Intracellular pH homeostasis in *Candida glabrata* in infection-associated conditions

Azmat Ullah, Maria Inès Lopes, Stanley Brul and Gertien J. Smits

Department of Molecular Biology and Microbial Food Safety, Swammerdam Institute for Life Sciences (SILS), University of Amsterdam, The Netherlands

*Candida glabrata* is an opportunistic fungal pathogen which is a growing concern for immunocompromised patients. It is ranked as the second most common cause of candidiasis after *Candida albicans*. For pathogenic yeasts, intracellular pH (pHi) has been implicated in proliferation, dimorphic switching and virulence. We expressed the pH-sensitive green fluorescent protein variant ratiometric pHluorin in the cytosol of *C. glabrata* to study pHi dynamics in living cells. We evaluated the response of pHi to the various growth and stress conditions encountered during interaction with the host and during antifungal treatment. *C. glabrata* maintained a pHi higher than that of *Saccharomyces cerevisiae* in all growth conditions. The pHi of *S. cerevisiae* cells appeared better controlled than the pHi in *C. glabrata* when the cells were exposed to food and fermentation-associated conditions. *C. glabrata* in turn maintained its pHi better when exposed to host-associated conditions.

**INTRODUCTION**

*Candida glabrata* is a haploid asexual yeast, belonging to the genus *Candida*, although phylogenetically it is more closely related to the non-pathogenic *Saccharomyces cerevisiae* than to, for instance, *Candida albicans* (Kaur *et al.*, 2005; Roetzer *et al.*, 2011). *Candida* species are the major cause of fungal bloodstream infections, which are the cause of more than 8% of all hospital-acquired infections (Edmond *et al.*, 1999). *Candida* species usually reside in healthy human hosts as commensals but can become pathogenic in immunocompromised patients. *C. glabrata* was long considered to be a non-pathogenic saprophyte of the normal flora of healthy individuals (Fidel *et al.*, 1999). However, in the last two decades, the number of incidences of *C. glabrata* infections has increased significantly, especially in immunocompromised individuals such as patients who are HIV-positive, elderly or subject to transplantation, and in the US is the cause of some 26% of bloodstream infections (Horn *et al.*, 2009). In genito-urinary tract infections, in some countries *C. glabrata* has become more abundant than *C. albicans* (Achkar & Fries, 2010). Unfortunately, bloodstream infection caused by *C. glabrata* leads to a high mortality, because of the species’ innate resistance to most commonly prescribedazole antifungals such as fluconazole (Fidel *et al.*, 1999). Hitherto, the mechanisms involved in pathogenicity of *C. glabrata* and its resistance against azoles are poorly understood compared with *C. albicans*. *C. glabrata* is a ubiquitous organism, which has diverse environmental and host niches enabling it to infect a wide range of host sites. These include the gastrointestinal tract, respiratory tract, urogenital tract and various skin locations (Fidel *et al.*, 1999).

Adaptation to the host environment is essential for *C. glabrata* to infect various anatomical sites of the human host. The conditions of these anatomical sites vary widely, especially with respect to ambient pH, which ranges from very low (stomach, vagina) to very high (blood, saliva) (Bairwa & Kaur, 2011). In many pathogenic fungi, ambient pH has been considered as a potent virulence-determining factor. For example, the phenotypic switching of *C. albicans* from a budding yeast cell to filamentous hyphae, an essential trait for virulence, is mediated by ambient pH (Lo *et al.*, 1997; Vylkova *et al.*, 2011). Although such extracellular pH (pHex)-dependent phenotypic switching is absent in *C. glabrata*, intracellular pH (pHi) regulation under these varying ambient pH conditions is essential for survival in and colonization of the host (Penalva & Arst, 2002). Interestingly, a recent study revealed the role of the cell-wall-bound yapsin aspartyl-proteases, which are key virulence factors in *C. glabrata*, in pHi homeostasis and fitness upon exposure to low pHex conditions (Bairwa & Kaur, 2011). pHi regulation is vital for cellular functioning, because almost all cellular activities are directly or indirectly dependent on pHi (Orij *et al.*, 2011). In *S. cerevisiae*, pHi is clearly related to growth (Orij *et al.*, 2009, 2012).

To understand how pHi is maintained and regulated under different environmental conditions, it is important to first monitor the dynamics of pHi in living cells. Understanding

**Abbreviations:** HA, acetic acid; HL, lactic acid; HS, sorbic acid; pHex, extracellular pH; pHi, intracellular pH; SC, synthetic complete medium; WOA, weak organic acid.

