RESEARCH ARTICLE

Protective effect of ions against cell death induced by acid stress in *Saccharomyces*

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Abstract

Saccharomyces boulardii is a probiotic used to prevent or treat antibiotic-induced gastrointestinal disorders and acute enteritis. For probiotics to be effective they must first be able to survive the harsh gastrointestinal environment. In this work, we show that *S. boulardii* displayed the greatest tolerance to simulated gastric environments compared with several *Saccharomyces cerevisiae* strains tested. Under these conditions, a pH 2.0 was the main factor responsible for decreased cell viability. Importantly, the addition of low concentrations of sodium chloride (NaCl) protected cells in acidic conditions more effectively than other salts. In the absence of *S. boulardii* mutants, the protective effects of Na^+ in yeast viability in acidic conditions was tested using *S. cerevisiae* Na^+-ATPases (*ena1-4*), Na^+/H^+ antiporter (*nha1*Δ) and Na^+/H^+ antiporter prevacuolar (*nhx1*Δ) null mutants, respectively. Moreover, we provide evidence suggesting that this protection is determined by the plasma membrane potential, once altered by low pH and low NaCl concentrations. Additionally, the absence or low expression/activity of Ena proteins seems to be closely related to the basal membrane potential of the cells.

Introduction

Living microorganisms have long been used as dietary supplements to enhance growth and performance or in the prevention and treatment of intestinal infections of humans and other animals (Czerucka *et al.*, 1991, 2007; Czerucka & Rampal, 2002; Mitterdorfer *et al.*, 2002a). Among the biotherapeutic/probiotic agents most commonly used are select strains of bacteria (e.g. *Lactobacillus* and *Bifidobacteria*) and yeasts (e.g. *Saccharomyces boulardii* and *Saccharomyces cerevisiae*). Several mechanisms have been proposed to explain the protective effects conferred by these organisms, including their potential to add nutritional value to food products (Mitterdorfer *et al.*, 2002a, b), the competitive exclusion of undesirable microorganisms (Nader-de-Macias *et al.*, 1993), the production of antimicrobial substances, the inhibition of pathogen adhesion, immune response modulation (Qamar *et al.*, 2001) or the inactivation of toxins and/or toxin receptors (Castagliuolo *et al.*, 1996, 1999).

Saccharomyces boulardii, a yeast isolated from the lychee fruit from Indochina has been used to treat diarrhea caused by several pathogens. Therefore, the protective effects conferred by this yeast against experimental diseases have been extensively studied (Toothaker & Elmer, 1984; Czerucka *et al.*, 1991; Dias *et al.*, 1995; Rodrigues *et al.*, 2000; Czerucka & Rampal, 2002). Previously, we have demonstrated that yeast cells are able to internalize cholera toxin, triggering cAMP-mediated responses such as trehalase activation (Brandão *et al.*, 1998), suggesting that toxin sequestration could be an additional mechanism by which yeast cells exerted protective effects.

Although comparative molecular studies have shown that *S. boulardii* could be considered a strain of *S. cerevisiae*, it is metabolically and physiologically very different (Mitterdorfer *et al.*, 2002a, b; Fietto *et al.*, 2004; Czerucka *et al.*, 2007), for example, *S. boulardii* can survive the stress of a simulated gastric environment and bile salts toxicity (Fietto *et al.*, 2004).

In order to function as effective probiotics, yeast cells must have the ability to sense and respond to changes in the host environment, including temperature, bile salt concentrations and low gastric pH levels. These are common properties used for the selection of probiotic strains and are considered critical to both survival of the microorganism and for their capacity to extend potential health benefits to the host (Charteris *et al.*, 1998; van der Aa Kuhle *et al.*, 2005).

The capacity to tolerate low pH levels by maintaining cytoplasmic pH at physiologically favorable levels is critical to survival in the gastrointestinal environment. By pumping protons out of the cells the H^+ pump Pma1 H^+-ATPase contributes, in concert with proton/cation antiporters, to intracellular pH regulation (Serrano *et al.*, 1986). Ion homeostasis in yeast is achieved by the coordinated activity of plasma membrane efflux and influx systems and by sequestration systems (Gaber, 1992; Navarre & Goffeau, 2000). Moreover, the transmembrane H^+-gradient generated by H^+-pumping is the driving force for active nutrient uptake directly affecting cell survival.

Taking into account these considerations, we investigated the *S. boulardii* and *S. cerevisiae* responses to separate elements of a simulated gastric environment and the effect of different salts [especially sodium chloride (NaCl)] in cellular tolerance to an acidic environment. Despite being a *S. cerevisiae* strain (Fietto *et al.*, 2004), this work confirms that *S. boulardii* is metabolically and physiologically different from other *S. cerevisiae* strains and exhibits a higher tolerance to acid stress. We found that the presence of Na^+ ions at the low concentrations found in gastric fluids confers protection to the yeast cells exposed to acid stress. Moreover, we provide evidence suggesting that systems involved in maintaining the plasma membrane potential (Pma1p H^+-ATPase and secondary transporters systems) are linked to the acidic stress response and that the plasma membrane potential established by the presence of NaCl is the major determinant of Na^+-mediated protection against acidic stress.

Materials and methods

Yeast strains and growth conditions

Saccharomyces boulardii (Floratil®, Merck S.A., Rio de Janeiro, Brazil) and other yeast strains listed in Table 1 were grown in an orbital shaker (200 r.p.m.) at 30 °C in YP medium [1% (w/v) yeast extract and 2% (w/v) peptone] containing 4% (w/v) glucose (YPD). Cellular growth was monitored by measuring $OD_{600\,nm}$.

