UNIVERSITAT ROVIRA I VIRGILI
DEHYDRATION TOLERANCE IN YEAST
Boris Alejandro Rodríguez Porrata
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UNIVERSIDAD ROVIRA I VIRGILI
Departamento de Bioquímica y Biotecnología
Facultad de Enología

Dehydration Tolerance in Yeast

Memoria presentada por

BORIS ALEJANDRO RODRÍGUEZ PORRATA

para optar al título de Doctor por la Universidad Rovira i Virgili

Director de Tesis: Dr. Ricardo Cordero Otero

Codirector: Dr. Xavier Escoté Miró

Tarragona, 2010

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A mis padres

A mis hijos Arley y Néstor

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Facultad de Enología de Tarragona
Marcel·lí Domingo s/n
43007 – TARRAGONA

Els sotasignants, Dr Ricardo Cordero Otero, Professor-Investigador del Departament de Bioquímica i Biotecnologia de la Universitat Rovira i Virgili, i

El Dr Xavier Escotè Miró, investigador del Hospital Universitario de Tarragona "Joan XXIII", grup de recerca biomèdica HJ23

FAN CONSTAR,

Que el present treball, amb títol **'*Dehydration Tolerance in Yeast*'** que presenta el Sr. **Boris Alejandro Rodríguez Porrata,** per optar al Grau de Doctor per la Universitat Rovira i Virgili ha estat realitzat sota la nostra direcció, i que tots els resultats obtinguts són fruit dels experiments duts a terme per l'esmentat doctoran.

I perquè se'n prengui coneixement i tingui els efectes que correspongui, signem aquesta certificació.

Dr. Ricardo Cordero Otero Dr. Xavier Escotè Miró

Tarragona, 1 de març de 2010

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Boris Alejandro Rodríguez Porrata **Date**

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OBJETIVES AND OUTLINE OF THE THESIS

Objetives

The aime of this project is to reduce the rate of problems during wine yeast re-hydration: (i) Analysing the occurrence and causes of cell-death, using a intracellular viability prove, during yeast re-hydration; (ii) Identify target genes essential for efficient *S. cerevisiae* re-hydration, understanding their patterns of induction and integrating that information into a metabolic model that will enable us to interpret the overall flow of metabolites, and predict necessary changes to maximize cell viability during re-hydration.

1. Determination of optimal conditions for drying and rehydration and subsequent use in alcoholic fermentations of a laboratory yeast *Saccharomyces cerevisiae* Study of different drying conditions and application times of each method (freeze drying, ventilation, vacuum). In the same study, addition of various protective substances for cells under drying and rehydration stress. Application of different rehydration conditions to study the effect on the viability and vitality in a commercial active dry yeast.

2. Study the effect of intracellular trehalose and glycogen acumulation. In the same way, study accumulation and role of different lipid during growth on cellular viability after rehydration.

3. Study phenotypic response to drying and rehydration of each single *knock-out* mutants for *Saccharomyces cerevisiae*. Gene characterization whose deletion leads to increase or decrease viability in the process of drying and rehydration. Gene over-expression, which deletions causes high mortality during the process. Study phenotypic response to drying and rehydration of transformants obtained, and examination subcellular localization of encoded proteins.

4. Determination which important genes in drying and rehydration are linked to cell death (Necrosis and Apoptosis) through studies of molecular markers of cell death in yeast.

Outline of the thesis

1.-Vitality enhancement of the rehydrated active dry wine yeast

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OBJETIVES AND OUTLINE OF THE THESIS

The main aim of this study was determine the best rehydration conditions for yeast ensuring a proper cell viability and vitality before inoculation into the must. Experimental rehydration media in this study can be divided into four groups: carbon and nitrogen compounds, metallic ions, oxidant and antioxidant agents, and membrane fluidity agents. We studied the biochemical and biophysical behaviour of Active Dry Wine Yeast (ADWY) after rehydration in a range of media under standard oenological conditions. Yeast cell viability rehydratation process was evaluated counting yeast cells under microscopy and plating these cells before and after this process. Besides, cell viability was assessed by fluorescence microscopy and flow cytometry. In addition, vitality of rehydrated cells was estimated by an indirect measure of dioxide carbon production.

Results are reported and discussed in **Chapter I**

2.-Lipids effect on the desiccation tolerance of yeasts

Next, we focused our attention in changes in metabolite features during the dehydration-rehydration process for different yeast species, in order to determine whether metabolites might play a roll in cell viability. These species were genetically closely related to *Saccharomyces.* In addition, we checked whether desiccation tolerance across the species was correlated or not with the intracellular content of trehalose or glycogen. We also investigated potential changes in cell lipid composition during this process, because triacylglycerol-and phospholipid content may vary across the species. Then, we studied cell viability after artificially increased intracellular lipid content before dehydration.

