

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

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

Correspondence
Gertien J. Smits
g.j.smits@uva.nl

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

Received 17 September 2012
Revised 10 January 2013
Accepted 29 January 2013

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 (pH_i) 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 pH_i dynamics in living cells. We evaluated the response of pH_i to the various growth and stress conditions encountered during interaction with the host and during antifungal treatment. *C. glabrata* maintained a pH_i higher than that of *Saccharomyces cerevisiae* in all growth conditions. The pH_i of *S. cerevisiae* cells appeared better controlled than the pH_i in *C. glabrata* when the cells were exposed to food and fermentation-associated conditions. *C. glabrata* in turn maintained its pH_i 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 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

Abbreviations: HA, acetic acid; HL, lactic acid; HS, sorbic acid; pH_{ex} , extracellular pH; pH_i , 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_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) prior to transformation. Transformed cells were cultured in low fluorescence synthetic complete (SC) medium (YNB without folic acid and riboflavin, 0.5% $(\text{NH}_4)_2\text{SO}_4$, 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 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 pHl-F-*Xba*I (5'-GAAGTCTAGAATGAGTAAAGGAGAAGAAC-3') and pHl-R-*Eco*RI (5'-GTGCAGAATTCTTATTGTATAGTTCATCCAT-3'). The PCR product was cloned into *Xba*I/*Eco*RI 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-*Eco*RI (5'-GTATCGGAATTCCATAAAGCACGTGGCCTCT-TAT-3') and pHl-R-*Xho*I (5'-GTGCAGCTCGAGTTATTGTAT-ATTCATCCATGC-3'), and subcloned into pGRB 2.2-pHluorin digested with *Eco*RI/*Xho*I, 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 spectrofluorometer.

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 (μ_{max}) were calculated from the slopes of log-transformed corrected OD_{600} growth curves.

Table 1. Yeast strains used in this study

Strain	Genotype	Reference or source
<i>C. glabrata</i> BG14	<i>ura3ΔTn903</i> G418 ^R (derived from BG2 strain)	Cormack & Falkow (1999)
CG-pHluorin (+)	<i>ura3Δ</i> pGRB 2.2-pHluorin	This study
CG-pHluorin (++)	<i>ura3Δ</i> pGRB 2.2-pHluorin (2 copies)	This study
<i>S. cerevisiae</i> BY4741	<i>MATa</i> ; <i>his3Δ1</i> ; <i>leu2Δ0</i> ; <i>met15Δ0</i> ; <i>ura3Δ0</i>	Euroscarf
SC-pHluorin	BY4741 <i>ura3Δ0</i> pYES-ACT-pHluorin	This study

Table 2. Plasmids used in this study

Plasmid	Description	Reference or source
pYES-ACT-pHluorin	<i>S. cerevisiae</i> 2 μ m-based vector containing <i>S. cerevisiae</i> URA3, Amp ^r marker for selection in <i>E. coli</i>	Orij <i>et al.</i> (2009)
pGRB 2.2	<i>C. glabrata</i> centromere and autonomously replicating sequence-based plasmid, containing <i>S. cerevisiae</i> PGK1 promoter and URA3, Amp ^r marker for selection in <i>E. coli</i>	Frieman <i>et al.</i> (2002)
pGRB 2.2-pHluorin +	pGRB 2.2 carrying pHluorin gene	This study
pGRB 2.2-pHluorin + +	pGRB 2.2 carrying two pHluorin genes	This study

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 OD₆₀₀ 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 ($R_{390/470}$) against the buffer pH (Fig. 1) as described previously (Orij *et al.*, 2009).

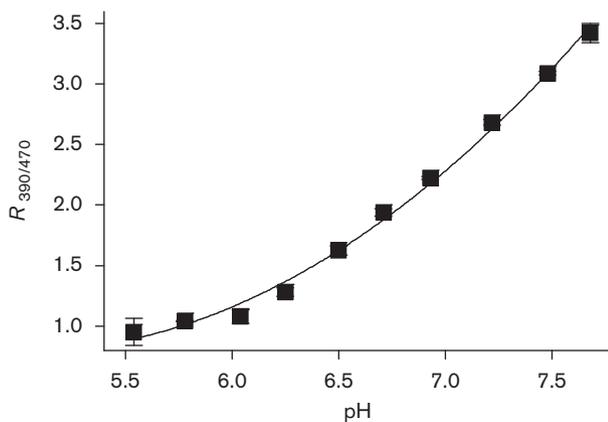


Fig. 1. Calibration curve relating fluorescence intensity ratios to pH. Permeabilized cells were suspended in citric acid/Na₂HPO₄ buffer of pH 5.5–8.0 and fluorescence emission was determined after subtraction of background. The curve represents a quadratic polynomial fit which was used to transform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

