Molecular mechanisms underlying tolerance to acetic acid in vaginal *Candida glabrata* clinical isolates: role of the CgHaa1-dependent system

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Abstract

To successfully colonize the acidic vaginal tract (pH 4.0 ± 0.5) *Candida glabrata* needs to adapt to multiple environmental insults including the presence of acetic acid which is produced, together with other organic acids, by the co-colonizing bacterial flora. The objective of this work was to obtain mechanistic insights into the adaptive responses used by *C. glabrata* clinical isolates to cope with acetic acid at low pH related with the CgHaa1 transcription factor (ORF CAGL0L09339g), recently found to confer tolerance to this acid by promoting the reduction of acetic acid internal accumulation through the up-regulation of the activity of the plasma membrane H^+-ATPase CgPma1 and of the drug efflux pump CgTpo3[3]. In a first analysis it was demonstrated that, despite some inter-strain variability, *C. glabrata* isolates harvested from the vaginal tract are significantly more tolerant to acetic acid compared to laboratory strains or with isolates recovered from the GI tract. The extreme tolerance of vaginal *C. glabrata* isolates to acetic acid did not correlated with a generalized resilience to stress indicating that specific responses have been evolved by these isolates to cope with acetic acid at low pH. Tolerance of more acetic-acid tolerant vaginal *C. glabrata* isolates was correlated with a reduced accumulation of the acid inside these cells, partly attributed to a reduced permeability of these cells to the undissociated form through the cell envelope, and to a higher activity of the proton pump CgPma1. All the tested strains were able to co-consume glucose and acetic acid indicating that this could be an important mechanism by which *C. glabrata* cells detoxify the presence of acetic acid in the growth medium. Notably, vaginal isolates exhibiting higher tolerance to acetic acid exhibited much faster consumption rates of glucose and acetic acid, compared to the more susceptible isolates. Finally, five new CgHaa1-target genes were identified as determinants in the tolerance to acetic acid stress, being highlighted the role of a second positive regulator of the PM-H^+-ATPase CgPma1, the gene CgHRK1.

Keywords: *C. glabrata*; CgHaa1; acetic acid stress; response and tolerance to weak organic acids

Introduction

Candida spp. are common opportunistic commensals in human gastrointestinal (GI) and genitourinary (GU) tracts. Although these microorganisms are part of the normal microbial flora of human mucosal surfaces, they can cause either superficial or disseminated infections when in dysbyosis and/or immunocompromising

situations[14]. Bloodstream and systemic infections, known as Invasive Candidiasis, have today a high mortality rate (ranging from 46 to 75%) and high morbidity in patients who survive the infection[14], therefore representing serious public health challenges with increase medical and economic importance[14, 21]. Mucosal candidiasis is frequent even among the healthy population, vulvovaginal candidiasis being the more common as it is estimated that around 75% of all women experience at least one episode during their life [28]. In the last few decades the relative incidence of infections caused by *C. glabrata* has particularly increased being nowadays the second most frequent cause of invasive and vulvovaginal candidiasis (5-24% and 7-20% of incidence) after *C. albicans*[1, 11, 26, 34]. *C. glabrata* infections are especially difficult to treat since this pathogen is innately resistant to azole drugs[22] and is showing a persistent increase in resistance to echinocandins[20]. This exacerbates the need to develop new effective antifungal agents and one way to develop these is by understanding the pathogenicity mechanisms of *C. glabrata*[32].

The progress of candidiasis is naturally restrained in human infection sites by the activity of the commensal bacterial microflora[12]. The predominance of homo-fermentative lactic acid bacteria (e.g. *Lactobacillus spp.*) in the vaginal communities suggests that production of organic acids, in particular of lactic acid, is critical to control overgrowth of pathogens in this niche [4, 23, 35]. Acetic acid is also present in vaginal tract particularly in conditions of dysbiosis however its precise origin has not been not fully elucidated, being presumed to be produced by anaerobic bacteria[6]. Acetic acid is also found in the GI tract as result of the activity of the human gut microbiota that ferment the non-digestive carbohydrates releasing short-chain fatty acids, being the dominant component[29]. Although effective, the presence these organic acids is not sufficient to eradicate *C. glabrata* suggesting that this yeast is equipped with appropriate mechanisms of defense to surpass the deleterious effects of these weak acids.

The antimicrobial effect exerted by weak organic acids is different from the inhibitory effect exerted by low pH itself. Due to the lipophilic character of the undissociated forms of organic acid (RCOOH), these can easily permeate the membrane by simple diffusion. At the acidic environment of vaginal tract (pH 3.6-4.5, depending on dominant *Lactobacillus* spp.)[5] approximately 80% of acetic acid and 42% of lactic acid (values estimated for a pH of 4.0) are expected to be found in their undissociated form (pk_a acetic acid=4.76; pk_a lactic acid=3.86). Once in the near-neutral cytosol the organic acids will dissociate leading to the consequent accumulation of protons (H^+) and of the negatively charged counter-ion ($RCOO^-$)[17]. Besides affecting internal pH homeostasis, weak acids also have an impact on the lipid organization and function of cellular membranes, increasing cell permeability to ions and to other small metabolites and leading to the dissipation of the electrochemical potential and reduction of intracellular pH[9, 17]. In *S. cerevisiae*, to offset this increased flux of protons and to prevent membrane permeabilization cells rely on the activity of two proton pumps: PM-H^+-ATPase and V-ATPase located in the plasma membrane and vacuolar membrane, respectively. While Pma1 excretes the exceeding protons to the cell exterior, V-ATPase catalyze their efflux to the lumen of the vacuole. Both proteins were already described to have a role in yeast response and resistance to lactic and acetic acids[13, 16]. Also, the transcription factor Haa1 is considered the main player in the control of acetic acid tolerance[9]. Haa1 effect in reducing the acetic acid-induced lag phase was correlated with its role in reducing the internal accumulation of the acid by regulating the expression of the drug:efflux pumps *TPO2* and *TPO3*[9, 15]. Plus, under acetic acid stress, Haa1 induce the up-regulation of *HRK1* gene, encoding a positive regulator of Pma1 activity[15].

