In vivo measurement of cytosolic and mitochondrial pH using a pH-sensitive GFP derivative in *Saccharomyces cerevisiae* reveals a relation between intracellular pH and growth

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The specific pH values of cellular compartments affect virtually all biochemical processes, including enzyme activity, protein folding and redox state. Accurate, sensitive and compartment-specific measurements of intracellular pH (pHi) dynamics in living cells are therefore crucial to the understanding of stress response and adaptation. We used the pH-sensitive GFP derivative ‘ratiometric pHluorin’ expressed in the cytosol and in the mitochondrial matrix of growing *Saccharomyces cerevisiae* to assess the variation in cytosolic pH (pH_{cyt}) and mitochondrial pH (pH_{mit}) in response to nutrient availability, respiratory chain activity, shifts in environmental pH and stress induced by addition of sorbic acid. The *in vivo* measurement allowed accurate determination of organelle-specific pH, determining a constant pH_{cyt} of 7.2 and a constant pH_{mit} of 7.5 in cells exponentially growing on glucose. We show that pH_{cyt} and pH_{mit} are differentially regulated by carbon source and respiratory chain inhibitors. Upon glucose starvation or sorbic acid stress, pHi decrease coincided with growth stasis. Additionally, pHi and growth coincided similarly in recovery after addition of glucose to glucose-starved cultures or after recovery from a sorbic acid pulse. We suggest a relation between pHi and cellular energy generation, and therefore a relation between pHi and growth.

**INTRODUCTION**

Microbes are able to adapt to a wide range of stressful environments such as deviant temperature, high or low osmotic pressure, oxidative stress and exposure to weak organic acids. The mechanisms by which they adapt to these environments are often poorly understood. To study these adaptive responses we rely on techniques that focus on various levels of cellular regulation, such as transcription profiles, protein levels and metabolic flux analysis. However, global physiological parameters such as intracellular pH (pH_{i}) affect nearly all processes in a living cell. pH_{i} directly influences the redox state of the cell by influencing the NAD^{+}/NADH equilibrium (Veine et al., 1998) and determines pH gradients necessary for transport over membranes (Goffeau & Slayman, 1981; Wohlrab & Flowers, 1982). Additionally, the effect of pH_{i} is very prominent in enzyme kinetics, as pH_{i} influences ionization states of acidic or basic amino acid side-chains and thereby influences the structure, solubility and activity of most, if not all, enzymes.

The different organelles in the cell all maintain their own specific pH, which is used to define and maintain the processes associated with each organelle. Yeast vacuoles, for instance, are reported to have an acidic pH (Preston et al., 1989; Brett et al., 2005; Martínez-Muñoz & Kane, 2008). The proton gradient across the vacuolar membrane has been shown to be essential for the transport of various compounds (Nishimura et al., 1998; Ohsumi & Anraku, 1981). The pH of the mitochondrial matrix on the other hand is reported to be alkaline, with a pH of 8.0 (Llopis et al., 1998). This is the result of electron transport chain activity, which pumps protons from the mitochondrial inner matrix, to generate a proton gradient (∆pH) and an electrochemical gradient (∆ψ) constituting the proton-motive force used for ATP synthesis. The pH of the peroxisomal lumen is reported to be 8.2; this coincides with the pH optimum for most peroxisomal enzymes, which lies between 8 and 9 (van Roermund et al., 2004). Lastly the secretory pathway has been shown to acidify from 7.2 in the endoplasmatic reticulum to 5.2 in the secretory granules. This acidification is necessary for proper protein sorting and modification (Paroutis et al., 2004).

These examples illustrate that organelle-specific pH is a crucial parameter in cell physiology. Therefore, an accurate organelle-specific tool to monitor changes in pH is...
required to fully understand cellular functioning. Current techniques used to measure the pH are \( ^{31} \text{P} \) NMR (Gillies et al., 1981; Ogino et al., 1983), probing with pH-sensitive fluorescent dyes (Bracey et al., 1998; Lanz et al., 1999), deploying radioactively labelled membrane-permeable weak acids or bases (Krebs et al., 1983; Siegumfeldt et al., 2000), as well as the equilibrium distribution of benzoic acid (Kresnowati et al., 2007). However, none of these techniques are organoleptic and thus they are bound to result in measurement of an average cellular pH. Additionally, most techniques require extensive manipulation of cells, which itself can affect \( \text{pH}_i \) (Karagiannis & Young, 2001). A promising method for \( \text{pH}_i \) measurements is based on \textit{in situ} expression of the pH-sensitive green fluorescent protein ratiometric pHluorin (Miesenböck et al., 1998), which is also functional in yeast species (Karagiannis & Young, 2001; Brett et al., 2005; Martinez-Muñoz & Kane, 2008). In this study we expressed pHluorin in the cytosol and mitochondria of \textit{Saccharomyces cerevisiae} and showed that this allows direct and time-resolved monitoring of the cytosolic and mitochondrial pH (\( \text{pH}_{\text{cyt}} \) and \( \text{pH}_{\text{mit}} \)) in yeast cells. We studied the effect of nutrient availability on \( \text{pH}_i \) to verify previously reported data on this subject, and studied how \( \text{pH}_{\text{mit}} \) is affected in fermentative versus respiratory metabolism, and by inhibition of respiratory energy generation. Subsequently, the response of the \( \text{pH}_i \) to external pH changes was studied, as well as the response to the weak acid sorbic acid. All of these experiments suggested a clear relation between \( \text{pH}_i \), the capacity for energy generation and the ability of cells to grow.

