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 prescribed azole 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 (pH_{ex})-dependent phenotypic switching is absent in *C. glabrata*, intracellular pH (pH_{i}) regulation under these varying ambient pH conditions is essential for survival in and colonization of the host (Peñalva & 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 pH_{i} homeostasis and fitness upon exposure to low pH_{ex} conditions (Bairwa & Kaur, 2011). pH_{i} regulation is vital for cellular functioning, because almost all cellular activities are directly or indirectly dependent on pH_{i} (Orij et al., 2011). In *S. cerevisiae*, pH_{i} is clearly related to growth (Orij et al., 2009, 2012).

To understand how pH_{i} is maintained and regulated under different environmental conditions, it is important to first monitor the dynamics of pH_{i} in living cells. Understanding
the relevance of these responses for host colonization and virulence could not only lead to a better understanding of pH$_i$ 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$_i$ 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$_i$ (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$_i$ 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 organelle specificity (Dechant et al., 2010; Lasorsa et al., 2004; Orij et al., 2009), allow single-cell pH$_i$ 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$_i$ 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) or SC medium (YNB without folic acid and 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 the indicated pH values in 15 ml glass tubes at 30 °C. All media contained 1.5% (w/v) agar. All chemicals were purchased from Sigma-Aldrich, unless stated otherwise in the text.

Table 1. Yeast strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td>C. glabrata BG14</td>
<td>ura3ΔTn903 G418$^+$ (derived from BG2 strain)</td>
<td>Cormack &amp; Falkow (1999)</td>
</tr>
<tr>
<td>CG-pHluorin (+)</td>
<td>ura3A pGRB 2.2-pHluorin</td>
<td>This study</td>
</tr>
<tr>
<td>CG-pHluorin (+ +)</td>
<td>ura3A pGRB 2.2-pHluorin (2 copies)</td>
<td>This study</td>
</tr>
<tr>
<td>S. cerevisiae BY4741</td>
<td>MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0</td>
<td>Euroscarf</td>
</tr>
<tr>
<td>SC-pHluorin</td>
<td>BY4741 ura3Δ0 pYES-ACT-pHluorin</td>
<td>This study</td>
</tr>
</tbody>
</table>

Overnight cultures were harvested by centrifugation (5000 r.p.m.) and diluted into fresh SC-ura to OD$_{600}$ 1.0. Growth (OD$_{600}$) 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$_i$ measurement. The pHluorin gene was PCR-amplified from plasmid pYES-ACT-pHluorin, using primers primi-F and XhoI (5’-GAAGTCTAGAATGAGTAAAGGAGAAGAAC-3’) and pHl-R-EcoRI (5’-GTGCAGAATTCATATTGTATGTCATCCATCATC-3’). The PCR product was cloned into XbaI/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’-GTATCGGATCCATAAAAGCAGTGCGCTCTAT-TAT-3’) and pHl-R-XhoI (5’-GTGCACGTCGATATTGTATAGTTCATCCATGC-3’), and subcloned into pGRB 2.2-pHluorin digested with EcoRI/XhoI, generating 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 OD$_{600}$ 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 OD$_{600}$ 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 OD$_{600}$ was registered. These linear OD$_{600}$ values measured in the spectrophotometer were used to correct the OD$_{600}$ 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 ($\mu$$_{\text{max}}$) were calculated from the slopes of log-transformed corrected OD$_{600}$ growth curves.
Table 2. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
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<tbody>
<tr>
<td>pYES-ACT-HLuorin</td>
<td>S. cerevisiae 2 µm-based vector containing S. cerevisiae URA3, Amp' marker for selection in E. coli</td>
<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>
<td>Frieman et al. (2002)</td>
</tr>
<tr>
<td>pGRB 2.2-</td>
<td>pGRB 2.2 carrying pHluorin gene</td>
<td>This study</td>
</tr>
<tr>
<td>pHluorin +</td>
<td>pGRB 2.2 carrying two pHluorin genes</td>
<td>This study</td>
</tr>
</tbody>
</table>

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).