Four supplementary figures are available with the online version of this paper.
the relevance of these responses for host colonization and virulence could not only lead to a better understanding of pH regulation but also generate new leads for antifungal targets (Monk & Perlin, 1994). So far, pH homeostasis in C. glabrata has been addressed in only a few studies (Schmidt et al., 2008; Bairwa & Kaur, 2011). Currently, there are several methods and techniques available to measure pH in yeast such as phospho-nuclear magnetic resonance (Hesse et al., 2000), radiolabelled membrane-permeable weak acids or bases (Anand & Prasad, 1989; Krebs et al., 1983; Ramos et al., 1989), probing with pH-sensitive fluorescent dyes (Bairwa & Kaur, 2011; Bracey et al., 1998) and equilibrium distribution of benzoic acid (Kresnowati et al., 2007). The above-mentioned methods require extensive manipulation of cells, which in itself may perturb pH (Brett et al., 2005; Karagiannis & Young, 2001; Orij et al., 2009). In recent years, the pH-sensitive green fluorescent protein (GFP) derivative pHluorin (Miesenböck et al., 1998) has been successfully used to measure pH in S. cerevisiae (Brett et al., 2005; Dechant et al., 2010; Maresová et al., 2010; Orij et al., 2009; Young et al., 2010). Ratiometric pH-sensitive GFPs are accurate and reliable, have the advantages of organanelle specificity (Dechant et al., 2010; Lasorsa et al., 2004; Orij et al., 2009), allow single-cell pH analysis (Bagar et al., 2009; Pineda Rodó et al., 2012), are non-invasive and do not affect normal physiological activity of yeast (Orij et al., 2009).

In this study, we have measured pH of C. glabrata using the pH-sensitive GFP ratiometric pHluorin. We studied the effect of different environmental conditions associated with various phases of host colonization and of antifungal treatment.

**METHODS**

**Strains and culture conditions.** All strains used in this study are described in Table 1. Yeast cells were maintained and propagated in YPD medium (1% yeast extract, 2% peptone and 2% glucose) prior to transformation. Transformed cells were cultured in low-fluorescence synthesis complete (SC) medium (YNB without folic acid and riboflavin, 0.5% (NH₄)₂SO₄, 2% glucose, 1 g sodium glutamate, 2 g uracil dropout mix). Medium was buffered at either pH 7.4 with MOPS or pH 4.0 with 75 mM tartaric acid as described (Sorgo et al., 2010). Pre-cultures were grown overnight from single colonies in 5 ml SC-ura medium at indicated pH values in 15 ml glass tubes at 30 or 37 °C. All solid media contained 1.5% (w/v) agar. All chemicals were purchased from Sigma-Aldrich, unless stated otherwise in the text.

Overnight cultures were harvested by centrifugation (5000 r.p.m.) and diluted into fresh SC-ura to OD₆₀₀ 1.0. Growth (OD₆₀₀) and fluorescence (excitation at 390 and 470 nm, emission at 510 nm) were assayed by transferring aliquots of fresh cultures (200 μl per well) into CELLSTAR black polystyrene clear-bottom 96-well plates (Greiner) using a Fluostar Optima Spectrophotometer (Isogen, BMG Labtech) in conditions as indicated. Plates were shaken at 200 r.p.m. for 2 min before each reading.

**Plasmid construction.** Plasmids used in this study are listed in Table 2. Plasmid pGRB2.2 was used to express pHluorin in C. glabrata for pH measurement. The pHluorin gene was PCR-amplified from plasmid pYES-ACT-pHluorin, using primers pH-F-Xbol (5'-GAAGTCTAGAAGGTAAAGAAGAAC-3') and pH-R-EcoRI (5'-GTCGACATCTTATTTGGATGTTCATCATTCAT3'). The PCR product was cloned into Xbol/EcoRI sites downstream of the S. cerevisiae phosphoglycerate kinase 1 (PGK1) promoter in plasmid pGRB 2.2, yielding plasmid pGRB 2.2-pHluorin + . To insert another copy of PGK1-pHluorin into plasmid pGRB 2.2-pHluorin, a PCR fragment was amplified from pGRB 2.2-pHluorin, using primers pgk1-F-EcoRI (5'-GATGGAGATCCTCATATAACCAGTGCCCTCTAT3') and pH-R-Xbol (5'-GTGCACTGATTTATGGATGTTCATCATTCATGC3'), and subcloned into pGRB 2.2-pHluorin digested with EcoRI/Xbol, generating plasmid pGRB 2.2-pHluorin + +. PCR amplifications were done using Pwo DNA polymerase (Roche) and later analysed by sequencing analysis. Basic DNA manipulation procedures were performed essentially as described by Sambrook et al. (1989). Unless otherwise indicated, all the restriction and modification enzymes used in this study were from Fermentas. For plasmid isolation and purification of PCR products we used Qiagen kits.