A recent study performed in our laboratory demonstrated the involvement of *C. glabrata* transcription factor CgHaa1 (Haa1 orthologue, encoded by the ORF CAGL0L09339g) in tolerance of this species to acetic and lactic acids[3]. A transcriptomic analysis demonstrated that, under acetic acid stress, expression of CgHaa1 contributes to reduce the internal accumulation of the acid by up-regulating the activity of the plasma membrane proton pump CgPma1 and other transporters such CgTpo2/3 and also for having a positive effect in biofilm formation[3]. Additionally, a highest number of CgHaa1-regulated genes was found to have a function related with stress response, transport, cell wall maintenance, signaling, RNA synthesis, regulation of glycolysis and internal pH homeostasis[3].

The objective of the present work was to examine the role of other genes o CgHaa1-regulon in mediating *C. glabrata* tolerance to acetic acid was examined extending the analyses that had been previously performed. Furthermore, it was also aimed to examine the relevance of the CgHaa1-system in tolerance to acetic acid of vaginal clinical isolates in order to see if this signaling system could play a role in improving adaptation of the isolates to the vaginal environment.

Results

CgCMR3, CgPPZ1, ORF CAGL0E03740g, CgHRK1 and CgPEP1 genes are required for acetic acid tolerance

Susceptibility assays of CgHaa1-regulated genes *CgCMR3*, *CgPPZ1*, *ORF CAGL0E03740g*, *CgHRK1* and *CgPEP1* showed that these genes were found to increase *C. glabrata* tolerance to acetic acid (50 and 60 mM), although this protective effect was below the one registered for *CgHAA1* (Fig.1). The protective effect exerted by *CgFPS1* and *CgFPS2* was also assessed. Interestingly, the individual elimination of the two copies of the *CgFPS1* and *CgFPS2* genes had no significant effect in tolerance of *C. glabrata* to acetic acid (results not shown). This result suggests that these Fps aquaporins might not be involved in mediating the entry of undissociated acetic acid into the *C. glabrata* cytosol, different from the function described for its *S. cerevisiae* counter-partner [18].

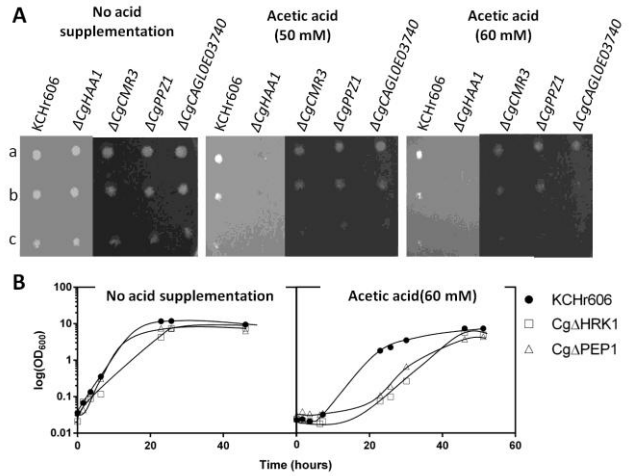

Fig 1 - A: Growth of the deletion mutants *ΔCgSUT2*, *ΔCgRSB1, ΔCgCMR3, ΔCgPPZ1* and *ΔCAGL0E03740g* and laboratory and parental strain KCHr606 at 50 and 60 mM of acetic acid. (a) OD_{600}=0.05; (b) and (c) are 1:5 and 1:25 dilutions of (a); **B**: growth curves of the parental strain KCHr606 and of the deletion mutants *ΔCgHRK1* and *ΔCgPEP1* in MM4 growth medium with 20 mg/l of tetracycline at pH 4.0, supplemented or not with 60 mM of acetic acid.

C. glabrata clinical isolates recovered from the vaginal tract are highly tolerant to acetic acid, compared to laboratory strains or clinical isolates recovered from other infection sites

The screening to tolerance to acetic acid at pH 4.5 of a cohort of *C. glabrata* clinical isolates, based on spot assays, shows that the vaginal isolates are significantly more tolerant to acetic acid than the isolates recovered from the GI tract or the laboratory strains KCHr606 or CBS 138, despite the inter-strain variability observed (Fig. 2). Interestingly, the deleterious effect of acetic acid is much less pronounced at pH 6.4 than at pH 4.5 for the both sets of clinical isolates (from GI and GU tracts) and the extreme tolerance of the vaginal clinical isolates does not seem to result from an increased activity of a general stress-response mechanism but it rather seems to result from the development of specific adaptive responses (results not shown).

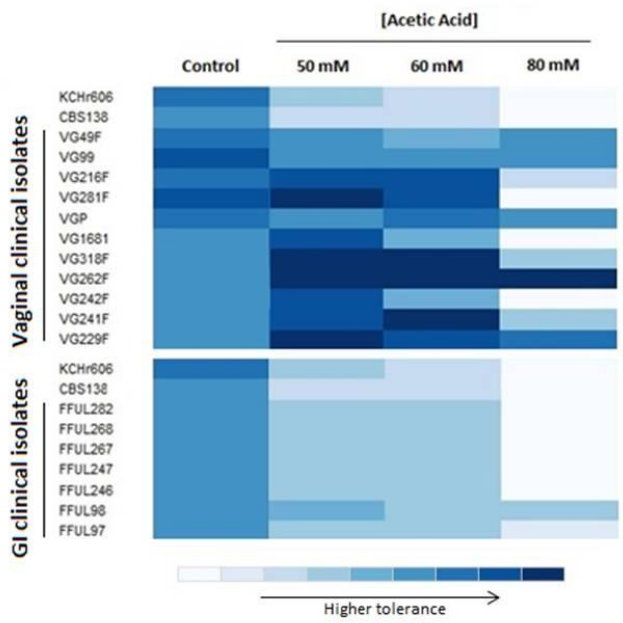

Fig 2 - Screening of *C. glabrata* clinical isolates recovered from GI and GU tracts and of laboratory strains KCHr606 and CBS138 to acetic acid tolerance (50, 60 and 80 mM) at pH 4.5. Cell density of each spot (OD_{600nm}=0.05) was measured using the software ImageJ and for the heat-map construction the cell density values were rearrange in colors by intensity through the programming software R.

