The role of intracellular pH in environmental adaptation of yeasts
Ullah, A.

Citation for published version (APA):
Ullah, A. (2012). The role of intracellular pH in environmental adaptation of yeasts Ridderprint
Chapter 5

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

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Submitted for publication
Abstract

*Candida glabrata* is an opportunistic fungal pathogen which is a growing concern for immunocompromised patients. It is ranked as second most common cause of candidiasis after *Candida albicans*. For pathogenic yeasts, intracellular pH (pH\textsubscript{i}) has been implicated in proliferation, dimorphic switching and virulence. We expressed the pH-sensitive green fluorescent protein (GFP) variant ratiometric pHluorin in the cytosol of *C. glabrata* to study pH\textsubscript{i} dynamics in living cells. We evaluated the response of pH\textsubscript{i} to the various growth and stress conditions encountered during interaction with the host and during antifungal treatment. *C. glabrata* maintained a pH\textsubscript{i} higher than that of *Saccharomyces cerevisiae* in all growth conditions. The pH\textsubscript{i} of *S. cerevisiae* cells appeared better controlled than the pH\textsubscript{i} in *C. glabrata* when the cells were exposed to food and fermentation associated conditions. *C. glabrata* in turn maintained its pH\textsubscript{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 related to the non-pathogenic *S. cerevisiae* than to for instance *C. albicans* [1, 2]. *Candida* species are the major cause of fungal bloodstream infections, which are the cause of more than 8% of all hospital-acquired infections [3]. *Candida* species are usually residing in healthy human hosts as commensals but can become pathogenic in immune compromised patients. For a long time, *C. glabrata* was considered as a non-pathogenic saprophyte of the normal flora of healthy individuals [4]. However, in the last two decades, the number of incidences of *C. glabrata* infections has increased significantly, especially in immunocompromised individuals such as patients with HIV or the elderly. Unfortunately, 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 [4]. Hitherto, the mechanisms involved in pathogenicity of *C. glabrata* and its resistance against azoles are poorly understood compared to *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 places of skin [4].

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 acidic (stomach, vagina) to highly alkaline (blood, saliva) [5]. In many pathogenic fungi, ambient pH has been considered as a potent virulence determining factor. For example, *C. albicans'* phenotypic switching from a budding yeast cell to filamentous hyphae, an essential trait for virulence, is mediated by ambient pH [6, 7]. Although such pH_{ex} dependent phenotypic switching is absent in *C. glabrata* intracellular pH regulation under these varying ambient pH conditions is essential for survival in and colonization of the host [8]. 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 [5]. Intracellular pH regulation is vital for cellular functioning, because almost all cellular activities are directly or indirectly dependent on intracellular pH [9]. In *S. cerevisiae*, pH_{i} is clearly related to growth [10].
To understand how intracellular pH 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 lead not only to a better understanding of pH$_i$ regulation but also generate new leads for antifungal targets [11]. So far, pH homeostasis is very poorly studied in *C. glabrata* [12]. Currently, there are several methods and techniques available to measure intracellular pH in yeast such as phospho-nuclear magnetic resonance [13], radiolabelled membrane-permeable weak acids or bases [14-16], probing with pH-sensitive fluorescent dyes [5, 17] and equilibrium distribution of benzoic acid [18]. The above mentioned methods require extensive manipulation of cells, which in itself may perturb intracellular pH [10, 19, 20]. In recent years, the pH sensitive GFP derivative pHluorin [21] has been successfully used to measure pH$_i$ in *S. cerevisiae* [10, 19, 22-24]. Ratiometric pH-sensitive GFPs are accurate and reliable, have as advantages their organelle specificity [10, 24, 25], allow single cell pH$_i$ analysis [26, 27], are non-invasive and do not affect normal physiological activity of yeast [10].

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.