Results are reported and discussed in **Chapter II**

3-Yeast cell death during drying and rehydration

To understand how yeasts cells have evolved to survive after drying, we addressed controversial issues such as cell age, longevity, the structural and biochemical properties of anhydrous cytoplasm, and metabolic stasis. Capacity of desiccation tolerance was evaluated after dry and rehydratation process in a collection of 4850 different single *knock-out* strains, by colony-counting assay. Taken in account that drying and rehydration cause high mortality, we hypothesized that some of relevant genes studied could be related to the cell death pathways. Therefore, we studied the

hallmarks of apoptosis to determine whether these stress conditions induced apoptosis and necrosis.

Results are reported and discussed in **Chapter III**

4- Sip18 hydrophilin prevents cell death during drying and rehydration process

The yeast *Saccharomyces cerevisiae* is able to survive dehydration and long periods in a dry state; the metabolic activity of the cell is arrested during this time, but resumes after rehydration. We have used a yeast gene deletion set (YGDS) of 4850 viable mutant haploid strains to perform a genome-wide screen. Among the essential genes we characterized to overcome the cell-drying/rehydration process, six of them belong to the group of very hydrophilic proteins known as hydrophilins. Although the functional role of hydrophilins remains speculative, there is evidence to support their participation in acclimation and/or in the adaptive response to stress. The over-expression of *SIP18* in *S. cerevisiae*, which encodes the yeast's hydrophilin, increases cell viability during the dehydration process. The apoptotic hallmarks of over-expressing *SIP18* and Δ*sip18* strains, such as the accumulation of reactive oxygen species (ROS), phosphatidylserine externalization (Annexin V/PI staining) and DNA-strand breaks (TUNEL assay), were analysed before desiccation and after rehydration. DHE staining showed that the dehydration and rehydration process promotes a higher number of ROS-accumulating cells in the Δ *sip18*strain as compared to the over-expressed and reference strains. Furthermore, double staining Annexin V/PI reveals, for the Δs*ip18*strain, an elevated percentage of necrotic- and late apoptotic/secondary necrotic-cells after stress imposition. Further evaluations using the over-expressing strain suggest that SIP18 prevents cell death during dehydration stress by acting as an antioxidant.

Results are reported and discussed in **Chapter IV**

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1. ANHYDROBIOTES

1.1 Anhydrobiotes

All cells are exposed to water loss and this water deficiency generates stress that has likely been imposed since very early on in the evolutionary process**.** Desiccation-tolerant organisms called *anhydrobiotes* tolerate drought by drying up but not dying. Some of them can even survive in a dry state even for years, during which time their metabolism and life processes come to a halt. Interestingly, when water again becomes available, these organisms rapidly swell and return to active life.

Desiccation tolerance is also remarkable because it means that an organism must be able to suspend animation. Life has never been possible without water, thus cell metabolism requires that enzymes and membranes to be surrounded by water. Desiccation-tolerant cells must be able to cease metabolism and then start it again, as Baker proposed in the Royal Society of London in 1743.

Cell metabolism probably stops by the time a cell has dried to about 0.1·g H_2O g^{-1} dry mass, because at that point there would be too little water to form a monolayer around proteins and membranes (Clegg, 1973; Billi and Potts, 2002). Drying to 10% absolute water content is roughly equivalent to equilibration with air at 50% relative humidity at 20°C and to dropping to a water potential of 100·MPa. These thresholds also clearly separate desiccation-sensitive from desiccation-tolerant species (Alpert, 2005). However, there is a gap in the literature about minimum water content that living organisms need to survive. Except for a small proportion of seeds, almost all species tested for drying die if they are dried to 20 % or less water content (Tweddle *et al*., 2003), making them desiccation sensitive. On the other hand, organisms that are able to survive drying to 10% water content are considered desiccation-tolerant.

Van Leewenhoek confirmed this property in nematodes and rotifers in 1702 (Keiling, 1959). Today, it is well known that anhydrobiotes are found across all biological kingdoms. Anhydrobiotes are well-represented among the tardigrades, crustaceans, arthropods, algae, fungi and bacteria.

However, it is in the plant kingdom where most anhydrobiotes can be found with around around 350 species of flowering plants, lichens, bryophytes, and some ferns able to survive in a dehydrated state. Among the vascular plants, poikilohydric or resurrection plants (a small group of angiosperms) can tolerate extreme dehydration

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(Gaff, 1987). Upon watering, dried resurrection plants rapidly revitalize and become fully photosynthetically active in hours (Bernacchia *et al.,* 1996).

Group		Known occurrence	Selected references
Nematodes		Many species	Wharton (2003); Treonis and Wall (2005)
Rotifers		Many species, including most species tested in the order *Bdelloidea*	Ricci and Caprioli (2005)
Tardigrades		Many species	Wright (2001); Jönsson and Järemo (2003)
Crustaceans		Encysted embryos of several genera of anostracans (brine and shrimps)	Wright (2001); Jönsson and Järemo (2003)Mitchell (1990); Clegg (2005)
Arthropods		Larva of the fly *Polypedilum vanderplanki*	Watanabe *et al.* (2004); Kikawada *et al.* (2005)
Lichens		Most species tested	Kappen and Valladares (1999)
Yeasts		Some species	Garay-Arroyo *et al.* (2000)
Mosses		Most species tested	Proctor and Tuba (2002)
Liverworts		Many species	Proctor and Tuba (2002)
Pteridophytes		Perhaps 50 species as sporophytes, many as spores, some as gametophytes	Pence (2000); Porembski and Barthlott (2000)
Gymnosperms		No adults but some seeds and pollen	Porembski and Barthlott (2000); Dickie and Prichard (2002); Hoekstra (2002)
Angiosperms		About 300 species as adults, 95% of species as seeds and pollen	Porembski and Barthlott (2000); Hoekstra(2002); Tweddle *et al.* (2003)
Bacteria		Many species, including cyanobacteria	Billi and Potts (2002); Buedel *et al.* (2002); de la Torre *et al.* (2003)
Terrestrial microalgae		Many species	Trainor and Gladych (1995)
Marine macroalgae		The intertidal red alga *Porphyra dentata*	Abe *et al.* (2001)

