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Vacuolar control of subcellular cation distribution is a key parameter in the adaptation of *Debaryomyces hansenii* to high salt concentrations

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ABSTRACT

Debaryomyces hansenii is a halotolerant and Na^+-includer yeast that can be isolated from different food and low-water activity products. It has also been defined as a marine-occurring yeast but key aspects for this salt tolerant behavior are far from being understood. Here, we searched for clues helping to elucidate the basis of this ability. Our results on growth, Rb^+ transport, total K^+ and Na^+ content and vacuolar fragmentation are compatible with a yeast species adapted to cope with salt stress. On the other hand, we confirmed the existence of *D. hansenii* strategies that are generally observed in sensitive organisms, such as the production of glycerol as a compatible solute and the efficient vacuolar sequestration of Na^+. We propose a striking role of *D. hansenii* vacuoles in the maintenance of constant cytosolic K^+ values, even in the presence of extracellular Na^+ concentration values more than two orders of magnitude higher than extracellular K^+. Finally, the ability to deal with cytosolic Na^+ levels significantly higher than those found in *S. cerevisiae*, shows the existence of important and specific salt tolerance mechanisms and determinants in *D. hansenii*.

1. Introduction

Although it is generally accepted that sodium is toxic for living cells, there are multiple exceptions to this assertion (Kronzucker et al., 2013). In the case of yeasts, it has been shown that sodium can partially replace potassium requirements improving cell performance under certain conditions (Camacho et al., 1981). Furthermore, there are yeast types that display halotolerant or halophilic behavior (Gunde-Cimerman et al., 2009). The model yeast *Saccharomyces cerevisiae* accumulates high amounts of potassium necessary for key functions such as regulation of pH, compensation of negative charges or maintenance of membrane potential (Ariño et al., 2010; Cyert and Philpott, 2013). On the other hand, it is relatively sensitive to sodium. Although the putative targets and the molecular basis of *S. cerevisiae* salt sensitivity are not fully understood, several determinants related to this behavior have been described, including ion transporters, plasma membrane composition, enzymes or intracellular sodium distribution (Ariño et al., 2010; Cyert and Philpott, 2013). We have recently determined subcellular location of K^+ and Na^+ in *S. cerevisiae* (Herrera et al., 2013). In that work, we showed that the cytosol contained such low amounts of sodium that it remained virtually free of this cation even when yeast growth was impaired by the presence of high NaCl concentrations, suggesting the highly toxic effect of this cation when present in the cytosolic fraction.

Numerous peculiarities found in the so-called non-conventional yeasts in comparison to the model yeast, hinder extrapolation of some observations (Ramos et al., 2011). *Debaryomyces hansenii* is a non-conventional ascomycetous yeast with high biotechnological interest (Breuer and Harms, 2006; Prista et al., 2016). Its genome was sequenced by the Genolevures consortium (Dujon et al., 2004); it is considered a rare human fungal pathogen (Desnos-Ollivier et al., 2008); it is abundant in cheeses and sausages and it can cope with salt stress and proliferate in salty environments (Prista et al., 1997; Prista et al., 2005; Calahorra et al., 2009; Norkrans, 1966; Norkrans, 1968). In contrast to the general behavior of most fungi, *D. hansenii* is a sodium ion-includer yeast. Although multiple salt tolerance determinants previously identified in *S. cerevisiae* have been found in *Debaryomyces*, their real contribution to the whole process remains obscure. Several differential determinants between the two yeast species have been reported, such as the existence of ion transporters absent in *Saccharomyces* (Hak1) (Prista et al., 2007; Martínez et al., 2011), the high salt tolerance of some *D. hansenii* enzymes (Hal2 or Ppz1) (Aggarwal and Mondal, 2006; Minhas et al., 2012) or specific

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changes in *D. hansenii* plasma membrane composition in response to salt (Turk et al., 2007). In this regard, we previously showed that the ability to transport and sequester sodium into the vacuole must be important for sodium halotolerance in *D. hansenii* (Montiel and Ramos, 2007). We proposed that while 50% of intracellular sodium corresponded to the cytoplasmic fraction, the remaining 50% was ascribed to the vacuole. These experiments were performed by treating the cells with cytochrome C which permeabilizes the plasma membrane (Lichko et al., 1980; Okorokov et al., 1980). However this approach turned to be too simplistic in *S. cerevisiae* since it considers only two important compartments (cytosolic and vacuolar fractions) where the cations are present in significant amounts and ignores other organelles such as nuclei in which almost 20% of intracellular K^+ or Na^+ are kept (Herrera et al., 2013). Therefore our initial proposal should be reconsidered.

In this work we have followed physiological, biochemical and microscopic approaches that provide new clues to understand the halotolerant character of *D. hansenii*. We show that, although high NaCl concentrations create osmotic stress, Na^+ tolerance is not a relevant concern for *Debaryomyces* as deduced from transport experiments and its minor effects on growth and vacuolar fragmentation. Furthermore, accumulation of Na^+ in the cytosol reaches values around three times higher than those reported in *S. cerevisiae* (Herrera et al., 2013) without seriously toxifying *D. hansenii* cells. In addition we demonstrate that cells respond to changes in external NaCl concentration by efficiently adapting their intracellular distribution of K^+ and Na^+.