**METHODS**

**Strains, media and growth conditions.** Strains used in this work are listed in Table 1. Strains were grown at 30 °C in a defined mineral medium as described by Verdun et al. (1992) using either 2 % glucose, or different carbon sources specified in the text and legends. The medium was selected because of its low background fluorescence; it is similar to the medium containing yeast nitrogen base (YNB) without folic acid and riboflavin as described by Sheff & Thorn (2004). Pre-cultures were grown in Erlenmeyer flasks on a rotary shaker at 200 r.p.m., in the same medium buffered with 25 mM (2004). Pre-cultures were grown overnight in mineral medium as described by Sheff & Thorn, but in this case containing 7.5 g glucose l\(^{-1}\). The stirrer speed was set to 800 r.p.m. while the pH was set to 5.0 and kept constant by automatic titration with 1 M KOH. The temperature of the chemostat was controlled with a heat jacket and a temperature probe. Stirring rate, pH and temperature were kept constant using an Applikon ADI 1010 Biocontroller. The chemostat was aerated by flushing air at 30 l h\(^{-1}\) through the culture. Steady states were verified by off gas analysis for oxygen and carbon dioxide and by dry weight measurements.

Dry weight concentration was determined in triplicate by filtering 10.0 ml broth on pre-washed and pre-weighed cellulose acetate membrane filters (pore size 0.45 μm, Schleicher & Schuell MicroSciences). Each filter was washed with 10 ml demineralized water and dried in a 450 W microwave (Whirlpool Promicro 825) for 15 min. Filters were cooled in a desiccator and weighed on an electronic analytical balance (Mettler-Toledo AB104).

Oxygen and carbon dioxide levels in the exhaust gas of the fermenters were monitored online using an oxygen analyser (Serdex Paramagnetic O\(_2\) transducer) and a carbon dioxide analyser (infrared Servomex Xenta 4100 gas purity analyser). To analyse glucose, ethanol, glycerol, succinate, acetate and trehalose, 1.0 ml broth was quickly quenched in 100 μl 35 % perchloric acid. Samples were subsequently neutralized with 55 μl 7 M KOH. Culture samples and media samples were analysed by HPLC on a Phenomenex Rezex ROA-Organic Acid H+ column using 7.2 mM H\(_2\)SO\(_4\) as mobile phase. Data were included if the carbon balance closed within a 10 % error.

**Strain construction.** Cloning procedures were carried out following standard protocols (Sambrook et al., 1989). All constructs were verified by DNA sequence analysis. To clone the \textit{ACT1} promoter 472 bp of sequence preceding the ATG of the \textit{ACT1} ORF were amplified using primers pACT1-N-Spe (GAACATGTCAAAAAC-CTTAAAAACATATGC) and pACT1-C-Hin (AGAAAGCCTTGT-TAAATCCAGTAAATTTTGATGC) on genomic DNA of the CEN.PK 113-7D yeast strain, introducing \textit{Spel} and \textit{HindIII} sites. Plasmid pYES2-mtGFP (a kind gift from Dr B. Westermann), containing a mitochondrial targeting sequence (Westermann & Neupert, 2000) was digested with \textit{Spel} and \textit{HindIII} to introduce the \textit{ACT1} promoter. The pHluorin ORF (Miesenböck et al., 1998) was PCR amplified using primers pGS0012 (AGGACACCATGATAAGGGAAAC-ACTGTC) and pGS0011 (AGAAATGTTATGATAGTCCATAGTGGAGGAAAC-TTTCA) on genomic DNA of the CEN.PK 113-7D yeast strain, introducing \textit{Spel} and \textit{HindIII} sites. This fragment was cloned into pYES2-\textit{pACT1}-mtGFP, generating pYES2-\textit{pACT1}-

<table>
<thead>
<tr>
<th>Strain PK</th>
<th>Genotype</th>
<th>Source</th>
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<tbody>
<tr>
<td>CEN.PK 113-7D (WT)</td>
<td>MATa MAL2-&amp;c SUC2</td>
<td>P. Kötter (Frankfurt, Germany)</td>
</tr>
<tr>
<td>CEN.PK 113-5D</td>
<td>MATa MAL2-&amp;c SUC2 ura3</td>
<td>P. Kötter</td>
</tr>
<tr>
<td>ORY001</td>
<td>MATa MAL2-&amp;c SUC2 ura3; pYES-\textit{pACT1}-pHluorin (URA3)</td>
<td>This study</td>
</tr>
<tr>
<td>ORY002</td>
<td>MATa MAL2-&amp;c SUC2 ura3; pYES-\textit{pACT1}-mtGFP (URA3)</td>
<td>This study</td>
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</table>
mtpHluorin. After sequencing this construct we noticed that the L220F mutation described by Miesenböck et al. (1998) was not there. However, we were still able to achieve proper ratiometric determinations (see below). The mitochondrial targeting sequence was removed by digestion with HindIII and BamHI, sequential blunting with T4 polymerase and religation, yielding pYES2-P*ACT1-pHluorin. Yeast transformation was carried out according to Schiestl & Gietz (1989).