![Fig. 1. Calibration curve relating fluorescence intensity ratios to pH.](http://mic.sgmjournals.org)

For pH₆–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 pHᵢ 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; Peñalva 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\textsubscript{i} in \textit{C. glabrata} based on the pH-sensitive GFP pHluorin. This method has been successfully used to measure pH\textsubscript{i} in different yeasts (Bagar \textit{et al.}, 2009; Dang \textit{et al.}, 2012; Orij \textit{et al.}, 2011). For cytoplasmic expression of pHluorin, we cloned two separate copies of ratiometric pHluorin (Miesenböck \textit{et al.}, 1998) into plasmid pGRB 2.2 (Frieman \textit{et al.}, 2002) each independently under the control of the PKGI promoter. This double copy construct, although still yielding lower fluorescence than a single-copy construct (Fig. S2), was sufficient for accurate pH\textsubscript{i} determination. High pHluorin expression does not interfere with the morphology and physiology of \textit{S. cerevisiae} (Orij \textit{et al.}, 2009). We compared growth and morphology of \textit{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\textsuperscript{-1} and exposed them to buffers in a range of known pH between 5.5 and 8.0 as described previously (Orij \textit{et al.}, 2009). Fluorescence ratios were plotted against pH (Fig. 1), and this calibration curve was used for pH conversion of all fluorescence data.

\textbf{\textit{C. glabrata} pH\textsubscript{i} is well adapted to host-associated conditions}

To study the association of pH\textsubscript{i} with growth in \textit{C. glabrata}, we monitored both aspects in \textit{C. glabrata} under different growth conditions, using \textit{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 exterior niches (Sobel, 2007; Whiteway \& Bachewich, 2007). \textit{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, \textit{S. cerevisiae} showed a clear preference for low pH (5.0 and 4.0) at both temperatures. We also monitored pH\textsubscript{i} of both yeasts during growth (Fig. 2b). \textit{C. glabrata} maintained a higher pH\textsubscript{i} than \textit{S. cerevisiae} in all conditions. Interestingly, we observed an apparent inverse correlation between growth rate and pH\textsubscript{i} in \textit{C. glabrata}. This is in contrast to \textit{S. cerevisiae}, where varying temperature and external pH did not reveal a condition-independent relationship between growth and pH\textsubscript{i}.

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

\textbf{pH\textsubscript{i} responses to commonly encountered organic acids}

WOAs are present in various ecological niches of both yeasts. \textit{S. cerevisiae} evolved in association with fruits containing high levels of organic acids and produces acetic acid as a by-product of fermentation. \textit{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 \textit{et al.}, 2012). We therefore studied pH\textsubscript{i} and growth of \textit{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

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**Fig. 2.** Comparison of growth and pH* in different conditions. Maximum specific growth rate (a) and pH (b) of *C. glabrata* (black bars) and *S. cerevisiae* (grey bars) in diverse pH*ex*/temperature combination. pH* data represent the cytosolic pH of cells at the time of maximum specific growth. Full growth and pH* profiles can be found in Fig. S3.

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**Fig. 3.** pH* response to glucose. pH* 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*ex* 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* was monitored at 1 s intervals for 2 min. ▼, Control culture pH during continuous growth on glucose; ▽, pH*ex* 7.4; ▲, pH*ex* 4.0. Asterisks indicate statistically significant differences (*P<0.05, **P<0.01, ***P<0.001) between pH* at *t*=0 and *t*=10 or 60 min.
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 ~80 and ~12%, respectively, compared with only ~40 and ~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. S4), and pH$_i$ recovered faster after acetic acid exposure than after sorbic acid exposure. In conclusion, even though acid entry rates and pH$_i$ 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* (Orij 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 in *S. cerevisiae* (Orij et al., 2011). It has been shown that pH regulation is important for virulence of *C. albicans* (Davis et al., 2000; Stewart et al., 1989). However, unlike *C. albicans*, *C. glabrata* does not rely on morphological switching for virulence. In this work, we have studied the pH of *C. glabrata* under different growth conditions present in host niches. Temperature and ambient pH are known to be major determinants of growth patterns in *Candida* species and are considered virulence factors (Cottier & Mühlh sl egel, 2009). *C. glabrata* was reported to grow faster than *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 *C. glabrata*