**Materials and 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% dextrose) prior to transformation. Transformed cells were cultured in low fluorescence synthetic complete (SC) medium (YNB without folic acid and riboflavin, 0.5% (NH$_4$)$_2$SO$_4$, 2% glucose, 1g sodium glutamate, 2g uracil dropout mix). Medium was buffered at either pH 7.4 with MOPS [3-(N-Morpholinyl)-2-hydroxypropanesulphonic acid] or pH 4.0 with 75 mM tartaric acid as described [28]. 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°C or 37°C. All solid media contained 1.5% (w/v) agar. All chemicals were purchased from Sigma-Aldrich Germany, unless stated otherwise in the text.
Overnight cultures were harvested by centrifugation (5000 rpm) and diluted into fresh SC-ura to an OD<sub>600</sub> of 1.0. Growth (OD<sub>600</sub>) and fluorescence (excitation at 390 and 470 nm, emission at 510 nm) were assayed by transferring aliquots of fresh cultures (200µl/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 rpm for 2 minutes 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<sub>i</sub> measurement. The pHluorin gene was PCR amplified from plasmid pYES-ACT-pHfluorin, using primers pHl-F-XbaI 5´-GAAGTCTAGAATGAGTAAAGGAGAAGAAC-3´ and pHl-R-EcoRI 5´-GTG-CAGAATTCTTTATTGTATAGTT- CATCCAT-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-pHfluorin+. To insert another copy of *PGK1*-pHfluorin into plasmid pGRB 2.2-pHfluorin, a PCR fragment was amplified from pGRB 2.2-pHfluorin, using primers pgk1-F-EcoRI 5´-GTATCGGAATTCCATAAAGCACGTGGCCTCTTAT-3´, pHl-R-XhoI 5´-GTGCAGCTCGAGTTATTTGTATAGTTCATCCATGC-3´, and sub cloned into pGRB 2.2-pHfluorin digested with EcoRI/XhoI, generating pGRB 2.2-pHfluorin++. PCR amplifications were done using Pwo DNA polymerase (Roche) and later analysed by sequencing analysis. Basic DNA manipulation procedures were performed essentially as described earlier [29]. Unless otherwise indicated, all the restriction and modification enzymes used in this study were from Fermentas (The Netherlands). For plasmid isolation and purification of PCR products we used Qiagen kits (Qiagen, The Netherlands).

Transformation of *S. cerevisiae* and *C. glabrata*
*S. cerevisiae* was transformed using the lithium acetate method as described in literature [30]. A modified lithium acetate protocol was used to transform *C. glabrata* [31, 32].

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

In spectrophotometers, the OD\textsubscript{600} measured is not linear with cell number at higher densities. To correct for this non-linearity overnight cultures of \textit{C. glabrata} and \textit{S. cerevisiae} were serially diluted, and OD\textsubscript{600} was measured in the microplate reader [33]. The same samples were diluted to within the linear range of the spectrophotometer (Pharmacia LKB Biochrom) and OD\textsubscript{600} was registered. These linear OD\textsubscript{600} values measured in the spectrophotometer were used to correct the OD\textsubscript{600} values determined in the microplate reader (Fig. S1), 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\textsubscript{600} growth curves.

\textbf{pHluorin calibration and pH measurement}

For \textit{S. cerevisiae}, a calibration curve of pHluorin fluorescence at different pH values was generated as described earlier [10]. The same protocol was optimized for \textit{C. glabrata}. Briefly, cultures growing exponentially in SC-ura were harvested at OD\textsubscript{600} 3.0 by centrifugation at 5000 rpm for 5 min and transferred to phosphate buffered saline (PBS) containing 300 mg/ml digitonin. The cell suspensions were incubated for 15 min at room temperature, harvested, washed with PBS buffer, and the permeabilized cells were suspended in citric acid/Na\textsubscript{2}HPO\textsubscript{4} buffer with pH values 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 the 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 [10].

\textbf{Conditions}

To determine the effect of glucose starvation and readdition, \textit{S. cerevisiae} and \textit{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 hour starvation cells were transferred to 96-wells plates and pH$_i$ was monitored at 1 second intervals for 10 seconds. 50 mM glucose was injected into the plates, and fluorescence was registered at 1-second intervals during 2 minutes.

To determine the effect of weak organic acids, *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-second intervals during 1 minute. For long-term experiments, growing cultures were challenged with weak acid stress in 96-well plates, and growth (OD$_{600}$) and pH$_i$ were monitored every 10 minutes over a period of 16 hours.