Gastric and acid stress conditions

Yeast cell cultures ($OD_{600\,nm}$ 1.0–1.2) were harvested by centrifugation at 3000 ***g*** for 5 min, washed with YP media twice and incubated in an orbital shaker at 37 °C for 1 h under the following conditions: (1) a simulated gastric environment composed of an aqueous solution containing 3 g L^{-1} pepsin (Sigma, St. Louis, MO) (3200–4500 U mg^{-1} protein), 86 mM NaCl, pH adjusted to 2.0 with 1 M HCl (Charteris *et al.*, 1998); (2) an aqueous solution at pH 2.0 (acid tolerance condition). This condition was supplemented with a variety of salts (see figure legends), as well as cycloheximide, to assess the effects of, respectively, different ions and protein synthesis on cell survival. Milli-Q water was utilized as a negative control. Aliquots were collected after 0, 5, 10, 15, 30 and 60 min of incubation and cell viability was determined microscopically using a Neubauer chamber and vital staining with methylene blue (Mills, 1941).

Na^+ and K^+ content

Cell cultures ($OD_{600\,nm}$ 1.0) were washed with YP media and incubated in HCl (pH 2.0), 86 mM NaCl in a rotary shaker at 37 °C. At various time intervals, cells were collected on Millipore (Billerica, MA) membrane filters and rapidly washed with a 20 mM $MgCl_2$ solution. Then, acid extraction was performed with 0.1 M HCl, overnight at 10 °C. The Na^+ and K^+ content of the extracts were assessed by atomic emission spectrometry (Rodríguez-Navarro & Ramos, 1984). Results were expressed in nanomoles of Na^+ or K^+ mg^{-1} of dry weight.

Salt tolerance

Yeast cells were grown to mid-log phase in liquid YPD media (2% glucose) and then diluted to an $OD_{600\,nm}$ of 1.0. Three microliters of this dilution were used to inoculate solid YPD (2%) medium supplemented with different concentrations of NaCl. Plates were visualized after 72 h at 30 °C.

Intracellular pH

The intracellular pH of *S. cerevisiae* cells was assessed based on the relative distribution of [2-^{14}C]-propionic acid ($K_a = 1.35 \times 10^{-5}$) between the cell's interior and the extracellular medium (Rottenberg, 1979) as described previously (Viegas & Sá-Correia, 1991). Cell culture samples (20 mL) were harvested, washed with YP media, and incubated in HCl (pH 2.0) containing 86 mM NaCl and 4 μL of 18.56 mM [2-^{14}C]-propionic acid solution (37 MBq mL^{-1}, Amersham, Pittsburg, PA) at 30 °C for 25 min. This incubation time necessary for achieving equilibrium between the

Table 1. *Saccharomyces cerevisiae* strains

Yeast strains	Genotype	Sources
UFMG 20	Wild type	Martins *et al*. (2008)
UFMG 24	Wild type	Martins *et al*. (2008)
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 511	*W303 Matα nha::LEU2*	Hana Sychrová
LBCM 500	*W303 Matα ena1::HIS3::ena4 nha1::LEU2*	Hana Sychrová
LBCM 480	W303 Matα *ena1::HIS3::ena4 nha1::LEU2 nhx1::TRP1*	Hana Sychrová

labeled propionic acid in the extracellular medium and in the cytoplasm was previously determined. The cells (4 mL) were then passed through a glass fiber filter (Whatman GFC) and washed with cold water (10 mL). The filters were suspended in a scintillation cocktail and placed in a Beckman LS 6000SC scintillation counter. To determine the total concentration of extracellular propionic acid, 500 μL of the cell suspension was centrifuged for 2 min at 10 000 ***g***. and the radioactivity of 20 μL of the supernatant was also measured. The total intracellular concentration of propionic acid was determined using 2.2 μL mg^{-1} dry biomass as the value of the internal cell volume (Viegas & Sá-Correia, 1995). External pH was measured using a pH Meter (Orion Model 720A). Values for pH_i were calculated as the means of at least two determinations using cells from at least three independent cultures.

Measurement of H^+-ATPase activity

Measurements of H^+-ATPase activity were made using 375 mg cells (wet weight) grown in YPD (4% glucose) to an $OD_{600\,nm}$ of 1.0–1.2. After collection on glass fiber filters, the cells were removed from the filters and immediately frozen in liquid nitrogen and stored until use. Plasma membrane isolation and determination of ATPase activity was performed as described previously (Becher dos Passos *et al*., 1992). Protein content was determined using the method developed by Lowry *et al*. (1951).

RNA isolation and Real-Time (RT) PCR

For the shift from glucose growth to an acidic environment (pH 2.0, 86 mM NaCl) yeast cells were grown in 50 mL YPD media 4% (w/v) to an $OD_{600\,nm}$ 1.0–1.2. The sample was split in two and washed quickly by centrifugation with Milli-Q water. One of the cellular pellets was used for RNA extraction (control). The other was resuspended in an aqueous solution (pH 2.0, 86 mM NaCl), rapidly mixed and incubated at 37 °C for 30 min. Samples were collected and washed by centrifugation with Milli-Q water. The pellet was used for RNA extraction (stressed cells). Total RNA was extracted from yeast cells following the protocol of Kohrer & Domdey (1991) and stored at −80 °C. RNA quantity and integrity was monitored by spectrophotometry and agarose gel electrophoresis, respectively.

Total RNA (4 μg) was treated with DNAse (Promega, Madison, WI). Reverse transcription was carried out using M-MLV Reverse Transcriptase (Promega) and oligo-dT(18) primers (Promega) according to the manufacturer's instructions. RT-PCR reactions were performed on an ABI7500 instrument (Applied Biosystems, Foster City, CA), using Platinum® qPCR SuperMixes (Invitrogen). The amplification reactions were performed as follows: 2 min at 50 °C, 10 min at 95 °C and 40 cycles at 94 °C for 15 s and 59 °C for 1 min. To confirm primer specificity, the dissociation curves of all amplification products were analyzed on the ABI7500 instrument. Fold variation in gene expression was quantified using the comparative C_t method: $2^{-[\Delta C_t(\text{Treatment})-\Delta C_t(\text{Control})]}$, which is based on a comparison of expression of the target gene (normalized to the endogenous control) between the experimental and control samples. Actin RNA was used to normalize all values in the RT-PCR assays because its expression levels do not change between treatments. Primers *ENA1* (forward: 5′-AGTTGGCGGTATTGCTTTTCTG-3′; reverse: 5′-TTGAGAGGCCATGACGATGAT-3′), *NHA1* (forward: 5′-CGCGCCGCCACATTTT-3′; reverse: 5′-CATTCCAATCTCCTCATTTACGTTGT-3′), and *ACT1* (forward: 5′-GCCGAAAGAATGCAAAAGGA-3′; reverse: 5′-TCTGGAGGAGCAATGATCTTGA-3′) were used for amplification.