Table 1. The taxonomic range of desiccation tolerance. Decication tolerance capacity it is not evolutionarily conserved and anhydrobiotes are found across all biological kingdoms in isolated species.

A distinctive feature of pluricellular organisms that tolerate dehydration is their size. Desiccation-tolerant organisms may be small because of the physical stresses associated with drying. Their cells shrink as they dry, and the whole organism must shrink with them. All pluricellular organisms that tolerate desiccation as adults adopt distinctive, balled or curled shapes as they dry (Alpert, 2006) (Fig.2).

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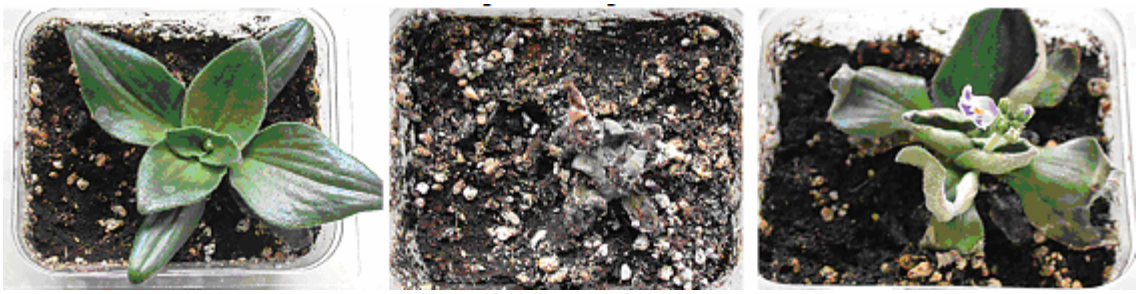

Fig. 1 *Craterostigma plantagineum* A representative example of a resurrection plant (adapted from Physiol. Plant. 121, 2004). The figure shows three states of a plant under water stress (left panel in optimal conditions, the central panel after 15 days of dehydration andoin the right one week after being rehydrated).

A variety of problems are associated with desiccation and the mecanisms that organisms employ to solve such problems are wide ranging. For instance, plants respond to mechanical damage due to shrinkage by changing cell wall composition to increase flexibility (Jones and McQueen-Mason, 2004; Vicre *et al.,* 2004) folding cell walls (Vander Willigen *et al.,* 2004), replacing water in vacuoles with non-aqueous compounds and fragmenting vacuoles (Farrant, 2000; Vicre *et al.,* 2004).

Desiccation rates affect the survival of many tolerant organisms. Rapid drying may preclude the induction of mechanisms needed for desiccation tolerance (Ricci *et al*., 2003; Clegg, 2005). A hypothetical solution to fast drying may be to slow down the process of water loss (Kikawada *et al*., 2005). However, very slow drying may prolong the time spent at water content levels just above those at which metabolism ceases, thus causing possible damage. A specific mechanism for facilitating the loss of water from cells during drying may be the up-regulation of aquaporins to increase the water-permeability of membranes (Berjak and Pammenter, 2001; Proctor, 2003; Walters *et al*., 2005).

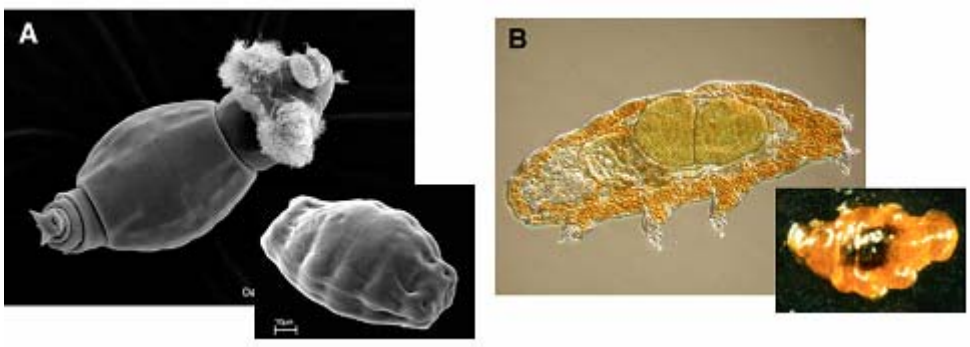

Fig. 2 Representative examples of desiccation-tolerant organisms: (A) Scanning Electron Micrographs (SEMs) of the rotifer *Macrotrachela quadricornifera* hydrated (right panel) (bar length

