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Na⁺-mediated piezoprotection in *Rhodotorula rubra*

Received: 14 May 2003 / Accepted: 1 August 2003 / Published online: 1 October 2003
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Abstract Sodium concentrations as low as 2 mM exerted a significant protective effect on the high-pressure inactivation (160–210 MPa) of *Rhodotorula rubra* at pH 6.5, but not on two other yeasts tested (*Shizosaccharomyces pombe* and *Saccharomyces cerevisiae*). A piezoprotective effect of similar magnitude was observed with Li⁺ (2 and 10 mM), and at elevated pH (8.0–9.0), but no effect was seen with K⁺, Ca²⁺, Mg²⁺, Mn²⁺, or NH₄⁺. Intracellular Na⁺ levels in cells exposed to low concentrations of Na⁺ or to pH 8.0–9.0 provided evidence for the involvement of a plasma membrane Na⁺/H⁺ antiporter and a correlation between intracellular Na⁺ levels and pressure resistance. The results support the hypothesis that moderate high pressure causes indirect cell death in *R. rubra* by inducing cytosolic acidification.

Keywords High hydrostatic pressure · pH · Piezo-(baro-)protection · *Rhodotorula rubra* · Sodium

Introduction

Inactivation of various types of microorganisms by high hydrostatic pressure (HHP) is well known and has stimulated research into the possible application of high pressure as a non-thermal food pasteurization or sterilization technique (Hoover et al. 1989; Knorr 1995). This application requires the efficient inactivation of all vegetative organisms in the case of pasteurization and of all spores in the case of sterilization. Pressure tolerance of

microorganisms depends on intrinsic factors such as genus, species, and physiological state of the cells, as well as environmental factors such as pH and water activity. However, these effects are poorly understood because relatively few studies are available on the mode of action of HHP on microorganisms at the cellular level.

Pressure affects many cellular structures and processes, including transcription (Erijman and Clegg 1998), ribosomes (Niven et al. 1999), microtubules (Kobori et al. 1995), membranes (Pagan and Mackey 2000; Ganzle and Vogel 2001), and membrane proteins (Ritz et al. 2000). Although the contribution of each of these effects to cell death by high pressure remains unknown, the disruption of membrane structure and function generally is considered to be the most important. Structural membrane damage has been observed in eukaryotic cells by electron microscopy, with organelle membranes damaged at lower pressures than the cytoplasmic membrane (Shimada et al. 1993; Kobori et al. 1995; Sato et al. 1995). Leakage of cytosolic components, e.g., amino acids, peptides, and metal ions, from high-pressure-treated cells suggests that minor defects, which are not directly observable by electron microscopy, occur in the cytoplasmic membrane at relatively low pressure (Shimada et al. 1993). In *Escherichia coli*, not a eukaryote, increased membrane fluidity is correlated with increased pressure tolerance (Casadei et al. 2002). Due to their role in cellular homeostasis, many studies have addressed the stability of membrane ATPases from various organisms under high pressure. In general, ATPases appear to be quite pressure sensitive, probably because of their complex multi-subunit structure. In *in vitro* studies with isolated components, several cation-transporting proteins, e.g., (Na⁺, K⁺) ATPases or Ca²⁺ ATPases, were irreversibly inactivated by pressures above 200 MPa (Chong et al. 1985). Lower pressures caused reversible conformational changes, either directly or indirectly, by altering membrane fluidity (Kato et al. 2002). In studies with intact cells of *Saccharomyces cerevisiae* subjected to sublethal pressure (40–60 MPa), Abe and Horikoshi (1997, 1998) suggested

Communicated by K. Horikoshi

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that cytoplasmic acidification occurred because hydration and subsequent dissociation of CO_2 are stimulated under pressure and could not be counteracted by the plasma membrane H^+ -ATPase because it is inactivated. Pressure inactivation of a bacterial F_0F_1 -ATPase also was demonstrated in *Lactobacillus plantarum* (Wouters et al. 1998). In this case, the authors suggested that the destruction of the ATPase was the direct cause of cell death, because it imposed a severe limitation on the cellular energy status. In the same organism, HHP also inactivated a membrane-bound, ATP-dependent, multidrug resistance transporter (Ulmer et al. 2002).