For pH_i–growth inhibition relationships, we determined the average pH_i 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 pH_i 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 pH_i 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 pH_i 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 pH_i regulation in different host conditions. We therefore developed the technique to

monitor pH_i in *C. glabrata* based on the pH-sensitive GFP pHluorin. This method has been successfully used to measure pH_i 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 (Frieman *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_i 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_i is well adapted to host-associated conditions**

To study the association of pH_i 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 exterior 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_i of both yeasts during growth (Fig. 2b). *C. glabrata* maintained a higher pH_i than *S. cerevisiae* in all conditions. Interestingly, we observed an apparent inverse correlation between growth rate and pH_i 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_i .

pH_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 *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_i (Orij *et al.*, 2009, 2012). In response to the addition of glucose to glucose-starved yeast, the pH_i suddenly 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_i 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_i response to glucose withdrawal (Fig. 3a, b) as well as the pH_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 *C. glabrata*, both glucose starvation and re-addition affected pH_i in a pH_{ex} -independent fashion, with a reduction of ~ 0.5 pH units after 1 h of starvation, and a small transient pH_i decrease upon glucose re-addition. In contrast, the pH_i 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_i 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_i caused by glucose re-addition was not pH_{ex} dependent: a glucose pulse led to an additional decrease of pH_i of ~ 0.7 units within 20 s, and pH_i 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_i 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_i and growth of *C. glabrata* in the presence of acetic, sorbic

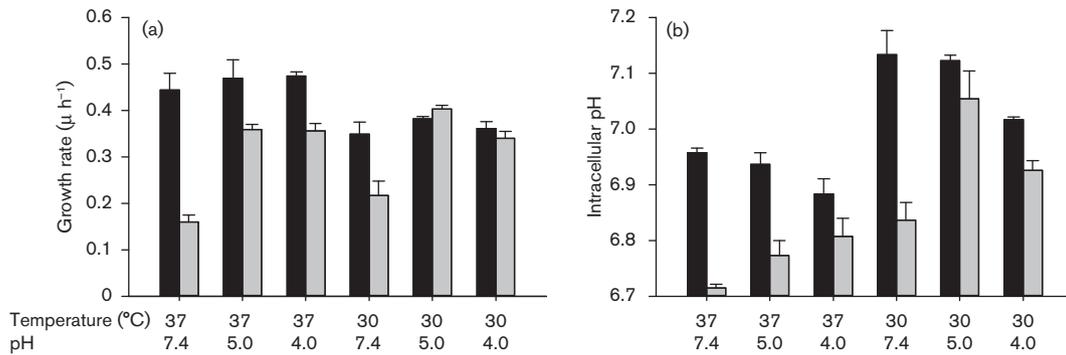


Fig. 2. Comparison of growth and pH_i in different conditions. Maximum specific growth rate (a) and pH_i (b) of *C. glabrata* (black bars) and *S. cerevisiae* (grey bars) in diverse pH_{ex} /temperature combination. pH_i data represent the cytosolic pH of cells at the time of maximum specific growth.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