The effect of three different antifungal drugs, fluconazole, amphotericin B and caspofungin (a kind gift from Merck Research Laboratories, Rahway, N.J.) on pH$_i$ was studied. Multiple stocks of fluconazole (20X in water), caspofungin (100X in water), and amphotericin B (20X in DMSO) were prepared depending on the stress concentration. Growing cultures were exposed to various concentrations of antifungal drugs mentioned in literature [34].

**Data Analysis**

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

**Figure 1. Calibration curve relating fluorescence intensity ratios to pH.**

Permeabilized cells were suspended in to citric acid/Na$_2$HPO$_4$ buffer of pH values ranging from 5.5 to 8.0 and fluorescence emission was determined after subtraction of background. The black line represents a quadratic polynomial fit which was used to transform fluorescence ratios to intracellular pH values.
Chapter 5

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* are defined by ambient pH [35-38]. *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 [9, 27, 39]. For cytoplasmic expression of pHluorin, we cloned two separate copies of ratiometric pHluorin [21] into plasmid pGRB 2.2 [40, 41] 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* [10] led to a doubled fluorescence intensity compared to 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* [10]. We compared growth and morphology of *C. glabrata* strains with and without pHluorin and equally observed no differences (Fig. S2B & data not shown).

To calibrate the fluorescent signal to pH values, we permeabilized cells with 300 mg digitonin ml$^{-1}$ and exposed them to buffers in a range of known pH values between 5.5-8.0 as described earlier [10]. 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 high temperature corresponding to the vaginal mucosa, and both pH values at low temperature, for exterior niches. We observed that at 37°C and pH 7.4 *C. glabrata* showed maximum growth (Fig. 2A). Moreover, we did not see a significant difference in growth in response to ambient pH. In contrast, baker’s yeast showed highest growth at low pH (4.0) at both temperatures. During these growth experiments (Fig. 2B), we also monitored
intracellular pH of both yeasts. pHᵢ profiles were different for both yeasts: C. glabrata maintained a higher pHᵢ than S. cerevisiae upon exposure to all growth conditions. Interestingly, we observed that C. glabrata had a low pHᵢ when cells were exposed to conditions where high growth (rates) were observed and vice versa. This is in contrast to S. cerevisiae, where we found no condition independent relation between growth and pHᵢ.

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

**pHᵢ response to glucose withdrawal and readdition**
In is a nutrient-limited environment, and the ability to withstand starvation and adapt to diverse nutrients is essential for species’ survival [42]. C. glabrata encounters nutrient limited conditions in diverse niches. Mucosal areas in the mammalian host or the interior of a macrophage are both glucose deficient [43]. Glucose is the preferred carbon/energy source for most of the cells and was shown to be a morphogen in C. albicans where it influences yeast-to-hypha transitions [44].

The initial acidification and subsequent alkalinisation in response to the addition of glucose to glucose starved yeast is a well-studied physiological behaviour in S. cerevisiae [10, 18]. However, this phenomenon has not been characterized in C. glabrata. We therefore studied pHᵢ dynamics in C. glabrata upon addition of glucose and compared this with baker’s yeast. We used exponentially growing cultures, which were starved for one hour 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 readdition affected pH\textsubscript{i} in a pH\textsubscript{ex} independent fashion, with a reduction of ~0.5 pH units after 1 hour of starvation, and a small transient pH\textsubscript{i} decrease upon glucose readdition. 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 minutes at pH\textsubscript{ex} 7.4, compared to a strong and rapid reduction reaching 1.0 unit after 10 minutes at pH\textsubscript{ex} 4.0. After one hour, 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 readdition was not pH\textsubscript{ex} dependent: A glucose pulse led to an additional decrease of pH\textsubscript{i} of ~0.7 units within 20

![Figure 3. Intracellular pH response to glucose. Intracellular pH in glucose deprived cells of \textit{C. glabrata} (black bars) and \textit{S. cerevisiae} (grey bars). Cells were starved for either 10 or 60 minutes at pH\textsubscript{ex} 7.4 (A) or 4.0 (B). 50 mM glucose was pulsed to 1 hour starved cultures of \textit{C. glabrata} (C) and \textit{S. cerevisiae} (D) at time 0 and pH\textsubscript{i} was monitored at 1-second intervals for 2 minutes. Asterisks indicate statistically significant differences (*=p<0.05; **=p<0.01; ***=p<0.001) between pH\textsubscript{i} at t=0 and t=10 or 60 minutes.](image-url)
seconds, and \( p_{H_i} \) recovered to neutral in approximately 2 minutes. The \( p_{H_{ex}} \) 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.