**Microscopy.** Cells were grown in Erlenmeyer flasks to an OD\textsubscript{600} of approximately 1.0 in Verduyn medium containing 2 % glycerol and 2 % ethanol as carbon source; an OD\textsubscript{600} of 1.0 corresponded to 3.0 \times 10\textsuperscript{6} cells. Then 25 nM MitoTracker Red CMXRos (Invitrogen) was added to the medium and cells were cultured for an additional 30 min. After washing with PBS, cells were transferred to agarose-coated glass slides. Images were obtained using a Canon A620 camera on an Axiovert 40 CFL microscope (Carl Zeiss) with a Plan Neofluar 100 \times/NA 1.3 oil objective, using Endow GFP and Cy3 narrow-band excitation filter sets for fluorescent images. Merge images of MitoTracker and pHluorin signals were created using Photoshop software.

**In situ pHluorin calibration and pHluorin measurements.** Strains were grown in Erlenmeyer flasks to an OD\textsubscript{600} of approximately 1.0 in Verduyn medium, centrifuged for 5 min at 4000 r.p.m. and resuspended in PBS containing 100 \(\mu\)g digitonin ml\textsuperscript{-1}. After 10 min cells were washed with PBS and put on ice. Cells were transferred to CELLSTAR black polystyrene clear-bottom 96-well microtitre plates (Greiner Bio-One) to an OD\textsubscript{600} of 0.5 in citric acid/Na\textsubscript{2}HPO\textsubscript{4} buffer of pH values ranging from 5.0 to 9.0. pHluorin fluorescence emission was measured at 512 nm using a SpectraMax Gemini XS microtitre plate spectrofluorometer (Molecular Devices) providing excitation bands of 9 nm centred around 390 and 470 nm. Background fluorescence for a wild-type culture not expressing pHluorin was subtracted from the measurements. The ratio of emission intensity resulting from excitation at 390 and 470 nm was calculated \((R_{390/470})\) and plotted against the corresponding buffer pH. In all experiments a wild-type culture was grown simultaneously as a reference for background fluorescence at both separate excitation wavelengths. pH values are always represented as means \(\pm\) SD. All pH determination experiments were repeated at least three times (biological replicates) and figures show one representative experiment in which error bars represent the standard deviation of at least three technical replicates.

**Perturbations.** Cells were pre-cultured in Erlenmeyer flasks to an OD\textsubscript{600} of approximately 1.0 in Verduyn medium with 2 % of the carbon source indicated. Cells were reinoculated in batch fermenters in Verduyn medium with controlled pH. For chitosan treatment this pH was 6.7. When cultures reached an OD\textsubscript{600} of 0.2 fragmented chitosan was added to a final concentration of 50 \(\mu\)g ml\textsuperscript{-1}. Chitosan was kindly provided by Dr A. Zakrzewska and fragmented as previously described (Zakrzewska et al., 2005). For pH-shift experiments cells were reinoculated in batch fermenters at the pH indicated. At an OD\textsubscript{600} of 0.2, the pH was changed to the post-shift value by titration with 1 M HCl or 1 M KOH. For sorbic acid treatment cells were reinoculated in batch fermenters with a pH of 5.0. When cultures reached an OD\textsubscript{600} of 0.2 potassium sorbate was added in given concentrations. OD\textsubscript{600} and pH were registered over time. For the studies concerning carbon source and the effect of respiratory inhibitors, cells were reinoculated in shake flasks with Verduyn medium buffered at pH 5.0 with 25 mM sodium citrate, with 2 % glucose, 2 % galactose or 2 % glycerol and 2 % ethanol. At an OD\textsubscript{600} of 0.2, samples were transferred to microtitre plates, in the presence or absence of 10 \(\mu\)M antimycin A or carbonyl cyanide m-chlorophenylhydrazone (CCCP, Sigma).

**Glucose pulse experiments.** Cells were grown to an OD\textsubscript{600} of approximately 1.0 in shake flasks in Verduyn medium and washed twice with Verduyn medium without glucose. Subsequently, cells were inoculated in batch fermenters to an OD\textsubscript{600} of 0.2 in Verduyn medium without glucose at pH 5.0 and starved for at least 1 h. Glucose was administered to the batch fermenters to a final concentration of 10 mM at time point \(t=0\). For short-term glucose pulse experiments, cells were taken from the batch fermenter prior to glucose addition and transferred to microtitre plates; a 10 mM glucose pulse was given in the plate. Time point \(t=0\) was measured just before glucose addition.

**RESULTS**

**pHluorin expression in cytosol and mitochondria**

To differentially determine pH\textsubscript{mit} and pH\textsubscript{cyt}, pHluorin was subcloned into multicopy plasmids either with or without a mitochondrial targeting signal. For strong, constitutive pHluorin expression we introduced the promoter region of the ACT1 gene. The mitochondrial targeting signal consisted of the first 69 amino acids of subunit 9 of the F\textsubscript{0} ATPase of Neurospora crassa, comprising the N terminus of the pHluorin fusion protein. Localization of a GFP protein to the mitochondrial network by means of this targeting signal was previously described by Westermann & Neupert (2000). To confirm proper localization of the pHluorin in both strains, the subcellular localization of the green fluorescence emitted from the proteins was compared to that of the red fluorescence of a MitoTracker dye in cells grown on glucose (not shown) and on glycerol/ethanol to obtain maximal mitochondrial networks (Fig. 1).