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

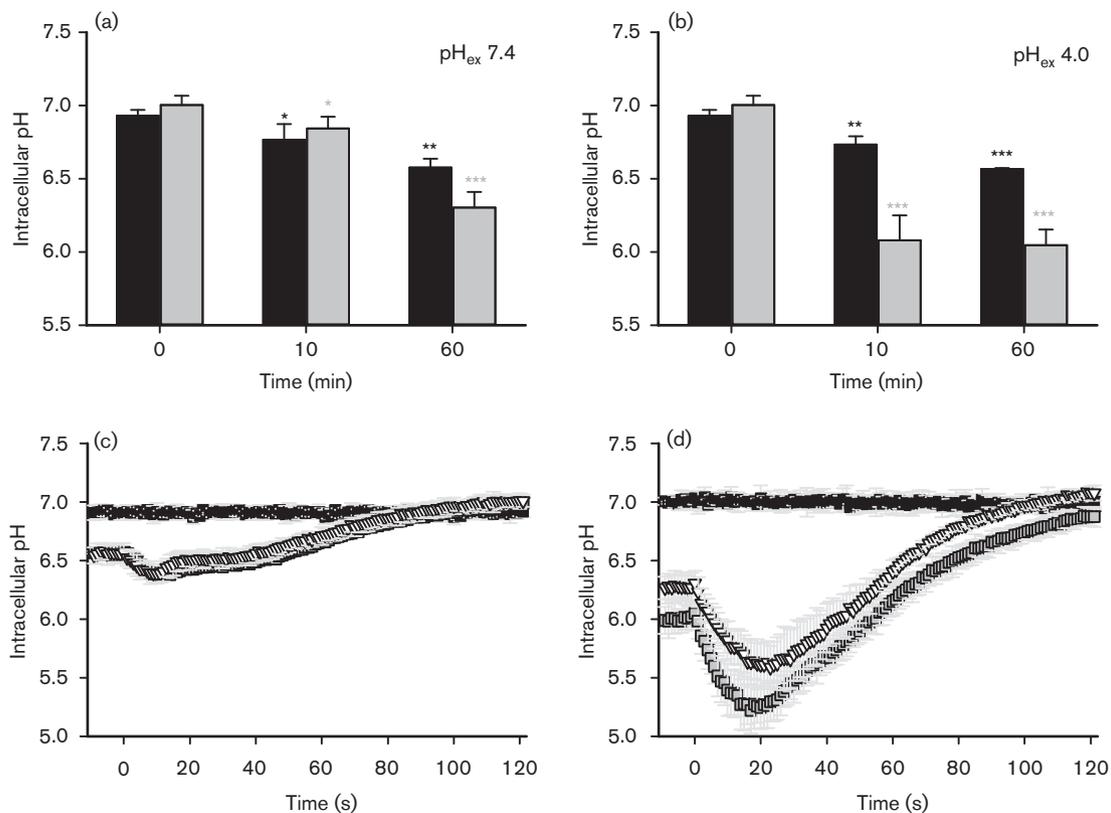


Fig. 3. pH_i response to glucose. pH_i in glucose-deprived cells of *C. glabrata* (black bars) and *S. cerevisiae* (grey bars). Cells were starved for either 10 or 60 min at pH_{ex} 7.4 (a) or 4.0 (b). Glucose (50 mM) was pulsed to 1 h starved cultures of *C. glabrata* (c) and *S. cerevisiae* (d) at time 0 and pH_i 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_i at $t = 0$ and $t = 10$ or 60 min.

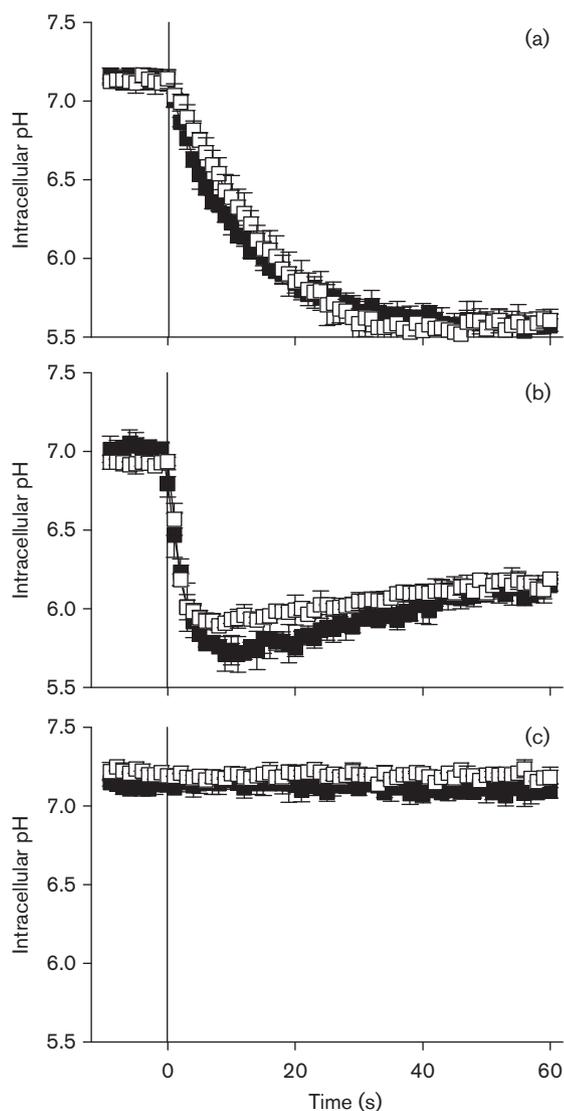


Fig. 4. Effect of weak acid preservatives on pH_i of both yeasts. Effect of 30 mM acetic acid (a), 1 mM sorbic acid (b) and 30 mM lactic acid (c) on pH_i of *C. glabrata* (open symbols) and *S. cerevisiae* (filled symbols). At $t=0$ weak acids were added and pH_i was monitored at 1 s intervals for 1 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 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 ($64 \mu\text{g ml}^{-1}$) 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 – $0.25 \mu\text{g ml}^{-1}$) and amphotericin B (0.5 – $2 \mu\text{g ml}^{-1}$) 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).