In the present work we report on a unique and highly specific piezoprotective effect of low concentrations of Na^+ and Li^+ on the yeast *Rhodotorula rubra*, which we demonstrate to be connected to Na^+/H^+ ion exchange, and which we propose to be based on improved pH homeostasis. This effect is different from all previously described solute effects on the inactivation of microorganisms by HHP or other physical stresses.

Materials and methods

Culture conditions

Cultures of *Rhodotorula rubra* (MUCL 30589; Belgian Coordinated Collections of Microorganisms BCCM), *Saccharomyces cerevisiae* W303, and *Schizosaccharomyces pombe* (laboratory collection) were grown to early stationary phase in yeast glucose peptone broth (1% yeast extract, 2% glucose, 2% peptone) at 25°C with shaking for 25 h.

Pressurization

Cells were washed twice and resuspended in water, potassium phosphate buffer (10 mM, pH 6.0), or Tris-HCl buffer (40 mM, pH 6.5–9.5). One-milliliter samples were sealed in sterile polyethylene bags without inclusion of air and pressurized at 20°C. Two types of pressure equipment were used, depending on the experiment: a cylindrical (height 300 mm, diameter 50 mm) stainless steel pressure autoclave (EPS International, Sint-Niklaas, Belgium) and a system with eight parallel thermostatically controlled 8-ml vessels, each of which could be compressed at different pressures (Resato, Roden, The Netherlands). It should be noted that the temperature in the vessels could not be kept constant due to adiabatic compression and decompression. However, temperature measurements with thermocouples inside the pressure vessels, which we conducted previously under identical conditions, showed a temperature increase <5°C upon pressurization to 170 MPa. Pressure-treated samples and an untreated control were serially diluted and plated on OGYE basal medium (LabM, International Medical, Brussels, Belgium). Inactivation was expressed as a \log_{10} of the reduction factor (RF):

$$\text{RF} = \frac{\text{cfu(untreated sample)}}{\text{cfu(pressure - treated sample)}}$$

Measurement of intracellular Na^+ content

A sample of the culture was washed three times with 40 mM Tris-HCl pH 6.5 and resuspended in ice-cold deionized water. After lysis of the cells at 95°C for 30 min, the suspension was centrifuged

(6,000 g, 15 min), and the supernatant was retained and stored at 4°C. The concentration of the Na^+ ions in the supernatant was determined by atomic absorption spectroscopy (PU9200 Series Atomic Absorption, Philips, The Netherlands) and rescaled to the number of cells previously in the suspension.

Reproducibility of results

The magnitude of the biological variation for the high-pressure inactivation of *R. rubra* in our experiments was determined by conducting particular experiments in sixfold and calculating the coefficient of variation (CV, standard deviation divided by the mean) for the results expressed as \log_{10} (RF); observed CV was <0.1. All experiments were conducted at least twice. For experiments that were conducted three or more times, mean values and error bars representing standard deviations are given in the figures. For other experiments, representative results are shown.

Results

Effect of Na^+ on high-pressure inactivation of *Rhodotorula rubra*

During a study on the activity of various bacteriocins in combination with high hydrostatic pressure (HHP) on different yeasts, we observed significantly lower reductions of *R. rubra* at 170 MPa in the presence of 100–1000 IU/ml Nisaplin, a commercial preparation of the bacteriocin nisin. Reconstruction experiments with dialyzed Nisaplin, alkali-inactivated Nisaplin, and Nisaplin components in K^+ phosphate and Na^+ phosphate buffer allowed us to ascribe the protective effect entirely to Na^+ (data not shown). Remarkable from these experiments were the low concentration of Na^+ required for piezoprotection (100 IU/ml Nisaplin corresponds to 0.4 mM NaCl) and the apparent ion specificity of Na^+ over K^+ .