2. Materials and methods

2.1. Yeast strains and growth conditions

Debaryomyces hansenii CBS767 was used throughout the work. Strain CBS767 is commonly used as *D. hansenii*-type strain since its genome is the only one so far been completely sequenced in this yeast species (Dujon et al., 2004). *Saccharomyces cerevisiae* (BY4741, *MATa his3Δ1 leu2 Δ met15 Δ ura3Δ*) was used in some experiments for comparison with *D. hansenii*. Cells were routinely grown in complex YPD [1% ($w.v^{-1}$) yeast extract, 2% ($w.v^{-1}$) peptone, 2% ($w.v^{-1}$) glucose] medium at 26 °C (*D. hansenii*) or at 28 °C (*S. cerevisiae*).

To study Na^+ tolerance, 5 μl water droplets containing yeast cells at an initial OD_{600} of 1, as well as the appropriate serial dilutions, were spotted onto YPD plates supplemented with several concentrations of NaCl and incubated at 26 °C during 48 h.

2.2. Cation content and transport measurements

To determine total K^+ and Na^+ content of yeast cells, liquid YPD medium (50 ml) supplemented with different NaCl concentrations was inoculated to OD_{600} 0.02. Flasks were incubated in a rotation shaker (200 rpm) at 26 °C until OD_{600} reached values of 0.4–0.6.

In order to study the time course of Rb^+ uptake, YPD cell cultures at an OD_{600} value of 0.4–0.6 were supplemented with 50 mM RbCl and, after ten minutes, 50 mM NaCl was also added. At different time points after cation addition, samples were extracted and treated for cation extraction as previously described (Ramos et al., 1990; Martínez et al., 2011). Whole cells were extracted with acid and the cations were analyzed by atomic emission spectrophotometry. Cation content in the different organelles was calculated related to the percentage isolated. In all cases values are expressed as nmol of cation per mg of cell dry weight (Ramos et al., 1990; Martínez et al., 2011; Herrera et al., 2013).

2.3. Measurement of intra- and extracellular glycerol

Intra- and extracellular glycerol concentrations were determined enzymatically using a commercial glycerol determination kit (Glycerin Glycerol Kit. Boehringer Mannheim Ref. 10148270035), as described previously (Albertyn et al., 1994). Briefly, cells were grown overnight in 50 ml YPD supplemented with the required amounts of NaCl. When OD_{600} reached values close to 1, cultures were centrifuged (5000 rpm, 4 °C, 5 min) and cells and supernatant were separately treated following the recommendations of the manufacturer.

2.4. Isolation of main organelles from D. hansenii and determination of K^+ and Na^+ content

The main yeast organelles were isolated as described previously for *S. cerevisiae* (Herrera et al., 2013; Gelis et al., 2015) with some modifications. All centrifugations were carried out at 4 °C in plastic tubes and all buffers were kept on ice. We always tested our solutions before and after use and we confirmed that the tubes were neither contaminating nor affecting their ion content. K^+- and Na^+-free buffers were used in all experiments. In order to obtain and stabilize protoplasts from *D. hansenii*, two major changes were introduced in the mentioned protocol: (1) treatment with lyticase was performed during 55 min using 6 mg lyticase 100 KU per g of cells, and (2) sorbitol concentration in the reaction buffer was increased to 2 M.

2.5. Vacuole staining with FM4-64 and fluorescence microscopy

Vacuoles were stained using the fluorescent probe FM4-64 as previously described for *S. cerevisiae* with some modifications (Ogita et al., 2010). Two different sets of experiments were performed. On the one hand, *S. cerevisiae* and *D. hansenii* were grown overnight in complex medium and fragmentation was induced by supplementing the suspension with 0.4 M NaCl. After 10 min of incubation at room temperature cells were examined (Michaillat and Mayer, 2013). On the other hand, *D. hansenii* cells were grown overnight in YPD supplemented with the appropriate NaCl concentrations and when OD_{600} reached 0.5–0.6, cultures were analyzed. Briefly, cells were suspended into 0.5 ml of fresh YPD containing 20 μM FM4-64 and incubated at 28 °C for 15 min. Cells were centrifuged and incubated in fresh medium (1 ml) during 45 additional minutes. Finally cells were collected, resuspended in PBS (137 mM NaCl; 2.7 mM KCl; 10 mM Na_2HPO_4; 1.8 mM KH2PO4; pH 7.4) and observed under optical and epifluorescence microscopy.

For optical and epifluorescence microscopy analyses cell aliquots were observed using the Nomarsky technique or the appropriate filter set, respectively, in a Zeiss Axio Imager M2 Dual Cam microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). Examination using epifluorescence was performed with UV-light 340–380 nm and a filter block (BP 560/40, FT 585, BP 630/75). Images were captured with an Evolve Photometrics PV Cam digital camera using the Axiovision 4.8 software. Images were processed using Adobe Photoshop CS3 (Adobe Systems, Mountain View, CA, USA).

2.6. Statistical analyses

In general, all the experiments were repeated at least two times. Cation determination experiments were performed at least in triplicate. The agreement among repetitions was usually higher than 90%. The data reported are an average of the independent experiments ± standard deviation.