Membrane potential

To carry out membrane potential (m.p.) measurements we used the bis[1,3-dibutylbarbituric acid(5)trimethine oxonol diBA-C_4-(3)] dye. The flow cytometric method was based on the assumption that the dye is distributed across the cytoplasmic membrane according to the Nersnt equation (Krasznai *et al*., 1995). The Di-BaC-4(3) dye (Sigma) was dissolved in 1% DMSO and stored at −20 °C in the dark. Fresh dilutions of the stock were prepared for each experiment.

A Becton Dickinson FACS-Calibur flow cytometer with an argon ion laser was utilized. Oxonol fluorescence was excited

with the 488-nm line at 300–700 mW power. The output optics contained a combination of a 560-nm longpass filter to block the scattered excitation light and a 540-nm band filter. Small-angle forward-scattered light was also detected and used for electronic gating of the data collection. Data collected from cellular debris were excluded from the analysis.

Cells grown in YPD medium containing 4% (w/v) glucose to an $OD_{600\,nm}$ 1.0–1.2 were washed twice with YP and resuspended in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and KH_2PO_4, pH 7.4). Cells were fixed overnight at 4 °C (W303 and *ena1-4*Δ strains) and at room temperature (RT) (*S. boulardii*). Ice-cold or RT solutions of 0.33 M paraformaldehyde, 1 M NaOH, 0.05 M sodium cacodylate and 0.11 M NaCl, pH 7.2, were slowly added with mild stirring to an equal volume of cells (5×10^6 cells mL^{-1}). The fixation was monitored by staining with methylene blue. Fixed cells were washed twice with PBS and resuspended in PBS containing various extracellular dye concentrations at 5×10^6 cells mL^{-1} for 30 min in the dark. Means of the fluorescence measured with the flow cytometer were used to construct a calibration curve of fluorescence intensity measured from stained cells vs. extracellular dye. This curve allows the evaluation of the m.p. in millivolts using fluorescence readings of the cells both in the depolarized state and in the state of interest (Krasznai *et al.*, 1995). One calibration curve was constructed for each strain (*S. boulardii*, W303 and ena1-4 mutant).

The determination of the absolute potential of yeast cells exposed to acid pH and 86 mM NaCl was assessed after dilution of the cells in PBS (5×10^6 cells mL^{-1}) containing 500 nM of the dye and measuring the fluorescence intensity values for the sample of interest (FI^S) and when completely depolarized (FI^0). These values allowed the determination of the intracellular and extracellular concentrations of the dye (D_i^S and D_e^S). The value of the D_i/D_e ratio was used to calculate the transmembrane potential (Krasznai *et al.*, 1995). Stain with propidium iodide allowed the identification of the population of cells to be analyzed because there is a parallel between patterns of staining with PI and Di-BaC-4(3).

Reproducibility of results

All experiments were performed at least three times with consistent results. SDs are indicated in each figure.

Results and discussion

Tolerance to the gastric environment and gastric constituents

Survival of probiotic microorganisms is dependent upon their capacity to withstand passage through the digestive tract. For this reason, tolerances to low gastric pH and bile salts have been used as a selection criteria of microorganisms with probiotic potential (Ouwehand *et al.*, 1999). Our previous studies with *S. boulardii* in simulated gastric, pancreatic and intestinal environmental conditions, revealed that this yeast had high tolerance to different stress conditions, primarily gastric stress (Fietto *et al.*, 2004). In the current study, this analysis was extended to different wild-type strains and specific mutants of *S. cerevisiae*, adequate for unraveling the physiology of this tolerance.

Saccharomyces boulardii tolerance to a simulated gastric environment (an aqueous solution containing 86 mM NaCl, 3 g L^{-1} pepsin at pH 2.0) was determined by measuring yeast viability over time in comparison to *S. cerevisiae* wild-type strains W303 (a laboratory strain), UFMG20 and UFMG24. The UFMG20 and UFMG24 strains were selected from a yeast collection based on their high tolerance to mild heat shock and high trehalose accumulation following stress treatments (Martins *et al.*, 2008). The rationale underlying this selection was that strains isolated from nature are usually more resistant to stress and that trehalose accumulation is generally used as an general indicator of stress resistance (Voit, 2003; Martins *et al.*, 2008).

All strains remained viable for 1 h poststress exposure with *S. boulardii* having the highest tolerance (Fig. 1a). Cell viability was evaluated upon separated exposures to the constituents of the simulated gastric environment, i.e. 85 mM NaCl, 3 g L^{-1} pepsin, pH 2.0. The results showed that neither NaCl nor pepsin significantly affected cell viability (Fig. 1b). In contrast, cellular survival decreased dramatically at pH 2.0, with the exception of *S. boulardii* that had previously been shown to resist low pH levels (Fietto *et al.*, 2004; van der Aa Kuhle *et al.*, 2005; Edwards-Ingram *et al.*, 2007). Additionally, the results obtained at pH 2.0 in the presence of NaCl (Fig. 1b) revealed that the addition of NaCl protected both *S. cerevisiae* and *S. boulardii* cells from deleterious low pH effects. Nevertheless, this protective effect (after a 1-h exposure) appeared higher in *S. boulardii* (84% cell survival) than in *S. cerevisiae* W303 (33% cell survival). The protective effect of NaCl was negatively influenced by the presence of cycloheximide (5 μg mL^{-1}), suggesting a partial role for protein synthesis in the NaCl-mediated protective effect (Fig. 2a and b).

