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Research Article

Investigating Acid Stress Response in Different *Saccharomyces* Strains

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Yeast cells need to respond to a variety of stresses found in such different conditions as gastrointestinal tract after probiotic ingestion or fermentation vat during ethanol production. In the present study, H^+ neutralisation capacity, membrane fatty acid composition, H^+-ATPase activity, and cytosolic Ca^{2+} concentration were evaluated in yeast cells used for probiotic (*Saccharomyces boulardii*) and laboratory (*Saccharomyces cerevisiae* W303) purposes, as well as in some W303 mutant strains for *ENA1* gene and *S. cerevisiae* BY4741. Results show that the H^+ internal concentration of yeast is regulated by several systems, including the plasma membrane H^+-ATPase, and that Ena1p has an important but undefined role in the cellular response to acid. Membrane fatty acid composition of *S. cerevisiae* W303 strain was affected by exposure to acidic pH, but the presence of 86 mM NaCl prevented this effect, whereas membrane fatty acid composition of *S. boulardii* was unaffected by acidic pH. We also demonstrated that the acid stress response is dependent on calcium metabolism and blocked by FK 506.

1. Introduction

To survive and proliferate, free-living organisms must adapt to changes in their environment. Exposure of the *Saccharomyces cerevisiae* to environmental stresses, such as toxic ions [1, 2], ethanol [3], or changes in temperature [4] or pH [5], triggers biochemical and gene expression changes [6]. Yeast rapid exposure to inorganic acids is of interest, because such exposure occurs under environmental (e.g., yeast probiotics passing through the gastrointestinal system) and industrial conditions (e.g., sulphuric acid to eliminate bacterial contamination in yeast cultures that are to be reused for fermentation [7]).

Saccharomyces cerevisiae grows well over a wide range of pH but grows better in acidic than in alkaline pH [5, 8]. Studies have demonstrated changes in the expression of hundred genes in *S. cerevisiae* following alterations in pH [9–11]. The responses of *S. cerevisiae* to alkaline pH have been reviewed by Ariño [5] and involve various signalling pathways. In particular, the role of calcineurin on alkaline stress was suggested early on, and the involvement of calcium signalling in this response was reported in subsequent works [5, 10, 12, 13]. Responses to alkaline pH have also been described for *Candida albicans* [14] and *Aspergillus nidulans* [15, 16].

Responses to acid stress have been studied in yeast cells that were artificially exposed to weak organic acids [9, 17], food preservatives [17, 18], and herbicides [19]. In response to exposure to weak acids, yeast cells show decreased membrane permeability, anion extrusion, an increased ability to catabolize preservatives [20], and altered gene expression [9]. Results of genomewide analysis and functional screening of

Table 1: *Saccharomyces cerevisiae* strains used in this study.

Strain	Genotype	Source
S. boulardii	Wild type	FLORATIL, Merck S.A.
W303	*Matα leu2-3, 112 ura3-1 trp1-1 his3-11 15 ade2-1 can1-100 GAL mal SUC2*	Johan M. Thevelein
LBCM 479	*W303 Matα ena1::HIS3::ena4*	José Ramos
LBCM 690	LBCM479 [p417]	This work
LBCM 691	LBCM479 [p427]	This work
LBCM 692	LBCM479 [p417::*ENA1*]	This work
LBCM 693	LBCM479 [p427::*ENA1*]	This work
BY4741	MATa his3Δ1 leu2Δ0 lis2Δ0 ura3Δ0	Euroscarf
YKL190W	BY4741 *cnb1::KanMX4*	Euroscarf
YNL027W	BY4741 *crz1::KanMX4*	Euroscarf

genes involved in response to lactic, acetic, and hydrochloric acids suggest that acidic conditions affect cell wall architecture, the expression of genes involved in metal metabolism, vacuolar H^+-ATPase (V-ATPase), and HOG MAPK protein levels [17].

Similar to the response to weak acids, resistance to inorganic acid exposure involves changes in the membrane conductivity to H^+, active extrusion of protons from the cell [21], and the modulation of gene expression [8, 17]. Previous studies have shown that the protein kinase C (PKC) pathway involved in cell integrity in *S. cerevisiae* is activated in response to acid stress and that this activation is dependent on the cell wall sensor Mid2p [22–24]. Claret et al. have suggested the existence of two distinct pathways, involving Mid2p (cell wall sensor) or Rgd1p (Hog pathway) that act together to induce cell integrity pathway and to increase acidic tolerance [22].

