Quantitative Analysis of Glycerol Accumulation, Glycolysis and Growth under Hyper Osmotic Stress

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Abstract

We provide an integrated dynamic view on a eukaryotic osmolyte system, linking signaling with regulation of gene expression, metabolic control and growth. Adaptation to osmotic changes enables cells to adjust cellular activity and turgor pressure to an altered environment. The yeast Saccharomyces cerevisiae adapts to hyperosmotic stress by activating the HOG signaling cascade, which controls glycerol accumulation. The Hog1 kinase stimulates transcription of genes encoding enzymes required for glycerol production (Gpd1, Gpp2) and glycerol import (Stl1) and activates a regulatory enzyme in glycolysis (Pfk26/27). In addition, glycerol outflow is prevented by closure of the Fps1 glycerol facilitator. In order to better understand the contributions to glycerol accumulation of these different mechanisms and how redox and energy metabolism as well as biomass production are maintained under such conditions we collected an extensive dataset. Over a period of 180 min after hyperosmotic shock we monitored in wild type and different mutant cells the concentrations of key metabolites and proteins relevant for osmoadaptation. The dataset was used to parameterize an ODE model that reproduces the generated data very well. A detailed computational analysis using time-dependent response coefficients showed that Pfk26/27 contributes to rerouting glycolytic flux towards lower glycolysis. The transient growth arrest following hyperosmotic shock further adds to redirecting almost all glycolytic flux from biomass towards glycerol production. Osmoadaptation is robust to loss of individual adaptation pathways because of the existence and upregulation of alternative routes of glycerol accumulation. For instance, the Stl1 glycerol importer contributes to glycerol accumulation in a mutant with diminished glycerol production capacity. In addition, our observations suggest a role for trehalose accumulation in osmoadaptation and that Hog1 probably directly contributes to the regulation of the Fps1 glycerol facilitator. Taken together, we elucidated how different metabolic adaptation mechanisms cooperate and provide hypotheses for further experimental studies.

Introduction

Upon increase in external osmolarity, cells first shrink and subsequently recover volume by accumulating compatible solutes [1]. Different processes contribute to adaptation, establishing negative feedback loops for the regulation of osmotic pressure, cell volume, and turgor [2–5]. The yeast Saccharomyces cerevisiae employs glycerol as compatible solute for osmo-regulation. Upon hyperosmotic shock the High Osmolarity Glycerol (HOG) pathway is activated, resulting in phosphorylation of the stress-activated protein (SAP) kinase Hog1. Phosphorylated Hog1 stimulates expression of genes encoding enzymes involved in glycerol production and uptake. Hyperosmotic stress also leads to rapid closure of the glycerol facilitator Fps1 preventing glycerol outflow. The contributions to glycerol accumulation of the different processes and regulatory mechanisms – including central metabolism - have not been systematically studied in a quantitative and time-resolved manner.

In the absence of hyperosmotic stress, glycerol production is required for maintaining the redox balance [1,6] and excess glycerol leaks out freely through the glycerol facilitator Fps1 [7,8]. The glycerol production pathway starts with the reduction of the glycolytic intermediate di-hydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P) catalyzed by the NAD+-dependent
glycerol-3-phosphate dehydrogenase. This enzyme is encoded by two isogenes, where osmostress controls expression of \textit{GPD1} and cellular redox potential controls expression of \textit{GPD2} [6,9–11]. Stimulated expression of \textit{GPD1} [12,13] enhances glycerol production under hyperosmotic conditions. G3P is transformed to glycerol by the G3P phosphatases Gpp1/Rhr2 and Gpp2/Hor2 [6,9,10,14–16]. Hog1 may also control the amount of DHAP available for glycerol production: it appears that Hog1 stimulates the 6-phosphofructo-2-kinase Pfk26, which, together with its isoform Pfk27, produces fructose-2,6-diphosphate (F26DP), an allosteric activator of the glycolytic enzyme phosphofructokinase (Pfk1) [17]. Glycerol transmembrane transport is facilitated by the aquaglyceroporin Fps1. Its rapid closure upon osmostress prevents glycerol outflow [18]. Active glycerol uptake is mediated by the glycerol-proton symporter Stl1, whose expression is strongly up-regulated upon osmoshock [19,20] and down-regulated by glucose repression. Fig. 1 presents an overview of the known mechanisms involved in osmoadaptation.