**\( p_{H_i} \) responses to commonly encountered organic acids**

Weak 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 [45]. One of the major antifungal mechanism of these weak acids is cytosolic acidification [9]. We therefore studied \( p_{H_i} \) and growth of \textit{C. glabrata} in the presence of acetic, sorbic and lactic acid and compared the results with \textit{S. cerevisiae}. We selected concentrations of sorbic and acetic acid which cause similar acidification. In case of lactic acid, we used a range of concentrations (30, 60, 120 mM), but even at high concentrations, acidification was very limited.

**Figure 4. Effect of weak acid preservatives on the intracellular pH of both yeasts.**

Effect of (A) 30 mM of acetic acid, (B) 1 mM sorbic acid, and (C) 30 mM of lactic acid on the intracellular \( p_{H_i} \) of \textit{C. glabrata} (open symbols) and \textit{S. cerevisiae} (closed symbols). At \( t=0 \) weak acids were added and \( p_{H_i} \) was monitored at 1-second intervals for 1 minute.
compared to the other two WOAs (data not shown). We therefore decided to use 30 mM of undissociated acid, corresponding to 71 mM of total lactate, since higher concentrations might additionally give osmotic stress [46]. First, we studied the immediate effects of acid exposure. We challenged growing cultures of \textit{C. glabrata} and \textit{S. cerevisiae} with WOAs and monitored pH\textsubscript{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 \textit{et al.}, submitted). Sorbic acid and acetic acid inhibited growth of \textit{C. glabrata} by ~80% and ~12% compared to only ~40% and ~5% in \textit{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\textsubscript{i} recovery was quite similar (Fig. S4), and pH\textsubscript{i} recovered faster after acetic acid exposure than after sorbic acid exposure. Concluding, even though acid entry rates and pH values reached were virtually identical for both yeasts, \textit{C. glabrata} showed a much stronger sensitivity to weak organic acids.

\textbf{Figure 5.} Effect of weak acid preservatives on the growth and pH\textsubscript{i} of the yeasts. (A) Effect of acetic acid (30 mM) and sorbic acid (1 mM) on the growth of \textit{C. glabrata} and \textit{S. cerevisiae}. The effect on growth is presented as growth inhibition, calculated from growth rates of stressed and control as described in material and methods. (B) The effect of the acids on pH\textsubscript{i} is represented as initial acidification (white bars), which indicates the pH\textsubscript{i} 10 minutes after the addition of the weak acids to growing culture of yeasts, and recovered pH\textsubscript{i} (grey bars), indicating the pH\textsubscript{i} at the time of maximally recovered growth. Full growth and pH\textsubscript{i} profiles can be found in figure S4.

\textbf{Perturbation of pH\textsubscript{i} by antifungal drugs}

Yeast cells are eukaryotes with a physiology and cell biology similar to those of
Intracellular pH of Candida glabrata human cells. Therefore, it is difficult to design antifungals drugs without side effects [47, 48]. For C. glabrata the treatment options are even more limited, owing to the species’ exceptional resistance to azoles [43]. Therefore, new antifungal targets are required to improve medication. In literature, Pma1p has been proposed as antifungal target [49] because it is a master regulator of pH$_i$ and responsible for nutrient uptake by generating an electrochemical proton gradient [11].

Figure 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 the intracellular pH of C. glabrata at two ambient pH values.

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 concentration of fluconazole, amphotericin B, and caspofungin and pH$_i$ was monitored. Growth inhibitory concentrations were selected from literature [34] and retested by measuring turbidity after 16 hours. It is well known that yeast’s susceptibility to antifungal drugs varies at different pH$_{ex}$ [34] 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 concentration (64 µg/ml) of fluconazole did not affect growth and pH\textsubscript{i} of \textit{C. glabrata} or \textit{S. cerevisiae} at either pH\textsubscript{ex} (our unpublished data). In contrast, growth was impaired by caspofungin (0.06-0.25µg/ml) and amphotericin B (0.5-2µg/ml) in agreement with literature [34]. Interestingly, we observed different pH\textsubscript{i} behaviour at different ambient pH values. Both drugs acidified the cells at acidic pH\textsubscript{ex}, but at alkaline pH\textsubscript{ex} cells maintained a high pH\textsubscript{i} (Fig. 6), with even a slight initial alkalinisation.