In *Saccharomyces*, cytosolic calcium plays an essential role in the control of the cell cycle, budding process, and mating. Additionally, cytosolic calcium is an important second messenger in eukaryotic cells. Yeast cells growing under standard laboratory conditions exhibit low cytosolic calcium levels and minimal calcineurin activity [25]. Exposure to various environmental stresses, such as salt, oxidative, alkali, or heat stress, causes immediate changes in the cytosolic calcium. Calcium levels first increase and then decline rapidly when yeast cells are exposed to these conditions [10, 14, 26–28]. Crz1 is the major effector of calcineurin-regulated gene expression in *S. cerevisiae*, but unidentified targets for calcineurin with roles in stress adaptation also exist [5]. The Cch1 and Mid1 proteins, components of membrane calcium channel, and effectors of Slt2p (PKC pathway) are involved in cell viability at low pH in *Saccharomyces* [22]. However, calcium-mediated signalling mechanisms have not been studied under the acid-shock response.

In a previous work carried out in our laboratory, we established that low concentrations of sodium ions conferred protection to yeast cells exposed to acid stress [29]. Our observations also suggested that the systems involved in maintaining the plasma membrane potential (PMA1p H^+-ATPase and secondary transporter systems) were linked to the acid stress response. In the present study, we focused experiments on plasma membrane H^+-ATPase participation and on calcium signalling events observed in yeast cells in response to acid stress.

2. Materials and Methods

2.1. Yeast Strains and Growth Conditions. Table 1 lists the yeast strains used in this study. Yeast strains were grown in an orbital shaker (200 rpm) at 30°C in YPD medium containing (w/v) 1% yeast extract, 2% peptone, and 2% glucose. Cellular growth was monitored by optical density (OD) at 600 nm.

2.2. Acid Stress Condition and Viability Assays. Yeast cell cultures ($OD_{600\,nm} \sim 1.0$) were harvested by centrifugation at 4,750 ×g for 5 min and washed twice with YP media. Then pellets were suspended at an OD600 nm of 1.0 in an aqueous solution, previously adjusted to pH 2.0 with 1 M HCl and supplemented with 86 mM NaCl. This condition was applied to every acid treatment performed in this study. Yeast samples were then incubated in an orbital shaker at 30°C for 1 h. Aliquots were collected after 0 (control cells, before acid stress), 15, 30, and 60 min of incubation. Cells were immediately washed twice with an equal volume of YP and diluted 20,000 ×. Then, 50 μL of cell suspension was seeded on YPD agar. Plates were incubated at 30°C for 48 to 72 h. The results were expressed as the percentage (%) of colony forming units (cfu) relative to the control sample ($t = 0$).

The growth of yeast cells in the presence of Na^+ was tested on a series of YPD plates supplemented with increasing concentrations of NaCl (200–500 mM). Tenfold serial dilutions of cell suspensions were prepared, and 5 μL aliquots of each were spotted onto YP plates. Growth was monitored over 3 days.

2.3. H^+ Neutralisation Capacity of the Yeast Cells. The capacity of cells to neutralise added H^+ to a cell suspension was determined by an acid-pulse technique with a pH meter. Briefly, the pH was measured after the addition of acid pulses [30]. To estimate the extracellular capacity to neutralise added H^+, the cells were grown as previously described, washed, and either exposed (stressed cells) or not (control

cells) to acid stress. Aliquots of 50 mL were collected by centrifugation, washed twice with Milli-Q water, and resuspended in a 2 mL volume to obtain a dense cell suspension. The pH of cell suspension was previously adjusted in pH 6.8 with NaOH and the first acid pulses were performed with small volumes of 10 mM HCl, because near the neutral pH range even the addition of small amounts of protons results in large pH changes. To estimate the total cell capacity to neutralise H^+ (meaning the ability of cell surface-derived chemical groups plus internal metabolites), the cells were previously disrupted with liquid nitrogen in Milli-Q water and resuspended as described before.