Ion specificity was subsequently studied by using low concentrations (2 or 10 mM) of NaCl, LiCl, KCl, NH_4Cl , MgCl_2 , CaCl_2 , and MnCl_2 added to a 10 mM potassium phosphate buffer pH 6.0. In addition to Na^+ , Li^+ also increased survival of *R. rubra* by several log units, although the level of protection tended to be somewhat lower than for Na^+ (Fig. 1).

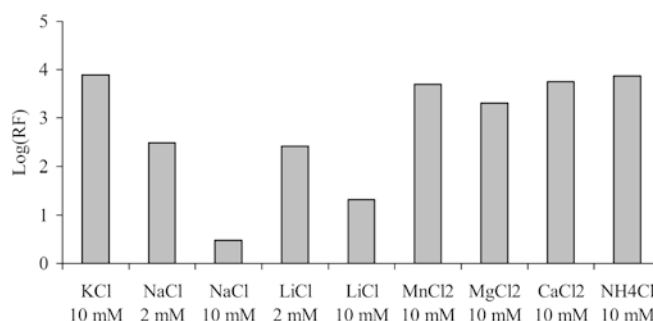


Fig. 1 Effect of different cations on pressure inactivation (160 MPa, 15 min) of *Rhodotorula rubra*. Cells were suspended in 10 mM K^+ -phosphate buffer pH 6.0 with chloride salts added as indicated

Effect of pH on pressure inactivation

The Na^+/Li^+ specificity and the occurrence at low concentrations of the piezoprotective effect suggested the involvement of a Na^+ -specific membrane transport process. Na^+ transport is ubiquitous in prokaryotic and eukaryotic organisms, and various types of transporters have been identified, such as Na^+ ATPases, Na^+/H^+ exchangers, and Na^+ /solute symporters. In many organisms, Na^+ transport plays a role in pH homeostasis, and therefore we studied the effect of pH on the pressure inactivation of *R. rubra*. Tris-HCl buffer was used in this experiment because the pH of this buffer is less pressure dependent than that of potassium phosphate buffer (Kitamura and Itoh 1987). Pressure tolerance of *R. rubra* increased from pH 6.5 to 8.0 and at pH 8.0–9.0 was similar to that seen at pH 6.0 in the presence of 10 mM Na^+ (Fig. 2). Provision of additional Na^+ at pH 8.0 did not increase piezoprotection (data not shown).

Intracellular Na^+ levels are correlated to pressure resistance

In the above experiments, piezoprotection was observed with cells exposed to Na^+ or elevated pH during the actual pressure treatment. However, we also observed a smaller piezoprotective effect when cells were pretreated with millimolar concentrations of Na^+ and subsequently washed and pressure treated in the absence of Na^+ (data not shown). No piezoprotection was found when Na^+ was added after pressure treatment of cells in a Na^+ free buffer. This prompted us to investigate the possible correlation of intracellular Na^+ concentration and pressure tolerance.

R. rubra cells were pre-treated with 5 mM NaCl in Tris-HCl (40 mM, pH 6.5) for 45 min then transferred to Na^+ free Tris-HCl buffer (40 mM, pH 6.5). Over a 2-h period, samples were taken for pressure treatment (170 MPa) and for determination of intracellular Na^+ content (Fig. 3). Immediately after pretreatment, the intracellular Na^+ content increased more than twofold from 1.18×10^{-13} to 2.84×10^{-13} mmol/cell compared with

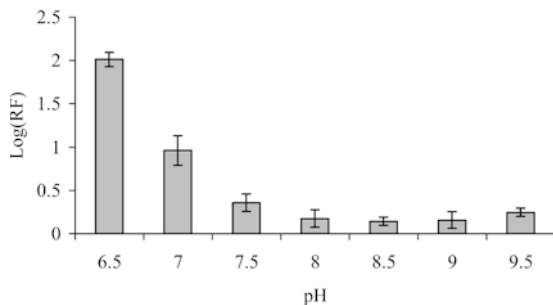


Fig. 2 Effect of pH on pressure inactivation (170 MPa, 15 min) of *R. rubra*. Cells were suspended in 40 mM Tris-HCl at pH as indicated

cells that were not pretreated. During the subsequent incubation in Na^+ -free buffer, the intracellular Na^+ concentration decreased exponentially to 1.93×10^{-13} mmol/cell after 2 h. The piezotolerance of the cells correlated with the intracellular Na^+ concentration.