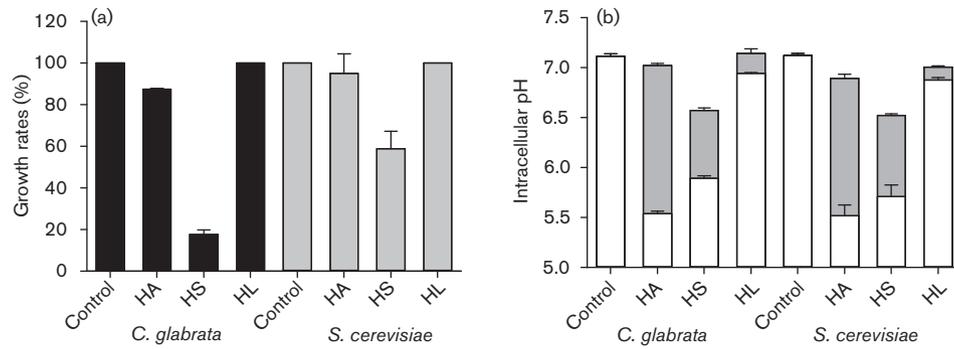


Fig. 5. Effect of weak acid preservatives on the growth and pH_i of the yeasts. (a) Effect of acetic acid (30 mM) and sorbic acid (1 mM) on the growth of *C. glabrata* and *S. cerevisiae*. The effect on growth is presented as growth inhibition, calculated from growth rates of stressed and control cultures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

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_i in *S. cerevisiae* (Oriji *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_i 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 & Mühlshlegel, 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*

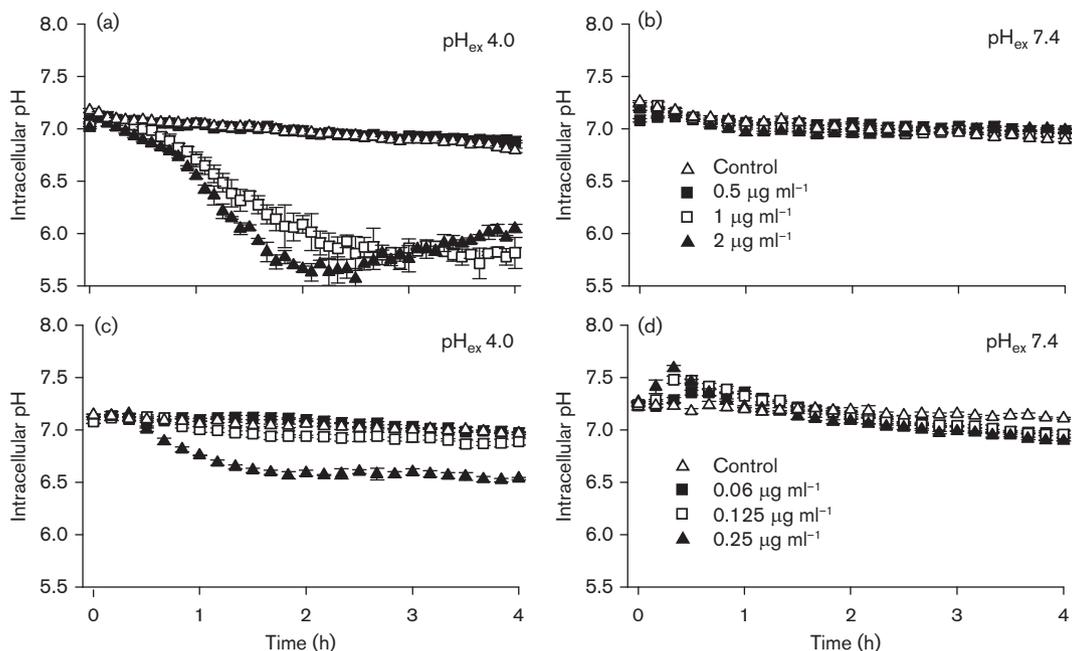


Fig. 6. Effect of antifungal drugs on the pH_i of *C. glabrata*. Effect of increasing concentration of caspofungin (a, b) and amphotericin B (c, d) on pH_i of *C. glabrata* at two ambient pH values.

displayed maximum cell growth rate, regardless of pH_{ex} , confirming earlier observations (Luo & Samaranyake, 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-Muñoz & 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-Muñoz & 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 alkalization. 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 to escape 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 & Mårdh, 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 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 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 Spi1p, 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-Laborín & 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 alkalinization 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.