Genetic tools to study osmoadaptation include a range of knockout mutants and several strains with alterations in regulatory nodes. The strain \textit{FPS1-A1} contains a mutation that prevents the stress dependent closure of Fps1; this strain produces glycerol but loses it by diffusion through Fps1 [8]. \textit{HOG1-att} contains a tag that tethers Hog1 to the plasma membrane. Such cells are osmo-resistant but do not mount a Hog1-dependent gene expression response [21].

In this study we integrate analysis of five distinct control mechanisms for glycerol accumulation and their link to glycolysis: (i) regulation of \textit{GPD1} expression by nuclear Hog1, (ii) activation of Pfk26 by cytosolic Hog1, (iii) regulation of glycerol transport through Fps1, (iv) volume and associated concentration changes of all cellular compounds, and (v) metabolic reconfiguration resulting in rerouting of fluxes. To study the interplay between these mechanisms, we employed an iterative approach of modeling and experimentation. This model is based on a far more extensive dataset than several previous models including experimental data on a range of metabolites as well as the Hog1 and Gpd1 proteins in wild type and different mutants. The interaction between the different glycerol accumulation processes can be considered as a network in which each node represents one means of control. We tested the robustness of this network to genetic perturbations by model simulation and experimentation.

This work for the first time provides an overview of the dynamics of a eukaryotic osmolyte system, integrating biophysical changes with signaling, control of gene expression, and regulation of metabolic flux. Specifically, we provide evidence that (i) rerouting of glycolytic flux from growth to glycerol production is a major effector of osmoadaptation, (ii) different glycerol accumulation control mechanisms can (partly) compensate for each other, providing robustness and flexibility, (iii) long-term adaptation may involve other osmolytes than glycerol and (iv) Fps1-mediated glycerol efflux is down-regulated by active Hog1.

\textbf{Results}

\textbf{Model}

Based on previous work [22], pilot experiments, and a previous model [2] we constructed a refined mathematical model (Supple-
mentary Information (SI) and Fig. S10). We included carbon flux to biomass, the influence of Hog1 on glycolytic flux via Pfk26/27, and Stl1-mediated glycerol uptake. This model comprises the following modules:

- **Biophysical changes:** changes in cell volume (basal solid volume, \( V_\text{b} \), and osmotically active volume, \( V_\text{os} \) [23,24]), cell surface area, osmotic pressure, and turgor pressure (where changes depend on \( V_\text{os} \) [2] and external osmolality).
- **Glycolysis module:** metabolic reactions from glucose uptake via phosphorylated intermediates to glycerol, trehalose, ethanol, and acetate. The module is based on models [2,25] where reactions have been lumped to reduce the number of parameters (see Text S1 for details). The \( V_\text{max} \) of the Pfk1 reaction is modulated by F26DP, which depends on the Hog1-regulated activity of Pfk26/27.
- **Transport module:** glucose, trehalose, ethanol, acetate transport, glycerol exchange via Fps1, and irreversible glycerol uptake via Stl1. We corrected rate laws of transport reactions for cell density increase in time-course experiments.
- **Biomass module:** biomass production is necessary for describing the interplay between osmotic regulation and glycolysis, but also to maintain correct carbon balance. Biomass is measured as cell number or cell density. Upon osmoshock, Hog1 arrests cell cycle progression [26,27] and hence proliferation. Hog1-activity affects metabolism by altering expression of metabolic genes [20,28–30]. A Hog1-dependent drop in growth rate is included in the model (\( v_\Delta \)).
- **Adaptation module:** Hog1 phosphorylation, GPD1, and STL1 mRNA, translation to Gpd1 and Stl1, activation of Pfk26/27, and closure of Fps1. Hog1 activity is correlated with volume changes and turgor [24]. The Hog1 signaling system is described with only two reactions.

In order to ensure that the available data support the model, which covers different major processes, metabolism is highly condensed for this specific study, compared to metabolic reconstructions suitable for steady state analyses. For example, we refrained from including redox balance dynamics since they are affected by cellular changes beyond the scope of this model, such as detailed biosynthetic pathways (see Text S1). We do not assume a steady state for the entire model because we consider external metabolites and cell density. Although most intracellular concentrations can be considered constant before osmotic stress, the increasing external concentrations of ethanol, acetate and glycerol and the decreasing external glucose level together with the increase in OD slightly affect model dynamics. Therefore, the model is not in steady state in a strict sense.

Models for the different mutant strains are generated by implementing known effects of the respective genetic perturbation (see Text S1). Besides these modifications, all models use the same parameter set.