**Transformation of S. cerevisiae and C. glabrata.** S. cerevisiae was transformed using the lithium acetate method as described by Schiestl & Gietz (1989). A modified lithium acetate protocol was used to transform C. glabrata (Schmidt, 2007; Walther & Wendland, 2003).

**Determination of growth inhibition.** All growth determinations were carried out in 96-well microtitre plates, where growth was measured by following the change in optical density at 600 nm in a Fluostar Optima spectrophotometer.

In spectrophotometers, the OD₆₀₀ measured is not linear with cell number at higher densities. To correct for this non-linearity overnight cultures of C. glabrata and S. cerevisiae were serially diluted, and OD₆₀₀ was measured in the microplate reader (Warringer & Blomberg, 2003). The same samples were diluted to within the linear range of the spectrophotometer (Pharmacia LKB Biochrom) and OD₆₀₀ was registered. These linear OD₆₀₀ values measured in the spectrophotometer were used to correct the OD₆₀₀ values determined in the microplate reader (Fig. S1, available with the online version of this paper), and these corrected values were used to determine growth. Specific growth rates (μmax) were calculated from the slopes of log-transformed corrected OD₆₀₀ growth curves.

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<th>Table 1. Yeast strains used in this study</th>
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<tr>
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<td>ura3ΔTn903 G418K (derived from BG2 strain)</td>
<td>Cormack &amp; Falkow (1999)</td>
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<td>ura3Δ pGRB 2.2-pHluorin</td>
<td>This study</td>
</tr>
<tr>
<td>CG-pHluorin (+ +)</td>
<td>ura3Δ pGRB 2.2-pHluorin (2 copies)</td>
<td>This study</td>
</tr>
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<td>Euroscarf</td>
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<tr>
<td>SC-pHluorin</td>
<td>BY4741 ura3Δ0 pYES-ACT-pHluorin</td>
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Intracellular pH of Candida glabrata

Table 2. Plasmids used in this study

<table>
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<th>Plasmid</th>
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<td>pYES-ACT-pHluorin</td>
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<td>Orij et al. (2009)</td>
</tr>
<tr>
<td>pGRB 2.2</td>
<td>C. glabrata centromere and autonomously replicating sequence-based plasmid, containing S. cerevisiae PGK1 promoter and URA3, Amp' marker for selection in E. coli</td>
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<td>This study</td>
</tr>
<tr>
<td>pGRB 2.2-pHluorin+</td>
<td>pGRB 2.2 carrying two pHluorin genes</td>
<td>This study</td>
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For maximal growth rate determination, growth rates were determined with a sliding window of 1 h (seven time points), over single 16 h time-courses. To eliminate outliers, we discarded the three highest values. Means and SDs of these fourth highest values of all biological replicates are depicted in the figures.

**pHluorin calibration and pH measurement.** For *S. cerevisiae*, a calibration curve of pHluorin fluorescence at different pH was generated as described previously (Orij et al., 2009). The same protocol was optimized for *C. glabrata*. Briefly, cultures growing exponentially in SC-ura were harvested at an OD 600 of 3.0 by centrifugation at 5000 r.p.m. for 5 min and transferred to PBS containing 300 mg digitonin ml⁻¹. The cell suspensions were incubated for 15 min at room temperature, harvested and washed with PBS buffer, and the permeabilized cells were suspended in citric acid/Na₂HPO₄ buffer with pH ranging from 5.5 to 8.0 in 96-well plates. Fluorescence intensities were recorded using a Fluostar Optima spectrofluorometer by excitation at 390 nm and at 470 nm with emission set at 510 nm. For elimination of background fluorescence, the same strains carrying an empty vector were grown in parallel in all experiments, and these background fluorescence values were subtracted from the fluorescence at each excitation wavelength separately. A calibration curve was generated plotting the ratio of emission at both excitation wavelengths (R₃₉₀/₄₇₀) against the buffer pH (Fig. 1) as described previously (Orij et al., 2009).

![Calibration curve relating fluorescence intensity ratios to pH. Permeabilized cells were suspended in citric acid/Na₂HPO₄ buffer of pH 5.5–8.0 and fluorescence emission was determined after subtraction of background. The curve represents a quadratic polynomial fit which was used to transform fluorescence ratios to pHᵢ.](http://mic.sgmjournals.org)

Fig. 1. Calibration curve relating fluorescence intensity ratios to pH. Permeabilized cells were suspended in citric acid/Na₂HPO₄ buffer of pH 5.5–8.0 and fluorescence emission was determined after subtraction of background. The curve represents a quadratic polynomial fit which was used to transform fluorescence ratios to pHᵢ.