0.2·mm) and desiccated (left panel) (B) Light picture of the tardigrade *Richtersius coronifer* before (large image) and after (small right corner image) desiccation; (adapted from Giulio Melone (A) and Ingemar Jönsson (B))

Other types of cellular damage caused by drying include membrane disintegration, macromolecule aggregation, lipid body coalescence and membrane leakage upon rehydration which are offset by various mechanisms, such as sugar accumulation. Such sugars include specially accumulated non-reducing disaccharides (*e.g*. trehalose or sucrose), which stabilize molecules, lower the melting temperature (*T*m) of the membrane phase, change from the liquid crystal to gel phase, and form glasses with high melting temperatures (Wingler, 2002; Bernacchia and Furini, 2004; Buitink and Leprince, 2004; Crowe *et al*., 2005). Moreover, the accumulation of these sugars promotes the synthesis of Late Embryogenesis Abundant (LEA) proteins, which act as molecular chaperones and interact with sugars to form glasses (Wise and Tunnacliffe, 2004; Goyal *et al*., 2005; Oliver *et al.,* 2005). Other small protein may act as chaperones or repair damage upon rehydration (Collins and Clegg, 2004; Crowe *et al*., 2005; Potts *et al*., 2005) supporting changes in lipid composition that stabilize membranes. These changes are involved in increases in phospholipid content and degree of saturation, and the accumulation of free sterols (Quartacci *et al*., 2002; Hoekstra, 2005).

The accumulation of Reactive Oxygen Species (ROS) is another harmful consequence of drying. Several studies have reported the synthesis of antioxidants during drying, maintenance of pools of reduced antioxidants and ROS-scavenging enzymes (Shirkey *et al.,* 2000; Augusti *et al.,* 2001; Espindola *et al.,* 2003; Kranner and Birtic, 2005). In plants, drying has been documented as an early down-regulation of photosynthesis (which causes ROS) (Jensen *et al.,* 1999; Deng *et al*., 2003; Hirai *et al*., 2004; Illing *et al*., 2005), folding leaves (Farrant *et al.*, 2003) and a programmed chlorophyll loss (Tuba *et al.,* 1996).

1.2 *Saccharomyces cerevisiae*

The model used in this study is *Saccharomyces cerevisiae*. Budding yeast (or baker's yeast) is able to tolerate desiccation and to recover methabolic life upon the presence of water (Garay-Arroyo *et al.*,2000). In addition, *S. cerevisiae* is the best characterized eukaryotic organism and is widely used as a research model for almost all biological processes. Yeast is easy to cultivate, fast dividing, non-pathogenic and particularly suitable for targeted genetic modification. Its importance in industry and scientific

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research is also highlighted by the fact that the *S. cerevisiae* genome was the first eukaryotic genome to be completely sequenced. Although *S. cerevisiae* is a single-celled fungus, it shares a lot of similarities with higher eukaryotes. Yeast is an excellent eukaryotic model and at least 40% of single-gene determinants of human genetic diseases have homologues in yeast (Oliver, 2002). This microscopic organism also exhibits various features generally assigned to complex animal organisms including mating, aging and death. *S. cerevisiae* is a type of yeast that has been extensively used in the manufacture of bread, beer and wine for centuries.

S. cerevisiae has two stable sexual phases depending on its DNA content: as a haploid cell and as a diploid cell. Two sexes, *Mat*a and *Mat*α, are present in the haploid phase. Working with haploid yeast allows mutants to be generated, isolated and characterized with great ease and a single gene copyto be interpreted. Haploid yeast contains 16 chromosomes (from 200 to 2200 kilobases). Haploid and diploid forms are reproduced by asexual budding. In certain conditions, the haploid *Mat* a can mate with the other sex, *Mat* α, generating a diploid yeast. On the other hand, diploid yeast is able to reproduce sexually. In these cases, meiosis occurs forming an asca that contains four haploid ascospores (two sex *Mat*a and two sex *Mat*α).

Growth in yeast is synchronized with budding growth, which reaches the size of the mature cell by the time it separates from the parental cell. In rapidly growing yeast cultures, all the cells can be seen to have buds since bud formation occupies the whole cell cycle. Both mother and daughter cells can initiate bud formation before cell separation has occurred. In yeast cultures which are grown more slowly, cells lacking buds can be seen and bud formation only occupies a part of the cell cycle.

The cell cycle basically consists of four distinct phases: G_1 phase, S phase (synthesis), G_2 phase (collectively known as interphase) and M phase (mitosis). Cells that have temporarily or reversibly stopped dividing are said to have entered a state of quiescence called the G_0 phase (Rubenstein *et al.,* 2008).