\textbf{Discussion}

\textit{C. glabrata} is an emerging fungal pathogen, closely related to \textit{S. cerevisiae} (Kaur \textit{et al.}, 2005, Roetzer \textit{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 [43]. Intracellular pH plays a vital role in yeast's physiology as it regulates a variety of cellular processes which are essential for proliferation and survival in \textit{S. cerevisiae} [9] and virulence in \textit{C. albicans} and \textit{Aspergillus} spp. [35]. Very little work has been done to understand the pH\textsubscript{i} regulation in the pathogenic yeast \textit{C. glabrata} compared to baker’s yeast [5, 50]. In this study, we have modified a method to determine pH\textsubscript{i} using GFP-pHluorin in \textit{C. glabrata}. We show that pHluorin expression does not interfere with cell physiology and is a reliable method to measure pH\textsubscript{i} in \textit{C. glabrata} [10]. Our pH\textsubscript{i} values corroborated a recent report measuring pH\textsubscript{i} with fluorescent probes [50].

Microbes are exposed to various insults associated with host niches, for instance high temperature, low pH, nutrient limitation and the presence of weak acids. The rapid adaptation of pathogens to these various conditions is critical for both fitness and virulence. The above mentioned environmental fluctuations have a profound effect on pH\textsubscript{i} in \textit{S. cerevisiae} [9]. It has been shown that pH regulation is important for virulence of \textit{Candida albicans} [51, 52]. However, unlike \textit{C. albicans}, \textit{C. glabrata} does not rely on morphological switching for virulence. In this work, we have studied the pH\textsubscript{i} of \textit{C. glabrata} under different growth conditions present in host niches. Temperature and ambient pH are known to be major determinants of growth patterns in \textit{Candida} species and are considered virulence factors [53]. Our findings confirmed that at host temperature \textit{C. glabrata} displayed maximum cell growth rate [54], regardless of pH\textsubscript{ex}. \textit{S. cerevisiae} showed highest growth rate at low pH\textsubscript{ex}, which is consistent
Intracellular pH of Candida glabrata

with the literature that baker’s yeast prefers low pH_{ex} [9, 55]. 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 [56]. We did not see a condition independent correlation of intracellular pH with growth [57]. It has previously been shown in S. cerevisiae that pH_{ex} does not directly affect pH_{i}. Here, we did observe different pH_{i} at different ambient pH values 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 temperature, consistent with the results of previous studies [58, 59]. 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 [58].

Glucose is the preferred carbon and energy source for most yeasts [60] and energy is required to maintain pH_{i} [10, 23]. Not surprisingly, upon glucose withdrawal the intracellular pH decreased in both yeasts, likely because of decreased activity plasma-membrane H^{+}-ATPase pump which is a major pH_{i} regulator in yeast [9, 61, 62]. 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 alkalinisation. 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, for instance because of H^{+}-coupled import of other nutrients, is also not the cause of the acidification. In contrast with 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 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 [43, 63].
Environmentally encountered weak organic acids may also affect intracellular pH and growth (Ullah et al., submitted). 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, likely through diffusion over the membrane. Interestingly, the pH response of the two yeasts to WOAs were quite similar, even while *S. cerevisiae* appeared more resistant. In nature, *S. cerevisiae* is adapted to colonize fruits, which contain high dosage of various WOAs. This may explain its resistance to WOA preservatives. *C. glabrata* showed a particularly high sensitivity to sorbic acid. These results corroborate our previous work that showed that sorbic acid does not inhibit growth by pH lowering (Ullah et al., submitted). The production of lactic acid and low pH of the vagina are considered beneficial for the prevention of pathogen growth. Remarkably, we did not see any growth or pH effects caused by lactic acid at vaginal pH 4.0.