Expression of CgHaa1 and of CgHaa1-regulated genes in the extreme tolerance to acetic acid exhibited by vaginal clinical isolates

Considering the previously demonstrated role of the CgHaa1-regulon in mediating response and tolerance of *C. glabrata* laboratory strains to acetic acid[3] it was examined if the higher tolerance to acetic acid of the vaginal clinical isolates VG216F (++), VG99 (++) and VG49F (++) could be attributed to a higher expression/activity of CgHaa1 and/or to CgHaa1-targets *CgTPO3* and *CgPMA1*. Overall, the real time RT-PCR results indicate that only the expression of

CgHAA1 correlates with increased tolerance to acetic acid. Surprisingly, the levels of *CgTPO3* and *CgPMA1* in the more tolerant isolates (VG216F, VG99 and VG49F) did not suffer a concomitant increase which might indicate that in these isolates *CgTPO3* and *CgPMA1* are indirectly regulated by CgHaa1 or that in these isolates the regulatory connection between CgHaa1 and *CgTPO3* was lost (Fig. 3).

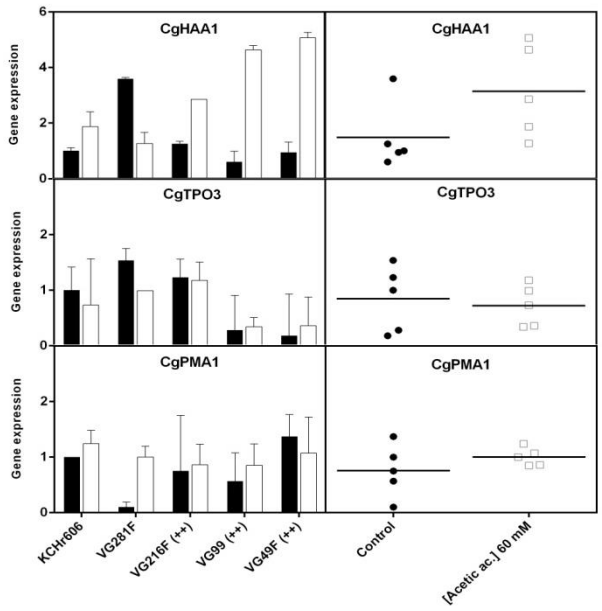

Fig 3 - Comparison by qRT-PCR of the transcript levels of *CgHAA1*, *CgTPO3* and *CgPMA1* genes in *C. glabrata* laboratory strain KCHr606 (-) and in the vaginal clinical under acetic acid-induced stress. Levels of mRNA of those genes were compared in all populations in exponential cells (dark bars) or after 1 hour of cultivation in MM4 growth media (at pH 4.0) supplemented with 60 mM of acetic acid (white bars). The values of the transcript levels were normalized using as internal control the levels of CgACT1 mRNA and the values presented are relative to those registered in unstressed KCHr606 cells which was considered to be equal to 1. The graphics on right show the distribution of gene expression in the population.

Acetic acid-tolerant vaginal C. glabrata clinical isolates accumulate less radiolabelled acetic acid, compared to susceptible isolates

In *S. cerevisiae* it was demonstrated that Haa1, and its target regulated gene Tpo3, contribute to reduce the internal accumulation of radiolabelled acetic acid when cells are cultivated in the presence of inhibitory concentration of cold acetic acid [9]. The results obtained by Bernardo (2013) showed that CgTpo3 and CgHaa1 are also required to reduce the internal accumulation of acetic acid in *C. glabrata* under acetic acid stress, although the effect exerted by CgHaa1 is marginal. No correlation was registered between the expression of CgTpo3 and the levels of acetic acid accumulated inside the different clinical isolates indicating that the reason why these isolates accumulate less acid is independent of the expression of this drug efflux pump. In the case of CgHaa1 a correlation is observed although not extendable to all the isolates (Fig. 3 and Fig. 4).

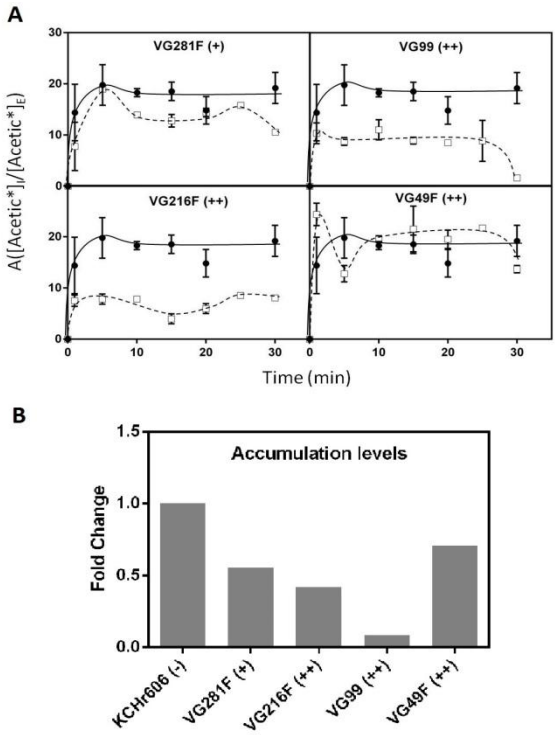

Fig 4 - A: Time-course representation of the accumulation ratio (A) of [1-^{14}C]-acetic acid in *C. glabrata* vaginal clinical isolates (VG281F, VG216F, VG99 and VG49F), represented by (□), in comparison with the susceptible laboratory strain KCHr606, represented by (•), during cultivation in MM4 (at pH 4.0) supplemented with 60 mM of cold acetic acid. **B:** Accumulation levels of each GU clinical isolates. The values of accumulation levels are relative to those registered in KCHr606 cells which were considered to be equal to 1. The results obtained were representative of, at least, three independent experiments.

