hog1—The cells were prepared as described previously (31). For AQP9, cells were grown to mid-logarithmic phase and were subsequently stressed with polyols. The samples were rapidly cooled in a dry ice/ethanol bath. The cells were resuspended in loading buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 20% glycerol, 0.2% bromophenol blue, 20 mM mercaptoethanol, 10 mM NaF, 0.1 mM sodium vanadate, Protease inhibitor (Complete EDTA-free Protease Inhibitor mixture tablets; Roche Applied Science)) and thereafter boiled at 100 °C for 10 min. The filters were blocked with 5% skimmed milk in Tris-buffered saline with Tween 20.

The antibody recognizing the dually phosphorylated Hog1 (phospho-p38 mitogen-activated protein kinase (Thr¹⁸⁰/Tyr¹⁸²); Cell Signaling) was diluted 1:1000 in 5% bovine serum albumin dissolved in Tris-buffered saline with Tween 20, and the membrane was incubated overnight at 4 °C.

An antibody against total Hog1 was used as a loading control (yC-20; Santa Cruz Biotechnology) and was diluted 1:200 in 5% milk, Tris-buffered saline with Tween 20. The membrane was incubated for 1 h at room temperature. The secondary antibodies (horseradish peroxidase-conjugated anti-rabbit IgG, Cell signaling; and donkey horseradish peroxidase-conjugated anti-goat IgG, Santa Cruz Biotechnology) were used in 1:2000 and 1:1500 dilutions, respectively. The Lumi-Light Western blotting substrate (Roche Applied Science) as well as the FUJIFILM LAS-1000 camera was used for visualization.

Northern Blot Analysis—RNA extraction and electrophoresis were performed as described previously (32). PCR fragments of the *GRE2* open reading frame and the 18 S RNA were prepared from genomic DNA template using primers with the following sequences (listed 5' to 3'): *GRE2*, TTCAGGTGCTAACGGGTTCA and AATTTGGGAGGCAG-TGTCGT; and 18 S RNA, CTATCAACTTCGATGGTAGG and TATG-GTTAAGACTACGACGGT. Probes were labeled with [³²P]dCTP using the Megaprime kit (Amersham Biosciences) purified on Nick columns (Amersham Biosciences) and employed with an activity of 1,000,000 cpm/ml. To detect and quantify signals the Molecular Imager FX, the Exposure cassette K (Bio-Rad) and the Quantity One software v 4.2.3 (Bio-Rad) were used. 18 S RNA was used to normalize transcript levels.

Water and Solute Transport Measurements—Yeast cells defective for the fusion of terminal secretory vesicles with the plasma membrane (*sec6*) at a nonpermissive temperature of 37 °C were first grown at 26 °C to a final $A_{600\text{ nm}}$ of ~1.0 in synthetic complete medium lacking uracil (see above) to maintain the wild type or mutant *FPS1* expression vector. Copper sulfate was added to a final concentration of 100 μM, and the cells were incubated at 37 °C with shaking for another 2 h. The cells were harvested, washed, and converted to spheroplasts as described (33). Carboxyfluorescein (CF)-loaded secretory vesicles were prepared as published (34, 35) and washed three times by centrifugation at $144,000 \times g$ to remove unincorporated CF.

Water transport was measured by stopped flow fluorescence as described previously (35). In brief, the vesicles were subjected to an abrupt doubling of external osmotic pressure, and the time course of vesicle shrinkage was monitored as a decrease in fluorescence intensity. The time course of volume change was fitted to a single exponential, and the osmotic water permeability was calculated as described (36). The transport of other polyols was measured after loading vesicles with the indicated polyol in a 1 M solution for 30 min, and the efflux of each polyol was monitored as a decrease in CF-mediated fluorescence over time after the vesicles were rapidly mixed into an iso-osmolar solution lacking the polyol. The permeability coefficients of various sugars were computed from a single exponential fit, as described (37) using equations formulated for each osmotic

² E. Klipp, B. Nordlander, R. Krüger, P. Gennemark, and S. Hohmann, submitted for publication.