2.4. Determination of H^+-ATPase Activity. Measurements of H^+-ATPase activity were made with 375 mg of cells (wet weight) grown in YPD (4% glucose) to an OD_{600} of 1.0–1.2 (control) or cells grown in YPD, washed, exposed to acid stress, as described above, and harvested after 10, 30, and 60 min. After recover on glass fiber filters, the cells were removed from the filters, immediately frozen in liquid nitrogen, and stored until use. Plasma membrane isolation and determination of ATPase activity, in the presence of inhibitors of mitochondrial ATPase and phosphatase, were performed as previously described [31]. Protein content was determined by the Lowry method [32].

2.5. Intracellular Free Calcium Concentration. The cytosolic free calcium concentration was measured by the aequorin-based method [33] with some modifications. The pVTU-AEQ plasmid was a gift from Marco Vanoni (Università di Milano-Bicocca, Milan, Italy). Briefly, exponentially growing cells transformed with the multicopy apoaequorin-expressing plasmid pVTU-AEQ were harvested by centrifugation at 4,750 ×g for 5 min, washed three times with Mes/Tris 0.1 M (pH 6.5), and resuspended at a density of ~10^9 cells/mL. To reconstitute functional aequorin, 50 μM coelenterazine (Molecular Probes; stock solution 1 μg/μL dissolved in methanol) was added to the cell suspension, and the cells were incubated for 30 min at room temperature in the dark. Excess coelenterazine was removed by washing the cells three times with Milli-Q water and centrifuging at 3,200 ×g for 3 min.

For each acid pulse (pH 2.0 plus 86 mM NaCl), 450 μL of cell suspension (in water) was transferred to a luminometer tube. Light emission was monitored in a Berthold Lumat LB 9501/16 luminometer at 2 s intervals starting 1 min before and lasting until >6 min after HCl addition to the final concentration indicated in the figures. The results summarise three replicates and are expressed in relative luminescence units per second (RLU/s).

2.6. Analysis of Membrane Fatty Acid Composition. The Microbial Identification System (MIS, Microbial ID Inc., Newark, DE, USA) was used to analyse the fatty acid composition of cytoplasmic membranes [34]. Methyl esters were obtained according to the MIDI method (Microbial ID Inc.). Methyl esters were identified and quantified by chromatography with the Sherlock software package (MIDI Inc., version 4.5). Arcsine square root transformations were performed. Data were analysed by analysis of variance (ANOVA) with the Scott Knott test at 5% significance. Transformations and analyses were performed with the Genes software package, version 2007.0.0, which allows for the study of microbial genetic diversity from membrane fatty acid composition [35]. Data were analysed by the standardized Euclidian distance, and the calculated matrix distance was used for clustering analysis by the UPGMA method (Unweighted Pair Group Method with Arithmetic Mean).

2.7. Molecular Biological Methods. Preparation and manipulation of nucleic acids (e.g., phenol extraction, ethanol precipitation, and electrophoresis) were performed as described by Sambrook et al. [36].

The plasmids p417-CYC (low copy) and p427-TEF (high copy), purchased from Dualsystems Biotech AG, Switzerland, were used to express *ENA1* (YDR040C, *Saccharomyces cerevisiae* Genome Database, http://www.yeastgenome.org/). The insert was prepared by using yeast genomic DNA from the W303 strain as a template. The primers were forward, G**ACTAGT**ATG GGCGAAGGAACTACTAAG; and reverse, TCC**CCCGGG**TCATTGTTTAT ACCAATATTAAC. The forward PCR primer contained a Spe1 site, and the reverse primer had a Sma1 site. The PCR product was cut with Spe1/Sma1 and inserted into Spe/Sma1 p417-CYC or p427-TEF vectors to produce the recombinant plasmids.

Competent *Escherichia coli Top10F'* strain cells were transformed with the recombinant plasmids, for expression of Ena1p or aequorin. The presence of the appropriate insert was determined by DNA extraction, followed by digestion and sequencing with the chain termination method (MegaBace 1000 Sequencing Analysis System and DynamicTM ET dye terminator kit). Nucleic acid sequences were compared to sequences in the *Saccharomyces* Genome Database.

For H^+-ATPase activity and bioluminescence assays, yeast cells were transformed by the lithium method [37].

2.8. Reproducibility of Results. All experiments were performed in triplicate. Means ± SD are indicated in each figure, except for H^+ neutralisation and calcium signal experiments.