Ion protection during acid stress and the role of Na^+

The specificity of the NaCl protective effect against low pH stress was investigated. Different ions (with the exception of lithium) protected *S. cerevisiae* W303 and *S. boulardii* against low pH stress (Fig. 3). Therefore, acid tolerance did not appear to have ion specificity. Nevertheless, the protective effect of sodium seemed to be considerably higher for *S. boulardii* cells and higher than any other ion examined (Fig. 3a). The absence of protection observed using lithium

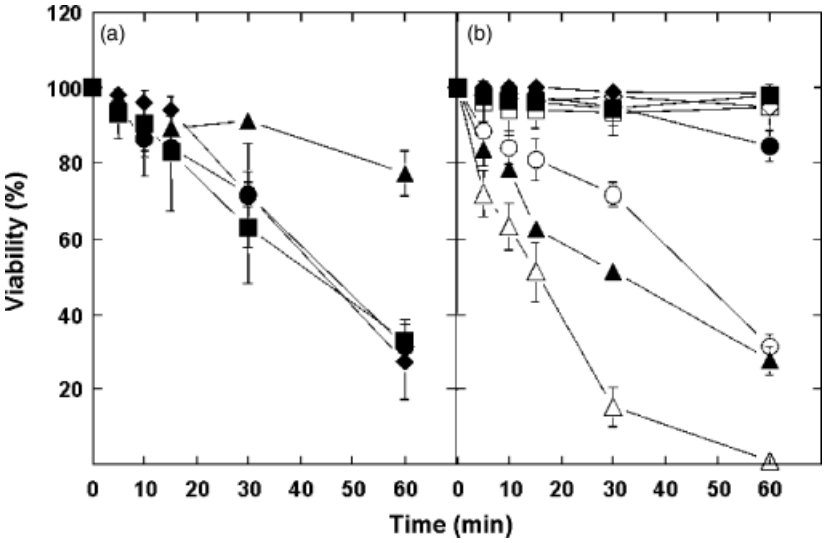

Fig. 1. Yeast cell viability. (a) Yeast cell tolerance to a simulated gastric environment. *Saccharomyces boulardii* (▲), W303 (●), UFMG 20 (■) and UFMG 24 (♦). (b) Isolated effects of gastric environment constituents and the protective effect of NaCl on yeast cell viability. *Saccharomyces boulardii* (closed symbols) and *Saccharomyces cerevisiae* W303 cells (open symbols) were exposed to isolated constituents of the gastric environment. Viability was evaluated during a 60-min time course; pH 2.0 (▲, △); pepsin 3 g L^{-1} (■, □); NaCl 86 mM (♦, ◇); NaCl+pH 2.0 (●, ○).

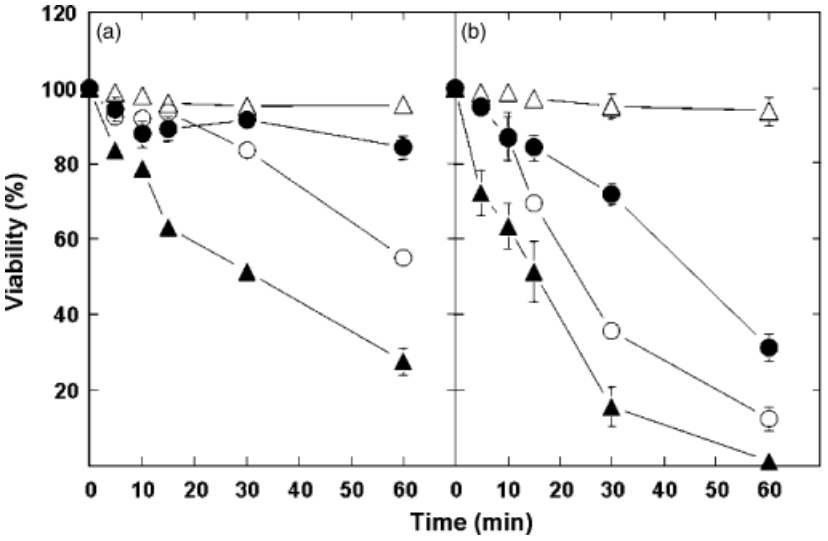

Fig. 2. Effect of cycloheximide on sodium protect effect. (a) *Saccharomyces boulardii* and (b) W303 were incubated at pH 2.0 (▲), pH 2.0+86 mM NaCl (●), pH 2.0+NaCl+CH (○) and H_2O (△) and percent viability determined over time.

could be related to the intrinsic toxicity of this ion (Ruiz *et al.*, 2006).

The chloride ion was common to all the salts examined, however, this ion was likely not responsible for the protective effects observed because Cl^- has been reported to be extremely toxic to yeast cells, which take up very limited amounts of this anion, maintaining low intracellular Cl^- levels (Conway & Downey, 1950; Coury *et al.*, 1999; Kuroda *et al.*, 2004).

In view of the absence of chemical specificity, the mode of action underlying the positive effects of salt on cells' survival at low pH is likely to involve global intracellular ion homeostasis. This is maintained as a delicate balance by the interplay of a large panoply of primary and secondary transporters at the plasma and vacuole membranes. *Saccharomyces cerevisiae* strains maintain cytoplasmic Na^+ and K^+ concentrations within specific ranges using systems that include plasma membrane H^+-ATPase (Pma1p), Na^+-ATPases (Ena 1-4p) (Benito *et al.*, 1997), Na^+/H^+ antiporters (Nha1p and Nhx1p) (Nass & Rao, 1998) and $K^+(Na^+)$ transporters (Trk1/2p) (Ko & Gaber, 1991). Na^+ efflux in *S. cerevisiae* is primarily mediated by the plasma membrane P2-type Na^+-ATPase encoded by the *PMR2/ENA* locus, containing a cluster of up to five *(ENA1-5)* tandemly-arranged and highly similar genes (Garciadeblas *et al.*, 1993). Therefore, and in order to gain a better understanding of the protection conferred by sodium upon acid stress, we evaluated the tolerance of yeast mutants defective in the primary Na^+ transporters, i.e. *ena1-4*, *nha1* and *nhx1* (Table 2).