**Data set**
To estimate model parameters and determine time scales, we measured intracellular and extracellular concentrations of glycerol, glucose, ethanol, acetate, and trehalose over 240 min for wild type and mutants lacking particular adaptation mechanisms (\( \text{hog}1\Delta \), \( \text{gpd}1\Delta \), \( \text{pfk}26\Delta \), \( \text{pfk}27\Delta \), \( \text{pfk}26/27\Delta \)) or with altered regulation nodes (\( \text{HOG}1\text{-att} \), \( \text{FPS}1\text{-att} \)) (Fig. 2, SI). While external glucose is consumed, glycerol, ethanol, and acetate as well OD\(_{600}\) and cell numbers (SI, Fig. S7) increased over time. Internal glycerol (Fig. 2G) accumulates transiently, while trehalose shows two peaks at 43 min and 180 min (Fig. S7G). We also measured GPD1 mRNA concentration, Gpd1 concentration, and Hog1 phosphorylation (Fig. 2A–B, Fig. S13B). The following characteristics were noted.

- In the \( \text{gpd}1\Delta \) mutant, the extracellular glycerol decreases, while the \( \text{FPS}1\text{-att} \) strain excretes large amounts of glycerol (Fig. S8).
- Intracellular glycerol (Fig. 2C) builds up with a delay in the \( \text{FPS}1\text{-att} \) and \( \text{hog}1\Delta \) mutants. In the \( \text{pfk}26/27\Delta \) and \( \text{HOG}1\text{-att} \) [21] strains, intracellular glycerol remains high over the entire period.
- The \( \text{pfk}26/27\Delta \) mutant displays slower glucose consumption (Fig. S7B).
- Trehalose dynamics at 180 min indicate that it may accumulate further in wild type; \( \text{pfk}26/27\Delta \) and \( \text{HOG}1\text{-att} \) strains do not display a late increase of trehalose (Fig. S7G).
• Hog1 phosphorylation (Fig. 2A) is slightly prolonged in cells lacking Gpd1 or Pfk26/27 or expressing constitutively open Fps1.
• The lowest Gpd1 levels are observed for the hog1Δ mutant (Fig. 2B).

Simulation of osmoadaptation
The model was fitted to experimental data (Figs. S12, S13, S14). In general, model fitting can follow two different paradigms: (i) either fit the model to part of the data and use the rest of the data to test model predictions or (ii) use all data for fitting to obtain the model best describing the observations. Since our quantitative time-resolved data of various compounds for wild type and different mutants comprehensively elucidates the potential network dynamics, we decided to eventually use all data from all yeast strains and conditions to parameterize the final model presented here. To reproduce the Gpd1-mediated increase of glycerol production, dimerization of Gpd1, as reported for human Gpd [21,31,32], was implemented. We further implemented a partial Hog1-independent increase of GPD1 mRNA to reproduce hog1A data, which is in accordance with experimental observations [21,22]. To fit gpd1A mutant data, we incorporated a small osmo-dependent increase in GPD2 transcription. Including regulation of biomass production [30] significantly improved model behavior (SI): A rapid drop in cell growth, dependent on stress and cell volume, is necessary to provide sufficient carbon for glycerol production and reproduce experimental data on cell density. To reproduce experimental data for intracellular glycerol in hog1A and HOGL-att strains, we included a negative regulation of open Fps1 by active Hog1.

Model analysis and predictions
To characterize the contribution of different processes to osmoadaptation (Fig. 3), we plotted the absolute fluxes to and from glycerol for wild type and gpd1A over time (Fig. 3A and B) and relative contributions of fluxes in ternary plots (Fig. 3C). We distinguish Fps1-reliant, Gpd1-reliant, and other contributions (‘Others’) to net glycerol flux in each strain. For wild type, immediate adaptation is influenced mainly by changes in volume (part of ‘Others’) and closure of Fps1. Within approximately 15 min after stress, glycerol production becomes the predominant contribution. After 30 min, Fps1 reopens and glycerol levels decrease. Open Fps1 in FPS1-1 strain results in constitutive glycerol efflux. Although the model underestimates the glycerol production for this mutant, it clearly shows that the distribution of fluxes remains at a state of sustained glycerol production. Sustained glycerol production occurs also in the hog1A mutant, but due to reduced Hog1-dependent GPD1 transcription its level remains low until 60 min after stress. Subsequently, the system moves to a state similar to that observed for the FPS1-1 strain.