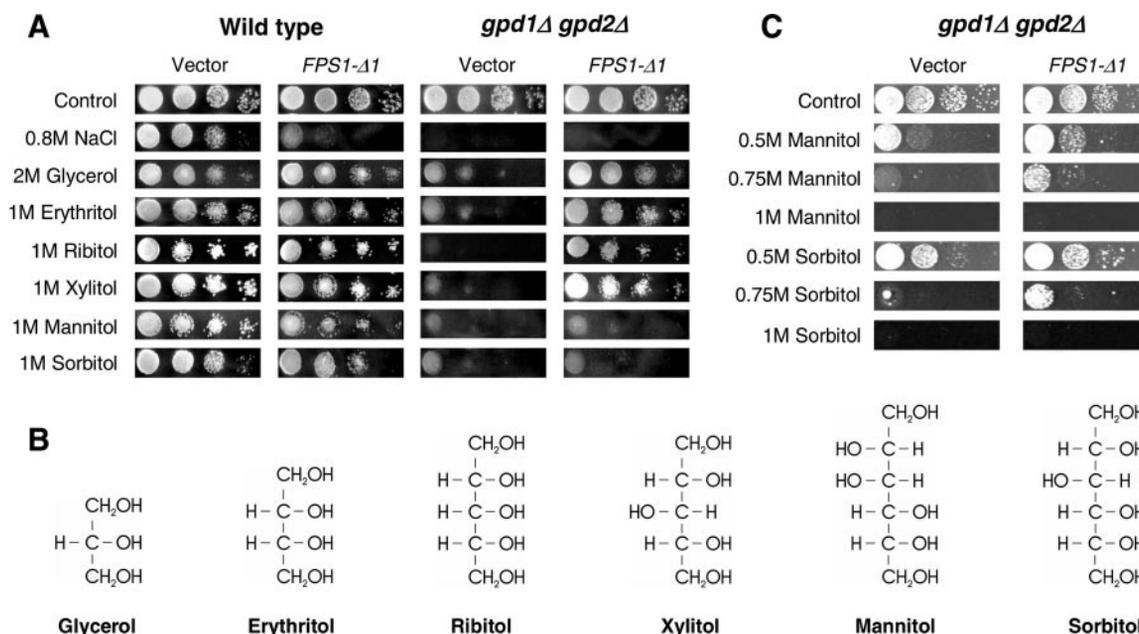


FIG. 1. Expression of Fps1- Δ 1 suppresses the growth defect of the *gpd1* Δ *gpd2* Δ mutant in the presence of high concentrations of different polyols. A and C, cells were spotted in 1:10 dilution series on synthetic medium supplemented with the indicated solute. The strains used were wild type and *gpd1* Δ *gpd2* Δ mutant transformed with an empty vector (YEplac181) and the same plasmid mediating expression of hyperactive Fps1- Δ 1. B, structures of the different polyols.

gradient ($d_{Vrel}/dt = (P_{solute})(SAV)(0.000763)(1611/Vrel-2117)$). The vesicle size (to calculate the surface area of the vesicle (SAV)) was measured by laser light scattering using a DynaPro particle sizer.

RESULTS

Expression of Hyperactive Fps1 Suppresses the Growth Defect of the *gpd1* Δ *gpd2* Δ Mutant on Polyols—A *gpd1* Δ *gpd2* Δ double mutant is unable to produce glycerol and hence does not grow in the presence of 0.8 M NaCl (6) (Fig. 1A). Growth was also inhibited in the presence of 2 M glycerol or 1 M erythritol, ribitol, xylitol, mannitol, or sorbitol (Fig. 1, A and B). When transformed with a plasmid mediating expression of hyperactive Fps1- Δ 1, the wild type strain grew poorly on 0.8 M NaCl (Fig. 1A) because it was unable to retain glycerol inside the cell (12). The Fps1- Δ 1-expressing *gpd1* Δ *gpd2* Δ double mutant grew almost like wild type in the presence of 2 M glycerol and 1 M erythritol, ribitol, and xylitol (Fig. 1A). In other words, Fps1- Δ 1 suppressed the growth defect of the *gpd1* Δ *gpd2* Δ double mutant on medium with high concentrations of polyols of five or fewer carbon atoms.

Growth curves in liquid culture (Fig. 2) both confirmed and added further insight to the observed pattern. Wild type and *gpd1* Δ *gpd2* Δ mutant grew equally well in synthetic growth medium (YNB). As expected, the *gpd1* Δ *gpd2* Δ mutant did not grow in medium containing 0.8 M NaCl, irrespective of the plasmid it contained. Growth of the wild type was reduced when transformed with hyperactive Fps1- Δ 1, as explained above. Interestingly, the effects conferred by polyols were less severe in liquid medium than on plates. Growth on 2 M glycerol resulted in a lag phase for the *gpd1* Δ *gpd2* Δ mutant, which was suppressed by hyperactive Fps1- Δ 1 (not shown). In a similar manner, xylitol caused a lag phase and slower growth for the *gpd1* Δ *gpd2* Δ mutant, but when expressing hyperactive Fps1- Δ 1 the growth profile was similar to that of wild type (Fig. 2). Hyperactive Fps1- Δ 1 allowed some growth even in the presence of mannitol (not shown) or sorbitol, although to a much lesser extent than in xylitol-containing medium (Fig. 2). However, hyperactive Fps1- Δ 1 caused a growth inhibition in wild type cells grown in the presence of sorbitol, probably because sorbitol uptake could not fully