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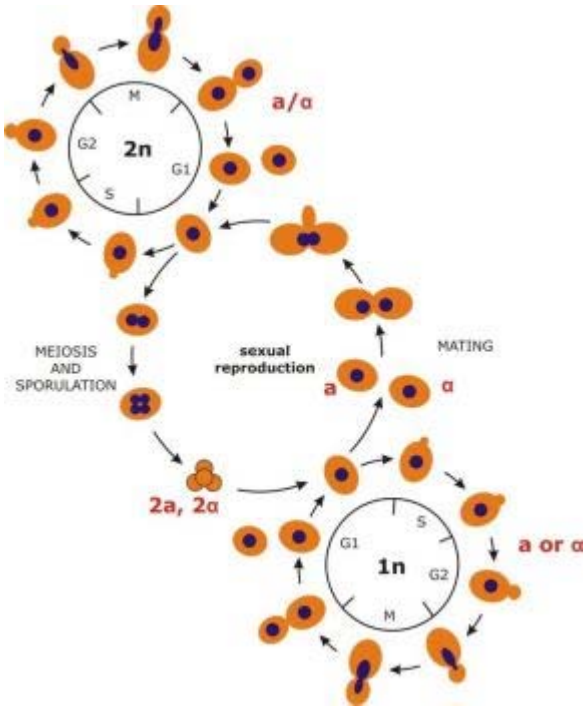

Fig. 3 The life cycle of *Saccharomyces cerevisiae*. Upper and lower panels represent an asexual diploid and haploid division cycles at different phases (G1, S, G2 and M) respectively. Under certain conditions these cycles are connected by meiosis and sporulation, and by mating. Adapted from (http://www.medils.hr/index.php/taddei-svetec-group/genetics-of-death-in-yeast/)

S.cerevisiae is a model organism because it scores favorably on a number of these criteria:

- As a single celled organism, yeast is small with a short generation period (doubling time 1.5-2 hours) and can be easily cultured. *S. cerevisiae* can be easily genetically modified.
- *S. cerevisiae* is a eukaryote, that shares the internal subcellular organelle complexity of an upper eukaryote (*e.g.*, Golgi apparatus, endoplasmic reticulum, mithocondria, nucleus, etc.).
- Originally research on *S. cerevisiae* was mainly driven by economic factors, due to its traditional use in the food and beverage industry (*e.g.* for beer, bread and wine).

In batch fermentation systems, the growth curve can be roughly divided into a few stages or phases: the lag phase, logarithmic growth phase, and stationary phase **(Fig. 4)**. During the second phase, cells preferentially metabolize sugar (usually glucose) which allows them to grow faster. Only after an essential nutrient has been exhausted do the cells switch to the stationary phase. “Diauxic shift” is represented by an inflection in the

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curve and corresponds to a lag period during which cells produce the enzymes needed to metabolize an alternative essential nutrient.

The lag phase is a period of adaptation to new growth conditions. Yeast cells need more or less time to acclimatize to a new environment depending on how it differs from the previous medium. The more different the new condition, the longer the lag phase lasts. Cells sense and interpret the new situation through synthesizing enzymes to consume nutrients and support growth. After the lag phase, there is a logarithmic or exponential growth phase, until a nutrient becomes restricted. During the logarithmic phase, cell population increases exponentially. Cell proliferation occurs by cell budding: individual cells increase in mass and volume to a certain size at which time new buds are formed. Cells gradually shift into the stationary phase as levels of sugar or other components fall. In this phase, cells do not divide. During this period, cells change their metabolisms, acquiring characteristics of stationary phase cells (thickening of the cell wall, accumulation of reserve carbohydrates, and acquisition of thermotolerance) (Werner-Washburne *et al.,* 1993).

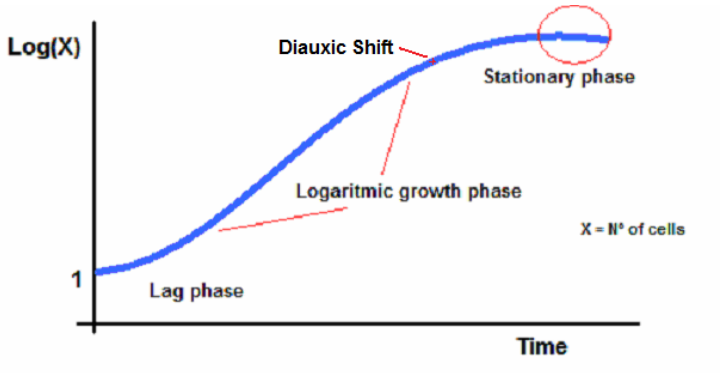

Fig. 4 Batch fermentation system curve showing the growth phases. The lag phase is a period of adaptation of the cells to a new media during which the enzymatic machinery is prepared for division. During logarithmic phase the division rate rich a maximum value. In the stationary phase yeast growth slow down.

The capacity of yeast to tolerate prolonged desiccation and restore metabolic functions once water is again available makes it very interesting from both a scientific and industrial perspective. Furthermore, due to the anhydrobiote qualities of *S. cerevisiae*, it is the starting point for deciphering the response mechanisms to Drying and Rehydration Stress (DRS) and the involvement of this type of stress in cell death

mechanisms such as apoptosis and necrosis. However, the use of ADWY in the food industry is the most important application from an industrial point of view.