3. Results

3.1. H^+ Neutralisation Capacity. Previous results of our laboratory showed that *S. boulardii* (a probiotic strain) is more resistant to low pH than *S. cerevisiae* W303 (laboratory non-probiotic strain). Therefore, we decided to test the behavior of these strains facing an acid pulse (pH 2.0 plus 86 mM NaCl or a simulated gastric environment without pepsin). As described before, low NaCl concentration seems to protect yeast cells against inorganic acid stress preventing exacerbated cell death due to extreme conditions. Figure 1 shows the extracellular and total (extracellular plus intracellular) H^+-neutralisation capacity of *S. boulardii* and *S. cerevisiae* W303 cells grown in YPD (control) and cells previously exposed to acid stress. The extracellular capacities of *S. cerevisiae* W303

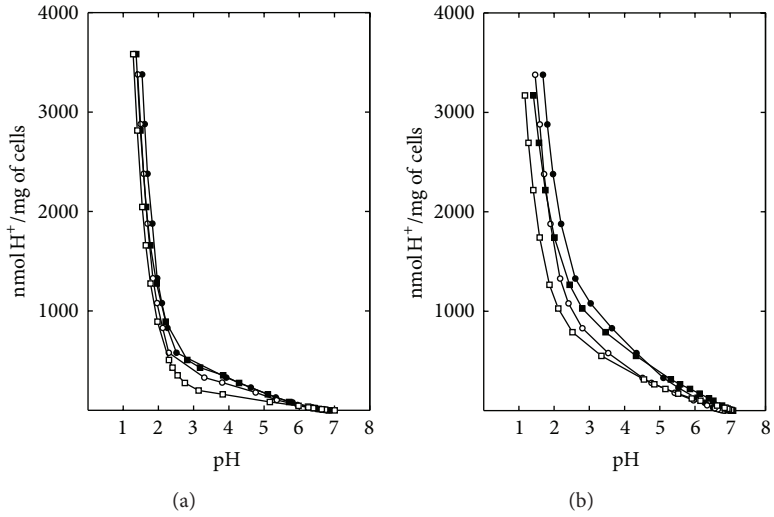

Figure 1: H^+ neutralisation capacity of yeast cells grown in YPD medium, as determined by an acid pulse technique. (a) Extracellular H^+ neutralisation capacity of *S. boulardii* (●), *S. boulardii* exposed to pH 2.0 + 86 mM NaCl (o), *S. cerevisiae* W303 (■), and *S. cerevisiae* W303 exposed to pH 2.0 + 86 mM NaCl (□). (b) Total H^+ neutralisation capacity of *S. boulardii* (●), *S. boulardii* exposed to pH 2.0 + 86 mM NaCl (o), *S. cerevisiae* W303 (■), and *S. cerevisiae* W303 exposed to pH 2.0 + 86 mM NaCl (□).

and *S. boulardii* to neutralise added H^+ were similar, but *S. cerevisiae* W303 was lightly more affected by acid pulses than *S. boulardii* (Figure 1(a)). Results also demonstrated that the extracellular capacity to neutralise added H^+ was smaller than the total capacity for both yeast strains (Figures 1(a) and 1(b)). This difference is particularly evident when we observe the resulting pH after the addition of a specific amount of H^+/mg of cells. *Saccharomyces boulardii* had a higher total H^+ neutralisation capacity and possibly a greater ability to maintain pH homeostasis after acid stress than *S. cerevisiae* W303 (Figure 1(b)), suggesting that the two strains have different compositions of intracellular buffering compounds. The resulting pH after the addition of 1,000 nmol H^+/mg of cells was 3.3 and 2.8 (in control cells or exposed to pH 2.0 + 86 mM NaCl, resp.) for *S. boulardii* and pH 2.6 and 2.2 for W303 (Figure 1(b)). This buffering mechanism is important for a rapid intracellular pH adjustment but cannot accommodate extreme changes in extracellular pH. In fact, a 0.5 pH unit difference in external medium, in low pH range, represents a pronounced stress condition.

3.2. Membrane Fatty Acid Composition. The composition and levels of fatty acids in the cell membrane were modulated in response to acid stress, as shown by a dendrogram shown in Figure 2. A passive increase in intracellular proton concentration may partially explain this cellular response to acid. This effect was particularly pronounced in the W303 strain, in which changes in fatty acid profile were observed after 30 min of acid exposure (pH 2.0). Small increases were observed for oleic acid and arachidic acid, whereas the percentage of gadoleic acid, a cis-icos-9-enoic acid related to oleic acid but has 20 carbon atoms, presented a large decrease. Figure 2 also shows that the addition of 86 mM NaCl prevented this effect in the W303 strain and that membrane fatty acid composition of *S. boulardii* was unaffected by acidic pH. It has been known that unsaturation of fatty acid chains has a profound effect on membrane fluidity, but further work will be needed to investigate if the changes in saturation and unsaturation degrees influence the acid tolerance.