For pHᵢ–growth inhibition relationships, we determined the average pHᵢ over the 1 h interval we selected as highest recovered growth rate period (see above). Means and SDs of these values over the independent biological replicates are depicted in the figures.

**Conditions.** To determine the effect of glucose starvation and re-addition, *S. cerevisiae* and *C. glabrata* were cultivated in SC-ura medium at pH 4.0 and 7.4 in shake flasks, harvested during exponential growth and washed twice with SD medium without glucose. Immediately after washing (~10 min) and after 1 h starvation cells were transferred to 96-well plates and pHᵢ was monitored at 1 s intervals for 10 s. Glucose (50 mM) was injected into the plates, and fluorescence was registered at 1 s intervals over 2 min.

To determine the effect of weak organic acids (WOAs), *S. cerevisiae* and *C. glabrata* were cultivated in SC-ura medium buffered at pH 4.0 in shake flasks. Exponentially growing cultures were transferred to 96-well plates, and exposed to lactic acid (HL), acetic acid (HA) and sorbic acid (HS). Intracellular pH was monitored at 1 s intervals over 1 min. For long-term experiments, growing cultures were challenged with weak acid stress in 96-well plates, and growth (OD₆₀₀) and pHᵢ were monitored every 10 min over a period of 16 h.

The effect of three different antifungal drugs, fluconazole, amphotericin B and caspofungin (a kind gift from Merck Research Laboratories), on pHᵢ was studied. Multiple stocks of fluconazole (20 × in water), caspofungin (100 × in water) and amphotericin B (20 × in DMSO) were prepared depending on the stress concentration. Growing cultures were exposed to various concentrations of antifungal drugs described by Danby et al. (2012).

**Data analysis.** Unless stated otherwise, all figures represent the mean ± SD of three independent (biological) experiments with each replicate consisting of three technical replicates.

**RESULTS**

**pHluorin expression in C. glabrata**

There is a body of evidence that appropriate responses to environmental pH govern fungal virulence. The physiological and pathological behaviour of *C. albicans* is defined by ambient pH (El Barkani et al., 2000; Fonzi, 2002; Penalva et al., 2008; Porta et al., 2001). *C. glabrata* has diverse niches with respect to ambient pH but we know very little about its pHᵢ regulation in different host conditions. We therefore developed the technique to
monitor pH in *C. glabrata* based on the pH-sensitive GFP pHluorin. This method has been successfully used to measure pH in different yeasts (Bagar *et al.*, 2009; Dang *et al.*, 2012; Orij *et al.*, 2011). For cytoplasmic expression of pHluorin, we cloned two separate copies of ratiometric pHluorin (Miesenböck *et al.*, 1998) into plasmid pGRB 2.2 (Frieran *et al.*, 2002) each independently under the control of the PGK1 promoter. This double copy construct, although still yielding lower fluorescence than 2µ-based expression in *S. cerevisiae* (Orij *et al.*, 2009), led to a doubled fluorescence intensity compared with a single-copy construct (Fig. S2), which was sufficient for accurate pH determination. High pHluorin expression does not interfere with the morphology and physiology of *S. cerevisiae* (Orij *et al.*, 2009). We compared growth and morphology of *C. glabrata* strains with and without pHluorin and observed no differences (Fig. S2b and data not shown).

To calibrate the fluorescent signal to pH, we permeabilized cells with 300 mg digitonin ml⁻¹ and exposed them to buffers in a range of known pH between 5.5 and 8.0 as described previously (Orij *et al.*, 2009). Fluorescence ratios were plotted against pH (Fig. 1), and this calibration curve was used for pH conversion of all fluorescence data.

### C. glabrata pH is well adapted to host-associated conditions

To study the association of pH with growth in *C. glabrata*, we monitored both aspects in *C. glabrata* under different growth conditions, using *S. cerevisiae* for comparison. We selected growth conditions that are associated with various host niches, namely high pH and high temperature corresponding to the oral cavity or blood, low pH and high temperature corresponding to the vaginal mucosa, and low and high pH at low temperature corresponding to external niches (Sobel, 2007; Whiteway & Bachewich, 2007). *C. glabrata* showed maximum growth at 37 °C (Fig. 2a). Moreover, we did not see a significant difference in growth in response to ambient pH. In contrast, *S. cerevisiae* showed a clear preference for low pH (5.0 and 4.0) at both temperatures. We also monitored pH of both yeasts during growth (Fig. 2b). *C. glabrata* maintained a higher pH than *S. cerevisiae* in all conditions. Interestingly, we observed an apparent inverse correlation between growth rate and pH in *C. glabrata*. This is in contrast to *S. cerevisiae*, where varying temperature and external pH did not reveal a condition-independent relationship between growth and pH.