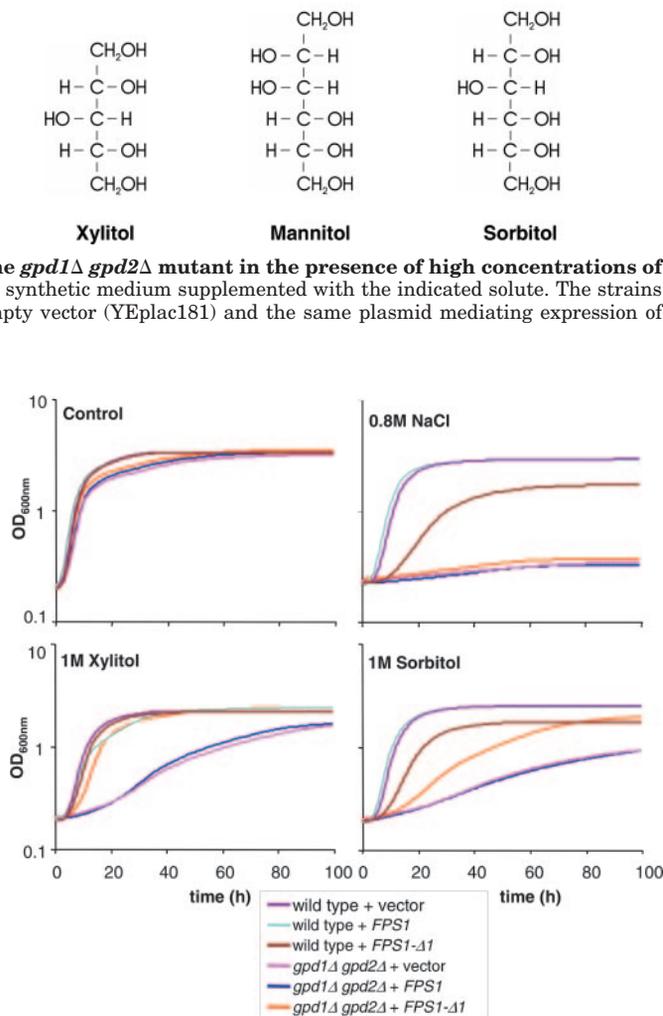


FIG. 2. Suppression of the growth defect of the *gpd1* Δ *gpd2* Δ mutant by expression of Fps1- Δ 1 is more pronounced in liquid medium. Growth curves monitored in a Bioscreen automatic reader. The strains used were wild type and *gpd1* Δ *gpd2* Δ mutant transformed with an empty vector (YEplac181) and the same plasmid mediating expression of wild type Fps1 and hyperactive Fps1- Δ 1.

compensate for the simultaneous glycerol loss (see below for a description of relative uptake rates).

Because the Fps1- Δ 1-expressing *gpd1* Δ *gpd2* Δ mutant grew in the presence of 1 M sorbitol or mannitol in liquid medium but not on plates, we tested plate growth with lower polyol concentrations. Indeed, at 0.75 M sorbitol or mannitol, Fps1- Δ 1 suppressed the growth defect of the *gpd1* Δ *gpd2* Δ mutant. At 0.5 M, the vector control grew slowly, and this growth was clearly improved by expression of Fps1- Δ 1. Hence, Fps1- Δ 1 weakly suppressed the growth defect of the

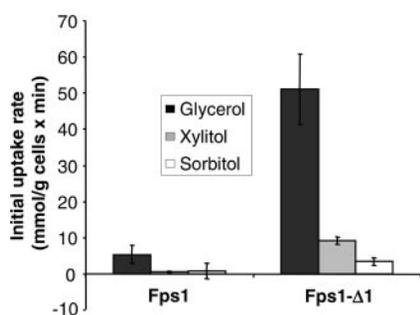


FIG. 3. Initial uptake rate of glycerol, xylitol, and sorbitol in an *fps1Δ* mutant transformed with a plasmid mediating expression of wild type Fps1 and Fps1-Δ1. Substrates were used at a concentration of 100 mM. The values are corrected for a control strain transformed with an empty plasmid and represent the averages of at least four independent experiments.

gpd1Δ gpd2Δ mutant in the presence of sorbitol and mannitol.

Fps1-mediated Polyol Transport—We surmised that suppression of the growth defect of the *gpd1Δ gpd2Δ* mutant by expression of Fps1-Δ1 was due to influx of polyols, resulting in equilibration across the plasma membrane and relief from osmotic stress. Influx of polyols may be more effective in liquid medium than on plates because cells are fully surrounded by medium. Indeed, the extent to which Fps1-Δ1 suppressed the growth defect of the *gpd1Δ gpd2Δ* mutant seemed to correlate well with the reported relative transport rates for these substrates through aquaglyceroporins (18). To directly measure transport rate, the uptake of three commercially available radiolabeled polyols, glycerol, xylitol, and sorbitol, was monitored (Fig. 3). Fps1-Δ1 transported glycerol with a relatively high rate, whereas wild type Fps1 mediated only moderate glycerol transport, as observed previously (12, 14). Fps1-Δ1 also mediated xylitol uptake, although at a clearly lower rate than glycerol uptake, whereas transport of sorbitol was barely detectable. Wild type Fps1 did not mediate detectable uptake of xylitol or sorbitol in this assay. Hence, Fps1-Δ1 mediates uptake of glycerol, xylitol, and sorbitol with progressively decreasing rates.