The piezoprotective effect of elevated pH also was linked to intracellular Na^+ levels. In this case, cells grown in YGP were incubated for 30 min in Na^+ -free Tris-HCl buffers (40 mM) of different pH (6.5–8.5) and then subjected to high-pressure treatment (170 MPa) and intracellular Na^+ analysis (Fig. 4). Higher pH in the resuspending buffer correlated with a higher intracellular Na^+ level and increased piezotolerance.

Specificity of the piezoprotective effect

An HHP inactivation experiment with *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* in addition to *R. rubra*, in water and in 10 mM and 100 mM Na^+ and K^+ phosphate buffer (pH 6), revealed a Na^+ -

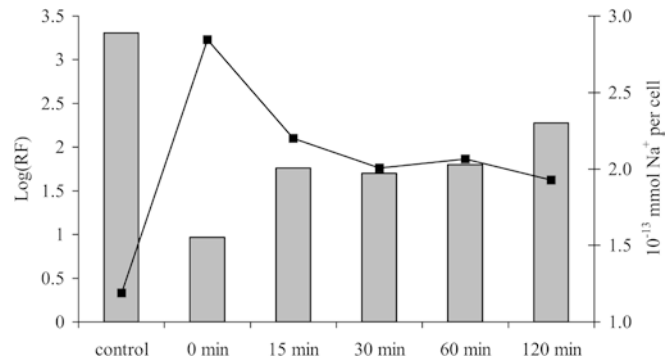


Fig. 3 Correlation between intracellular Na^+ content (■) and pressure inactivation (170 MPa, 15 min) of *R. rubra* (bars). Cells were first incubated for 45 min in 40 mM Tris-HCl (pH 6.5) with 5 mM NaCl then transferred to the same buffer without NaCl and analyzed at different times thereafter as indicated. Control refers to cells not preincubated in Na^+ -containing buffer and analyzed immediately upon resuspension in 40 mM Tris-HCl (pH 6.5)

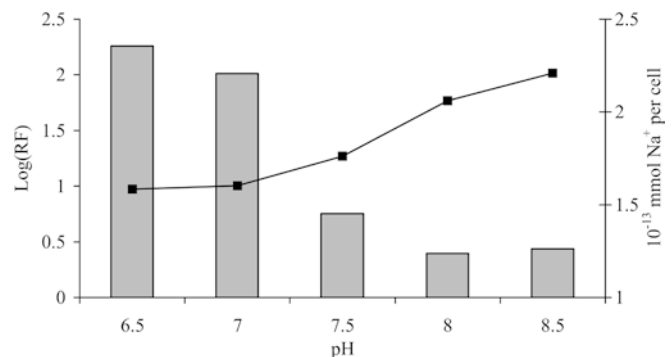


Fig. 4 Correlation between intracellular Na^+ content (■) and pressure inactivation (170 MPa, 15 min) of *R. rubra* (bars), resuspended after growth in YGP in Na^+ -free 40 mM Tris-HCl buffers of different pH as indicated

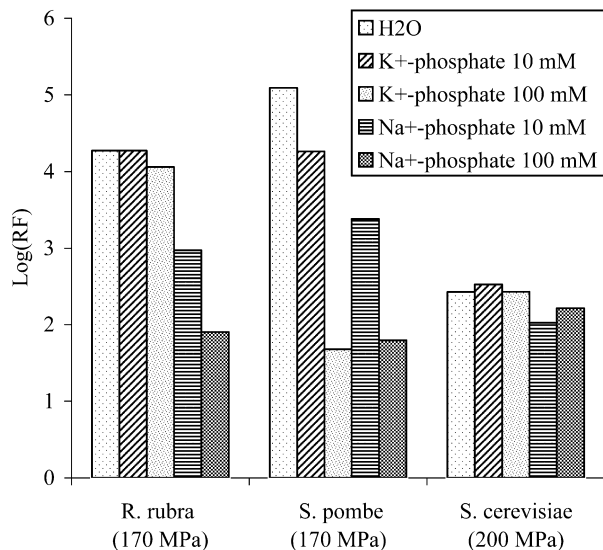


Fig. 5 Effect of Na⁺ and K⁺ -phosphate buffer (pH 6.0) concentrations on pressure inactivation (15 min, pressure as indicated) of different yeasts. Water was used as a comparison