Although the relative composition of fluxes approaches pre-stress values in wild-type, we do not observe perfect adaptation [33] (Fig. 4). For a more comprehensive analysis, the complete state of cells has to be considered, including changes in reactions or pathways indirectly affecting glycerol concentrations, e.g. changes in biomass production. To do so, we employed scaled time-dependent response coefficients (RCs), $R_i^\tau(t) = c \ln(s(t)) / \ln(q)$ for a compound concentration $s(t)$ and a parameter $q$ [34]. RCs express the relative change in $s(t)$ given a small change in $q$, serving as quantitative measure for the effect of a parameter perturbation on a time course taking all direct and indirect effects into account. Positive or negative values indicate that the time course increases or decreases upon parameter increase, respectively. Scaled RCs (Fig. 4B–C) show that regulation of Pfk26/27 does not have a pronounced effect on intracellular glycerol (Fig. 4B), which is in accordance with experimental data for pfk26/27Δ (Fig. 2C). Additionally, other reactions downstream of Pfk1 are positively affected while glycolytic reactions upstream of Pfk1 (trehalase and biomass production) are reduced. This indicates that Pfk26/27 might be part of a rerouting mechanism of metabolic flux ensuring that the influx to each branch of glycolysis is adjusted to demands and that ATP-production downstream of pyruvate is maintained during osmoadaptation.

We use RCs to compare the role of Sst1 in different experiments (Fig. 4C): apparently the contribution of Sst1, though negligible in most strains until 90 min after stress, is significant in gpd1Δ. In silico predictions show an early decrease in intracellular glycerol accumulation in the gpd1A sst1A double mutant compared with gpd1Δ but a late decrease in the sst1A mutant compared with wild type (Fig. 4D). The different roles of Sst1 in wild type and gpd1A cells highlight context specificity in osmoadaptation.

The regulation of biomass production significantly contributes to glycerol accumulation (Fig. 5, Fig. S15B). Rerouting of glycolytic flux can be assumed to result from Hog1-mediated cell-cycle arrest and glycolytic regulation. The cost of maintaining a certain cell volume by producing more glycerol is compensated by a decrease in growth rate. Changes in doubling times before and after stress are plotted in Fig. 5A. The observed decrease in growth rate is similar for wild type, pfk26/27Δ, and HOGL-att strains. This indicates that the main contribution of Hog1 activity to osmoadaptation is not the transcriptional activation of GPD1, which is absent in the HOGL-att strain. Instead, control of cytosolic or membrane-bound targets constitutes the main contributions of Hog1. A strong growth rate drop is observed in FPS1-1 and hog1A, while this drop is relatively low in the gpd1A mutant. This supports the idea that a Hog1-effect on Fps1 is mainly responsible for the prominent role of Hog1 in osmoadaptation. Fig. 5B shows model simulations of the relative carbon fluxes from glycolysis to glycerol or biomass production, respectively, for wild type and different mutants before 20 min and 90 min after stress induction, indicating a trade-off of cellular adaptation versus growth.

The model is fitted to data covering a period until 180 min after stimulation. However, cellular processes beyond the scope of the model may interfere with the adaptation process at later stages, hence model results and hypotheses derived from those may be less reliable beyond 120 min.

Discussion
For the first time we present a data-driven analysis of the dynamic regulation of a eukaryotic osmolyte system mediating adaptation to hyperosmotic stress that integrates different regulatory layers. Our mathematical model, supported by a detailed dedicated dataset, provides novel insight into the quantitative contributions of regulatory processes underlying glycerol accumulation during the yeast osmostress response.

The main conclusions are:

(i) The Hog1 SAPK mediates regulation of glycolysis via indirect activation of the enzyme Pfk1 and increased expression of glycerol-producing enzymes.

(ii) Pfk1 activation serves stabilization of glycolytic flux as much as increased glycerol accumulation.

(iii) Glycerol accumulation occurs at the expense of biomass production.
Glycerol accumulation is achieved by different fluxes comprising basal (Gpd2-dependent) and Hog1-regulated (Gpd1-dependent) production, glycerol exchange over the plasma membrane as well as concentration increase through volume loss; these fluxes contribute to varying extent during different periods of adaptation.

The parameter set was fitted to reproduce data from seven different strains and experimental observations on 14 of 29 model variables, providing the model with an unprecedented coverage [35]. This coverage was achieved employing coordinated model reduction and experimentation with the intention to maintain all regulatory nodes while lumping metabolites and proteins for which no experimental data was gathered.

Deleting individual nodes of the glycerol accumulation network (Figs. 1, S10) results in slightly prolonged HOG pathway activation. For FPS1-Δ1, this prolongation is less pronounced than in previous data [2], which is probably due to different copy numbers of the expression construct. In contrast to Dihazi et al. [17], we found that only the double deletion mutant, pfk26Δpfk27Δ, shows prolonged Hog1 phosphorylation. The mutation causes a transient delay of glycerol accumulation (Fig. 2C).