The viability of the mutant strain *ena1-4* at pH 2.0 in the absence or presence of sodium ions was higher than that of *S. cerevisiae* W303 parental strain and similar to that of *S. boulardii* (Table 2). These results suggested that Na^+-

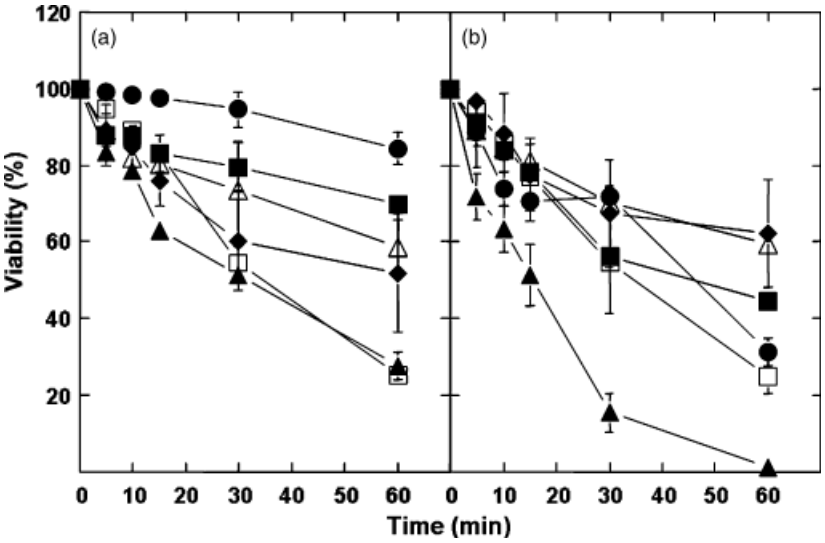

Fig. 3. Effect of salts (86 mM) on yeast cell viability at pH 2.0. *Saccharomyces boulardii* (a) and W303 (b) were incubated at pH 2.0 (▲), pH 2.0, NaCl (●), pH2.0, KCl (■), pH 2.0, $CaCl_2$ (♦), pH 2.0, $MgCl_2$ (△) and pH 2.0, LiCl (□) and percent viability determined over time.

Table 2. Effect of pH 2 and Na^+ on strain viability

	%Viability				
Conditions		pH 2		pH 2+NaCl	
Strains	Control	30 min	60 min	30 min	60 min
S. boulardii	98.8 ± 0.8	51.3 ± 3.7	25.5 ± 3.5	95.3 ± 0.3	84.6 ± 3.7
W303	99.1 ± 0.8	12.2 ± 3.4	3.6 ± 1.3	70.1 ± 5.0	32.9 ± 4.1
LBCM 479 (*ena 1-4*Δ)	100.0 ± 0.0	44.6 ± 9.0	5.0 ± 0.7	84.4 ± 4.3	67.3 ± 3.1
LBCM *511* (*nha1*Δ)	98.8 ± 1.2	9.6 ± 2.6	2.7 ± 0.6	73.0 ± 1.4	27.2 ± 2.9
LBCM 500 (*ena1-4*Δ*nha1*Δ)	99.1 ± 0.2	10.0 ± 1.6	3.2 ± 0.9	72.8 ± 7.5	20.2 ± 9.2
LBCM 480 (*ena1-4*Δ*nha1*Δ*nhx1*Δ)	98.2 ± 2.4	15.3 ± 1.5	6.7 ± 2.5	53.0 ± 0.3	38.0 ± 2.2

Yeast cells were harvested during exponential growth and subjected to 1 h of acidic stress (pH 2) with or without 86 mM NaCl. The data are expressed as the mean ± SDs of three separate experiments.

ATPase silencing was not only important to improved survival in acidic conditions but also in the presence of NaCl. Our hypothesis was that a minimal intracellular ion concentration was needed to resist low pH as well as to achieve the salt protection effect and that low ion levels, probably higher than the one found under regular environmental circumstances (20–25 mM), must be maintained independent of the molecular mechanism mediating protection.

Taking into account that *S. boulardii* presents a phenotype very similar to that of the *ena1-4*Δ strain (high resistance to low pH levels in the presence of sodium ions, Table 2) it seems plausible that *S. boulardii* could express constitutively low *ENA* gene products resulting in higher sensitivities to high sodium environments. In fact, as shown in Fig. 4a, *S. boulardii* displayed higher sensitivity to Na^+ ions compared with the W303 wild-type strain. Consistently, an increase in NaCl concentrations (up to 0.2 M) had a negative effect on acid tolerance by *S. boulardii* (data not shown). Also, analysis of *ENA1* expression showed that *S. boulardii* had lower *ENA* transcript levels compared with *S. cerevisiae* W303 and that transcription of *ENA* was affected by low pH/NaCl, in both yeast strains (Fig. 4b). *ENA1* transcription has been reported previously to be stimulated by alkaline but not by acidic conditions (Garciadeblas *et al.*, 1993; Bañuelos *et al.*, 1998). Additionally, these authors mention a salt-mediated induction, which is unlikely in the present case, due to the very low amounts of salt mandatory to obtain protection against low pH-induced death.

Additionally Nha1p Na+/H+ antiporter was investigated. Nha1p mediates both Na^+ and K^+ efflux while Nhx1p, localized to the prevacuolar compartment, mediates Na^+ compartmentalization (Nass & Rao, 1998). The transcription of *NHA1* is important to sodium extrusion at low pH (Bañuelos *et al.*, 1998). We found that *NHA1* transcripts at pH 2.0 in the presence of NaCl were lower in *S. boulardii* than in *S. cerevisiae* W303 (Fig. 4b). Unlike observed with ENA mutants above, the expression of *NHA1* did not correlate with viability results (Table 2). The *nha1* mutant presents a viability phenotype identical to *S. cerevisiae* parental strain, but different from that of *S. boulardii*. One