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

- Aabo, T., Glückstad, J., Siegumfeldt, H. & Arneborg, N. (2011). Intracellular pH distribution as a cell health indicator in *Saccharomyces cerevisiae*. *J R Soc Interface* **8**, 1635–1643.
- Achkar, J. M. & Fries, B. C. (2010). *Candida* infections of the genitourinary tract. *Clin Microbiol Rev* **23**, 253–273.
- Anand, S. & Prasad, R. (1989). Rise in intracellular pH is concurrent with 'start' progression of *Saccharomyces cerevisiae*. *J Gen Microbiol* **135**, 2173–2179.
- Ariño, J. (2010). Integrative responses to high pH stress in *S. cerevisiae*. *OMICS* **14**, 517–523.
- Bagar, T., Altenbach, K., Read, N. D. & Bencina, M. (2009). Live-cell imaging and measurement of intracellular pH in filamentous fungi using a genetically encoded ratiometric probe. *Eukaryot Cell* **8**, 703–712.
- Bairwa, G. & Kaur, R. (2011). A novel role for a glycosylphosphatidylinositol-anchored aspartyl protease, CgYps1, in the regulation of pH homeostasis in *Candida glabrata*. *Mol Microbiol* **79**, 900–913.
- Boskey, E. R., Cone, R. A., Whaley, K. J. & Moench, T. R. (2001). Origins of vaginal acidity: high D/L lactate ratio is consistent with bacteria being the primary source. *Hum Reprod* **16**, 1809–1813.
- Bracey, D., Holyoak, C. D. & Coote, P. J. (1998). Comparison of the inhibitory effect of sorbic acid and amphotericin B on *Saccharomyces cerevisiae*: is growth inhibition dependent on reduced intracellular pH? *J Appl Microbiol* **85**, 1056–1066.
- Brett, C. L., Tukaye, D. N., Mukherjee, S. & Rao, R. J. (2005). The yeast endosomal $\text{Na}^+\text{K}^+/\text{H}^+$ exchanger Nhx1 regulates cellular pH to control vesicle trafficking. *Mol Biol Cell* **16**, 1396–1405.
- Cardenas, M. E., Cruz, M. C., Del Poeta, M., Chung, N. J., Perfect, J. R. & Heitman, J. (1999). Antifungal activities of antineoplastic agents: *Saccharomyces cerevisiae* as a model system to study drug action. *Clin Microbiol Rev* **12**, 583–611.
- Chirife, J. & Ferfontan, C. (1980). Prediction of water activity of aqueous-solutions in connection with intermediate moisture foods – experimental investigation of the a_w lowering behavior of sodium lactate and some related-compounds. *J Food Sci* **45**, 802–804.
- Cohen, B. E. (2010). Amphotericin B membrane action: role for two types of ion channels in eliciting cell survival and lethal effects. *J Membr Biol* **238**, 1–20.
- Colombo, S., Ma, P., Cauwenberg, L., Winderickx, J., Crauwels, M., Teunissen, A., Nauwelaers, D., de Winde, J. H., Gorwa, M. F. & other authors (1998). Involvement of distinct G-proteins, Gpa2 and Ras, in glucose- and intracellular acidification-induced cAMP signalling in the yeast *Saccharomyces cerevisiae*. *EMBO J* **17**, 3326–3341.
- Coote, P. J., Jones, M. V., Seymour, I. J., Rowe, D. L., Ferdinando, D. P., McArthur, A. J. & Cole, M. B. (1994). Activity of the plasma membrane H^+ -ATPase is a key physiological determinant of thermotolerance in *Saccharomyces cerevisiae*. *Microbiology* **140**, 1881–1890.
- Cormack, B. P. & Falkow, S. (1999). Efficient homologous and illegitimate recombination in the opportunistic yeast pathogen *Candida glabrata*. *Genetics* **151**, 979–987.
- Cottier, F. & Mühlischlegel, F. A. (2009). Sensing the environment: response of *Candida albicans* to the X factor. *FEMS Microbiol Lett* **295**, 1–9.
- Danby, C. S., Boikov, D., Rautemaa-Richardson, R. & Sobel, J. D. (2012). Effect of pH on *in vitro* susceptibility of *Candida glabrata* and *Candida albicans* to 11 antifungal agents and implications for clinical use. *Antimicrob Agents Chemother* **56**, 1403–1406.
- Dang, T. D., De Maeseneire, S. L., Zhang, B. Y., De Vos, W. H., Rajkovic, A., Vermeulen, A., Van Impe, J. F. & Devlieghere, F. (2012). Monitoring the intracellular pH of *Zygosaccharomyces bailii* by green fluorescent protein. *Int J Food Microbiol* **156**, 290–295.
- Davis, D., Edwards, J. E., Jr, Mitchell, A. P. & Ibrahim, A. S. (2000). *Candida albicans* RIM101 pH response pathway is required for host-pathogen interactions. *Infect Immun* **68**, 5953–5959.
- de Lucena, R. M., Elsztein, C., Simões, D. A. & de Moraes, M. A., Jr (2012). Participation of CWI, HOG and Calcineurin pathways in the tolerance of *Saccharomyces cerevisiae* to low pH by inorganic acid. *J Appl Microbiol* **113**, 629–640.
- Dechant, R., Binda, M., Lee, S. S., Pelet, S., Winderickx, J. & Peter, M. (2010). Cytosolic pH is a second messenger for glucose and regulates the PKA pathway through V-ATPase. *EMBO J* **29**, 2515–2526.
- Deresinski, S. C. & Stevens, D. A. (2003). Caspofungin. *Clin Infect Dis* **36**, 1445–1457.
- Edmond, M. B., Wallace, S. E., McClish, D. K., Pfaller, M. A., Jones, R. N. & Wenzel, R. P. (1999). Nosocomial bloodstream infections in United States hospitals: a three-year analysis. *Clin Infect Dis* **29**, 239–244.