1.3 Industrial production of ADWY

Regardless of how yeast is used (in bread, beer or wine production), the production of yeast for drying follows the Zulauf process (**Fig. 5**), which dates back to the early 1900s. Yeast feed-stock, molasses, is thick syrup containing about 50% fermentable sugars and is a by-product of sugar processing. Before yeast is propagated, it is diluted, centrifuged and sterilized to ensure its microbiological cleanliness. Predictably, molasses must be supplemented with other nutrients in order to become a proper yeast growth culture. Such nutrients include nitrogen sources (typically ammonia or ammonium sulfate) and trace substances such as vitamins, salts and metals. Finally pH is adjusted with sulfuric acid (pH 4.5-5) (Quain, 2006).

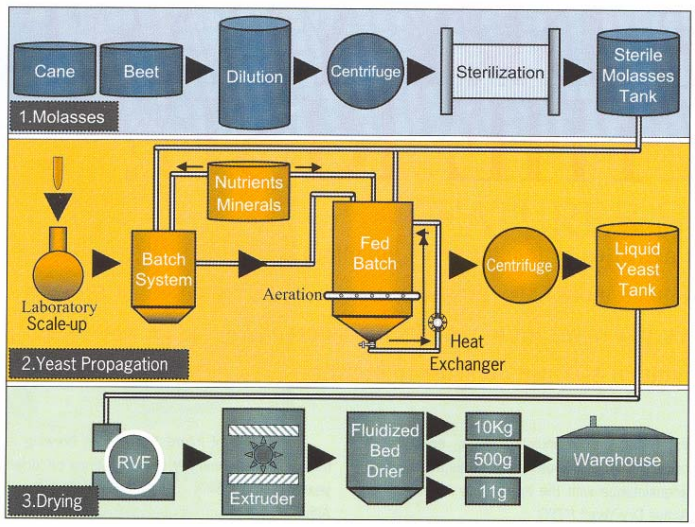

Fig. 5. **Schematic overview of the Zulauf process for drying yeast**. 1. The top image shows the preparation of the media, molasses dilution, separation of solids and sterilization. 2. The middle image depicts yeast propagation, Feed Batch Fermentation process and centrifugation to separate the yeast from the liquid media. 3. The bottom image shows the drying process by rotary vacuum filtration followed by extruction, air drying in fluidized bed and packing.

ADWY propagation is tightly controlled, maintaining low levels of sugar availability (0.1%). This is achieved using a continuous variable volume 'feed batch' process where yeast is fed/topped-up with fresh molasses. The process is controlled to ensure the yeast cells are in the correct physiological state required for drying. Yeast propagation occurs through several steps from the laboratory to the final vessel culminating in huge batch

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fermenters. High oxygen concentrations are sustained, promoting rapid yeast growth which results in a respiratory metabolism that produces a large amount of biomass and undetectable ethanol (Bauer and Pretorius, 2000; Attfield, 1997). Moreover, high oxygen concentrations also induce a higher production of unsaturated fatty acids. When biomass reaches a preferred level, the yeast is briefly deprived of an oxygen and carbon source. This deficiency induces some stress responses increasing the intracellular content of trehalose and stress protection proteins (Pretorius, 2000; Roustan and Sablayrolles, 2002).

Subsequent centrifugation steps remove any existing water. Rotary vacuum filtration concentrates the yeast to 30% solids ('cake' or 'crumb'). It is then extruded to form thin strands of spaghetti-like yeast that are dried in an air-lift fluidized dryer. During drying, the balance between temperature and time is established to minimize yeast damage while ensuring an efficient process. Although water removal is reasonably fast until the moisture content reaches 80%, slower, more careful drying is required to achieve the typical in-pack moisture content of 95%. In the final stage, the ADWY is vacuum-packed to ensure a shelf life of several years.

Quality of ADWY is essential to avoid stuck or sluggish fermentations that could affect the taste of the wine. By definition, a stuck fermentation is a fermentation that has stopped before all the available sugar in the wine has been converted to alcohol and CO_2 (http://www.grapestompers.com/articles/stuck_fermentation.htm). Aditionally, nutrient deficiencies in the natural fermentation media, inhibitory substances or certain technological practices (*e.g.* fermentation at low temperatures) may lead to stuck or sluggish fermentations (Alexandre *et al.*, 1998, Bisson, 1999).

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2. GENERAL STRESS RESPONSE IN *SACCHAROMYCES CEREVISIAE*

As a single-cell organism, yeast is exposed to many types of environmental aggression. In order to hold their own against such aggression, yeast cells have evolved a wide range of responses to many different types of stresses, including osmotic stress, temperature, oxidative stress, starvation, low pH and organic solvents (along with ethanol). Some of these defenses are constitutive and depend on the previous physiological or metabolic conditions under which yeast has been grown. The growth phase is especially important in this respect.