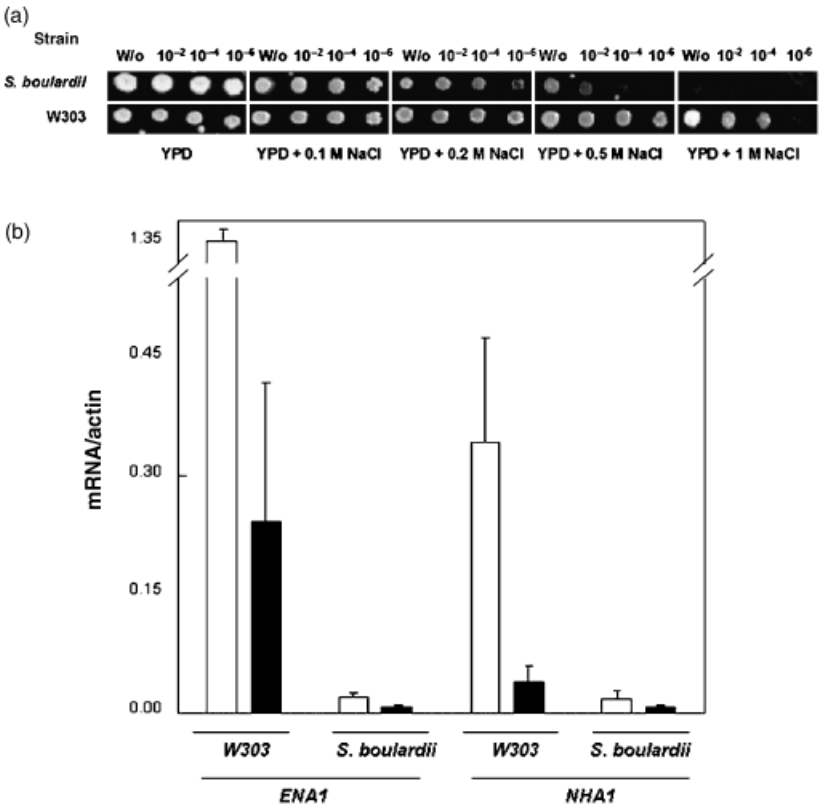

Fig. 4. Salt tolerance and transcript levels of *ENA1* and *NHA1*. (a) Exponentially grown cells were diluted and spotted onto YPD media supplemented with different NaCl concentrations. Results photographed after 3 days of growth. (b) Transcript levels of *ENA1* and *NHA1* in *Saccharomyces boulardii* and *Saccharomyces cerevisiae* W303 by RT-PCR. Basal transcription defined by cells grown in YPD media (□); stressed cells, 30 min in pH 2.0, 86 mM NaCl (■). Constitutive expression of actin (ACT1) was used as control.

can thus reason that NHA1 is not a major player in this process.

Intracellular pH

Following the lead of intracellular ion homeostasis, we measured the intracellular pH of the several strains at external pH 2.0 and 86 mM NaCl. *Saccharomyces boulardii*, the *S. cerevisiae* wild type and the *ena1-4* mutant, presented very similar internal pH of 6.08 ± 0.01, 6.04 ± 0.09 and 6.04 ± 0.03, respectively. Under similar conditions, the *nha1*Δ mutant showed a lower intracellular pH (4.98 ± 0.20) than the double and triple mutants (respectively, 6.58 ± 0.11 and 6.78 ± 0.11). The pH values observed for the *nha1* mutants were quite different from the 6.08–6.09 found in the other strains (including the *ena* mutants).

Under regular circumstances, the low viability of yeast cells at low pH could be linked to changes in intracellular pH (Sychrova *et al.*, 1999; Kinclova-Zimmermannova *et al.*, 2006). For this reason, the lower pH values observed for the *nha1* mutants could likely be deleterious for vital cellular functions (Imai & Ohno, 1995; Fernandes & Sá-Correia, 2001). Yet, the viability of this strain did not suffer accordingly (Table 2), neither did any of the other mutants. Therefore, it follows that the pH variations observed cannot account for either the low pH/low salt stress response or protective effects.

Because the Nha1p is a Na^+/H^+ antiporter, its activity should be higher in cells with higher proton motive forces for example, the intracellular pH of the *nhpa1*Δ strain was higher compared with the wild-type strain (Sychrova *et al.*, 1999). In our study, the *nha1* mutant grown in YPD showed a higher internal pH (6.72 ± 0.04). The internal pH value found for the *nha1* mutant grown at pH 2.0, 86 mM NaCl was pH 4.98, suggesting that in this mutant the passive influx of protons could affect ion efflux and influx systems that contribute to internal pH stability. In addition, no information has been published relative to the effect of external pH levels as low as pH 2.0 on the internal pH and on yeast viability.

Participation of P-type ATPases in the gastric stress response

The yeast viability data suggested the participation of P-type ATPases in gastric stress tolerance (Table 2 and Fig. 4b). Screening of the *S. cerevisiae* genome identified nine P_2-ATPases, including the Pma1p, Pma2p and the Ena1-5p Na^+-ATPases (Morsomme *et al.*, 2000). In low-pH environments, yeast cells need an effective H^+-efflux system. It is also well known that Pma1p generates the electrogenic

proton gradient that drives secondary transport, contributing to the intracellular pH regulation that consequently affects cell viability. For this reason, the activity of the PMA1-encoded H^+-ATPase is fine-tuned at different levels to match its physiologic function. Therefore, we investigated the involvement of the H^+-ATPase in acid tolerance in the presence of low-Na^+ concentrations by measuring the H^+-ATPase activity in *S. boulardii*, W303 and *ena1-4* cells exposed to pH 2.0 plus NaCl (Fig. 5). The results showed that there is clear H^+-ATPase activation in *S. boulardii* and *ena1-4*Δ cells; however, this enzyme seems not to be activated by pH 2.0 in *S. cerevisiae* W303. Activation of the H^+-ATPase *in vivo* is essential to counteract several types of stress, including a decrease in internal pH (Carmelo *et al.*, 1996). Accordingly, acidic pH levels have been reported to regulate H^+-ATPase activity *in vivo* both positively (Eraso & Gancedo, 1987) or negatively (Carmelo *et al.*, 1996). This apparent contradiction could be a function of the acid used to lower pH, i.e. succinic or hydrochloric acids, respectively. Our results suggested that the most pronounced tolerance to extreme acidic stress condition in the probiotic strain and *ena1-4* mutant was due partly to a higher H^+-ATPase activity under such condition. Nevertheless, the results of H^+-ATPase activity did not reflect any differences on the maintenance of internal pH in *S. boulardii* and W303 cells. The acid-resistant green alga *Dunaliella acidophila* grown in a medium at pH 1.0 shows a cytoplasmic pH close to 7 (Gimmler *et al.*, 1989). Below, we briefly discuss adaptations necessary in order to maintain a large transmembrane H^+-gradient.