![Fig. 5. Effect of weak acid preservatives on the growth and pH of the yeasts. (a) Effect of acetic acid (30 mM) and sorbic acid (1 mM) on the growth of *C. glabrata* and *S. cerevisiae*. The effect on growth is presented as growth inhibition, calculated from growth rates of stressed and control cultures as described in Methods. (b) The effect of the acids on pH is represented as initial acidification (white bars), which indicates the pH 10 min after the addition of the weak acids to growing culture of yeasts, and recovered pH (grey bars), indicating the pH at the time of maximally recovered growth. Full growth and pH profiles can be found in Fig. S4. HA, acetic acid; HL, lactic acid; HS, sorbic acid.](http://mic.sgmjournals.org)

![Fig. 6. Effect of antifungal drugs on the pH of *C. glabrata*. Effect of increasing concentration of caspofungin (a, b) and amphotericin B (c, d) on pH of *C. glabrata* at two ambient pH values.](http://mic.sgmjournals.org)
displayed maximum cell growth rate, regardless of pH_{ex}, 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 pH_{ex}, consistent with the literature that baker's yeast prefers low pH_{ex} (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 pH_{i} with growth (Orij et al., 2012). It has previously been shown in *S. cerevisiae* that pH_{ex} does not directly affect pH_{i} in defined media. Effects of pH_{ex} on pH_{i} are usually observed in rich media, in which many compounds are weak acids. Here, we did observe different pH_{i} 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 pH_{ex}-dependent in *S. cerevisiae*. Also, we observed low pH_{i} 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 pH_{i} 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 pH_{i} (Orij et al., 2009; Young et al., 2010). In buffers without glucose present, pH_{i} 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 pH_{i} decreased in both yeasts, probably because of decreased activity of the plasma-membrane H^{+}-ATPase pump, which is a major pH_{i} regulator in yeast (Lecchi et al., 2005; Martínez-Munoz & Kane, 2008; Orij et al., 2011). In *S. cerevisiae* acidification was high and pH_{ex} dependent upon starvation, while in *C. glabrata* acidification was significantly less and no effect of pH_{ex} was observed. This observation was consistent with our other data (Fig. 2) with *C. glabrata* maintaining a higher pH_{i} 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 pH_{ex}. In contrast to *S. cerevisiae*, in *C. glabrata*, cytosolic acidification was similar at high and low pH_{ex}. *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 when it is engulfed by macrophages (Jandric & Schüller, 2011; Roetzer et al., 2010).

Environmentally encountered WOAs may also affect pH_{i} 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 pH_{i} 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 & Márđh, 1991) are considered advantageous in the prevention of pathogen growth. Remarkably, we did not see any growth or pH_{i} 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 differentazole 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 pH_{i} was affected even using high concentrations of the drug. Interestingly both caspofungin and amphotericin B perturbed pH_{i} 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 pH_{ex} to cell-wall biogenesis, and it appears likely that pH_{i} 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 pH_{ex} (Ariño, 2010), which may increase the tolerance to echinocandin (miyazaki et al., 2010). Moreover, extracellular stimuli that acidify cells (low pH_{ex} in complex media, WOA) activate the HOG-pathway, leading to the expression of the
glycosylphosphatidylinositol-anchored cell-wall protein Spil1p, 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 pH_{ex} dependent, and the interaction of pH_{ex} and caspofungin led to a very strong decrease of pH_{i}.

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 pH_{ex}-dependent effect on pH_{i}, causing a cytoplasmic acidification at low pH_{ex} and a slight alkalization at high pH_{ex}. The reduction of pH_{i} 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 pH_{i} signals to control growth rate too (Orij et al., 2012). This emphasizes the potential use of pH_{i} homeostasis as an antifungal drug target.

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

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Intracellular pH of Candida glabrata


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