Other defense systems are triggered in reaction to stress. Inducible responses differ from one stress to another, but can overlap. The development of resistance to one stress leads to cross-resistance to others. Several studies have identified a sequence element common to the promoters of stress-induced genes, referred to as a Stress Response Elements (STRE), strongly suggesting that these genes are co-regulated by a common factor (Kobayashi and McEntee, 1990). The hypothetical STRE binding factor was proven to be one of two related zinc-finger transcription factors, Msn2p or Msn4p (Martinez-Pastor *et al.,* 1996; Schmitt and McEntee, 1996). Deletion of these factors renders cells sensitive to a variety of stressful conditions, and it was shown that Msn2p and Msn4p govern the induction of a large number of genes in response to many different stresses (Boy-Marcotte *et al*., 1998; Moskvina *et al*., 1998). Thus, these factors have become known as the "general stress" transcription factors and are reported to be generically activated in response to cellular stress to induce a set of genes that defend against environmental aggressions. However, it was noted that under certain conditions genes identified as targets of these factors were normally induced regardless of *MSN2* and *MSN4* deletion, hinting that regulation of the stress response was more complicated than the initial model suggested (Schuller *et al.,* 1994).

2.1 Drying and Rehydration Stress (DRS)

Drying and rehydration are the sum of several stresses, among which the most important are temperature, oxidative and osmotic stress. We will therefore specifically treat these forms of stress in the next section.

2.1.1 Heat shock stress

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Organisms have a range of temperatures within which they can proliferate. In cells exposed to supra-optimal temperatures there are numerous target sites for heat-induced injury, including protein aggregation or denaturation, cell membrane damage leading to permeability changes and ion leakage, ribosome breakdown, and DNA strand breakage. An important component of heat injury is its effect on membranes. Recently, it has been speculated that the plasmatic membrane is the major target site for thermal damage, since temperature-sensitive mutants lose viability and even block membrane synthesis or function (Dawes, 1976). High temperatures increase membrane fluidity and permeability to protons and other ions, resulting in the alteration of membrane composition as well as lipid saturation. Moreover, the activation of pathways stimulated by Ca^{2+} is affected at high temperatures (Piper, 1997).

A cell's sensitivity to heat stress is dependent on its physiological state. Thus, those in the post-diauxic or stationary phase are intrinsically much more resistant to a temperature shift, as they are to a number of other conditions. Heat stress generated by a shift in temperature (e.g. from 23 ºC to 37 ºC) leads to an increase in cell thermotolerance (Miller *et al*., 1979; McAlister and Holland, 1985). This period is accompanied by the accumulation of trehalose in the cytoplasm, which has been proposed as a marker for thermotolerance (Wiemken, 1990). Subsequently, adapted cells resume growth at a higher temperature. A major change observed in cells exposed to heat shock is the rapid broad-spectrum shutdown of protein synthesis, with the concomitant up-regulation of a set of genes encoding a limited set of Heat-Shock Proteins (HSPs). Many HSPs function as protein chaperones, so named because of their ability to bind to partially unfolded proteins and protect them from degradation or aggregation (Santoro M.G., 2000; Borges *et al.,* 2005). The group of HSPs includes Hsp104 (Hsp100 family), Hsp83 (Hsp 90 family), Hsp70 y Hsp60 and the smaller Hsp30, Hsp26 and Hsp12 (Schlesinger, M.J., 1990).

2.1.2 Oxidative stress

Aerobic metabolism has allowed an efficient energy generation process with aerobic respiration to evolve. However, aerobic respiration is intimately associated with the generation of Reactive Oxygen Species (ROS). ROS can react with DNA, lipids and proteins, altering their functions. To solve this problem, cells have evolved a variety of antioxidant defense mechanisms to satisfactorily deal with ROS content.

2.1.2.1 Reactive Oxygen Species

ROS is a collective term that includes both oxygen radicals and certain non-radicals that are oxidizing agents and/or are easily converted into free radicals (HOCl, HOBr, O_3, $ONOO^-$, H_2O_2). In other words, all oxygen radicals are ROS, but not all ROS are oxygen radicals (Ghibelli *et al.,* 1995). ROS are highly reactive due to the presence of unpaired valence shell electrons. 'Reactive' is not always an appropriate term, because H_2O_2, NO^- and O_2^- can react quickly with only a few molecules, whereas OH^- reacts quickly with almost everything. In contrast, RO_2^-, RO^-, HOCl, HOBr, NO_2^-, $ONOO^-$, and O_3 have intermediate reactivity (Halliwell *et al*., 2004).

Additionally, upon exposure to various environmental stresses (e.g. exposure to UV light or heat-shock) ROS levels increase dramatically, resulting in cell structure damage. The accumulation of intracellular ROS produces an oxidative stress which can trigger death (Hockenbery *et al*., 1993; Kane *et al*., 1993; Greenlund *et al*., 1995; Slater *et al*., 1995). Normally cells are able to protect themselves against ROS damage through the use of detoxifying enzymes such as superoxide dismutases, catalases, glutathione peroxidases and peroxiredoxins to diminish ROS levels.

Singlet oxygen ($^1O_2$) is the most reactive form of ROS. It is generated by photoexcited chlorophyll molecules in the triplet state, air pollutants and pathogenic fungi, and it causes mutagenesis, membrane lipid peroxidation and photo-oxidation of amino acids (Scandalios, 1987).