The structure of cell envelop of vaginal C. glabrata clinical isolates susceptible and tolerant to acetic acid is differently resistant to lyticase activity

The fact that after only 5 minutes of incubation the VG281F, VG216F and VG99 clinical isolates already accumulate a lower concentration of radiolabelled acetic acid (see Fig. 4) suggests that these isolates might have intrinsic characteristics that turn them less permeable to the acid. The lyticase susceptibility assay show that the

highly acetic acid tolerant vaginal clinical isolates (VG99, VG49F and VG216F) have an increased resistance to lyticase activity while the susceptible strain KCHr606 and the clinical isolate VG281F exhibit a high susceptibility showing a reduction in the OD_{600nm} of about 20% (see Fig 5). This different susceptibility of these isolates suggest that the composition of their cell walls is considerably different, at least to what concerns to the β-1,3-glucan contents, which might modulate their different permeability to acetic acid.

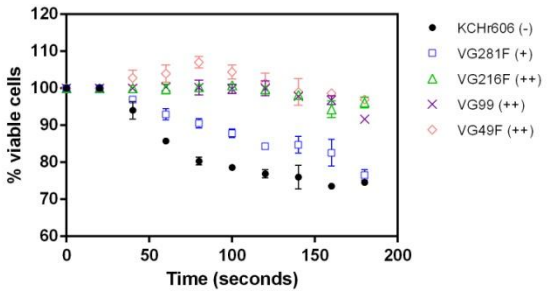

Fig. 5 - Comparison of the susceptibilities to lyticase of *C. glabrata* laboratory strain KCHr606 and vaginal clinical isolates (VG281F, VG216F, VG99 and VG49F). Cells were cultivated in unsupplemented MM4 liquid medium until mid-exponential phase (OD600nm of 0.8±0.05) and then re-inoculated into 0.1 mM sodium phosphate buffer (pH 7.0) supplemented with 10 µg/ml lyticase from *Arthrobacter luteus*.

The higher tolerance to acetic acid of vaginal clinical isolates correlates with their increased ability to consume the acid in the presence of glucose

In the absence of glucose *C. glabrata* cells are capable of assimilate acetate, pyruvate and, preferentially, lactate[31]. In HPLC quantification (Fig. 6 - Appendix) it is visible the co-consumption of glucose and acetic acid by all the clinical isolates, being evident that the more tolerant isolates (VG216F, VG99 and VG49F) start to consume the acid as soon as they are inoculated in the growth medium. Despite this, the results obtained indicate that the consumption of the acid does not serve as itself as a detoxifying mechanism since for example strain KCHr606 starts to actively consume acetic acid after approximately 20 hours, a period at which the culture is already exponentially growing in the presence of the acid. This is also observed for the intermediate tolerant isolate VG216F which starts consumption of acetic acid much later than it begins to grow in the presence of the acid.

Estimation of in vivo activity of PM-H$^+$ATPase in the differently acetic acid-tolerant vaginal isolates

The addition of glucose to the cell suspensions of laboratory strain KCHr606 and vaginal clinical isolates VG281F, VG216F, VG99 and VG49F led to an immediate activation of CgPma1 reflected in the acidification of the medium, although it is clear that the vaginal isolates exhibited higher rates of acidification, being the moderately acetic acid tolerant clinical isolate VG281F and highly tolerant VG49F those exhibiting the higher acidification rates (Fig. 7). Supplementation of cell suspensions with increasing concentrations of acetic acid (0.4 and 0.8 mM) led to the inhibition of the activity of the *C. glabrata* proton pump, consistent with the permeabilization of the plasma membrane induced by the acid, causing the dissipation of the plasma membrane potential required for maximal PM-H$^+$-ATPase activity.

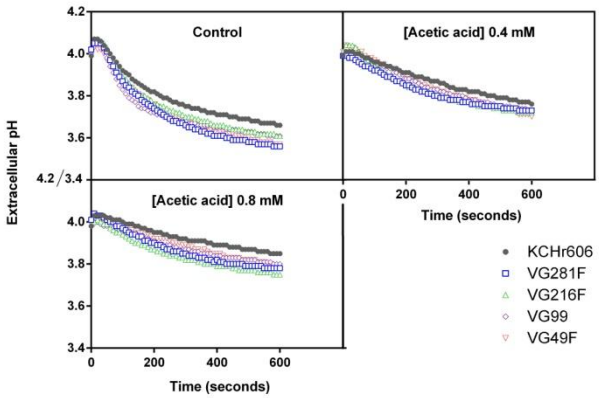

Fig. 7 - External medium acidification promoted by PMA1 H+-ATPase in laboratory strain KCHr606 vaginal clinical isolates (VG281F, VG216F, VG99 and VG49F) before acetic acid-induced stress. External acidification of the growth medium was taken as an in vivo measurement of the enzyme activity.

Discussion

Previous work developed in our laboratory has demonstrated the involvement of the transcription factor CgHaa1 (orthologue of *S. cerevisiae* transcription factor Haa1, encoded by the *ORF CAGL0L09339g*) in conferring tolerance to acetic and lactic acids in *C. glabrata*[3]. A transcriptomic analysis revealed that a high number of CgHaa1-regulated genes is involved in stress response, transport, cell wall maintenance, signaling, RNA synthesis, regulation of glycolysis and internal pH homeostasis[3], being some of these features explored in this work.