3.3. H^+-ATPase Activity in Ena Mutants. To confirm the involvement of systems involved with maintaining the plasma membrane potential (PMA1/H^+-ATPase and secondary transport systems) during the acid stress response, we constructed yeast strains expressing different levels of Ena1p. While Pma1p is involved in protons active extrusion, ENA proteins act in the responses to saline or alkaline stress [38] and help to regulate the plasma membrane potential [29]. *Saccharomyces cerevisiae* contains several genes encoding ENA proteins but the *ENA1* gene is the most functionally relevant component of the gene cluster [38]. Figure 3 shows the in vivo activation of the plasma membrane H^+-ATPase by pulse acid (pH 2.0 + 86 mM NaCl) in yeast strains with different levels of Ena1p. The deletion strain (W303 *ena1::HIS3::ena4*), the W303 *ena1::HIS3::ena4* strain transformed with the empty vectors, and the strain expressing single copy of ENA1 (W303 *ena1::HIS3::ena4* + *p417::ENA1*) showed acid-induced H^+-ATPase activation. In contrast, no activation was observed in *Saccharomyces* strains expressing high levels of Ena1p (W303 and W303 *ena1::HIS3::ena4* + *p427::ENA1*). Table 2 shows that *ena1*Δ mutant presented higher tolerance to acid stress when viability of strains with different Ena1p levels was compared to wild-type *S. cerevisiae* W303 (high ENA expression) over time. These results were confirmed by spot tests when tenfold serial dilutions of control and acid stressed cells were spotted onto YPD plates (results not shown). The more negative membrane potentials under standard growth conditions of *ena* null mutants and cells with low Ena1p expression could explain the correlation between

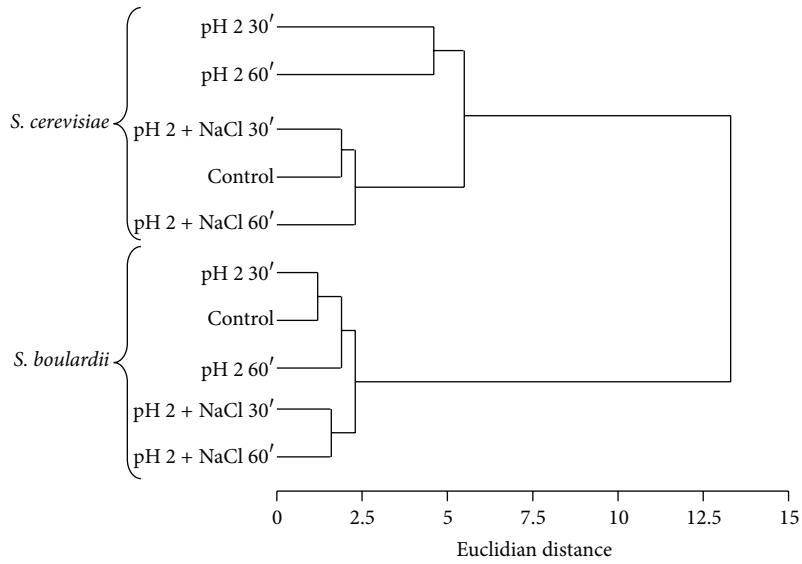

Figure 2: Dendrogram of the fatty acid composition of yeast cytoplasmic membranes. Data were analysed by the standardized Euclidian distance, and the calculated matrix distance was used for clustering analysis by the UPGMA method. The composition and levels of fatty acids in the cell membrane were modulated in response to acid stress, particularly in the W303 strain, and this effect was prevented by addition of 86 mM of NaCl as shown by the dendrogram.