Purified and reconstituted Fps1 is so far not available. To study transport through Fps1 and Fps1-Δ1 in a more defined system, we purified secretory vesicles from a *sec6* mutant (expressing Fps1 or Fps1-Δ1) following a shift to restrictive temperature, which induces vesicle accumulation. Such vesicles indeed contained the proteins (Fig. 4A), and secretory vesicles for further functional studies were isolated at the time point at which expression was maximal. Transport assays were performed by monitoring the kinetics of osmotic shrinkage of polyol and CF loaded vesicles upon dilution into an iso-osmolar buffer lacking the polyol. Polyol efflux causes fluorescence quenching of CF. Using this system, we found that wild type Fps1 mediated glycerol transport as well as transport of xylitol and ribitol, although the two pentitols were transported at a rate ~1 order of magnitude lower than glycerol (Fig. 4B). Moreover, Fps1-Δ1 mediated an ~2–3-fold higher transport rate than wild type Fps1 for all substrates tested. Hence, these data confirmed and were fully consistent with the data obtained using whole yeast cells.

For Fps1 to function properly as an osmotic glycerol channel in osmoregulation it would be important not to transport significant amounts of water simultaneously. The yeast vesicle system allowed testing of whether Fps1 facilitates water transport. For this, osmotic shrinkage of vesicles containing Fps1 or Fps1-Δ1 was compared with that of secretory vesicles containing AQP1, which is specific for water transport (15). We first found that AQP1 mediated significant water transport (Fig.

4C), as described previously (35), whereas neither Fps1 nor Fps1-Δ1 mediated significant water uptake. Hence, it appears that Fps1 mediates no or only minimal water transport and that the truncation of the Fps1 N terminus, which strongly increases the transport rate for polyols, does not affect water transport.

Expression of AQP9 Allows Growth of the *gpd1Δ gpd2Δ* Mutant on Different Polyols—We surmised that expression of other active aquaglyceroporins should also suppress the growth defect of the *gpd1Δ gpd2Δ* mutant on polyols and therefore tested rat AQP9 (38). Western blot analysis confirmed expression and membrane localization (Fig. 5A). AQP9 appears as monomers as well as dimers, a common feature of aquaglyceroporins (39). The *gpd1Δ gpd2Δ* mutant expressing AQP9 grew in the presence of 2 M glycerol as well as 1 M erythritol, ribitol, and xylitol (Fig. 5B). As was the case with Fps1-Δ1, 1 M sorbitol or mannitol prevented growth, but at lower concentrations suppression of the growth defect of the *gpd1Δ gpd2Δ* mutant was observed. Hence, a heterologous aquaglyceroporin also suppresses the growth defect of the *gpd1Δ gpd2Δ* mutant on different polyols.

Like Fps1-Δ1, AQP9 was also able to mediate uptake of glycerol into whole yeast cells and to a lesser extent xylitol, whereas sorbitol transport was not detectable in this assay (Fig. 5D). The uptake rates measured for AQP9 using this assay were clearly lower than those obtained for Fps1-Δ1 but higher than for wild type Fps1. These differences might be due to different levels of active proteins expressed, a lower fraction of cells maintaining the plasmid, or specific properties of the hyperactive Fps1-Δ1 allele.

Signaling through the HOG Pathway—We reasoned that when *gpd1Δ gpd2Δ* cells expressing Fps1-Δ1 or AQP9 were stressed with different polyols, they experienced osmotic stress for different periods of time. This period would depend on the time it takes for the polyol to equilibrate across the plasma membrane (or to reach a certain level allowing cell reswelling; see “Discussion”), which in turn likely depends on polyol-specific transport rates. According to this hypothesis, the period of osmotic stress exposure should increase from glycerol to xylitol to sorbitol and should be longer for AQP9 than for Fps1-Δ1-expressing cells.

To assess whether such different periods of stress exposure are reflected in HOG pathway activity, the profile of Hog1 phosphorylation was monitored in Fps1-Δ1- and AQP9-expressing *gpd1Δ gpd2Δ* mutants. Upon osmotic shock such mutants show a sustained high level of Hog1 phosphorylation because of their inability to properly adapt to osmotic stress (7).² This was also the case when *gpd1Δ gpd2Δ* mutants expressing either empty control vectors or wild type Fps1 were exposed to 2 M glycerol or 1 M xylitol or sorbitol (Fig. 6). Similarly, when sorbitol, which is transported only slowly through Fps1-Δ1 and AQP9, was used as stress agent, expression of these aquaglyceroporins did not significantly change the Hog1 phosphorylation profile. However, when glycerol was used, Hog1 phosphorylation was not detectable in Fps1-Δ1-expressing cells and was observed for only 5–10 min in the AQP9-expressing cells. With xylitol, Fps1-Δ1-expressing cells exhibited Hog1 phosphorylation for ~40 min, whereas it declined in AQP9-expressing cells only after 120 min. Hence, the period of Hog1 phosphorylation in these experiments showed a correlation with the relative transport rates for these compounds: the more rapid the transport rate the shorter the period of Hog1 phosphorylation.