We hypothesize that, like human Gpd1 [32], also yeast Gpd1 dimerizes since this assumption results in significantly better fits to experimental data.

It has been shown that Hog1 interacts with and controls Fps1 under certain conditions [36–38], although the exact mechanism of Hog1-dependent control of Fps1 remains elusive. Experimental observations (e.g. [8]), as well as the simulations shown here and elsewhere [3], are consistent with a Hog1-dependent down-regulation of glycerol flux through Fps1 under osmostress. In hog1Δ cells the glycerol efflux is elevated while membrane-attached Hog1 results in very strict regulation.

In the gpd1Δ mutant the glycerol production rate is diminished. Our experimental data show that in this strain the extracellular glycerol concentration decreases over time, indicating glycerol uptake through the Stl1 glycerol-proton symporter.

The presented model underestimates the production of glycerol for FPS1-Δ1. This may have two reasons. First, FPS1-Δ1 cells adapt to the imposed genetic perturbation prior to stress, which can, for example, lead to an up-regulation of glycerol production. Second, the proposed interaction of the modified protein with Hog1 or other potential regulators [38] may be perturbed. Thus, the model indicates that FPS1-Δ1 has global effects beyond the

Figure 3. Contribution of glycerol accumulation mechanisms in different strains. (A, B) Absolute fluxes towards glycerol as well as relative contributions of specific mechanisms differ between wild type WT (A) and gpd1Δ (B). (C) Changes in relative contributions of Fps1, Gpd1, and other effects (basal glycerol production, uptake through Stl1, effects of volume change) over time are depicted for WT, fps1-Δ1 and hog1Δ. Colors in (C) indicate time as shown on the x-axis in (A).

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expected de-regulation of glycerol efflux that need to be investigated further.

In adapted yeast cells the intracellular glycerol level diminishes. We observed an increase in intracellular trehalose concentrations coinciding with the decline of intracellular glycerol (Fig. S7G). In the FPS1-D1 strain, which is unable to accumulate intracellular glycerol, the intracellular trehalose concentration is permanently high. Although the combination of high trehalose and glycerol concentrations has a synergistic osmoprotective effect [39], the observed trehalose concentrations are too small to significantly contribute to intracellular osmotic pressure. Hence, trehalose may have more specific roles in osmoadaptation by protecting proteins and membranes [40,41].

We do not observe perfect adaptation in the sense defined previously [33]. Although cell volume returns to its original values, other variables remain perturbed in adapted cells at constant high external osmolarity, such as Gpd1, trehalose or glycerol concentration, and especially growth rate.

Glycolysis serves three major fluxes resulting in the production of ethanol plus acetate, biomass, and glycerol. Although active Hog1 leads to cell cycle arrest [26,27], potentially decreasing carbon flux towards biomass, a role of glycolysis in the osmotic stress response has not been considered in detail. Data presented by Dihazi et al. [17] as well as recent gene expression data [42] indicate that glycolytic flux may play a role in osmoadaptation. Our preliminary theoretical work [22] indicated that Pfk26/Pfk27 participate in maintaining the flux towards pyruvate upon adaptation to hyper-osmotic conditions. Glucose consumption and ethanol production rates are similar in all strains studied here, regardless which node of the glycerol accumulation network was removed. The pfk26/pfk27 strain is the only exception: the slightly lower ethanol production and glucose consumption observed in this mutant indicate that Pfk26/27 influence glycolysis rather than osmotic adaptation. We conclude that under hyper-osmotic stress part of the carbon flux, which is normally directed towards biomass production, is used for increasing glycerol production. One of the elements in this metabolic prioritization mechanism is Pfk26/27, although it does not seem to be the only factor. The ratio between the production of glycerol and pyruvate (resulting in the formation of ethanol and acetate) is crucial for a proper energy and redox balance. In all strains, the flux towards pyruvate remains unaffected by osmoadaptation while flux towards biomass decreases. Apparently, energy production and redox balance are maintained while growth is temporarily stopped for faster adaptation. This pattern is likely to hold for a wide variety of stresses and adaptation mechanisms.

The relative contribution of each individual glycerol accumulation mechanism depends on the environmental conditions and the physiological state of the cell (Fig. 3A). In their natural environment, cells have to cope with combinations of stresses demanding for dynamic modulation of response mechanisms. The different mechanisms contributing to osmoadaptation might make the process robust but also allow choosing the ‘cheapest’ adaptation strategy under different conditions.