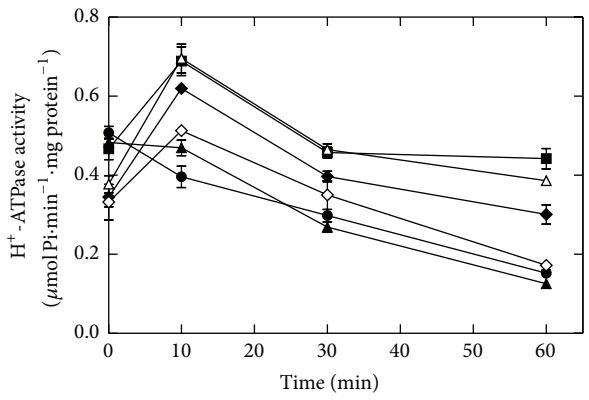

Figure 3: H^+-ATPase activity. Plasma membrane H^+-ATPase activation in wild *S. cerevisiae* W303 (●), the *ena1-4* mutant (■), *ena1-4* mutant + *p417::ENA1* (♦), *ena1-4* mutant + *p427::ENA1* (▲), *ena1-4* mutant + p417 (◊), and *ena1-4* mutant + p427 (Δ) *S. cerevisiae* W303 mutants subjected to pH 2.0 (HCl) + 86 mM NaCl for 60 min.

the ENA1 levels, the acid-induced plasma membrane H^+-ATPase activation, and the acid tolerance as demonstrated in a previous study [29]. In contrast, strains overexpressing Ena1p have more positive membrane potentials and are not susceptible to acid-induced H^+-ATPase activation. The strain expressing single copy (or maximum two copies of ENA1) showed lower viability compared to *S. boulardii* (low ENA1 expression and acid resistance, previous work [29]) possibly because W303 *ena1::HIS3::ena4* + *p417::ENA1* shows a different expression profile compared to *S. boulardii* (ectopic x eutopic expression). Subsequent experiments to understand the relationships between Ena1p expression and acid induced H^+-ATPase activation are necessary to confirm this hypothesis. In addition, other factors are involved in acid stress response.

3.4. Acid Induced Cytosolic Ca^{2+} Transient Increase. The calcium signal induced by glucose was well known and used as a positive control in the present experiment [39]. However, information on calcium transient signal in response to acid exposure is not available in the literature. As expected, yeast cells resuspended in Mes/Tris (0.1 M, pH 6.5) and submitted to glucose pulses showed clear glucose-induced calcium signal (Figure 4(a)). On the other hand, acid pulses produced very low cytosolic calcium signal which were pH dependent (Figure 4(b)). The effect of identical acid pulses was also measured in suspensions with lower cell density. We observed a higher calcium signal in these suspensions, likely because the denser cell suspensions had a higher buffering capacity (results not shown). However, the luminescence assay is pH sensitive, and other studies have demonstrated a relationship between pH and luminescence of coelenterazine [40]. To evaluate whether the transient acid-induced calcium signals were the result of pH changes, we performed a similar experiment using a 15 mM KCl pulse as a negative control. The KCl pulse did not induce pH change or stimulate luminescence (Figure 4(b)).

Figure 5 shows that calcineurin and CrZ1 were required to induce the acid stress response. This suggests that calcineurin, a protein phosphatase, is activated by exposure to acid and acts by modulating gene expression through dephosphorylation of the CrZ1p/Tcn1p transcription factor and its translocation to the nucleus [25]. This pathway was blocked by addition of 1 μg/mL FK 506 (calcineurin inhibitor) to yeast cells culture at 0.5 O.D. The acid stress condition was only applied when the cell concentration reached 1.0 O.D.

Table 2: Effect of acid stress on strain viability (% cfu).

Strains	Conditions			
	Control (nonstressful)	pH 2.0 + 86 mM NaCl		
		10 min	30 min	60 min
W303	100	82.2 ± 3.1	70.1 ± 5.0	32.9 ± 4.1
LBCM479 (*ena1-4*Δ)	100	96.6 ± 4.2*	84.4 ± 4.3*	67.3 ± 3.1*
LBCM479 [p417:: *ENA1*]	100	92.6 ± 2.6*	77.7 ± 0.6	37.0 ± 1.4
LBCM479 [p427:: *ENA1*]	100	88.0 ± 1.6	73.2 ± 0.9	38.9 ± 3.5

Yeast cells were harvested during the exponential growth phase and subjected to 1 h of acidic stress (pH 2.0 + 86 mM NaCl). Data are expressed as the mean ± standard deviations of three separate experiments. *(P values < 0.05 compared to W303 strain).