To monitor the effect on Hog1-dependent gene expression, the relative levels of *GRE2* mRNA were followed over time (Fig. 7). The addition of 2 M glycerol or 1 M xylitol or sorbitol to

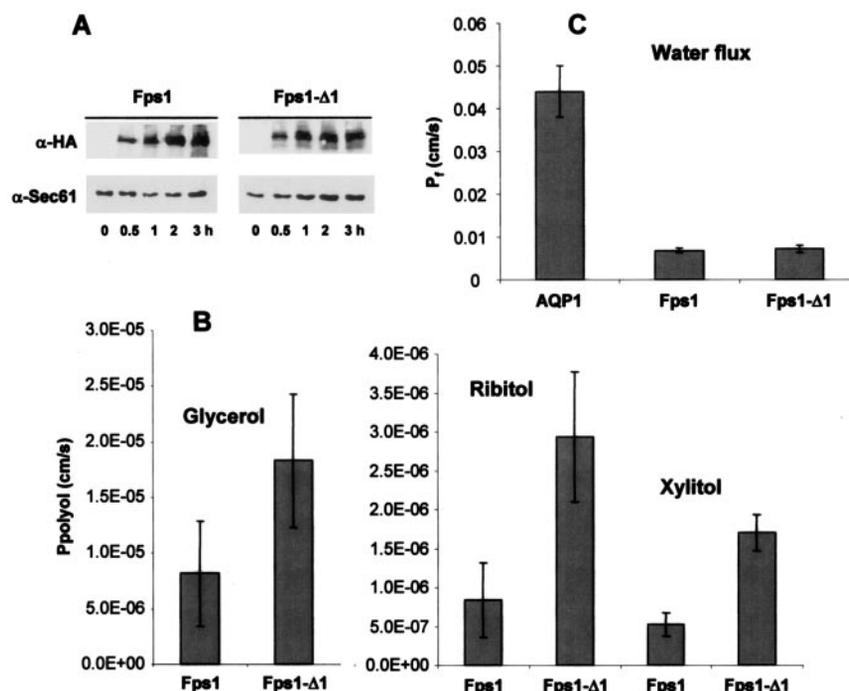
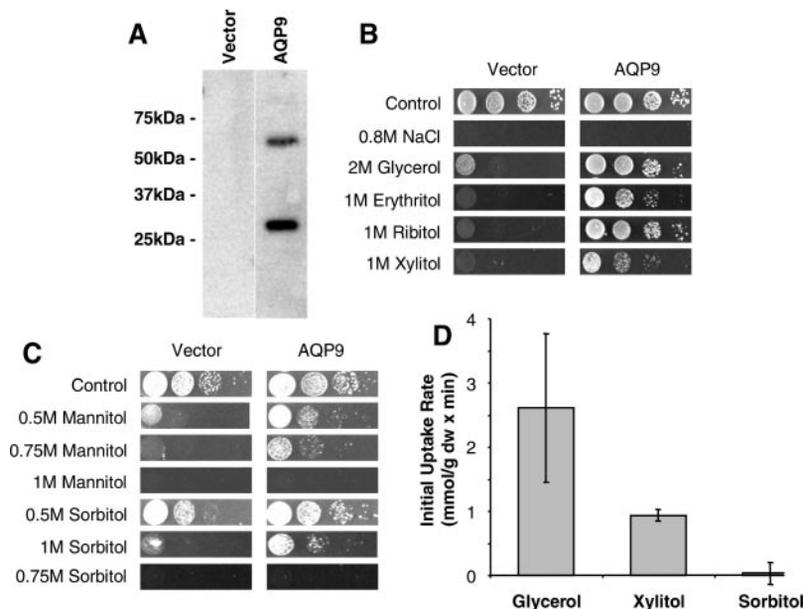