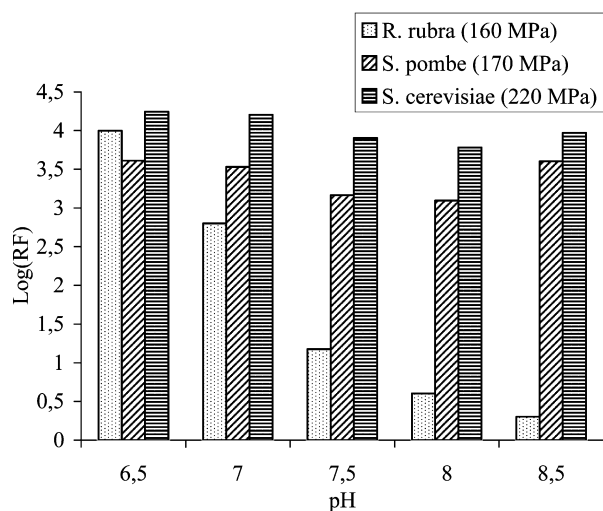


Fig. 6 Effect of pH on pressure inactivation (15 min, pressure as indicated) of different yeasts. Cells were suspended in 40 mM Tris-HCl at pH as indicated

specific piezoprotective effect only in *R. rubra* (Fig. 5). A protective effect also was observed in *S. pombe*, but it was not ion specific. No significant effect of the buffers (compared to water) on inactivation was found for *S. cerevisiae*. Furthermore, the piezoprotective effect of elevated pH was specific for *R. rubra* (Fig. 6).

The piezoprotective effect of Na⁺ on *R. rubra* inactivation was evaluated at different pressures and was restricted to pressures below approximately 210 MPa, with a maximum of around 180 MPa (Fig. 7). A similar upper limit also was found for piezoprotection by elevated pH. At 250 MPa elevated pH reduced the piezotolerance of *R. rubra* (Fig. 8; compare with Fig. 2). Remarkably, the inactivation of *R. rubra* as function of

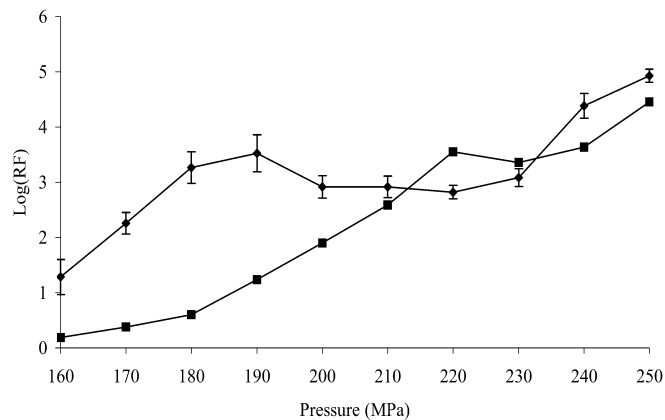


Fig. 7 Pressure inactivation (15 min, pressure as indicated) of *R. rubra* in the presence (■) or absence (◆) of 5 mM Na⁺. Cells were suspended in 40 mM Tris-HCl (pH 6.5)

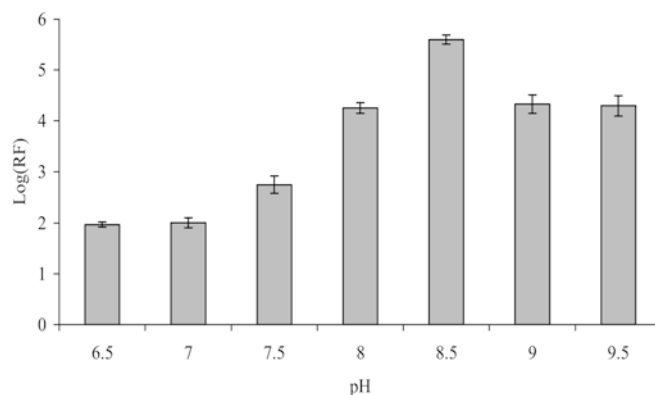


Fig. 8 Effect of pH of the resuspension buffer on pressure inactivation (250 MPa, 15 min) of *R. rubra*. Cells were suspended in 40 mM Tris-HCl at pH indicated

pressure shows a plateau or even a small depression between 190 and 230 MPa.