![Fig. 1. Targeting of pHluorin to cytosol and mitochondria. (a) Wild-type, (b) ORY001 and (c) ORY002 strains were grown in Verduyn medium with 2 % glycerol and 2 % ethanol to mid-exponential phase at 30 °C. Then 25 nM MitoTracker Red CMXRos was added to the medium and cells were cultured for an additional 30 min. Images show (I) phase-contrast, (II) pHluorin and (III) MitoTracker signals. Column IV shows merged images of pHluorin and MitoTracker signals.](https://example.com/fig1.jpg)
pHluorin expression in strain ORY001, which contained a multi-copy plasmid carrying the pHluorin construct (Table 1), was diffusely distributed over the cell (Fig. 1b). In this strain the protein was excluded from mitochondria as the green and red signals did not co-localize. Additionally, exclusion of both signals from another organelle, which probably corresponded to the vacuole, was also observed. These observations are consistent with pHluorin localization in the cytosol. Strain ORY002, containing a plasmid with the pHluorin gene preceded by a mitochondrial targeting sequence, showed pHluorin expression that clearly co-localized with the MitoTracker dye (Fig. 1c), consistent with pHluorin expression in the mitochondria.

To assess whether cellular functioning was affected by the overexpression of pHluorin in cytosol and mitochondria, we determined the physiological parameters of respiring, glucose-limited chemostat cultures of wild-type cells as well as ORY001 and ORY002 (Table 2). We found that, although expression of both cytosolic and mitochondrial pHluorin led to a small decrease in CO₂ and O₂ fluxes, it did not affect specific biomass yield, glucose flux or respiratory quotient. No significant differences were detected between the two pHluorin-expressing strains. These data show that overexpression of pHluorin in cytosol and mitochondria did not disturb cellular metabolism.

**pH monitoring in cytosol and mitochondria**

To obtain *in situ* calibration curves for pH measurements, cultures were grown to an OD₆₀₀ of approximately 1.0 and subjected to mild permeabilization by incubation with 100 µg digitonin ml⁻¹ for 10 min. Cells were resuspended in buffers of known pH in the range 5.0–9.0 and ratios of pHluorin emission by 390 and 470 nm excitation (R₃⁹⁰/₄⁷₀) in buffers of known pH in the range 5.0–9.0 and ratios of pHluorin led to a small decrease in CO₂ and O₂ fluxes, it did not affect specific biomass yield, glucose flux or respiratory quotient. No significant differences were detected between the two pHluorin-expressing strains. These data show that overexpression of pHluorin in cytosol and mitochondria did not disturb cellular metabolism.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dry weight (g g⁻¹)</th>
<th>Yield (g g⁻¹)</th>
<th>q₉glucose</th>
<th>q₀₂</th>
<th>q₀₂</th>
<th>pH₉cyt</th>
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<tbody>
<tr>
<td>CEN.PK 113-7D</td>
<td>3.8 ± 0.2</td>
<td>0.50 ± 0.03</td>
<td>1.1 ± 0.1</td>
<td>2.9 ± 0.3</td>
<td>2.9 ± 0.3</td>
<td>ND</td>
</tr>
<tr>
<td>ORY001</td>
<td>3.7 ± 0.2</td>
<td>0.50 ± 0.05</td>
<td>1.1 ± 0.1</td>
<td>2.5 ± 0.1</td>
<td>2.5 ± 0.1</td>
<td>6.9 ± 0.1</td>
</tr>
<tr>
<td>ORY002</td>
<td>3.8 ± 0.2</td>
<td>0.54 ± 0.03</td>
<td>1.0 ± 0.1</td>
<td>2.5 ± 0.1</td>
<td>2.5 ± 0.1</td>
<td>7.3 ± 0.1</td>
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</table>

Data are the means ± SD of three independent chemostat cultures for each strain. Ethanol was not detectable in these conditions. q₉glucose, q₀₂, and q₀₂ values are expressed as mmol (g dry wt)⁻¹ h⁻¹.

**Starved cells pulsed with glucose show a rapid acidification followed by alkalinization**

A well-known phenomenon in yeast is glucose pulse-induced initial acidification and subsequent alkalinization of glucose-starved cells (Martinez-Munoz & Kane, 2008; Kresnowati et al., 2007; Ramos et al., 1989; van Urk et al., 1989). While reports on the kinetics of this phenomenon are similar, the methods of culturing and pHdetermination vary greatly, probably significantly contributing to the differences in actual pH values reported. Glucose-starved cultures (Fig. 3b) showed pH values significantly lower than cultures growing on glucose-containing media (Figs 2c, 4a, b, 5a–c, 6c, d). Both pH₉cyt and pH₉mit values decreased to 5.7 during the starvation period, abolishing the pH difference between the two organelles. At t = 0 glucose was added, and an alkalinization of the batch culture was observed to pH values transiently higher than the pH of glucose-grown cultures. After approximately 60 min both pH₉cyt and pH₉mit recovered to those of glucose-growing cultures in mid-exponential phase (Fig. 3b). At the same time, the cultures resumed growth (Fig. 3a), indicative of a relation between pH and growth. The initial acidification reported in earlier experiments could not be observed, which could be explained by the fact that this is a fast effect that cannot be observed on a timescale of minutes.