Response coefficient analysis is an excellent tool to elucidate this kind of interdependences. We found that introducing an individual deletion into the glycerol accumulation network is compensated. Our experimental data show that the adaptation takes longer for
single knock-out strains, compared to wild type. The activation of the remaining glycerol accumulation mechanisms is prolonged (active component of the compensation) and the remaining mechanisms become more important (passive compensation component).

Minimalistic models of biological processes, such as the osmoadaptation model presented by Mettetal et al. [3] are of great value for understanding general principles underlying a given process. With a sufficient degree of generalization, conclusions drawn from minimalistic models can be applicable for characterizing other cellular events. This approach neglects the individual mechanisms contributing to a cellular function. On the other extreme, the approach of hierarchical control analysis [43] measures the effects of changes on a cellular process in an enormous amount of detail. This approach is crucial for understanding the role of individual components and events. Our work attempts to bridge between the two paradigms, which can be viewed as an intermediate stage of different magnifications of the same picture.

The principles of osmoregulation are conserved from yeast to mammalian cells. The mammalian stress-activated protein kinase p38 is a homolog of Hog1. Like Hog1 it plays a critical role in mounting the adaptive response to stress by controlling metabolism, gene expression and cell cycle progression [44]. Hence, the approaches employed in the present work as well as the conclusions drawn may have consequences for studies on mammalian cells as well.

By integration of experimental data for different strains into a medium-size model and reliably estimating its parameters, we were able to achieve a better understanding of the contribution of individual players to a cellular response – osmoadaptation – in a quantitative and time-resolved manner. Moreover, our analysis has revealed the trade-off between growth control and glycerol accumulation in the adaptation process.

**Materials and Methods**

**Experimental methods**

**Yeast strains and culturing.** Strains used in this work originate from W303-1A (MATa leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2 malt0) [45]. The strains include: wild type, gad1Δ, pfk26Δ/pfk27Δ, fps1-Δ1 (kindly provided by Markus Tamás, University of Gothenburg), stl1Δ, hog1Δ, HOG1-att (kindly provided by Jeremy Thorner, University of California at Berkeley). A complete list of strains used in this study is presented in Table 1. Yeast cultures were grown until mid-exponential phase (OD600 = 0.7–1.0) in YPD medium (Yeast Peptone D-glucose; 1% yeast extract (Bacto), 2% peptone (Bacto), 2% glucose), then NaCl was added from a stock solution of 5 M in water to a final concentration of 0.4 M at t = 0 min.

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

<table>
<thead>
<tr>
<th>Strain name</th>
<th>Strain genotype</th>
<th>Origin</th>
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<tbody>
<tr>
<td>W303-1a</td>
<td>MATa leu23/112</td>
<td>Thomas and Rothstein (1989) [45]</td>
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<tr>
<td>YMR84</td>
<td>W303-1A with gad1Δ::URA3</td>
<td>Martijn Rep (Amsterdam)*</td>
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<tr>
<td>YSH1583</td>
<td>W303-1A with pfk26Δ::KanMX</td>
<td>This study</td>
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<td>This study</td>
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<td>This study</td>
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<td></td>
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<td>This study</td>
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*Strain YMR84 was kindly provided by Martijn Rep (Amsterdam) and contains a replacement of the GPD1 upstream region (–883 to +91) by the URA3 gene. The strains was generated using a PCR approach and does not express the GPD1 gene product.

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Western blot analysis. Samples of 1 ml were collected at the indicated time points, sedimented and frozen in liquid nitrogen after removing the supernatant. Proteins were extracted by boiling for 10 min in extraction buffer [100 mM Tris-HCl pH 6.8, 20% glycerol, 200 mM DTT, 4% SDS, 10 mM NaF, 0.1 mM Na3VO4 (sodium orthovanadate), protease inhibitor (Complete EDTA-free Protease Inhibitor Cocktail tablets, Roche), and 20 mM mercaptoethanol]. The extracts were clarified by centrifugation (13 000 rpm in 4°C for 10 min). For each sample 40 μg of protein was separated by electrophoresis on a 10% polyacrylamide gel (SDS-PAGE) and transferred (semi-dry) to a nitrocellulose membrane (Hybond-ECL, Amersham). Membranes were blocked with Odyssey Blocking Buffer (Li-Cor Biosciences) and incubated sequentially with primary antibodies: first primary antibody - phospho-p38 MAPK (Thr180/Tyr182) monoclonal rabbit antibody (Cell Signalling), 1:1000 in Odyssey Blocking Buffer with TBST (1:1000), overnight at 4°C; second primary antibody - yC20 total Hog1 polyclonal goat antibody (Santa Cruz Biotechnology Inc.), 1:2000 in Odyssey Blocking Buffer with TBST (1:1000), 1 h at room temperature, third primary antibody - rabbit polyclonal antiserum Gpd1-A (Innovagen), 1:2000 in Odyssey Blocking Buffer with TBST (1:1000), 1 h at room temperature, and simultaneously with secondary antibodies: donkey anti-goat IR Dye 680, 1:12 500 and donkey anti-rabbit IR Dye 800CW 1:12500 (Li-Cor Biosciences), in Odyssey Blocking Buffer with TBST (1:1000), for 45 min at room temperature.