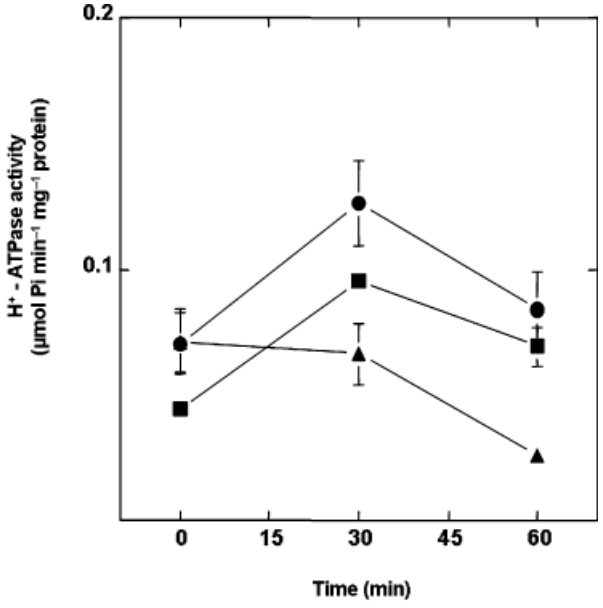

Fig. 5. H^+-ATPase activity. Plasma membrane H^+-ATPase activation in *Saccharomyces boulardii* (●), *Saccharomyces cerevisiae* W303 (▲) and the *ena1-4* mutant (■) strains subjected to pH 2.0 (HCl)+86 mM NaCl for 60 min.

Na^+ and K^+ content

Taking into consideration all the above it became mandatory to measure the intracellular concentrations of the main ionic players, Na^+ and K^+. Figure 6 shows the Na^+ and K^+ contents of different yeast cells grown in YPD media (control) and after exposure to pH 2.0, 86 mM NaCl. *Saccharomyces boulardii* and all *ENA1-4* mutants accumulated more Na^+ and K^+ when grown in YPD medium than the parental *S. cerevisiae* W303 strain. On the other hand, all the strains lost Na^+ to the external medium when exposed to pH 2.0, 86 mM NaCl until equilibrium was reached (internal concentration = external concentration, i.e. 85 mM).

Because Na^+ efflux was still observed in the *ena1-4*Δ *nha1*Δ double mutant, it is possible that in *S. cerevisiae* another permease may be involved in Na^+/K^+ extrusion in addition to Nha1p and Ena1-4p. It appeared that sodium protected cells against acidic stress indirectly, because although we detected low viability at pH 2.0 plus NaCl, there was still a considerable intracellular Na^+ accumulation (e.g. see viability data and Na^+ levels in the double mutant). More importantly, it appears that, regardless to the amounts/combinations of transport proteins expressed, the end result is the equilibrium between the inner and outer faces of plasma membrane, thus affecting $\Delta\psi$. On the other hand, ΔpH is less affected, as can be seen from results above. Considering that plasma membrane potential depends on these two parameters, it is conceivable that it must be very different in *S. boulardii* in comparison with *S. cerevisiae*.

Membrane potential

In view of all the above results, a major picture emerges, pointing to a putative major contribution of m.p. to the salt-mediated low pH response. In order to evaluate this hypothesis, we compared the m.p. of different strains using the Nernstian distribution of the monovalent dye Di-BaC-4(3). The m.p. in *S. cerevisiae* has been reported to be between -50 and -130 mV (Borst-Pauwels, 1981). Results show that *S. boulardii* naturally presents a more polarized m.p. than *S. cerevisiae* (Table 3). Upon 1-h transference to pH 2.0, *S. boulardii* m.p. depolarized *c.* 60%, while *S. cerevisiae* though polarity was inverted from negative to positive, changing from -48 to $+37$ mV (Table 3). Hofer & Kunemund (1984) used the distribution of TPP^+ (tetraphenyphosphonium ion) and SCN^- (thiocyanate ion) as indicators of m.p. in the yeast *Rhodotorula glutinis* and showed continuous transition from TPP^+ accumulation ($\Delta\psi$ negative) at pH values above 4.5 to SCN^- accumulation (indicating positive $\Delta\psi$) at pH values < 4.5, due to increasing diffusion of H^+ into the cells at low pH. Gimmler

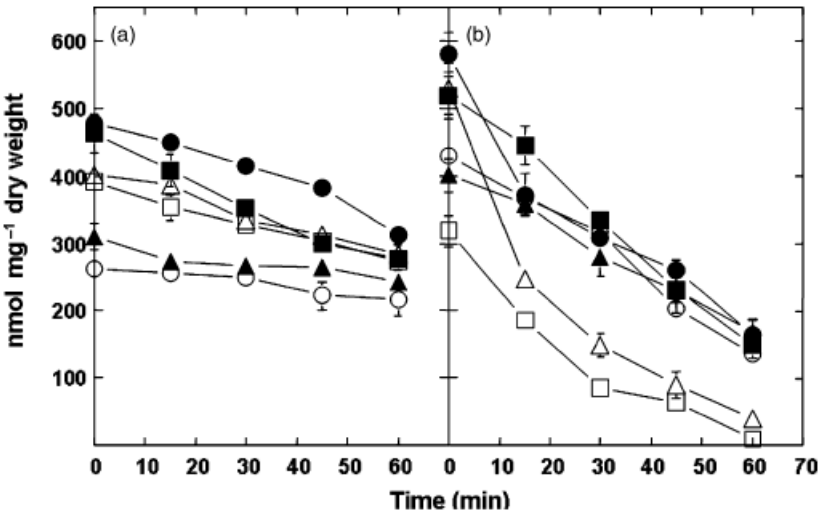

Fig. 6. Intracellular ions levels. Levels of (a) Na^+ and (b) K^+ in *Saccharomyces* strains grown in YPD medium and submitted to pH 2.0 (HCl)+86 mM NaCl. *Saccharomyces boulardii* (●), *Saccharomyces cerevisiae* W303 (▲), *ena 1-4* mutant (■), *nha1* mutant (○), *ena1-4 nha1* mutant (Δ) and *ena1-4 nha1 nhx1* mutant (□).