We also investigated the specificity of protection with respect to the applied stress factor. Hereto, *R. rubra* cells were resuspended in water and in 50 mM Na⁺ and K⁺ phosphate buffer (pH 6) and subjected to heat treatments at 54°C, 55°C, and 56°C. Only at 54°C was a slight protective effect of both buffers observed, but this effect was not ion specific and was much smaller in magnitude than the piezoprotective effect observed in the same buffer (data not shown).

Discussion

We identified a piezoprotective effect of Na⁺ on *Rhodotorula rubra* that is different from all previously described solute effects on the high-pressure inactivation of microorganisms in that: (1) the observed piezoprotection is highly ion specific, being restricted to Na⁺/Li⁺ among seven cations tested (Fig. 1); (2) it is induced by very low Na⁺ concentrations (<2 mM) (Fig. 1); (3) it

was not found in two other yeasts tested (Fig. 5); and (4) the effect seems to be restricted to pressure as opposed to heat treatment, and even only to pressures under 210 MPa.

The piezoprotective effects of solutes described hitherto are based on depression of water activity and hence occur only at relatively high solute concentrations and are neither solute nor organism specific (Oxen and Knorr 1993; Palou et al. 1997; Simpson and Gilmour 1997; Van Opstal et al. 2003). Reduced water activity also increases the thermotolerance of microorganisms, an effect that has been explained by reduced hydration and the resulting higher stability of cellular proteins (Kita et al. 1994) and/or by enhanced membrane stability (Crowe and Crowe 1988). For *R. rubra*, this type of piezoprotection was observed with NaCl and sugars in the molar concentration range (Oxen and Knorr 1993).

In the present work, we demonstrate large Na^+ - and pH-dependent variations in piezotolerance of *R. rubra* at low solute concentrations, and the combined results of several experiments are consistent with the view that this piezotolerance is mediated by cytoplasmic membrane Na^+/H^+ exchange. An important indication of such mechanism is the ion specificity (Na^+/Li^+ , not K^+) and millimolar concentration range at which the effects occur (Fig. 1) and which are typical of certain Na^+ -specific membrane transporters (Rodrígueznavarro et al. 1994; Prior et al. 1996; Padan et al. 2001). Further, piezotolerance was correlated with the intracellular rather than extracellular Na^+ concentration. This could be clearly shown by preincubating the cells either in 5 mM Na^+ or in Na^+ -free buffer and then subjecting them to pressure treatment in Na^+ -free buffer. Cells preincubated in Na^+ had higher intracellular Na^+ levels and higher piezotolerance than did cells preincubated in the absence of Na^+ (Fig. 3). Finally, the effect of extracellular H^+ concentration on intracellular Na^+ and piezotolerance was opposite to that of extracellular Na^+ : YGP grown cells retained higher intracellular Na^+ levels and piezotolerance when they were preincubated at pH 8.0–9.0 than at pH 6.0–6.5 (Figs. 2, 8). These results indicate that the loss of intracellular Na^+ that the cells had accumulated during growth in YGP (due to the natural Na^+ content of this medium) was dependent on extracellular pH, which is consistent with a mechanism based on a Na^+/H^+ antiporter. Indeed, assuming a constant intracellular pH around neutrality, the predicted Na^+ loss from the cell by Na^+/H^+ exchange will be facilitated when the H^+ concentration is higher outside the cell than inside, i.e., at pH 6.0–6.5, and vice versa for pH 8.0–9.0. In addition, from the data in Figs. 3 and 4, which express a quantity of Na^+ per cell, and an estimate of the average cell volume ($\pm 50 \mu\text{m}^3$, derived from microscopic observation of cell dimensions), we can calculate that the intracellular Na^+ concentration is on the order of 3–7 mM in our experiments. Again, this is consistent with our hypothesis of a Na^+/H^+ exchanger because it is in the same concentration