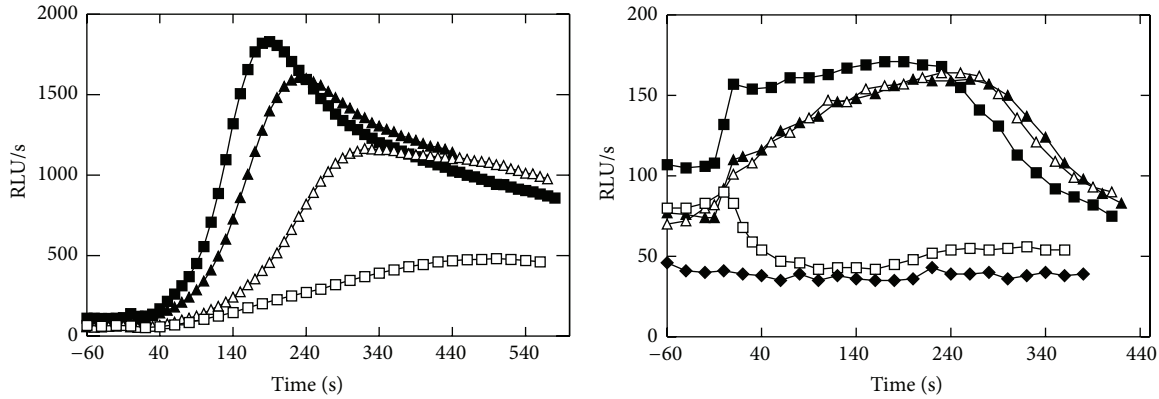

Figure 4: Calcium signalling in *S. cerevisiae* BY4741. (a) Glucose-induced calcium signalling at pH 6.5 (■), pH 3.7 (▲), pH 3.0 (Δ), and pH 2.0 (□); (b) acid-induced calcium signalling at pH 5.1 (■), pH 4.2 (▲), pH 3.0 (Δ), pH 2.0 (□), and 15 mM KCl (⧫).

These cells (incubated with FK506) were less resistant when compared to control cells (Figure 5). It is known that *S. cerevisiae* generates cytosolic calcium signals in response to diverse stimuli. These calcium transient signals mediate various responses in eukaryotic cells. For example, alkaline stress triggers calcium fluctuation in *S. cerevisiae* [5] and *C. albicans* [14]. Claret et al. [22] proposed the involvement of the cell calcium channel components Cch1 and Mid1 in cell viability at low pH.

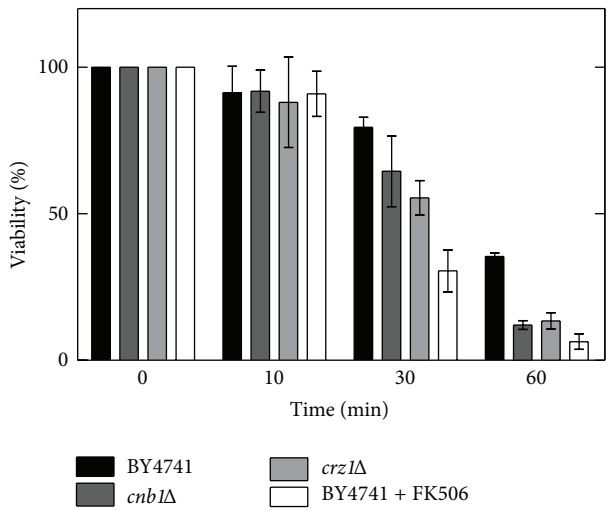

Figure 5: Effect of pH 2.0 (HCl) + 86 mM NaCl on calcineurin/*crz1* mutants. The results show that the acid stress response is dependent on calcium metabolism and is blocked by FK 506.

4. Discussion

Changes in extracellular pH have a negative effect on the yeast life cycle and the maintenance of internal pH homeostasis is important for cell viability. Yeast cells can maintain an appropriate internal pH by utilizing cell buffer systems and consuming H^+ through metabolic pathways [41], increasing proton extrusion by the plasma membrane H^+-ATPase [42, 43], transporting acid between the cytosol and organelles [44, 45]. Our results confirm that the cell buffer power might be part of the pH homeostatic mechanism involved in the acid stress response. The composition and levels of fatty acids in the cell membrane were also modulated in response to acid stress. Here, we propose that modulation of composition and level of membrane fatty acids interferes with H^+ conductivity into the cells. Genes involved in the biosynthesis of plasma membrane lipids, which are essential structural membrane components, have their expression modulated under stress [46, 47]. Plasma membrane structure is likely to affect yeast tolerance to low pH and other stresses [47]. We also showed an effect of sodium on the modulation of the fatty acid composition after acid stress (Figure 2). Our finding is in agreement with observations by Dos Santos