The membranes were scanned using Odyssey Infrared Imaging System (Li-Cor Biosciences) and quantified using Multi Gauge 3.0 (FujiFilm) software.

Metabolite measurements. Samples of 1 ml were collected at the indicated time points. Three types of samples were collected for each time point: intracellular - centrifuged cell pellet without supernatant, extracellular - pure supernatant, removed from intracellular samples, total - 1 ml cells in medium, and frozen in liquid nitrogen. Extracellular samples did not require further processing; total extracts were boiled for 10 min and cleared by centrifugation, cell pellets were extracted with sterile water by boiling for 10 min and cleared by centrifugation. The concentrations of glucose, trehalose, glycerol, acetate, succinate, pyruvate and ethanol were measured by high performance liquid chromatography (DIONEX) with an Aminex HPX-87H ion exchange column (Bio-Rad, Hercules, USA). An isocratic condition was performed with 5 mM H2SO4 as mobile phase at flow rate of 0.6 ml/min and oven temperature of 65°C. Glucose, trehalose, glycerol and ethanol were quantified by a refraction index detector with secondary antibodies: donkey anti-goat IR Dye 680, 1:12 500 and donkey anti-rabbit IR Dye 800CW 1:12500 (Li-Cor Biosciences), in Odyssey Blocking Buffer with TBST (1:1000), for 45 min at room temperature.

Data processing and additional data. Experimental data was processed to account for cell density increase and the reliability of HPLC measurements was assessed by comparison with enzyme assay quantification. For a full description of data processing and additional data, see Text S1.

Mathematical modeling

System dynamics were described by ordinary differential equations (ODEs). A complete list of model equations and parameter estimation procedures is provided in S1. Time-dependent response coefficients have been calculated as described in [34]. Temporal simulations were performed with Mathematica7 (Wolfram Research. Mathematica edition: Version 7.0, 2008). Parameter estimation was done with PottersWheel [46] and SBML-PET [47].

Supporting Information

Dataset S1 Raw and processed metabolite measurements for different strains, stress 0.4 M NaCl added at t=0.

Dataset S2 Overview of experimental data as used in fitting.

Dataset S3 Comparison of intracellular trehalose measurements by HPLC and enzyme assay, stress 0.4 M NaCl added at t=0.

Dataset S4 Western Blot data for different strains and stress strengths.

Dataset S5 Intracellular glycerol quantification by enzyme assay in different strains for different stresses added at t=0.

Dataset S6 Intracellular trehalose quantification by enzyme assay in different strains, stress 0.4 M NaCl added at t=0.

Dataset S7 Northern Blot results for CTT1, GRE2, STL1, 18S in different strains.

Figure S1 Cell density and optical density in stressed (0.4 M NaCl added at t=0) and control experiments (A) and normalized to the values at t=0 (B). Experimental setup as explained in main text.

Figure S2 Plot of OD versus cell density values as obtained from control and stressed time course experiments and the function for computing cell density from OD.

Figure S3 Measured (blue) and inferred (pink) intracellular glycerol for different experiments. Errors in the inferred values due to inconsistencies in measurements are visible in FPS1 ΔT1, log T1 and WT1.

Figure S4 Comparison of glycerol time courses for WT1 and WT4. A) intracellular glycerol, B) extracellular glycerol.

Figure S5 Comparison of enzyme assay (blue) and HPLC (pink) intracellular glycerol quantifications. Underlying data is given in Supplemental Dataset S2.

Figure S6 Comparison of intracellular trehalose levels obtained with different methods. Enzyme assay data is in g D-glucose/l per μg protein/ml, HPLC data in mol/l is normalized to the enzyme assay value at t=30. Underlying data is given in Supplemental Dataset S3.