- El Barkani, A., Kurzai, O., Fonzi, W. A., Ramon, A., Porta, A., Frosch, M. & Mühlischlegel, F. A. (2000). Dominant active alleles of RIM101 (PRR2) bypass the pH restriction on filamentation of *Candida albicans*. *Mol Cell Biol* **20**, 4635–4647.
- Fidel, P. L., Jr, Vazquez, J. A. & Sobel, J. D. (1999). *Candida glabrata*: review of epidemiology, pathogenesis, and clinical disease with comparison to *C. albicans*. *Clin Microbiol Rev* **12**, 80–96.
- Fonzi, W. A. (2002). Role of pH response in *Candida albicans* virulence. *Mycoses* **45** (Suppl. 1), 16–21.
- Frieman, M. B., McCaffery, J. M. & Cormack, B. P. (2002). Modular domain structure in the *Candida glabrata* adhesin Epa1p, a β 1,6 glucan-cross-linked cell wall protein. *Mol Microbiol* **46**, 479–492.
- Galdieri, L., Mehrotra, S., Yu, S. A. & Vancura, A. (2010). Transcriptional regulation in yeast during diauxic shift and stationary phase. *OMICS* **14**, 629–638.
- Gasch, A. P. & Werner-Washburne, M. (2002). The genomics of yeast responses to environmental stress and starvation. *Funct Integr Genomics* **2**, 181–192.
- Hesse, S. J. A., Ruijter, G. J. G., Dijkema, C. & Visser, J. (2000). Measurement of intracellular (compartmental) pH by ^{31}P NMR in *Aspergillus niger*. *J Biotechnol* **77**, 5–15.
- Horn, D. L., Neofytos, D., Anaissie, E. J., Fishman, J. A., Steinbach, W. J., Olyaei, A. J., Marr, K. A., Pfaller, M. A., Chang, C. H. & Webster, K. M. (2009). Epidemiology and outcomes of candidemia in 2019 patients: data from the prospective antifungal therapy alliance registry. *Clin Infect Dis* **48**, 1695–1703.
- Horowitz, B. J. & Mårdh, P. A. (1991). *Vaginitis and Vaginosis*. New York: Wiley-Liss.
- Jacobsen, I. D., Brunke, S., Seider, K., Schwarzmüller, T., Firon, A., d'Enfert, C., Kuchler, K. & Hube, B. (2010). *Candida glabrata* persistence in mice does not depend on host immunosuppression and is unaffected by fungal amino acid auxotrophy. *Infect Immun* **78**, 1066–1077.
- Jandric, Z. & Schüller, C. (2011). Stress response in *Candida glabrata*: pieces of a fragmented picture. *Future Microbiol* **6**, 1475–1484.
- Kapteyn, J. C., ter Riet, B., Vink, E., Blad, S., De Nobel, H., Van Den Ende, H. & Klis, F. M. (2001). Low external pH induces HOG1-dependent changes in the organization of the *Saccharomyces cerevisiae* cell wall. *Mol Microbiol* **39**, 469–480.
- Karagiannis, J. & Young, P. G. (2001). Intracellular pH homeostasis during cell-cycle progression and growth state transition in *Schizosaccharomyces pombe*. *J Cell Sci* **114**, 2929–2941.
- Kaur, R., Domergue, R., Zupancic, M. L. & Cormack, B. P. (2005). A yeast by any other name: *Candida glabrata* and its interaction with the host. *Curr Opin Microbiol* **8**, 378–384.
- Kaur, R., Ma, B. & Cormack, B. P. (2007). A family of glycosylphosphatidylinositol-linked aspartyl proteases is required for virulence of *Candida glabrata*. *Proc Natl Acad Sci U S A* **104**, 7628–7633.
- Klis, F. M., Boorsma, A. & De Groot, P. W. J. (2006). Cell wall construction in *Saccharomyces cerevisiae*. *Yeast* **23**, 185–202.
- Krebs, H. A., Wiggins, D., Stubbs, M., Sols, A. & Bedoya, F. (1983). Studies on the mechanism of the antifungal action of benzoate. *Biochem J* **214**, 657–663.
- Kresnowati, M. T., Suarez-Mendez, C., Groothuizen, M. K., van Winden, W. A. & Heijnen, J. J. (2007). Measurement of fast dynamic intracellular pH in *Saccharomyces cerevisiae* using benzoic acid pulse. *Biotechnol Bioeng* **97**, 86–98.