To be able to evaluate shorter timescales we next performed an experiment with glucose-starved cells from the fermenter using microscopy. After addition of glucose, we monitored the pH₉ four times for 100 s with 13 s intervals. To get optimal time resolution we started each subsequent measurement series 2 s after the previous one. The data were plotted in a single figure (Fig. 3c). Equipment limitations prevented measuring the kinetics of the first 20 s. Still, with this set-up we could observe the initial acidification. A rapid and transient drop in pH₉cyt could be observed from 5.7 to 5.3 within 20 s, followed by an immediate alkalinization to 6.5 within the first 100 s.

### Table 2. Physiological parameters and pH of pHluorin-expressing strains in glucose-limited continuous cultures

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dry weight (g g⁻¹)</th>
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<td>2.9 ± 0.3</td>
<td>2.9 ± 0.3</td>
<td>ND</td>
</tr>
<tr>
<td>ORY001</td>
<td>3.7 ± 0.2</td>
<td>0.50 ± 0.05</td>
<td>1.1 ± 0.1</td>
<td>2.5 ± 0.1</td>
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<td>6.9 ± 0.1</td>
</tr>
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<td>ORY002</td>
<td>3.8 ± 0.2</td>
<td>0.54 ± 0.03</td>
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<td>2.5 ± 0.1</td>
<td>7.3 ± 0.1</td>
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The pHmit did not show an acidification and increased to 7.2 in the first 100 s. Thus, organelle-specific expression of pHluorin allows a direct and distinctive kinetic monitoring of organellar pH.

Effect of respiratory chain inhibitors on pHcyt and pHmit depends on carbon source

While the pHcyt measurements recapitulate previous findings, the pHmit behaves differently. Also, there might be a difference in fermenting versus respiring cells. Indeed, in our chemostat cultures respiring glucose (Table 2) we determined a pHcyt of 6.9 and a pHmit of 7.3, a pH

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**Fig. 2.** Calibration curves and in situ determinations of pHcyt and pHmit. (a, b) Strains were grown in Erlenmeyer flasks to an OD600 of approximately 1.0 in Verduyn medium and permeabilized using 100 μg digitonin ml⁻¹. Cells were washed and resuspended in citric acid/Na2HPO4 buffer of pH values ranging from 5.0 to 9.0. Ratios of pHluorin emission at 512 nm upon excitation at 390 and at 470 nm (R390/470) were plotted against pH after wild-type background subtraction to provide calibration curves for (a) strain ORY001 and (b) strain ORY002. (c) Strains were grown to mid-exponential phase in batch fermenters in Verduyn medium at pH 6.7. At t=0 fragmented chitosan was added to a final concentration of 50 μg ml⁻¹. At t=80 and t=95 the pH of the medium was set to 6 and 7 using HCl and KOH respectively. Error bars represent standard deviation of three technical replicates. ●, pHcyt; ○, pHmit; dashed line, medium pH.

**Fig. 3.** Long- and short-term effects of an external glucose pulse on the pH of starved cells. (a, b) Cultures were starved as described in Methods and reinoculated in batch fermenters containing Verduyn medium without glucose. At t=0 batch cultures were pulsed with glucose to a final concentration of 10 mM. (a) Growth curves, (b) pH profiles. Error bars represent standard deviation of three technical replicates. (c) Samples of glucose-starved cells were transferred to microtitre plates followed by a glucose pulse to a final concentration of 10 mM. Time point t=0 was measured just before glucose addition. Error bars represent standard deviation of seven technical replicates. ●, pHcyt; ○, pHmit.
difference already much bigger than that in glucose-fermenting batch cultures. To observe how pH\textsubscript{cyt} and pH\textsubscript{mit} are differentially controlled, we cultured our strains on media with different carbon sources. Glucose-containing batch cultures are fully fermenting. Galactose can be metabolized in fermentative and respiratory metabolism, and does not cause glucose repression. In galactose-grown batch cultures \textit{S. cerevisiae} has fully functional mitochondria and respiratory activity (Lagunas, 1976). Lastly, glycerol/ethanol-containing media can only be used via respiration. On these different media, both pH\textsubscript{cyt} and pH\textsubscript{mit} change. In cultures grown on glucose, galactose and glycerol/ethanol, we measured pH\textsubscript{cyt} values of 7.0 ± 0.0, 7.1 ± 0.1 and 6.8 ± 0.0, respectively, while pH\textsubscript{mit} was registered at 7.5 ± 0.0, 7.30 ± 0.1 and 7.26 ± 0.0 (see Fig. 4, t=0). There was no significant effect of respiratory conditions on the pH difference between mitochondria and cytosol, however. We then assessed the effect of inhibition of mitochondrial respiration on organellar pH. We stressed cultures using the respiratory chain electron transfer inhibitor antimycin A (Fig. 4a, c, e), or the proton gradient uncoupler CCCP (which affects plasma membrane proton gradient as well; Fig. 4b, d, f). Antimycin A prevents energy generation using the respiratory chain, and causes a rapid drop in mitochondrial and pH\textsubscript{cyt} in cells grown on galactose (Fig. 4c) and glycerol/ethanol (Fig. 4e), but has very little effect on glucose-grown cells (Fig. 4a), which have repressed respiratory chain expression. Interestingly, in glucose-grown cells the ΔpH over the mitochondrial membrane is maintained (Fig. 4a), while it is lost in derepressed cells (Fig. 4c) and even reversed in fully respiratory cells (Fig. 4e). With all three carbon sources, addition of CCCP leads to a complete dissipation of the pH gradient over the mitochondrial membrane. Interestingly, the pH reached after 20 min of treatment depends on the culture carbon source, where respiratory, glycerol/ethanol-grown cells seem to equilibrate with the external pH (Fig. 4f), while galactose- and glucose-grown cells can control pH\textsubscript{i} for longer (Fig. 4b, d).