Sant'Ana et al. [29], who reported that the addition of sodium conferred protection to yeast cells against acid stress. In fact, addition of sodium slowed depolarization promoted by proton influx. The authors suggested that, alternatively, the sodium effect could be related to unknown signalling events in response to acid stress. For instance, Bollo et al. [48] reported an effect of 120 mM extracellular NaCl on Ca^{2+} mobilization from intracellular stores in *Trypanosoma cruzi*. The present work showed that sodium, through an indirect effect, is able to affect the modulation of membrane fatty acids in acid stress condition.

It is well known that cells of most organisms maintain high concentration ratios of extracellular to intracellular H^+ across the plasma membrane and that stress factors leading to dissipation of the H^+ gradient across the plasma membrane and to intracellular acidification induce the stimulation of plasma membrane H^+-ATPase activity. In this work we compared the activity of plasma membrane H^+-ATPase in different yeast strains exposed to low pH. The results indicate a H^+-ATPase activation following the acid exposure. However, the level of activation, as previously suggested, and the great tolerance to acid were dependent on expression of secondary transport systems involved in maintaining the plasma membrane potential. This relationship between acid H^+-ATPase activation and secondary systems needs to be clarified by studies of internal pH, of membrane potential, and H^+-ATPase activity in strains expressing different levels of a gene family, especially those encoding the most important ionic transporters.

In the yeast *S. cerevisiae*, calcineurin, a heterodimeric enzyme consisting of a catalytic (a) subunit and an associated calcium binding regulatory (b) subunit, is activated under specific environmental conditions and plays an important role in coupling Ca^{2+} signals to cellular responses. The sensitivity and response of the cells to various stresses are dependent on its ability to sequester and use Ca^{2+} from external and internal stores. Our results show that calcium signalling participates in acid stress response and that the stress response is calcineurin dependent. Crz1, a substrate phosphoprotein for calcineurin, functions downstream of calcineurin to effect calcineurin-dependent responses. Viability test results show similar phenotypes for *cnb1*Δ and *crz1*Δ mutants.

The response of the yeast *S. cerevisiae* to environmental stress results in remodelling of gene expression. In previous studies, the genome response to acid stress has been evaluated by DNA microarray analysis and a functional screening performed using a gene deletion collection [9, 17]. The expression analysis showed that genes involved in stress responses, such as *YGP1*, *TPS1*, and *HSP150*, were upregulated after acid shock and that genes involved in metal metabolism or regulated by Aft1p were induced under the acid adaptation [17]. The last study also indicated that loss of V-ATPase and Hog-MAPK proteins caused acid sensitivity. Mildly stressing acidic conditions (e.g., shift from pH 6 to pH 3) do not affect *S. cerevisiae* growth but instead may be slightly beneficial [8]. However, proteins in the cell wall have been shown to be upregulated in response to low pH [8, 17, 49].

Some studies on the effect of acidic pH on yeast show that PKC cell wall integrity pathway is activated in response to low pH and that RGD1p (Hog pathway) plays a role in response to acidic pH [22–24]. In more recent work, using screening deletion mutants and gene expression profile, de Lucena et al. [50] showed that the cell wall integrity pathway is the main mechanism for cell tolerance to sulphuric acid pH 2.5 and that Ca^{2+}-calmodulin pathway is also responsive to this type of stress. Here, we have shown that cytosolic free calcium levels increase under acid shock and that calcineurin is an important transducer of calcium signals in acid stress responses.

In conclusion, the results of the present study show that the H^+ internal concentration of yeast is regulated by several systems, including the plasma membrane H^+-ATPase, and that Ena1p has an important but undefined role in the cellular response to acid. We also demonstrated that the acid stress response is dependent on calcium metabolism and blocked by FK 506. RNA-seq and microarray studies using different *S. cereviseae* strains, including *S. boulardii*, are currently being performed to provide some insight into the mechanisms of acid stress response. In parallel, experiments aiming to characterize the calcium signal (external or internal) and the dependence of the acid response pathway on calcineurin/Crz1p are under way.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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