A screening of a new set of *C. glabrata* mutants allowed the identification of five new genes of the CgHaa1 regulon that emerged as novel determinants of tolerance to acetic acid this being *CgCMR3*, *CgPPZ1*, *CgHRK1*, *CgPEP1* and the *ORF CAGL0E03740g* (Fig. 1). From these new determinants stand out the CgPPZ1 and CgHRK1 genes. In *S. cerevisiae* Ppz1 has been found to function as a repressor of the activity of the high-affinity potassium import system mediated by the Trk system, playing no direct role in the activity of the PM-H^+-ATPase Pma1[33]. In strains lacking *PPZ1* and *PPZ2* it is observe an increase in intracellular pH due to the enhanced uptake of K^+ that leads to a higher efflux of H^+ in order to maintain the electrical balance[33]. The alkalinization of cytosol ultimately leads to a decrease in Pma1 activity. Having this in mind, the up-regulation of *CgPPZ1* by CgHaa1 under acetic acid stress may be determinant to maximize the activity of CgPma1 thereby counteracting the dissipation of the electrochemical potential and the intracellular acidification caused by acetic acid stress[33]. Another gene uncovered in this work as contributing for maximal *C. glabrata* tolerance to acetic acid is *CgHRK1*. This gene is an orthologue of *S. cerevisiae*' *HRK1,* which encodes a protein kinase involved in the activation of the plasma membrane H^+-ATPase Pma1 in response to glucose metabolism[10] but that has a negligible role in the regulation of this proton pump in acetic acid stressed yeast cells[15]. It remains to be evaluated whether CgHrk1 plays a role in the control of CgPma1 activity under stress imposed by acetic acid. As described in Introduction section, activation of Pma1 is important to offset the increased flux of protons during acetic acid stress. In *C. glabrata*, CgPma1 had been already described to be positively regulated under acetic acid stress by the yapsin CgYps1[2], also a CgHaa1-target[3]. The confirmation of three additional CgHaa1 targets involved in positive regulation of CgPma1, CgPpz1, CgHrk1 and CgYps1, emphasizes the relevance of CgHaa1 transcription in the regulation PM-H^+-ATPase pump. Consistently, elimination of CgHaa1 was found to reduce the activity of CgPma1 proton pump in response to acetic acid stress[3].

In the second part of this work it was investigated the mechanisms of tolerance to acetic acid of a cohort of *C. glabrata*' isolates recovered from the vaginal tract, a niche where pathogenic *Candida* spp. are expected to find acetic and also lactic acids. Under the conditions utilized for the phenotypic screening it was possible to observe that vaginal clinical isolates are significantly more tolerant to acetic acid than the isolates recovered from the GI tract or the laboratory strains CBS138 and KCHr606 (also recovered from intestinal source), although some inter-strain variability has been observed (Fig. 2). Among the cohort of vaginal isolates tested those found to be more tolerant to acetic acid were not those more tolerant to oxidative or to heat stresses (and vice-versa) indicating that the higher tolerance of these isolates does not correlate with a generalized stress resilience or increased fitness (results not shown). The existence of phenotypic diversity within the vaginal population studied is expected because these isolates were recovered from different patients and might therefore have been subjected to different selective pressures. Nevertheless, even within the same population of *C. glabrata* that colonizes a single host it may be expected to observe phenotypic heterogeneity since this should increase competitiveness by providing the colonizing population with the necessary versatility to survive in case sudden modifications of the environment occur. The vaginal isolates VG49F, VG99F and VG216F were those found to be more tolerant to acetic acid and this correlated with a reduced accumulation of the acid registered inside these cells, when compared with the more susceptible strains KCHr606 and the moderately tolerant isolate VG281F (Fig. 3). This reduced accumulation of the acid inside VG99 and VG216F isolates might result from an increased export of the acid anion or from a reduced entry of the undissociated form. Until so far only two transporters had been found to mediate acetic acid tolerance in *C. glabrata*: CgAqr1 and CgTpo3[7, 8]. No differences were found in the expression of the *CgTPO3* gene in the cohort of isolates tested (Fig. 3). It remains to be established if the activity of this drug efflux pump is higher in the VG99 and VG216F isolates, although this is hard to demonstrate. It also remains to be evaluated if these acetic acid-tolerant isolates exhibit different levels of expression of the *CgARQ1* gene. Notably, the cell wall structure of the VG99 and VG216F isolates was much more resistant to lyticase activity (Fig. 5), compared to the tolerant isolates, a difference that might lead to a reduced permeability of the cell envelope to the diffusion of undissociated acetic acid. The acetic acid-tolerant VG49F, VG99F and VG216F vaginal isolates exhibited an increased activity of the CgPma1 proton pump, in comparison with the activity that was registered in cells of the KCHr606 strain (Fig. 7), indicating that these isolates are better equipped to avoid the intracellular acidification imposed by acetic acid stress. The fact that the higher tolerance to acetic acid of vaginal isolates was correlated with their ability to prevent entry of the undissociated acid form and with an increased control of internal pH homeostasis is expected to provide them a competitive advantage when challenged with inhibitory concentrations of other organic

acids. On the overall the results obtained indicate in the vaginal tract *C. glabrata* cells evolve responses aiming to prevent the internal accumulation of these acids inside the cell and increasing control over internal pH homeostasis. Higher tolerance to acetic acid in the tested vaginal isolates correlated with these cells exhibiting a higher expression of *CgHAA1* gene under acetic acid stress although it did not resulted in a higher expression of *CgTPO3* nor of *CgPMA1* indicating that other targets of this transcription factor might be more relevant to mediate tolerance to acetic acid in the vaginal isolates (Fig. 3).