### pH response to glucose withdrawal and re-addition

The ability to withstand starvation and adapt to diverse nutrients is essential for species’ survival (Gasch & Werner-Washburne, 2002). A cycle of nutrient abundance followed by starvation is the natural condition for *S. cerevisiae*. *C. glabrata* encounters conditions with poor nutrient abundance in most niches, and is highly starvation resistant (Jandric & Schüller, 2011). Glucose is the preferred carbon/energy source for most yeasts and was shown to be a morphogen in *C. albicans* where it influences yeast-to-hypha transitions (Sabina & Brown, 2009). However, mucosal areas in the mammalian host or the interior of a macrophage are both glucose deficient (Jandric & Schüller, 2011), and the blood has only low glucose concentrations.

In *S. cerevisiae*, glucose availability strongly affects pH (Orij *et al.*, 2009, 2012). In response to the addition of glucose to glucose-starved yeast, the pH decreases, and subsequently the interior of the cells becomes relatively alkaline. This well-studied physiological behaviour in *S. cerevisiae* (Thevelein, 1991; Colombo *et al.*, 1998; Kresnowati *et al.*, 2007) has not been characterized in *C. glabrata*. We therefore studied pH dynamics in *C. glabrata* during glucose starvation and sudden replenishment. We used exponentially growing cultures, which were washed and starved for 1 h at two different ambient pH values. We recorded the immediate pH response to glucose withdrawal (Fig. 3a, b) as well as the pH decrease during starvation. Next, we pulsed the starved cells with glucose to see the rapid response to the initiation of glycolysis (Fig. 3c, d). In *C. glabrata*, both glucose starvation and re-addition affected pH in a pH-ex-independent fashion, with a reduction of ~0.5 pH units after 1 h of starvation, and a small transient pH decrease upon glucose re-addition. In contrast, the pH decrease upon glucose withdrawal was strongly pH-ex dependent in the case of *S. cerevisiae*, with a reduction of ~0.2 units after 10 min at pH-ex 7.4, compared with a strong and rapid reduction reaching 1.0 unit after 10 min at pH-ex 4.0. After 1 h, pH had decreased a further 0.6 units at pH-ex 7.4, whereas it remained stable at the 1.0 unit reduction at pH-ex 4.0. The reduction of pH caused by glucose re-addition was not pH-ex dependent: a glucose pulse led to an additional decrease of pH of ~0.7 units within 20 s, and pH recovered to neutral in approximately 2 min. The pH-ex independence of this profile (Fig. 3c, d) in both *C. glabrata* and *S. cerevisiae* renders it unlikely that the decrease is caused by a rapid influx of protons from the cellular environment.

### pH responses to commonly encountered organic acids

WOAs are present in various ecological niches of both yeasts. *S. cerevisiae* evolved in association with fruits containing high levels of organic acids and produces acetic acid as a by-product of fermentation. *C. glabrata* has to cope with weak acids during the establishment of infection, as they are naturally present at different sites of infections. The vaginal mucosa for instance has a low pH and high concentrations of lactate (Owen & Katz, 1999). One of the major antifungal mechanisms of weak acids is cytosolic acidification (Ullah *et al.*, 2012). We therefore studied pH and growth of *C. glabrata* in the presence of acetic, sorbic...
and lactic acid and compared the results with *S. cerevisiae*. We selected concentrations of sorbic and acetic acid which cause similar acidification. In the case of lactic acid, we used a range of concentrations (30, 60 and 120 mM), but even at high concentrations, acidification was very limited compared with the other two WOAs (data not shown). We therefore decided to use 30 mM of undissociated acid, corresponding to 71 mM of total lactate, as higher concentrations might additionally give osmotic stress (Chirife & Ferrofontan, 1980). First, we studied the immediate effects of

![Fig. 2. Comparison of growth and pH<i></i> in different conditions. Maximum specific growth rate (a) and pH<i></i> (b) of *C. glabrata* (black bars) and *S. cerevisiae* (grey bars) in diverse pH out/temperature combination. pH<i></i> data represent the cytosolic pH of cells at the time of maximum specific growth. Full growth and pH<i></i> profiles can be found in Fig. S3.](http://mic.sgmjournals.org)