range at which extracellular Na^+ affects intracellular concentration and piezotolerance (Fig. 3). We have not been able to find further evidence for this hypothetical Na^+/H^+ exchanger by making use of inhibitors specific for Na^+ transporters such as amiloride, cimetidine, and harmaline (Ganapathy et al. 1986; Kleyman and Cragoe 1988) (data not shown). However, these inhibitors have been used primarily on vertebrate cells, and, for example, the *Saccharomyces cerevisiae* vacuolar Na^+/H^+ antiporter is much less sensitive (Darley et al. 2000).

Na^+/H^+ antiporters have been identified in the vacuolar and cytoplasmic membranes of *S. cerevisiae* and other yeasts. Our results now suggest the presence of a plasmalemma Na^+/H^+ antiporter also in *R. rubra*, which is distinct from its counterparts in *S. cerevisiae* and *S. pombe* in that it confers piezotolerance. Both the vacuolar and plasmalemma Na^+/H^+ antiporters contribute to salt tolerance in *S. cerevisiae* and other yeasts by transporting Na^+ out of the cytosol (Prior et al. 1996; Quintero et al. 2000) and may be involved in pH homeostasis, but this is the first time that they have been implicated in tolerance to other types of stress.

The precise mechanism of piezotolerance remains a matter of speculation, but the observation that piezotolerance correlated with an elevated intracellular Na^+ level suggests a link with pH homeostasis. In *S. cerevisiae*, indications do indeed exist that pressure treatment results in cytosolic acidification due to inactivation of the plasma membrane H^+ ATPase, and this is believed to be one of the major causes of cell death (Abe and Horikoshi 1997, 1998). Since any increase in intracellular Na^+ is expected to be concentrated in the vacuoles, which serve as a Na^+ sink to protect the cell against toxic levels of Na^+ in the cytosol (Quintero et al. 2000), it can further be speculated these Na^+ -loaded vacuoles can serve as a reservoir for uptake of excess protons after pressure treatment, and thus counteract acidification of the cytosol. Since cytosol acidification is the result of a metabolic imbalance by a selective knockout of cell functions (e.g., the plasma membrane H^+ ATPase), it can be predicted that at some higher pressure, no acidification will take place anymore because all metabolic functions have been stopped. This prediction is supported by the data in Fig. 7, which show that Na^+ -dependent piezoprotection is limited to pressures below 215 MPa, and by the data in Fig. 8, which show that the effects of pH on pressure inactivation at pressures above and below 250 MPa are opposite. Interestingly, the inactivation of *R. rubra* as a function of pressure in Na^+ -free buffer shows a plateau or even a small depression between 190 and 230 MPa (Fig. 7). A similar observation was recently described for a clinical isolate of *Escherichia coli* O157:H7 H1071, showing a depression in HHP inactivation between 200 and 400 MPa (Pagan and Mackey 2000). Although very uncommon, this behavior may in fact support the acidification hypothesis as follows: at pressures up to 190 MPa, cell death may occur mainly as a result of metabolic imbalance and cytosolic acidification; at

180–230 MPa, the metabolic functions responsible for acidification are also knocked out, but other vital cell functions remain intact; as a result the cells survive better at 210–220 MPa than at 180–190 MPa pressure because the metabolic imbalance is relieved. Finally, pressures above 230 MPa affect other vital targets, resulting in increased inactivation.

In conclusion, we have described a novel and unique mechanism of piezoprotection in *R. rubra*, which provides insight into the mechanisms of cellular inactivation by high pressure. With respect to the application of high pressure for food preservation, these observations clearly underscore the need to investigate HHP inactivation on a sufficiently wide spectrum of different microorganisms and under different environmental conditions.

Acknowledgments This work was supported by a fellowship from the Flemish Institute for the Promotion of Scientific Technical Research (IWT) to A.A.

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