- Kresnowati, M. T., van Winden, W. A., van Gulik, W. M., Heijnen, J. J. (2008). Energetic and metabolic transient response of *Saccharomyces cerevisiae* to benzoic acid. *FEBS J* **275**, 5527–5541.
- Laniado-Laborín, R. & Cabrales-Vargas, M. N. (2009). Amphotericin B: side effects and toxicity. *Rev Iberoam Micol* **26**, 223–227.
- Lasorsa, F. M., Scarcia, P., Erdmann, R., Palmieri, F., Rottensteiner, H. & Palmieri, L. (2004). The yeast peroxisomal adenine nucleotide transporter: characterization of two transport modes and involvement in ΔpH formation across peroxisomal membranes. *Biochem J* **381**, 581–585.
- Lecchi, S., Allen, K. E., Pardo, J. P., Mason, A. B. & Slayman, C. W. (2005). Conformational changes of yeast plasma membrane H^+ -ATPase during activation by glucose: role of threonine-912 in the carboxy-terminal tail. *Biochemistry* **44**, 16624–16632.
- Lo, H. J., Köhler, J. R., DiDomenico, B., Loebenberg, D., Cacciapuoti, A. & Fink, G. R. (1997). Nonfilamentous *C. albicans* mutants are avirulent. *Cell* **90**, 939–949.
- Luo, G. & Samaranyake, L. P. (2002). *Candida glabrata*, an emerging fungal pathogen, exhibits superior relative cell surface hydrophobicity and adhesion to denture acrylic surfaces compared with *Candida albicans*. *APMIS* **110**, 601–610.
- Maresová, L., Hosková, B., Urbánková, E., Chaloupka, R. & Sychrová, H. (2010). New applications of pHluorin – measuring intracellular pH of prototrophic yeasts and determining changes in the buffering capacity of strains with affected potassium homeostasis. *Yeast* **27**, 317–325.
- Martínez-Muñoz, G. A. & Kane, P. (2008). Vacuolar and plasma membrane proton pumps collaborate to achieve cytosolic pH homeostasis in yeast. *J Biol Chem* **283**, 20309–20319.
- Miesenböck, G., De Angelis, D. A. & Rothman, J. E. (1998). Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature* **394**, 192–195.
- Miyazaki, T., Inamine, T., Yamauchi, S., Nagayoshi, Y., Saijo, T., Izumikawa, K., Seki, M., Kakeya, H., Yamamoto, Y. & other authors (2010). Role of the Slt2 mitogen-activated protein kinase pathway in cell wall integrity and virulence in *Candida glabrata*. *FEMS Yeast Res* **10**, 343–352.
- Monk, B. C. & Perlin, D. S. (1994). Fungal plasma membrane proton pumps as promising new antifungal targets. *Crit Rev Microbiol* **20**, 209–223.
- Orij, R., Postmus, J., Ter Beek, A., Brul, S. & Smits, G. J. (2009). *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. *Microbiology* **155**, 268–278.
- Orij, R., Brul, S. & Smits, G. J. (2011). Intracellular pH is a tightly controlled signal in yeast. *Biochim Biophys Acta* **1810**, 933–944.
- Orij, R., Urbanus, M. L., Vizeacoumar, F. J., Giaever, G., Boone, C., Nislow, C., Brul, S. & Smits, G. J. (2012). Genome-wide analysis of intracellular pH reveals quantitative control of cell division rate by pH_c in *Saccharomyces cerevisiae*. *Genome Biol* **13**, R80.
- Owen, D. H. & Katz, D. F. (1999). A vaginal fluid simulant. *Contraception* **59**, 91–95.
- Pappas, P. G., Kauffman, C. A., Andes, D., Benjamin, D. K., Jr, Calandra, T. F., Edwards, J. E., Jr, Filler, S. G., Fisher, J. F., Kullberg, B. J. & other authors (2009). Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. *Clin Infect Dis* **48**, 503–535.
- Peñalva, M. A. & Arst, H. N., Jr (2002). Regulation of gene expression by ambient pH in filamentous fungi and yeasts. *Microbiol Mol Biol Rev* **66**, 426–446.
- Peñalva, M. A., Tilburn, J., Bignell, E. & Arst, H. N., Jr (2008). Ambient pH gene regulation in fungi: making connections. *Trends Microbiol* **16**, 291–300.