![Fig. 3. pH<i></i> response to glucose. pH<i></i> in glucose-deprived cells of *C. glabrata* (black bars) and *S. cerevisiae* (grey bars). Cells were starved for either 10 or 60 min at pH out 7.4 (a) or 4.0 (b). Glucose (50 mM) was pulsed to 1 h starved cultures of *C. glabrata* (c) and *S. cerevisiae* (d) at time 0 and pH<i></i> was monitored at 1 s intervals for 2 min. •, Control culture pH during continuous growth on glucose; ▼, pH out 7.4; □, pH out 4.0. Asterisks indicate statistically significant differences (*<i>P</i> < 0.05, **<i>P</i> < 0.01, ***<i>P</i> < 0.001) between pH<i></i> at t=0 and t=10 or 60 min.](http://mic.sgmjournals.org)
acid exposure. We challenged growing cultures of *C. glabrata* and *S. cerevisiae* with WOAs and monitored pH$_i$ dynamics (Fig. 4). Acetic and sorbic acid immediately acidified the cytosol but lactic acid did not. Comparatively, sorbic acid reduced cytosolic pH faster than acetic acid, in agreement with previous work (Ullah *et al.*, 2012). Sorbic acid and acetic acid inhibited growth of *C. glabrata* by $\sim$80 and $\sim$12%, respectively, compared with only $\sim$40 and $\sim$5% in *S. cerevisiae* (Fig. 5a). Overall, both yeasts showed highest susceptibility to sorbic acid while no growth inhibition was observed in response to lactic acid. For both yeasts, pH$_i$ recovery was quite similar (Fig. 5b), and pH$_i$ recovered faster after acetic acid exposure than after sorbic acid exposure. In conclusion, even though acid entry rates and pH values reached were virtually identical for both yeasts, *C. glabrata* showed a much stronger sensitivity to WOAs.

**Perturbation of pH$_i$ by antifungal drugs**

Yeast cells are eukaryotes with a physiology and cell biology similar to those of human cells. Therefore, it is difficult to design antifungal drugs without side effects (Cardenas *et al.*, 1999; Shapiro *et al.*, 2011). For *C. glabrata* the treatment options are even more limited, owing to the species’ exceptional resistance to azoles (Jandric & Schüller, 2011). Therefore, new antifungal targets are required to improve medication. Pma1p has been proposed as an antifungal target (Soteropoulos *et al.*, 2000) because it is a master regulator of pH$_i$ and is responsible for nutrient uptake by generating an electrochemical proton gradient (Monk & Perlin, 1994). In the last part of this study we probed the pH$_i$ of *C. glabrata* in the presence of three commonly used antifungal drugs. Growing cultures of *C. glabrata* were challenged with increasing concentrations of fluconazole, amphotericin B and caspofungin and pH$_i$ was monitored. Growth-inhibitory concentrations were selected from the literature (Danby *et al.*, 2012) and retested by measuring turbidity after 16 h. It is well known that yeast's susceptibility to antifungal drugs varies at different pH$_{ex}$ (Danby *et al.*, 2012) which is why we used two different ambient pH values (4.0 and 7.4). In our experimental setup using liquid media rather than plate assays, the known MIC (64 μg ml$^{-1}$) of fluconazole did not affect growth and pH$_i$ of *C. glabrata* or *S. cerevisiae* at either pH$_{ex}$ (our unpublished data). In contrast, growth was impaired by caspofungin (0.06–0.25 μg ml$^{-1}$) and amphotericin B (0.5–2 μg ml$^{-1}$) in agreement with the literature (Danby *et al.*, 2012). Interestingly, we observed different pH$_i$ behaviour at different ambient pH. Both drugs acidified the cells at low pH$_{ex}$ but at high pH$_{ex}$ cells maintained a high pH$_i$ (Fig. 6), with even a slight initial alkalinization.