Another novel relevant aspect that comes from the results obtained in this thesis is the observation that all the tested *C. glabrata* strains are able to co-consume glucose and acetic acid, which was something that had not been previously described (Fig. 6). Little is known on the existence of a glucose repressive effect in the ability of *C. glabrata* to metabolize alternative carbon sources, although some preliminary studies indicated that this phenomenon might occur similarly to *S. cerevisiae*[19]. Transcriptomic profiling has suggested that *C. albicans* regulates its carbon assimilation in an analogous fashion to *S. cerevisiae* being observed a strong down-regulation of genes involved in metabolic pathways required for the use of alterative non-preferred carbon sources when cells where cultivated in a growth medium containing glucose[25]. However, these authors have demonstrated that there is a significant dislocation between the proteome and transcriptome in *C. albicans* which causes this yeast to be able to retain the activity of enzymes belonging to acetate metabolism even when this metabolic pathway is repressed at transcriptomic level, allowing it to continue to assimilate acetate even in the presence of glucose[25], unlike *S. cerevisiae*[30]. This metabolic flexibility of *C. albicans* during infection probably facilitates its rapid colonization in the GI and GU tracts where glucose can be limited, allowing it to use other available carbon sources like lactic and acetic acids even when glucose becomes available[25]. It is not known if in *C. glabrata* a similar mechanism is active, being required further studies, although this ability to co-consume acetic acid and glucose does not seem *per se* to play a main mechanism of detoxification employed by vaginal isolates since even the more susceptible isolates where able to trigger that.

In sum, the results obtained in this thesis show that *C. glabrata* cells are well adapted to acetic acid stress, especially those harvested from the vaginal tract. This high tolerance is probably justified by presence of high percentages of undissociated acetic acid in the vaginal tract that allow *C. glabrata* to evolve strong adaptive responses. Also, this adaptation seems to be dependent on the activity of the CgHaa1 regulatory systems as it is observed that clinical isolates have adopted some mechanisms that are controlled by CgHaa1 to counteract the antimicrobial effect caused by inhibitory concentrations of this weak acid such as the increased activity of the proton pump CgPma1. It was also demonstrated that, like *C. albicans*, *C. glabrata* cells apparently can co-consume glucose and acetic acid, a process that has not been previously described in the literature and that requires further investigation.

Materials and Methods

Strains and growth media

C. glabrata deletion mutant strains and clinical isolates used during the master thesis course are listed in Tables 1 and 2.

Table 1 - *C. glabrata* strains used in this study. [1] indicates that the gene expression is repressed by tetracycline-controlled transcriptional repression, by adding to the medium 20 mg/l of tetracycline; [2] indicates that the strain has an auxotrophy and requires an uracil supplementation of the medium (0.4 g/l).

Strain	Genotype/Description	Source
KCHr606	Laboratory strain derived from CBS 138/ATCC2001	Prof. Hiroji Chibana
CBS 138 or ATCC2001	Reference strain (intestinal source)	
CgΔHAA1	KCHr606_ΔCAGL0L09339g	
CgΔSUT2	KCHr606_ΔCAGL0I04246g	
CgΔRSB1	KCHr606_ΔCAGL0L10142g	
CgΔCMR3	KCHr606_ΔCAGL0L05786g	
CgΔPPZ1	KCHr606_ΔCAGL0H04851g	
CgΔHRK1	KCHr606_ΔCAGL0F03707g[1]	
CgΔCAGL0E03740g	KCHr606_ΔCAGL0E03740g	Prof Hiroji Chibana
CgΔPEP1	KCHr606_ΔCAGL0A01870g[1]	
CgΔFPS1	KCHr606_ΔCAGL0C03267g	
CgΔFPS2	KCHr606_ΔCAGL0E03894g	
Htu	Reference strain (Genotype: *his3Δ trp1Δ ura3Δ*) [2]	Prof. Christoph Schüller
CgΔMSN2	Htu_ΔCAGL0F05995g[2]	
CgΔMSN2/4	Htu_ΔCAGL0F05995g ΔCAGL0M13189g[2]	

Table 2 – *C. glabrata* clinical isolates used in this study

GU clinical isolates	GI clinical isolates (anal source)	Source
VG49F	FFUL24	Prof. Maria Manuel Lopes (Faculdade de Farmácia da Universidade de Lisboa)
VG79C	FFUL75	
VG95	FFUL76	
VG99	FFUL92	
VG102F	FFUL93	
VG111F	FFUL97	
VG124F	FFUL98	
VG137F	FFUL246	
VG216F	FFUL247	
VG229F	FFUL267	
VG241F	FFUL268	
VG242F	FFUL281	
VG262F		
VG281F		
VG318F		
VG1681		
VGP		

Both the *C. glabrata* laboratory stains and the clinical isolates were batch-cultured at 30°C, with orbital agitation (250 rpm), in minimal media MM4. MM4 contains, per liter, 1.70 g yeast nitrogen base (YNB) without amino acids and NH_4^+ (Difco Laboratories, Detroit, Mich.), 20 g Glucose (Merck Millipore, Darmstadt, Germany) and 2.65 g $(NH_4)_2SO_4$ (Merck Millipore). When required this growth medium was adjusted to pH 4.0 or to pH 6.4 using HCl or NaOH. The different *C. glabrata* strains were maintained at -80°C in rich growth medium Yeast Peptone Dextrose (YPD) (per liter, 20 g glucose (Merck Millipore), 10 g yeast extract (HiMedia Laboratories, Mumbai, India) and 20 g peptone (HiMedia Laboratories) and 30% glycerol (v/v) (Merck). Solid media were obtained by supplementing the liquid growth medium with 20 g per liter of agar (Iberagar).