*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. *C. glabrata* exhibited a high resistance to fluconazole, as neither growth nor pH were affected even using high concentrations of the drug. Interestingly both caspofungin and amphotericin B perturbed intracellular pH 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. It is thought that inhibition of β-1,3-glucan lowers the integrity of the cell wall, results in osmotic instability, which may lead to cell lysis and cell death.

There is a body of evidence linking pH to cell wall biogenesis, and it appears likely that pH would also affect cell wall biogenesis through a perturbed activity of the cell wall integrity pathway. Echinocandin antifungals appear more potent at low pH, because a high alkaline pH activates the cell wall integrity (Slt2) pathway to adapt to an elevated pH in complex media, WOA activate the HOG-pathway, leading to the expression of the glycosylphosphatidylinositol anchored cell wall proteinSpi1p which is thought to be involved in WOA resistance. 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 [72, 73]. Interestingly, we found that the effect of caspofungin was strongly pH\textsubscript{ex} dependent, and the interaction of pH\textsubscript{ex} and caspofungin led to a very strong decrease of pH\textsubscript{i}, which should by itself already cause a strong reduction in fitness [57]. Another possible explanation of a pH\textsubscript{ex} dependent pH\textsubscript{i} effect in caspofungin exposed cells could be osmotic stress. Caspofungin disrupts cell wall integrity, causing osmotic stress, which in itself acidifies cells (Ullah \textit{et al.}, submitted). Disruption of pH\textsubscript{i} homeostasis by caspofungin might contribute to its inhibitory activity.

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 [74, 75] resulting in depolarization of the membrane. This is consistent with our data where amphotericin B leads to a pH\textsubscript{ex} dependent effect on pH\textsubscript{i} causing a cytoplasmic acidification at acidic pH\textsubscript{ex} and a slight alkalinisation at alkaline pH\textsubscript{ex}. In a previous study a strong correlation between growth and pH\textsubscript{i} was identified, suggesting that amphotericin B inhibits growth by disrupting pH\textsubscript{i} homeostasis [17]. This emphasizes the potential use of pH\textsubscript{i} homeostasis as an antifungal drug target.

In conclusion, we have developed a tool that allows rapid and reliable determination of pH\textsubscript{i} of \textit{C. glabrata} when exposed to a number of important physiologically stressful conditions. We used the method to gain insight in the relation between pH\textsubscript{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, N.J.). We are also grateful to HEC, Pakistan for financial support (A.U.).
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Supplementary Material

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

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Supplementary Material

INDEX:

**Figure S1**: OD$_{600}$ calibration curve for *C. glabrata*.

**Figure S2**: Fluorescence signals increased with two copies of pHluorin.

**Figure S3**: Growth and intracellular pH profiles of yeasts.

**Figure S4**: Growth and intracellular pH profiles in the presence of weak organic acids.
Figure S1. OD\textsubscript{600} calibration curve for \textit{C. glabrata}.
The curve relates the real OD\textsubscript{600} and observed OD\textsubscript{600}. Overnight culture was serially
diluted into water and used for calibration. Real OD\textsubscript{600} was measured in standard
spectrophotometer and observed OD\textsubscript{600} measured in microtiter plate reader. A
polynomial function (black line) was used to translate the relation of real OD\textsubscript{600} and
observed OD\textsubscript{600}.

Figure S2. Expression and growth effect of pHluorin.
(a) Time course of fluorescence signals (390 nm) in \textit{C. glabrata} without pHluorin (black
triangles), with plasmid carrying one copy of pHluorin (white squares) and two copies of
pHluorin (grey circles) (b) Growth (OD\textsubscript{600}) curves of \textit{C. glabrata} without pHluorin (black
triangles), with plasmid carrying one copy of pHluorin (white square) and two copies of
pHluorin (grey circles).
Figure S3. Growth and intracellular pH profiles of yeasts.
Growth (A, C) and pH\textsubscript{i} (B, D) curves of C. glabrata (closed symbols) and S. cerevisiae (open symbols) at pH\textsubscript{ex} 7.4 (A, B) and 4.0 (C, D).
Figure S4. Growth and intracellular pH profiles in the presence of weak organic acids. Growth (a, c) and intracellular pH (b, d) of *C. glabrata* (a, b) and *S. cerevisiae* (c, d) in the presence of acetic acid (30 mM), sorbic acid (1mM) and lactic acid (30 mM).