- Pineda Rodó, A., Váchová, L. & Palková, Z. (2012). *In vivo* determination of organellar pH using a universal wavelength-based confocal microscopy approach. *PLoS ONE* 7, e33229.
- Porta, A., Wang, Z., Ramon, A., Mühlshlegel, F. A. & Fonzi, W. A. (2001). Spontaneous second-site suppressors of the filamentation defect of *prf1Δ* mutants define a critical domain of Rim101p in *Candida albicans*. *Mol Genet Genomics* 266, 624–631.
- Ramos, S., Balbín, M., Raposo, M., Valle, E. & Pardo, L. A. (1989). The mechanism of intracellular acidification induced by glucose in *Saccharomyces cerevisiae*. *J Gen Microbiol* 135, 2413–2422.
- Roetzer, A., Gratz, N., Kovarik, P. & Schüller, C. (2010). Autophagy supports *Candida glabrata* survival during phagocytosis. *Cell Microbiol* 12, 199–216.
- Roetzer, A., Gabaldón, T. & Schüller, C. (2011). From *Saccharomyces cerevisiae* to *Candida glabrata* in a few easy steps: important adaptations for an opportunistic pathogen. *FEMS Microbiol Lett* 314, 1–9.
- Sabina, J. & Brown, V. (2009). Glucose sensing network in *Candida albicans*: a sweet spot for fungal morphogenesis. *Eukaryot Cell* 8, 1314–1320.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schiestl, R. H. & Gietz, R. D. (1989). High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr Genet* 16, 339–346.
- Schmidt, P. (2007). *Molecular mechanisms of the human pathogen Candida glabrata involved in the interaction with the host*. PhD thesis, Georg August University Göttingen, Göttingen, Germany.
- Schmidt, P., Walker, J., Selway, L., Stead, D., Yin, Z., Enjalbert, B., Weig, M. & Brown, A. J. (2008). Proteomic analysis of the pH response in the fungal pathogen *Candida glabrata*. *Proteomics* 8, 534–544.
- Shapiro, R. S., Robbins, N. & Cowen, L. E. (2011). Regulatory circuitry governing fungal development, drug resistance, and disease. *Microbiol Mol Biol Rev* 75, 213–267.
- Simões, T., Teixeira, M. C., Fernandes, A. R. & Sá-Correia, I. (2003). Adaptation of *Saccharomyces cerevisiae* to the herbicide 2,4-dichlorophenoxyacetic acid, mediated by Msn2p- and Msn4p-regulated genes: important role of SPI1. *Appl Environ Microbiol* 69, 4019–4028.
- Simões, T., Mira, N. P., Fernandes, A. R. & Sá-Correia, I. (2006). The *SPI1* gene, encoding a glycosylphosphatidylinositol-anchored cell wall protein, plays a prominent role in the development of yeast resistance to lipophilic weak-acid food preservatives. *Appl Environ Microbiol* 72, 7168–7175.
- Sobel, J. D. (2007). Vulvovaginal candidosis. *Lancet* 369, 1961–1971.
- Sorgo, A. G., Heilmann, C. J., Dekker, H. L., Brul, S., de Koster, C. G. & Klis, F. M. (2010). Mass spectrometric analysis of the secretome of *Candida albicans*. *Yeast* 27, 661–672.
- Soteropoulos, P., Vaz, T., Santangelo, R., Paderu, P., Huang, D. Y., Tamás, M. J. & Perlin, D. S. (2000). Molecular characterization of the plasma membrane H⁺-ATPase, an antifungal target in *Cryptococcus neoformans*. *Antimicrob Agents Chemother* 44, 2349–2355.
- Stewart, E., Hawser, S. & Gow, N. A. (1989). Changes in internal and external pH accompanying growth of *Candida albicans*: studies of non-dimorphic variants. *Arch Microbiol* 151, 149–153.
- Thevelein, J. M. (1991). Fermentable sugars and intracellular acidification as specific activators of the RAS-adenylate cyclase signalling pathway in yeast: the relationship to nutrient-induced cell cycle control. *Mol Microbiol* 5, 1301–1307.
- Ueno, K., Matsumoto, Y., Uno, J., Sasamoto, K., Sekimizu, K., Kinjo, Y. & Chibana, H. (2011). Intestinal resident yeast *Candida glabrata* requires Cyb2p-mediated lactate assimilation to adapt in mouse intestine. *PLoS ONE* 6, e24759.
- Ullah, A., Orij, R., Brul, S. & Smits, G. J. (2012). Quantitative analysis of the modes of growth inhibition by weak organic acids in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* 78, 8377–8387.
- Vylkova, S., Carman, A. J., Danhof, H. A., Collette, J. R., Zhou, H. J. & Lorenz, M. C. (2011). The fungal pathogen *Candida albicans* autoinduces hyphal morphogenesis by raising extracellular pH. *mBio* 2, e00055–e11.
- Walker, G. M. (1998). *Yeast Physiology and Biotechnology*. Chichester: Wiley.
- Walther, A. & Wendland, J. (2003). An improved transformation protocol for the human fungal pathogen *Candida albicans*. *Curr Genet* 42, 339–343.
- Warringer, J. & Blomberg, A. (2003). Automated screening in environmental arrays allows analysis of quantitative phenotypic profiles in *Saccharomyces cerevisiae*. *Yeast* 20, 53–67.
- Whiteway, M. & Bachewich, C. (2007). Morphogenesis in *Candida albicans*. *Annu Rev Microbiol* 61, 529–553.
- Young, B. P., Shin, J. J. H., Orij, R., Chao, J. T., Li, S. C., Guan, X. L., Khong, A., Jan, E., Wenk, M. R. & other authors (2010). Phosphatidic acid is a pH biosensor that links membrane biogenesis to metabolism. *Science* 329, 1085–1088.
- Zhou, J., Liu, L. & Chen, J. (2011). Improved ATP supply enhances acid tolerance of *Candida glabrata* during pyruvic acid production. *J Appl Microbiol* 110, 44–53.

Edited by: J. Morschhäuser