FIG. 4. Transport properties as determined by stopped flow spectrometry in vesicles purified from a yeast *sec6* mutant. **A**, Western blot analysis using an anti-HA antibody of a time course of expression of HA-tagged Fps1 and Fps1- Δ 1 following the addition of copper and a temperature shift to 37 °C at time 0. Crude yeast cell extracts at each time point were prepared, and total protein was resolved by SDS-PAGE. Fps1 and Fps1- Δ 1 expression were assessed by immunoblot analysis using anti-HA antibody. The amount of Sec61p, an integral endoplasmic reticulum resident membrane protein serves as a loading control. **B**, apparent rate of vesicle shrinkage as a measure of polyol transport in yeast secretory vesicles prepared from Fps1 or Fps1- Δ 1-expressing cells (see **A**). Note that different scales were applied to illustrate glycerol and ribitol/xylitol transport, respectively. **C**, osmotic vesicle shrinkage as a result of water transport into CF-loaded AQP1, Fps1, or Fps1- Δ 1-containing vesicles was measured upon rapid mixing of a hyperosmotic solution in a stopped flow apparatus at 15 °C. The vesicles were prepared as in **B**. In all cases, measurements of transport across secretory vesicle membranes prepared from vector control yeast did not significantly increase transport rates beyond those observed in Fps1-containing vesicles (data not shown). The permeability coefficient P indicates the distance the solute travels per time unit, *i.e.* cm/s, where P_w refers to water permeability, P_{glycerol} refers to glycerol permeability, and P_{polyol} refers to ribitol and xylitol permeability.

FIG. 5. Expression of AQP9 suppresses the growth defect of the *gpd1Δ gpd2Δ* mutant in the presence of high concentrations of different polyols. **A**, Western blot analysis of a total cell membrane fraction using an anti-HA antibody. AQP9 monomers appear at ~30 kDa. The upper band likely represents dimers. **B** and **C**, cells of the *gpd1Δ gpd2Δ* mutant transformed with an empty vector (pYX242) and the same plasmid mediating expression of AQP9 were spotted in 1:10 dilution series on synthetic growth supplemented with the indicated solute. **D**, initial uptake rates of 100 mM glycerol, xylitol, and sorbitol in an *fps1Δ* mutant expressing AQP9. Values are corrected as in Fig. 3.



control *gpd1Δ gpd2Δ* cells caused very similar strong and sustained induction (shown only for sorbitol). In AQP9-expressing *gpd1Δ gpd2Δ* cells, the mRNA levels reached progressively lower levels in the following order: sorbitol, xylitol, and then glycerol. The amplitude of the mRNA level was also reached progressively earlier. Together, these data correlate the relative transport rates of each polyol with their ability to attenuate the HOG-dependent stress response.

DISCUSSION

Expression of an active aquaglyceroporin suppresses the osmosensitivity of the yeast *gpd1Δ gpd2Δ* mutant in the presence of high polyol levels. The growth defect is due to the inability of the mutant to produce glycerol; *S. cerevisiae* wild type cells accumulate up to 1 M glycerol to increase intracellular solute concentrations, thereby protecting biomolecules and driving water back into the cell (2). Suppression of osmosensitivity by

FIG. 6. Expression of Fps1- Δ 1 and AQP9 in the *gpd1 Δ gpd2 Δ* mutant and exposure to different polyols results in different periods of HOG pathway activation. HOG pathway activity was monitored in time courses following polyol addition using an antibody specific for the dual phosphorylated (active) p38 kinase (*p(T/Y)-Hog1*). An antibody against total Hog1 served as control. The vector is YEp181 in case of Fps1 and Fps1- Δ 1 and pYX242 for AQP9.

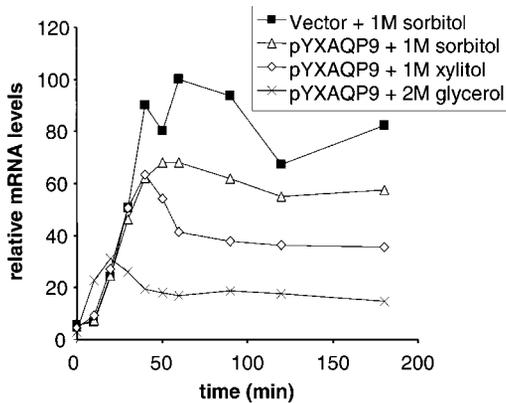
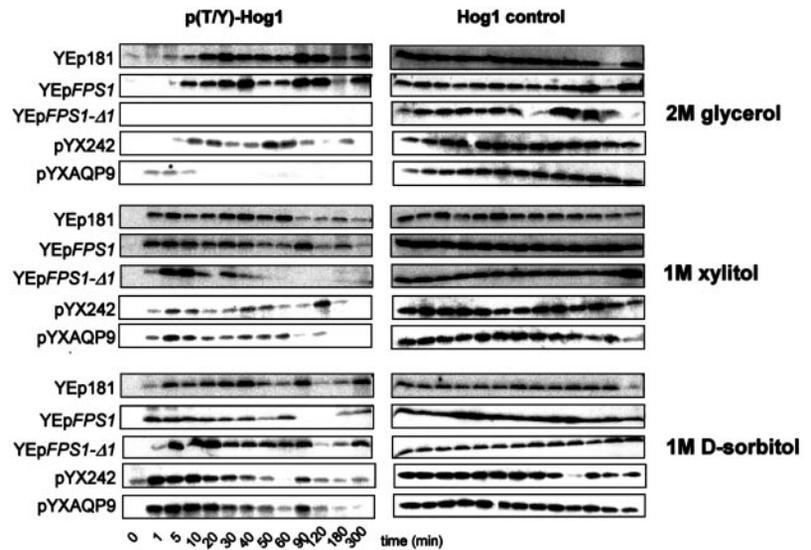