**pH\textsubscript{i} values are unaffected by external pH shifts between pH 3.0 and pH 7.5**

Baker’s yeast is very resistant to acidic environments, and can grow in cultures with a pH as low as 2.5 (Carmelo et al., 1996). We investigated how external pH changes

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**Fig. 4.** Effect of respiratory inhibitors on pH\textsubscript{i} depends on the extent of glucose repression. Cultures were grown in media containing 2% glucose (a, b), 2% galactose (c, d), or 2% glycerol and 2% ethanol (e, f) as carbon sources. (a, c, e) Effect of treatment with 10 μM antimycin A on pH\textsubscript{cyt} (●) and pH\textsubscript{mit} (○) on cultures grown to exponential phase on (a) glucose, (c) galactose, and (e) glycerol/ethanol. (b, d, f) Effect of treatment with 10 μM CCCP on pH\textsubscript{cyt} (●) and pH\textsubscript{mit} (○) on cultures grown to exponential phase on (b) glucose, (d) galactose, and (f) glycerol/ethanol. Error bars represent standard deviation of four technical replicates.
influence the pH$_i$ using our pHluorin-based method. As protons are charged we do not expect them to diffuse over the plasma membrane. However, changes in the pH of the medium affect the pH gradient over the plasma membrane, which affects ATPase activity (Carmelo et al., 1996), and previous reports have described the dependency of pH$_i$ on the extracellular pH (Valli et al., 2005). To investigate if pH$_i$ values were sensitive to changes in the external proton concentration, we shifted the pH of the medium of cells grown at pH 6.0 to pH 3.0 using HCl (Fig. 5a), and the pH of the medium of cells grown at pH 3.0 to pH 6.0 using KOH (Fig. 5b). All cultures showed a doubling time of 109 min and the shifts in external pH did not affect the growth rates of the cultures (unpublished data). Fig. 5(a) shows that the effect of a sudden acidification on pH$_{cyt}$ and pH$_{mit}$ is negligible on a timescale of minutes. A similar absence of response in pH profiles was observed when cultures were subjected to a sudden alkalinization (Fig. 5b). Additionally, we measured pH$_{cyt}$ in cultures shifted from pH 6.0 to pH values between 7.0 and 8.0 (Fig. 5c). Shifts to pH 7.0 and 7.5 did not lead to a change in pH$_i$. However, shifting to an external pH of 8.0, much higher than the stable internal pH of 7.2, did lead to decreased growth, and to an increase in pH$_i$ to a new (stable) value of 7.5. However, these cultures showed evidence of cell lysis; the cultures developed foam, and when we analysed the culture supernatant we detected fluorescence at pHluorin wavelengths, which we did not detect in supernatants from other cultures (unpublished data), suggesting leakage of possibly 50% of the pHluorin out of the cell. The pH$_i$ values determined are therefore unreliable, as they result from an average fluorescence of pHluorin in the culture supernatant and pHluorin in the cell. We conclude that external shifts in proton concentration in the pH range from 3.0 to 7.5 do not significantly affect pH$_i$ values. High pH values, causing a reversal of the pH gradient, caused cell lysis.

**pH$_i$ dynamics during sorbic acid stress and recovery coincide with the effects observed on growth**

Weak organic acids are presumed to cause pH$_i$ homeostasis stress (Bracey et al., 1997; Brett et al., 2005). The undissociated, uncharged molecule is thought to diffuse over the plasma membrane and dissociate at the higher pH encountered in the cell, which leads to acidification as well as anion accumulation (Piper et al., 2001). We assessed the effect of the food preservative sorbic acid on growth and pH$_i$. We used concentrations of 2.74 and 5.47 mM potassium sorbate, which at pH 5.0 corresponds to 1 and 2 mM undissociated sorbic acid respectively. Cultures maintained full viability under these stress conditions (unpublished data). The presence of 1 mM sorbic acid resulted in a reduction of exponential growth rate of 50–60% in both strains, whereas 2 mM resulted in a nearly complete growth stasis (>90% inhibition) within the time-frame studied (Fig. 6a, b).