Figure S7 Experimental data as used for model fitting. Measured entities are indicated on y-axes. Shown are representative experiments for each strain. Stress of 0.4 M NaCl is added at t=0. A: intracellular glucose, B: extracellular glucose, C: Optical density, D: intracellular ethanol, E: extracellular ethanol, F: extracellular acetate, G: intracellular trehalose, H: extracellular trehalose, I: extracellular glycerol.
Figure S8  Experimental data for extracellular glycerol (A,B) and Gpd1 [C] following addition of 0.4 M NaCl at \( t = 0 \). Close inspection reveals a decrease of extracellular glycerol in gpd1Δ strain. The decrease intracellular glycerol in gpd1Δ is on a similar timescale as the transcriptionally regulated increase of Gpd1 in other strains, indicating that a transcriptionally regulated mechanism is responsible for this decrease as well. (PDF)

Figure S9  Effects of HOG1-att [Hog1 attached to the plasma membrane] compared to wild-type. Under unstressed conditions, inactive Hog1 (dark red stars) is localized throughout the cell, residual active Hog1 (light red stars) is localized in the nucleus. Possible Hog1 interaction partners are depicted: Fps1 (brown triangles), Pfk26/27 (blue spirals) and genes (black waves). In wild-type, osmoadaptation leads to active Hog1 translocating to the nucleus to stimulate transcription. In HOG1-att, transcriptional regulation is abolished and cytosolic Hog1-concentration is reduced while possible interactions with membrane-bound proteins are increased. (PDF)

Figure S10  Model topology in SBGN syntax. The different modules are color coded (red: adaptation, yellow: biophysical, brown: transport, blue: glycolytic, green: growth). Measured entities are indicated by a green background. Perturbations to the model as stress [NaCl] or different mutations [I: hog1Δ, II: pfk26/27Δ, III: HOG1-att, IV: gpd1Δ, V: FPS1-Δ1 ]). (PDF)

Figure S11  Changes in cell density in a batch culture experiment. Cells are controlled by an ODE model highlighted in yellow. (PDF)

Figure S12  Agreement between main model variables and experimental data. Concentrations of intracellular glycerol (dashed) and phosphorylated Hog1 in different strains (A: WT, B: pfk26/27Δ, C: HOG1-att, D: FPS1-Δ1, E: gpd1Δ, F: hog1Δ) following hyperosmotic stress of 0.4 M NaCl at \( t = 0 \). (PDF)

Figure S13  Experimental data and simulated model variables, stress 0.4 M NaCl added at \( t = 0 \). A: phosphorylated Hog1, B: GPD1 mRNA, C: Gpd1, D: cell volume, E: abundance of open Fps1, F: intracellular trehalose. Concentrations in A and B are scaled as described in text. (PDF)

Figure S14  Simulation of model variables, stress 0.4 M NaCl added at \( t = 0 \). A: extracellular glycerol, B: intracellular glycerol, C: extracellular trehalose, D: extracellular glycerol, E: extracellular ethanol, F: extracellular acetate. (PDF)

Figure S15  Model Simulations and scaled response coefficients for models of different strains. A,D,G: Model variables (solid line: phosphorylated Hog1, dashed line: intracellular glycerol, dotted: abundance of open Fps1) for wild-type, gpd1Δ and hog1Δ, respectively. B,E,H: scaled response coefficients of osmoshock dependent parameters on intracellular glycerol for wild-type, gpd1Δ and hog1Δ, respectively. C,F,I: scaled response coefficients of glycolytic parameters on intracellular pyruvate for wild-type, gpd1Δ and hog1Δ, respectively. (PDF)

SBML Model S1  Annotated model of osmoadaptation in wild type. (XML)

SBML Model S2  Annotated model of osmoadaptation in pfk26/27Δ. (XML)

SBML Model S3  Annotated model of osmoadaptation in HOG1-att. (XML)

SBML Model S4  Annotated model of osmoadaptation in FPS1-Δ1. (XML)

SBML Model S5  Annotated model of osmoadaptation in gpd1Δ. (XML)

SBML Model S6  Annotated model of osmoadaptation in hog1Δ. (XML)

Table S1  Optical density (OD) and cell density (CD) for control and stressed WT cultures. OD in arbitrary units, cell density in 10⁶ cells/ml. (PDF)

Text S1  Materials and Methods, data processing, and details concerning modeling, parameter estimation, and response coefficients. (PDF)

Author Contributions

Conceived and designed the experiments: EK SH EPK CK BN. Performed the experiments: EPK CK BN JK KKH TJ PD. Analyzed the data: EPK CK BN JS EK SH JN. Wrote the paper: EPK CK EK SH JN.

References