Susceptibility assays

The susceptibility assays performed were based on spot assays and/or on the comparison of the growth curve of the different strains in liquid MM4 growth medium. For the spot assays cell suspension, strains were batch-cultured in MM4 liquid medium (adjusted at pH 4.0) at 30°C with orbital agitation (250 rpm) until mid-exponential phase (OD_{600nm} 0.8-1.0±0.05). The cellular suspension was diluted to a standardized OD_{600nm} of 0.05±0.005 in 1 ml of sterilized-deionized water and subsequent dilutions (1:5 and of 1:25) were prepared. The initial cell suspension and the dilutions prepared were applied as spots (4 µl) onto the surface of agarized MM4 plates (at pH 4.5) supplemented with different concentrations of acetic acid (50, 60 and 80 mM). After inoculation, the agar plates were incubated at 30°C for 1 to 2 days depending on the severity of growth inhibition. In order to have a quantitative analysis of the results obtained each spot density was estimated using ImageJ software and the results obtained were compiled in a matrix that was used to build the heat-maps shown which was prepared using the R software. Growth curves of selected strains was performed in liquid MM4 growth media with 20mg/l of tetracycline and either or not supplemented with 60 mM of acetic acid (at pH 4.0). Cells of the different strains were cultivated in MM4 growth medium (at pH 4.0) until mid-exponential phase (OD_{600nm} of the culture between 0.5 and 0.8±0.05) and then re-inoculated at a standardized OD_{600nm} into MM4 growth medium either or not supplemented with acetic acid. Cells were cultivated at 30°C with orbital agitation (250 rpm) and growth was followed by accompanying the increase in OD_{600nm} of the cultures.

Real time RT-PCR

Cells were cultivated under the same experimental conditions as for the microarray analysis RNA extraction was performed using the same kit. Conversion into cDNA was performed in a mixture of 10 µL using 1.0 µL of mRNA (500ng/µL), 2.2 µL MgCl2 25mM, 1.0 µL Buffer 10x, 2.0 µL dNTPs 2.5mM, 0.5 µL Random Hexamers, 0.2 µL RNAse inhibitor, 1.85 µL ddH20 and 0.25 µL reverse transcriptase. Amplifying conditions used: 10 min at 25°C, 30 min at 48°C and 5min at 95°C. Sample was diluted 1:2. Real time RT-PCR second step was performed in a mixture with the following composition: 2.5 µL cDNA, 12.5 µL SYBR® Select Master Mix (Applied Biosystems), 2.5 µL Primer Forward (4pmol/mL), 2.5 µL Primer Reverse (4pmol/mL) and 5 µL ddH20. The mixture was subjected to 40 cycles of PCR amplification using the following setup: 2min at 50°C, 10 min at 95°C, 15 seconds at 95°C, 1 min at 60°C. Primers were designed using Primer Express® Software (Applied BIosystems).

Table 3 - Primer sequences used to perform real time RT-PCR

Primer Identification	Primer Sequence
CgACT1 primer forward	5'-AGA GCC GTC TTC CCT TCC AT-3'
CgACT1 primer reverse	5'-TTG ACC CAT ACC GAC CAT GA-3'
CgHAA1 primer forward	5'-GCC GGA CAT AAA CGG AAT AGG-3'
CgHAA1 primer reverse	5'-AGG CCA GTC TTG AGC TGT TAA TG-3'
CgTPO3 primer forward	5'-GCC GAT ATG TTC CCA AGT GAA-3'
CgTPO3 primer reverse	5'-TGG AGC GAA AGC GAA GAA AG -3'
CgPMA1 primer forward	5'-CAC CTC AGG ACG TCT ACG AAG A -3'
CgPMA1 primer reverse	5'-TCG ATC AAG GCG TCG ATG T -3'

[1-^{14}C]-acetic acid accumulation assays

Cells of the different strains were cultivated in MM4 growth medium (at pH 4.0), harvested by centrifugation (8000xg, 5 min) when OD_{600nm} reached 0.5 ± 0.05, washed one time with fresh medium and finally resuspended in MM4 to obtain a 5 mL suspension with an OD_{600nm} = 0.7± 0.05. The cell suspension was incubated for 5 minutes at 30°C in a water bath with orbital agitation (150 rev/min). 60 mM of acetic acid and 21.12 µM of labeled [1-^{14}C]-acetic acid were added to the cell suspension. Culture samples were taken after 1, 5, 10, 15, 20, 25 and 30 minutes of acid supplementation. Each time, a 100 µl culture sample was collected and the supernatant was recovered by centrifugation to assess the extracellular [1-^{14}C]-acetic acid; a 200 µl culture sample was filtered through pre-wetted glass microfiber filters (Whatman GF/C) and washed with cold water to assess the intracellular [1-^{14}C]-acetic acid accumulation. Both supernatant and filter were added to 7 ml of scintillation liquid (Beckman) and their radioactivity was measured in a scintillation counter [24].

β-1,3 glucanase susceptibility assay

Susceptibility of the laboratory strain KCHr606 and of the vaginal clinical isolates VG216F, VG99, VG49F and VG281F to lyticase (β-1,3-glucanase, Sigma) was performed according to protocols found to be successful in *S. cerevisiae* [27]. Cells from all strains were grown in MM4 minimal medium (at pH 4.0), harvested at the exponential growth phase, until a culture OD_{600nm} of 0.8 ± 0.1 was reached. The harvested cells were washed with distilled water and ressuspended in 0.1 mM sodium phosphate buffer (pH 7.0). After the addition of 10µg/ml lyticase from *Arthrobacter luteus* (Sigma), cell lysis was monitored by measuring the percent decrease of the initial OD_{600nm} of the cell suspensions every 20 minutes for a total period of 3 hours.

Quantification of the consumption of glucose and acetic acid

Cells were cultivated in MM4 supplemented or not with 60mM of acetic acid at 30°C with orbital agitation (250 rpm) and growth was followed by accompanying the increase in OD_{600nm} of the cultures. Samples of culture supernatants were harvested by centrifugation and used for the quantification of acetic acid and glucose concentrations by HPLC. Cultures supernatants were analyzed on an Aminex HPX-87H column, eluted at room temperature with 0.005 M H_2SO_4 at a flow-rate of 0.6 ml/min during 30 minutes, using a refractive-index (RI) (for glucose quantification) and a UV (for acetic acid quantification) detector. Under the conditions used glucose and acetic acid had retention times of 9.2 and 14.3 minutes, respectively. Reproducibility and linearity of the method were tested and concentrations were estimated based on appropriate calibration curves.