FIG. 7. Expression of AQP9 in the *gpd1 Δ gpd2 Δ* mutant and exposure to different polyols results in different profiles of HOG pathway target gene expression. The mRNA level of *GRE2* was monitored in time courses following polyol addition. Vector (empty plasmid) control is shown only for sorbitol because the profiles for glycerol, xylitol, and sorbitol were essentially superimposable.

Fps1- Δ 1 and AQP9 is most probably due to polyol influx, because there is a correlation between the apparent rate of transport and the degree of suppression. Influx of polyols likely leads to an equilibration of the polyol concentration inside and outside of the cell, thereby relieving osmotic stress.

In this work different polyols were employed, all of which inhibited growth of the *gpd1 Δ gpd2 Δ* mutant unless it expressed Fps1- Δ 1 or AQP9. This means that high intracellular concentrations of these compounds are compatible with cell proliferation, despite that fact that *S. cerevisiae* is specialized to use glycerol as a compatible solute. Yeasts other than *S. cerevisiae* have been reported to accumulate different polyols, such as erythritol and mannitol (40), and it has been shown that mannitol and sorbitol can replace glycerol as solute, although these polyols did not function as well as glycerol at comparable levels (41). It has also been observed that accumulation of xylitol in a genetically engineered yeast strain improved resistance to drying (42). Hence, although this is not an unexpected observation, it is worth noting that high intracellular levels of many polyols are compatible with yeast growth.

The fact that the yeast *gpd1 Δ gpd2 Δ* mutant fails to grow in the presence of high polyol levels and that this defect is suppressed by expression of Fps1- Δ 1 and AQP9 suggests that the yeast plasma membrane is impermeable to glycerol. This makes sense, because the combination of a glycerol-tight

plasma membrane and an osmoregulated glycerol channel is the basis for the well studied efficient and flexible yeast osmolyte system used in osmoadaptation. The growth defect of the *gpd1 Δ gpd2 Δ* mutant and its suppression also indicates that yeast does not possess any other effective uptake systems for polyols, including glycerol, at least not under the conditions employed in this work. Finally, the observation also suggests that osmoshock-induced closure of Fps1 is mediated by an osmotic signal that is independent of the osmolyte used (NaCl as shown previously (14) or different polyols). Hence, its physiological substrate, glycerol, can also mediate channel closure at high concentrations, even though this is counterintuitive at first sight. However, in nature such high glycerol levels are not likely to occur.

The experimental set-up described here can be used to study polyol transport through aquaglyceroporins and probably any other transport protein that can be functionally expressed in yeast. There seems to be a good correlation between the apparent rate of transport as well as the growth rate in liquid culture, as illustrated for Fps1- Δ 1. This allows for at least qualitative estimates of relative transport rates of different compounds through a single transporter. We note that on plates there was no major difference in the degree of suppression conferred by Fps1- Δ 1 and rAQP9, although the observed transport rates were quite different. Hence, plate growth data alone may not be suitable to compare estimated transport rates between different proteins, probably because plate growth tests are evaluated only after several days. In addition, different levels of expression as well as different proportions of cells carrying the plasmid may influence the results. On the other hand, the different transport rates observed for Fps1- Δ 1 and AQP9 were well reflected by the period of Hog1 phosphorylation, which is taken as a measure of the period during which the cell “feels” osmotic stress, *i.e.* the period it takes to equilibrate the polyol to a sufficient extent across the plasma membrane (see below). Together with yeast-based tests for urea (43) or metalloids transport (44), the system to study polyol transport presented here provides a useful arsenal for functional studies of aquaglyceroporins in yeast.

The transport data obtained here for Fps1- Δ 1 are largely in agreement with those reported for purified and reconstituted GlpF (18, 45), the prototype aquaglyceroporin. GlpF transports polyols with rates decreasing in the order: glycerol, ribitol, xylitol, sorbitol, and mannitol (the last was below detection; erythritol was not tested in Ref. 18). Our data extend those studies in that we even observed conductance of

mannitol, based on growth assays, although at an apparently low rate. This substrate preference order follows the size (Fig. 1B) as well as the stereochemical properties of the polyols, with hydroxyl groups on the same side being preferred because of the different hydrophilic and hydrophobic surfaces of the pore (18). Although Fps1 has many similarities to GlpF, the canonical NPA motifs in loops B and E, which form the central pore constriction (18, 46), are NLA and NPS in Fps1 (47). However, our data indicate that these differences do not affect substrate specificity.