Similar to our previous experiments, cells in unstressed glucose-growing cultures maintained a pH$_{cyt}$ of 7.2 and a pH$_{mit}$ of 7.5 (Fig. 6c, d). When cultures were stressed with 1 mM sorbic acid, an immediate decrease in pH$_{cyt}$ to 6.2 could be observed, which, after a lag of approximately 1 h, increased to a value of 6.7 over the next 2 h and then stabilized (Fig. 6c). When cells were stressed with 2 mM sorbic acid, pH$_{cyt}$ dropped to 6.0 and did not increase for at least 4 h. Similarly, the pH$_{mit}$ decreased from 7.5 to 6.6 when cultures were stressed with 1 mM sorbic acid, and
recovered to a pH of 7.3 after a lag of 1 h and a recovery period of 90 min (Fig. 6d). When cultures were stressed with 2 mM sorbic acid, pHmit dropped to 6.3 and, like pHcyt, did not recover for at least 4 h.

Comparing these results to the growth curves we observed that cells stressed with 1 mM sorbic acid were also able to recover growth. These cultures partly recovered their pHi values (Fig. 6c, d) and showed a reduced growth rate of approximately 60% of their original growth rate (Fig. 6a, b). Cells stressed with 2 mM sorbic acid did not recover their pHi and showed no significant growth recovery. Interestingly, the ΔpH between cytosol and mitochondria was maintained during sorbic acid stress and even increased in cultures stressed with 1 mM sorbic acid. In these cells ΔpH was as large as 0.6 pH units after 3 h of stress as opposed to 0.3 in unstressed cultures (Fig. 6c, d). This suggests that cells may be able to at least maintain a level of oxidative phosphorylation under this level of sorbic acid stress. We conclude that pHi dynamics during sorbic acid stress and recovery coincide with the effects observed on growth. Whether pHi is causal with respect to growth rate, or simply generally associated with decreased growth rates, cannot yet be concluded.

**DISCUSSION**

The pH inside a compartment of any living cell is an important parameter. Changes in pH influence the ionization state of all weak acids and weak bases, which includes all peptides and proteins. Cellular processes that are affected by diverging pH values include transport of molecules over membranes (Nishimura et al., 1998) and key metabolic processes such as redox state (Veine et al., 1998). Not surprisingly, most organisms try to regulate the pH in specific organelles (Martinez-Munoz & Kane, 2008; Llopis et al., 1998; Roos & Boron, 1981). We set out to accurately determine the organelle-specific pH in live, unperturbed yeast cells. We therefore expressed ratiometric pHluorin (Miesenböck et al., 1998) in the cytosol and the mitochondrial matrix. This yielded fast (seconds timescale) and accurate pH measurements in cytosol and mitochondria. The range of pH values that can be accurately measured is limited, as the resolution of the calibration curve deteriorates below 5.5 and above 8.0, but it is sufficient to measure pH values currently reported in living cells (Brett et al., 2006). We determined a pHcyt of 7.2 ± 0.2 and a pHmit of 7.5 ± 0.2 in cells growing on glucose. These specific pH values appear to be tightly regulated and

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**Fig. 6.** Effects of sorbic acid on pH. Strains were grown to mid-exponential phase in batch fermenters in Verduyn medium at pH 5.0. Filled symbols, ORY001/pHcyt; open symbols, ORY002/pHmit. At an OD600 of 0.34 potassium sorbate was added (t = 0) to final concentrations of 0 mM (●, ○), 2.74 mM (■, □) and 5.74 mM (▲, △). (a) Growth curve of ORY001, (b) growth curve of ORY002, (c) pHcyt profiles, (d) pHmit profiles. Error bars represent standard deviation of three technical replicates.
maintained in a broad range of external pH values, ranging from pH 3.0 to 7.5 (Figs 2c, 5a–c and 6). Previously reported pHi values were lower (Bracey et al., 1998; Fernandes et al., 2003; Guldfeldt & Arneborg, 1998). This is most likely due to the methods used, which determine an average pH over whole cells including the vacuole. This will result in significantly lower values. Indeed, others using pHluorin report similar pHcyt values (Martínez-Muñoz & Kane, 2008; Brett et al., 2005). We have not determined vacuolar pH, even though this organelle is important for cellular pH homeostasis and is crucial in the weak acid stress response (Martínez-Muñoz & Kane, 2008; Makrantoni et al., 2007; Piper et al., 2001). pHluorin could not be used to report on vacuolar pH, because of both the reduced reliability of the reporter in the expected low-pH environment (Martínez-Muñoz & Kane, 2008; Brett et al., 2005; Smith et al., 2002) and the apparent instability of the pHluorin targeted to the vacuole (our unpublished data). Possibly other fluorescent proteins with lower pKav values or different maturation requirements will yield successful tools in the future (Shaner et al., 2005; van Roermund et al., 2004)