In vivo estimation of C. glabrata PM-H^+-ATPase CgPMA1 activity

Cells of the wild-type *C. glabrata* KCHr606 strain and of the ΔCgHaa1 mutant cultivated until mid-exponential phase in MM4 growth medium (at pH 4.0) were harvested by centrifugation (8000 rpm, 5 min, 4°C), washed twice with distilled water and incubated at 30°C in sorbitol solution (20g/L, pH4) for 30 minutes to deactivate the plasma membrane H_+-ATPase. Cells were centrifuged, washed with water resuspended in distilled water (pH4) to obtain a dense cell suspension (OD_{600nm} ~ 20.0 ± 2.0). The experiment was conducted in a water-jacketed cell, at 30°C, with agitation, by adding 3.0 ml of water (at pH 4.0), 1 ml of cell suspension and 0.4, 0.8 and 1.2 mM acetic acid, individually. After homogenization, the pH was adjusted to 4.0±0.05 using HCl or NaOH. Reaction was initiated upon addition of 1 ml of glucose (100 g/L, at pH4). External acidification of the suspension's pH was taken as a measurement of CgPma1 activity being the pH measured every 10 seconds.

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Appendix

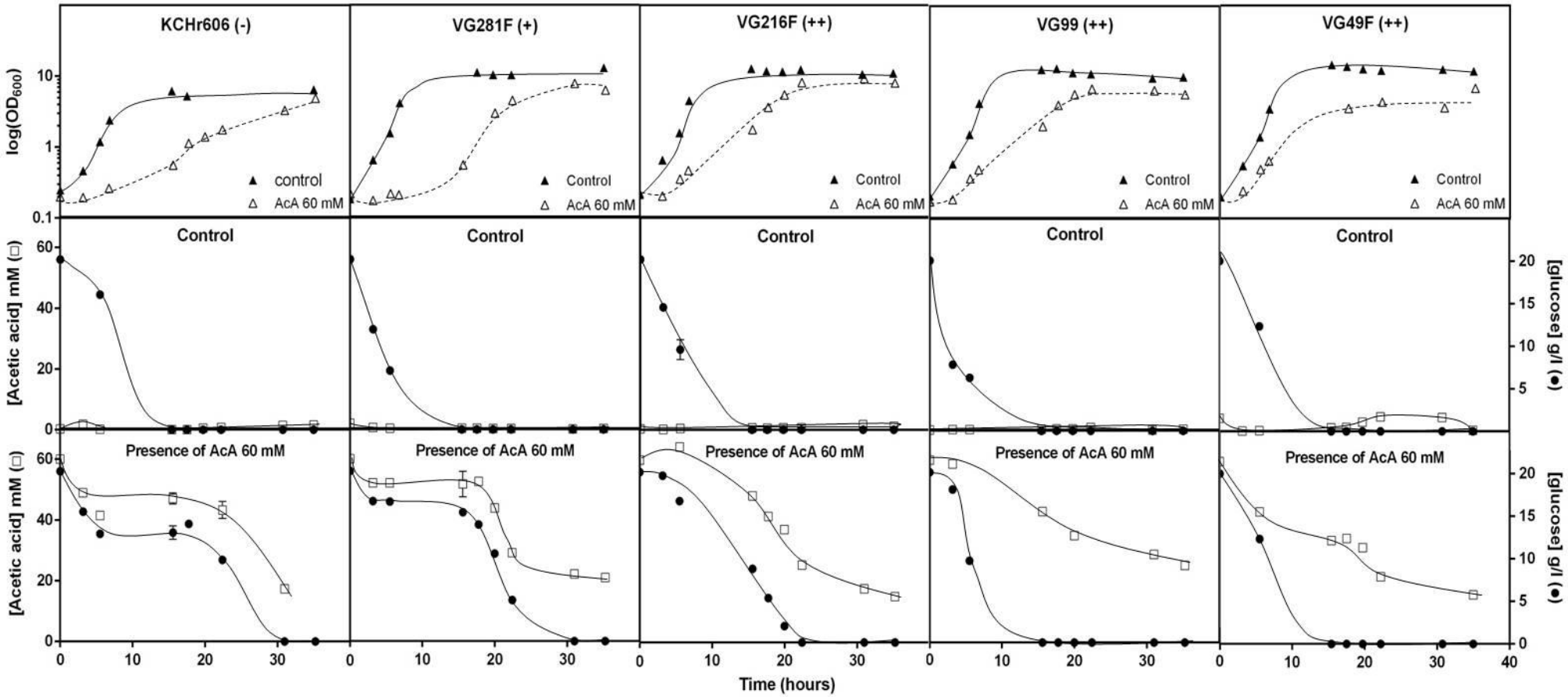

Fig. 6 - Time-course representation of glucose and acetic acid external concentrations during cultivation of the laboratory strain KCHr606 (-) and of the moderately (VG281F) and highly (VG216F, VG99 and VG49F) acetic acid tolerant vaginal clinical isolates during acetic acid-induced stress. Cells were cultivated in MM4 growth medium (at pH 4.0) until mid-exponential phase and then re-inoculated into MM4 growth medium either or not supplemented with 60 mM acetic acid. Samples of culture supernatants were harvested by centrifugation and used for the quantification of acetic acid and glucose concentrations by HPLC. The variation of glucose and acetic acid can be compared with the growth curves of each clinical isolate in absence and presence of acetic acid (60 mM). Legend: Growth in control conditions (▲); Growth in the presence of acetic acid (60 mM) (Δ); Variation of glucose concentration (•); Variation of acetic acid concentration (□). The results obtained were representative of two independent experiments.