**DISCUSSION**

*C. glabrata* is an emerging fungal pathogen, closely related to *S. cerevisiae* (Kaur *et al.*, 2005; Roetzer *et al.*, 2011). Unlike baker’s yeast it is well adapted to human commensalism and has a high resistance to certain antifungal agents, starvation and various stress conditions (Jandric & Schüller, 2011). pH$_i$ plays a vital role in the physiology of yeast as it regulates a variety of cellular processes which are essential for proliferation and survival in *S. cerevisiae* (Oriti *et al.*, 2011, 2012) and virulence in *C. albicans* and *Aspergillus* spp. (Peñalva *et al.*, 2008). Very little work has been done to understand the pH$_i$ regulation in the pathogenic yeast *C. glabrata* compared with baker’s yeast (Bairwa & Kaur, 2011; Zhou *et al.*, 2011). In this study, we have modified a method to determine pH$_i$ using GFP-pHluorin in *C. glabrata*. Our pH$_i$ values corroborated a recent report measuring pH$_i$ with fluorescent probes (Zhou *et al.*, 2011).
Microbes are exposed to various insults associated with host niches, for instance high temperature, low pH, nutrient limitation and the presence of weak acids. The rapid adaptation of pathogens to these various conditions is critical for both fitness and virulence. The above-mentioned environmental fluctuations have a profound effect on pH\textsubscript{i} in \textit{S. cerevisiae} (Orij et al., 2011). It has been shown that pH\textsubscript{i} regulation is important for virulence of \textit{C. albicans} (Davis et al., 2000; Stewart et al., 1989). However, unlike \textit{C. albicans}, \textit{C. glabrata} does not rely on morphological switching for virulence. In this work, we have studied the pH\textsubscript{i} of \textit{C. glabrata} under different growth conditions present in host niches. Temperature and ambient pH\textsubscript{i} are known to be major determinants of growth patterns in \textit{Candida} species and are considered virulence factors (Cottier & Mühlischlegel, 2009). \textit{C. glabrata} was reported to grow faster than \textit{S. cerevisiae} in rich media (Kaur et al., 2007; Jacobsen et al., 2010), and has higher numbers of viable cells per OD unit due to its smaller size (our unpublished data). Our findings confirmed that at host temperature \textit{C. glabrata}
displayed maximum cell growth rate, regardless of pHex, confirming earlier observations (Luo & Samaranayake, 2002; Jacobsen et al., 2010; Schmidt et al., 2008; Roetzer et al., 2010). S. cerevisiae showed highest growth rate at low pHex, consistent with the literature that baker’s yeast prefers low pHex (Ariño, 2010; Orij et al., 2011). High ambient pH reduces the electrochemical gradient across the cytoplasmic membrane and eventually impairs nutrient uptake as electrochemical gradient is a driving force for nutrient transport (Walker, 1998). We did not see a condition-independent correlation of pHi with growth (Orij et al., 2012). It has previously been shown in S. cerevisiae that pHex does not directly affect pHi in defined media. Effects of pHex on pHi are usually observed in rich media, in which many compounds are weak acids. Here, we did observe different pHi at different ambient pH on longer timescales, suggesting an effect of, for instance, the use of the electrochemical gradient or plasma membrane proton motive force. Indeed, the effect of nutrient status was strongly pHex-dependent in S. cerevisiae. Also, we observed low pHi at high temperatures, consistent with the results of previous studies (Aabo et al., 2011; Coote et al., 1994). The exact mechanism of this temperature-related acidification is not known but it has been suggested that increased temperature lowers pHi by changing membrane permeability and interruption of the activity of membrane transporters (Aabo et al., 2011).

Glucose is the preferred carbon and energy source for most yeasts (Galdieri et al., 2010) and energy is required to maintain pHi (Orij et al., 2009; Young et al., 2010). In buffers without glucose present, pHi of both S. cerevisiae (Martínez-Munoz & Kane, 2008) and C. glabrata (Bairwa & Kaur, 2011) are lower than those in the presence of glucose. Not surprisingly, upon glucose withdrawal the pHi decreased in both yeasts, probably because of decreased activity of the plasma-membrane H+-ATPase pump, which is a major pHi regulator in yeast (Lecceti et al., 2005; Martínez-Munoz & Kane, 2008; Orij et al., 2011). In S. cerevisiae acidification was high and pHex dependent upon starvation, while in C. glabrata acidification was significantly less and no effect of pHex was observed. This observation was consistent with our other data (Fig. 2) with C. glabrata maintaining a higher pHi than S. cerevisiae under acidic conditions. Addition of glucose to a starved culture caused a fast acidification followed by alkalinization. The cause of this acidification is unclear, and it was shown that the protons generated by the initial steps of glycolysis are not sufficient to explain the decrease in S. cerevisiae (Kresnowati et al., 2008). Our data show that an alternative, influx of protons from the environment, is also not the cause of the acidification, because such a mechanism should abolish intracellular acidification at high pHex. In contrast to S. cerevisiae, in C. glabrata, cytosolic acidification was similar at high and low pHex. C. glabrata has specific mechanisms to survive and proliferate under glucose-deficient conditions that are different from those of C. albicans, which play a critical role in virulence, as C. glabrata, in contrast to C. albicans, cannot switch to the hyphal morphology to escape when it is engulfed by macrophages (Jandric & Schüller, 2011; Roetzer et al., 2010).