Effect of external pH on pHi

There are several reports in the literature on the transcriptional and physiological effects of a shift of the extracellular pH to low pH (Causton et al., 2001; Kapteyn et al., 2001; Motizuki et al., 2008). External pH was also shown to affect pHi when pHluorin was assayed in buffers instead of media (Martínez-Muñoz & Kane, 2008). Remarkably, our experiments show that a medium pH of 3.0 does not lead to a significant change of pHcyt or pHmit (Fig. 5) and also has no apparent effect on growth. Two aspects contribute to this discrepancy. First, usually complex media which contain (unwanted) weak acids are used, or weak-acid buffers are added to the medium. Lowering the pH in these media causes protonation of these compounds, and thus induces a weak-acid stress that lowers pHi. Evidence of this is provided by our observation that a shift in pH from 6.0 to 4.2 in complex YPD medium results in a decrease in growth rate, with doubling times increasing from 77 to 88 min (our unpublished data), which is in marked contrast to the absence of any effect on growth rate upon a shift from pH 6.0 to 3.0 in defined medium. We conclude that extracellular pH does not affect pHi in growing cells, except when it is much higher than pHi, as evidenced by the cell lysis at pH 8.0 (see Fig. 5).

Effect of nutrient availability on pHi

pHcyt was reported to decrease transiently and then increase upon addition of glucose to glucose-repressed or starved cells (Martínez-Muñoz & Kane, 2008; Kresnowati et al., 2007; Ramos et al., 1989; van Urk et al., 1989). We observed the same (Fig. 3). The availability of nutrients also appears crucial for maintaining the pH gradients over organelar membranes, as exemplified by the difference between cytosolic and vacuolar pH values assayed in buffers compared with those measured in cells growing in defined media (Martínez-Muñoz & Kane, 2008; Brett et al., 2005), as well as the dissipation of the ΔpH over the mitochondrial membrane in glucose-starved cells (Fig. 3).

We generated a tool that allows an easy assessment of the pH gradient over the mitochondrial membrane, which is a crucial part of the energy-generating proton-motive force. Thus, we could determine that the proton ionophore CCCP led to a dissipation of the ΔpH over the mitochondrial membrane within minutes, irrespective of the carbon source used for cultivation. Interestingly, the equilibration of pHi with external pH, also expected to occur in CCCP-treated cells, did depend on the culture carbon source, which may be caused by the absence of any means of energy generation in glycerol/ethanol-grown cells without a proton-motive force. Blocking of electron transfer from ubiquinol to cytochrome b/c1 using antimycin A hardly had any effect on glucose-grown cells, which do not depend on a highly functional respiratory chain (Lagunas, 1976). In galactose-grown cells the gradient did dissipate, while it was even reversed in glycerol/ethanol-grown cells (Fig. 4). This suggests that in glucose-repressed cells the ΔpH, still important for uptake of compounds and import of proteins into the mitochondria, can be maintained independent of respiratory chain activity. Interestingly, again we see that pHcyt is affected most in glycerol/ethanol-grown cells, and least in glucose-grown cells, reflecting the need for energy-generating capacity in maintaining a neutral pHcyt.

We hypothesize that cytosolic acidification in cells grown on galactose or glycerol/ethanol, but also in glucose-starved cells (Fig. 3), is caused by protons that enter the cytosol during the uptake of various nutrients, for which the plasma membrane proton gradient is the driving force (Goffeau & Slayman, 1981; Van Leeuwen et al., 1992). This acidification would normally be counteracted by V-ATPases pumping protons into the vacuole (Martínez-Muñoz & Kane, 2008) or the plasma membrane ATPase, which translates protons out of the cytosol into the medium, both at the cost of ATP, to keep the pHcyt constant. A shortage of energy as a result of carbon starvation or respiratory chain inhibition would abolish the activity of the plasma membrane ATPase, resulting in a decrease in pHcyt ultimately to the pH of the environment (Karagiannis & Young, 2001). A decrease of pHcyt also affects the redox balance, shifting the NADH/NAD+ equilibrium to the oxidized form and thus lowering the cytosolic NADH concentration (Veine et al., 1998). Additionally, glucose starvation results in a severely reduced glycolytic flux, which also leads to a decrease in the cytosolic NADH concentration (Vemuri et al., 2007). This decrease of NADH in its turn leads to a reduced translocation of protons over the mitochondrial matrix membrane. Ultimately, pHmit will also equilibrate to the pH of the environment.
After the addition of glucose, pH\textsubscript{i} values were restored to values observed in growing cells (Fig. 3b) and cells resumed growth (Fig. 3a). Similarly, sorbic acid-stressed cells recovered their pH\textsubscript{i} when they resumed growth, whereas cells treated with concentrations of sorbic acid that caused a permanent growth stasis also did not show pH\textsubscript{i} recovery (Fig. 6) This raises the question whether the lowered pH\textsubscript{i} is actually causing the reduction in growth rate observed in the presence of sorbic acid. Interestingly, although both pH\textsubscript{cm} and pH\textsubscript{mit} are lowered, the weak-acid stress does not collapse the ΔpH over the mitochondrial inner membrane. How this gradient is linked to energy generation and cellular growth under these conditions remains to be elucidated.

Concluding remarks
We have shown that there is a remarkable coincidence between pH\textsubscript{i}, energy-generating capacity and growth rate. Our toolset will allow us to generate a deeper understanding of how pH\textsubscript{i} affects cellular metabolism and growth, and vice versa.

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