Using secretory vesicles purified from yeast and stopped flow spectrophotometry, we confirmed our polyol transport data. This experimental set-up also allowed us to measure water transport through Fps1 and Fps1- Δ 1. These experiments show for the first time that Fps1 has poor water permeability. GlpF has previously been shown to conduct water at a rate \sim 10-fold lower than glycerol (48), a remarkable but physiologically important specificity. Because Fps1, in contrast to GlpF, is involved in osmoregulation as an osmotic glycerol valve (2, 12), it is vital that this channel can distinguish between glycerol and water. In fact, expression of an active water channel renders yeast cells osmosensitive,³ and the expression of the yeast aquaporin Aqp2 is reduced under hyperosmotic stress.⁴ Remarkably, Fps1- Δ 1 did not mediate higher water transport than wild type Fps1. Fps1- Δ 1 is truncated for the large N-terminal extension, which might interact with the channel-forming B-loop to gate the channel (12, 49). Thus, we do not believe that this truncation causes a big conformational change in the protein, which would simply lead to a wider pore allowing unselective bulk flow through the channel. This also indicates that the substrate specificity observed here for Fps1- Δ 1 is identical to that of wild type Fps1. The sensitive gating mechanism of wild type Fps1 apparently reduces the observed transport rate for "slower" substrates such that it falls below the detection level of the assay.

Activation of the HOG pathway, which mediates transcriptional as well as post-transcriptional and post-translational responses in yeast (2, 20, 21), is transient and controlled by strict feedback mechanisms. This is important because active Hog1 kinase inhibits cell proliferation (50). The addition to the *gpd1 Δ gpd2 Δ* mutant of high external levels of glycerol, xylitol, and sorbitol caused strong and sustained activation of the HOG pathway, very similar to that observed with NaCl (7).² Moreover, it has been observed previously (7)² that the presence of hyperactive Fps1- Δ 1, which strongly delays glycerol accumulation, also caused prolonged activation of the HOG pathway. These and further observations (23)² suggested that feedback is linked to successful osmotic adaptation rather than intrinsic feedback mechanisms alone. This is in contrast to the generally accepted view that Hog1-dependent activation of protein phosphatases causes pathway deactivation during adaptation (51).

Data obtained here using the *gpd1 Δ gpd2 Δ* mutant transformed with Fps1- Δ 1 or AQP9 and incubated with different polyols provide strong evidence for pathway deactivation being closely coupled to osmotic adaptation. Glycerol, xylitol, and sorbitol, used as stress agents here, cause similar degrees of osmotic shock (*i.e.* initial cell water loss) and similar prolonged HOG pathway activation in the *gpd1 Δ gpd2 Δ* mutant. However, when the same mutant expressed Fps1- Δ 1 and AQP9, the period was diminished to an extent that correlated with the apparent transport rate for these compounds by the respective protein. Moreover, when comparing the same compound, such as xylitol, the period of HOG pathway activation was clearly

shorter in Fps1- Δ 1 than in AQP9-expressing cells, which is also consistent with slower xylitol transport rates for AQP9. In this experimental set-up it appears that adaptation occurs without the contribution of the yeast (because it cannot produce glycerol) but rather by polyol inflow. Hence it appears that in this set-up the pathway remains activated until a certain amount of the polyol used as stress agent has been taken up by the cell, which then mediates cell reswelling. In wild type cells we have observed that deactivation of the HOG pathway occurs once \sim 30% of the maximal internal glycerol has been reached.² This would be consistent with the idea that the osmosensors monitor osmotic changes rather than states, *i.e.* that they turn on HOG pathway signaling upon water and turgor loss and turn off signaling as soon as cells start to reswell and gain turgor. Taken together, it appears that consistent with its role as an osmosensing signaling pathway, both activation and deactivation of the HOG pathway are mediated by osmotic changes. Taken together, these data are consistent with a model in which the initial osmotic shock, causing water and turgor loss (see also Ref. 22) mediates HOG pathway activation, whereas subsequent reswelling during adaptation causes cessation of the signal.

In summary, then, this work has established an experimental "conditional osmotic stress" system. The system has been shown to be a reliable indicator not only of polyol specificity and transport rates but also the underlying osmosignaling occurring in the system itself. This will no doubt provide tools for unraveling the mechanisms responsible for cellular osmoadaptation. Furthermore, the experimental set-up using the *gpd1 Δ gpd2 Δ* mutant and its suppression by hyperactive Fps1 can be used to select for mutations that render Fps1 hyperactive. We have already used this concept to isolate a range of point mutations providing novel insight into channel control (49).

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Conditional Osmotic Stress in Yeast: A SYSTEM TO STUDY TRANSPORT THROUGH AQUAGLYCEROPORINS AND OSMOSTRESS SIGNALING
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