Environmentally encountered WOAs may also affect pHi and growth (Ullah et al., 2012). We studied initial acidification upon WOA exposure. Two different acids led to rates of acidification similar in both yeasts, suggesting that the acids use similar entry routes, probably through diffusion across the membrane. Interestingly, the pHi responses of the two yeasts to WOAs were quite similar, even though S. cerevisiae appeared more resistant. In nature, S. cerevisiae is adapted to colonize fruits, which contain high concentrations of various WOAs. This may explain its resistance to WOA preservatives. C. glabrata showed a particularly high sensitivity to sorbic acid. The production of lactic acid (Boskey et al., 2001) and low pH of the vagina (Horowitz & Mårdh, 1991) are considered advantageous in the prevention of pathogen growth. Remarkably, we did not see any growth or pHi effects caused by lactic acid at vaginal pH (4.0). Recent data showed, however, that in glucose-limited conditions, such as those in the intestine, C. glabrata assimilates lactate better than S. cerevisiae, even in the absence of high oxygen concentrations (Ueno et al., 2011). Growth on lactate was also better than growth on acetate or pyruvate, which corresponds to the acetate sensitivity we observed. This may provide C. glabrata with a growth benefit during host colonization.

C. glabrata has a high tolerance for different azole antifungals, a widely used class of antifungals to treat Candida infections. Therefore, cell-wall biogenesis inhibitors (caspofungin) and polyenes (amphotericin B) are preferred over azoles to treat C. glabrata infections (Pappas et al., 2009). C. glabrata exhibited a high resistance to fluconazole, as neither growth nor pHi was affected even using high concentrations of the drug. Interestingly both caspofungin and amphotericin B perturbed pHi as well as affecting growth. Caspofungin is a semi-synthetic lipopeptide inhibitor of 1,3-β-D-glucan synthase, which is a key enzyme required for the synthesis of β-1,3-glucan, the major structural component (30–45 %) of the fungal cell wall (Klis et al., 2006). It is thought that inhibition of β-1,3-glucan lowers the integrity of the cell wall and results in osmotic instability, which may lead to cell lysis and cell death (Deresinski & Stevens, 2003). There is a body of evidence linking pHex to cell-wall biogenesis, and it appears likely that pHi would also affect cell-wall biogenesis through a perturbed activity of the cell-wall integrity pathway (Bairwa & Kaur, 2011; de Lucena et al., 2012). Echinocandin antifungals appear more potent at low pH, because a high pH activates the cell-wall integrity (SLT2) pathway to adapt to an elevated pHex (Ariño, 2010), which may increase the tolerance to echinocandin (Miyazaki et al., 2010). Moreover, extracellular stimuli that acidify cells (low pHex in complex media, WOA) activate the HOG-pathway, leading to the expression of the
glycosylphosphatidylinositol-anchored cell-wall protein Spplp, which is thought to be involved in WOA resistance (Kapteyn et al., 2001; Simões et al., 2003). In addition, such cells became resistant to the cell-wall lytic enzyme 1,3-β-glucanase and had in general a more stress-resistant phenotype (Simões et al., 2003, 2006). Interestingly, we found that the effect of caspofungin on C. glabrata growth was strongly pHex dependent, and the interaction of pHex and caspofungin led to a very strong decrease of pHi.

Similarly, amphotericin B acts by binding the ergosterol in membranes, leading to the formation of aggregate structures which act as transmembrane channels. This leads to altered cell permeability to protons and monovalent cations (Cohen, 2010; Laniado-Laborin & Cabrales-Vargas, 2009) resulting in depolarization of the membrane. This is consistent with our data showing that amphotericin B leads to a pHex-dependent effect on pHi, causing a cytoplasmic acidification at low pHex and a slight alkalization at high pHex. The reduction of pHi upon amphotericin B exposure was previously interpreted to be the growth inhibitory mechanism of the compound (Bracey et al., 1998). In S. cerevisiae, we showed that lowering of pHi signals to control growth rate too (Orij et al., 2012). This emphasizes the potential use of pHi homeostasis as an antifungal drug target.

In conclusion, we have developed a tool that allows rapid and reliable determination of pHi of C. glabrata when exposed to a number of important physiologically stressful conditions. We used the method to gain insight into the relationship between pHi and fitness, virulence and drug tolerance of this opportunistic pathogen.

ACKNOWLEDGEMENTS

We thank Drs Kuchler (Vienna University) and Cormack (Johns Hopkins University) for strains and plasmids. We appreciate the gift of caspofungin from Merck (Rahway, NJ, USA). We are also grateful to HEC, Pakistan, for financial support (to A. U.).

REFERENCES


Intracellular pH of Candida glabrata


Edited by: J. Morschhäuser