Table 3. Membrane potential analysis

	Membrane potential (mV)				
		pH 2		pH 2+NaCl	
Conditions Strains	Control	30 min	60 min	30 min	60 min
S. boulardii	−98.42 ± 0.83	−76.50 ± 0.54	−40.16 ± 4.24	−90.43 ± 10.10	−53.16 ± 1.27
W303	−48.71 ± 0.12	+24.97 ± 0.96	+37.6 ± 7.81	−18.56 ± 1.55	+25.06 ± 3.01
LBCM 479 (*ena 1-4*Δ)	−83.75 ± 0.78	−69.80 ± 2.21	−18.57 ± 5.05	−88.54 ± 1.49	−36.16 ± 0.74

Yeast cells were harvested during exponential growth and subjected to 1 h of acidic stress (pH 2) with or without 86 mM NaCl. The data are expressed as the mean ± SDs of three separate experiments.

et al. (1989) showed that the alga *D. acidophila* grown in medium at pH 1.0 exhibited a positive m.p. of about pH+70 mV. Such positive potential has been postulated for acidophilic bacteria (Krulwich & Guffanti, 1983). Although a positive m.p. would be useful to decrease the influx of H^+ from the medium into the cell, it would be more difficult to maintain a proper Na and K balance (Gimmler *et al.*, 1989).

Saccharomyces boulardii has low levels of ENA1 expression, and ena1-4Δ mutants behaved similarly to *S. boulardii*. For this reason we compared the above results with this mutant. Again it accompanied closely the results and their variations observed with *S. boulardii* (Table 3). The effect of transferring the cells to pH 2.0 and salt yielded, at 30 min, a return to the control values of all strains. Yet, at 1-h incubation time, *S. boulardii* had again depolarized, though less intensively (±40%) and *S. cerevisiae* parental strain again inverted polarization this time presenting a lower m.p. than at pH 2.0 alone. Taken together, the results show that addition of 86 mM sodium delayed the pH 2.0-induced depolarization in all the strains studied. The positive m.p. seems to suppress the uptake of cations (Gimmler *et al.*, 1989). In addition, it appeared that *ENA1-4* deletions in the parental strain (W303) or low expression of Ena proteins (*S. boulardii*) had visible effects on the plasma membrane potential of yeast cells. Deletions of *ENA* genes increased the plasma membrane potential (Table 3). The higher polarization observed in *S. boulardii* and ENA mutants relates to these strains' higher sensitivity to salt as described above (Fig. 4). The relationship between membrane hyperpolarization and salt sensitivity was described before (Navarre & Goffeau, 2000). These authors suggested that the hyperpolarization of the plasma membrane potential due to the *PMP3* deletion promoted a nonspecific influx of monovalent cations.

Figure 7 shows the relationship between cell viability (Table 2) and plasma membrane potential (Table 3). The figure includes data from both conditions (absence and presence of 86 mM NaCl). The slope of the three curves was not significantly different ($P = 0.9995$), suggesting that the effect of NaCl on m.p. was the same for the three strains examined. Based on these data we conclude that the increased proton influx induced by HCl (pH 2.0) resulted in depolarization of the plasma membrane and decreased cell viability. It is likely that the increased resistance of *S. boulardii* and the *ena1-4* mutant to acidic stress (presence or absence NaCl) was due to the already high basal plasma m.p.

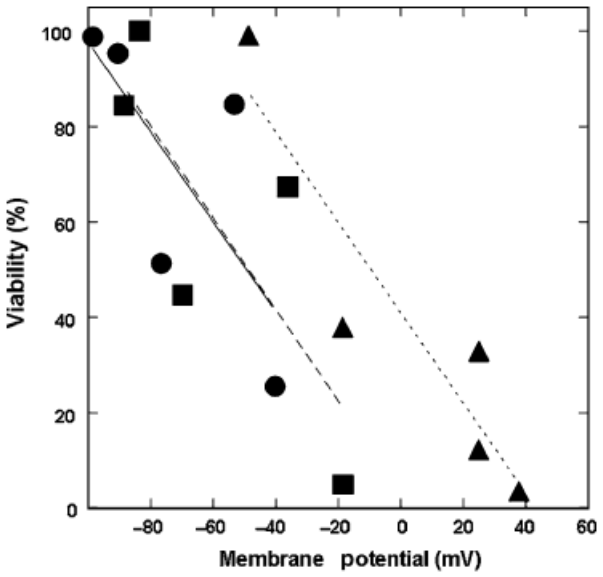

Fig. 7. Cell viability and membrane potential. Percent *Saccharomyces boulardii* (●), *Saccharomyces cerevisiae* W303 (▲) and *ena1-4* mutant (■) viability was plotted against the membrane potential.

of nonstressed cells, product of the relative activity of the Pma1p H^+-ATPase (Fig. 5) together with secondary transporters. The sensitivity of fluorescent dye distribution to membrane potential was used to assess yeast cell viability and predict fermentation activity of yeasts cells (Attfield *et al.*, 2000).

Final remarks

In the present work, we demonstrate that yeast resistance to an acidic environment is improved by the presence of very low salt concentrations in the medium. According to the present results, the salts protective ability over extreme acidic pH relates with the unspecific effects of a mono or divalent cation on membrane potential achieved by the activity of the Pma1p H^+-ATPase together with secondary transporters. The mechanism behind these observations is yet undefined, although it is plausible that the indirect ion effects on homeostasis were related to signaling events that might have triggered specific responses to acidic stress consistent with the dependence of this process on *de novo* protein synthesis. On the other hand, the mechanisms that allow the yeast to survive to a large transmembrane H^+-gradient involve a low permeability for H^+, high H^+-export capacity (which is energy dependent) and a high buffer capacity. Future work will focus on these aspects.

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