Superoxide anion (O_2^-) is a moderate reactive radical formed by an electron reduction of oxygen. It is mainly generated during respiration in mitochondria (Bouveris and Cadenas, 1982). Other sources of superoxide anion include electronic transport events in microsomes, and respiratory bursts produced by phagocytic cells. Superoxides, while abundant in cells, are not strongly reactive, although they can directly damage some proteins (Kuo *et al.,* 1987; Gardner and Fridovich, 1991).

The toxic effects of both superoxide anion and hydroxyl peroxide result from their complete conversion to the extremely reactive $^{\cdot}OH$ species. These reactions are catalyzed by metal ions, through the Haber-Weiss reaction, which generates a reduced form of metal ions from Fe(III) or Cu(II) reacting with superoxide; reduced forms of active metals (e.g. Fe(II), Cu(I) and Ti(III)) can participate in these reactions. The hydroxyl radical indiscriminately reacts, in a diffusion-limited manner, with sugars, amino acids, phospholipids, nucleotides and organic acids (Lesko *et al*., 1980; Halliwell, 1995).

2.1.2.2 Sources of oxidative stress

Sources of oxidative stress can be related to a decrease in antioxidants or an increase in oxidizing species. Diminished antioxidant levels can promote oxidative stress. For instance, mutations affecting the activity of enzymes such as superoxide dismutases or glutathione peroxidase reduce antioxidant defenses. Deficiencies in some minerals (e.g. Zn^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}) can alter the function of some of these enzymes due to their role as cofactors (Halliwell *et al*., 2004).. Furthermore, exposure to a number of toxins can decrease antioxidant levels. For example, many xenobiotics are metabolized by conjugation with glutathione (GSH) causing oxidative stress even if the xenobiotic is not itself a generator of ROS (Halliwell *et al*., 2004).

Furthermore, increased oxidants can be caused by the exposure of cells to elevated oxygen levels or toxins that are themselves reactive species (e.g. NO_2^-) or are metabolized to generate reactive species (e.g. paraquat), or by the excessive activation of 'natural' systems (e.g. inappropriate activation of phagocytic cells in chronic inflammatory diseases) (Halliwell *et al*., 2004; Perrone *et al.,* 2008).

The types of cell damage resulting from ROS during oxidative stress are:

1. Cell adaptation by up-regulation of defense systems, which may completely protect against damage or under/over-protect (Halliwell *et al*., 2004).

2. Cell injury to several molecular targets (lipids, DNA, protein, or carbohydrate). Not all damage caused by oxidative stress is oxidative damage (Halliwell *et al*., 2004). Biomolecular damage can also result from oxidative stress-related changes in ion levels (e.g. Ca^{2+}) or protease activation (Baines *et al*., 2005).

3. Cell death as a consequence of oxidative injury. Cells may recover from oxidative damage by repairing or replacing damaged molecules, survive with persistent oxidative damage or they may die or experience overall DNA damage through apoptosis or necrosis (Halliwell *et al.*, 2004).

In aerobic cells exposure to some degree of oxidative stress is common. Presumably this repetitive exposure has given rise to evolutionary oxidative pressure since the earth's atmosphere became aerobic. Organisms, in order to deal with ROS and its damaging consequences, have evolved many efficient systems. This is the case for facultative aerobic species such as *S cerevisiae*. Cells have evolved several systems to repair damaged molecules including ROS degradation and detoxifying, preservation of the metal ion homeostasis (to prevent free metal ions which generate hydroxyl radicals), as

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well as an ordinary system. Induction levels for some antioxidant systems can become pro-oxidant and therefore deleterious if they are in excess. Therefore, a delicate balance is needed between defense systems and harmful aggressions.

2.1.2.3 Defense mechanisms

Almost any chemical can exert antioxidant effects *in vitro* by choosing appropriate assay conditions.

Antioxidant	Characteristics	Function	References
Glutathione (GSH)	**Main low molecular mass thiol present in yeast cells**	**DNA and protein synthesis, protein folding, amino acid transport and as antioxidant**	**Penninckx, 2000; Muller, 1996**
Ubiquinol	**Reduced form of Ubiquinona present in many intracellular membranes (microsomes, lysosomes, peroxisomes and plasma membranes)**	**Potent lipid antioxidant capable of inhibiting lipid peroxidation**	**(rnser and Dallner, 1995; Kalen *et al.*, 1987**
D-Erythroascorbic acid, L-ascorbate	**Yeast produces ten times more D-erythroascorbic acid than ascorbic, indicating that ascorbate may only play a minor role in its metabolism.**	**Antioxidant activity**	**Halliwell, 1994; Kim *et al.*, 1993**
Superoxide dismutases	**Yeast cells contain two Super Oxide Dismutase (SOD) genes: *SOD1* encodes a Cu/Zn^{2+} SOD present in the cytoplasm; and *SOD2* encodes a Mn^{2+} SOD located in the mitochondrion.**	**Convert O_2^- into H_2O_2 and O_2 catalyzed by a transition-metal center in SOD which is iron, copper or manganese and does not require NADPH**	**Culotta, 2000; Sturtz *et al.*, 2001**
Catalases	**Homotetrameric iron containing enzymes, with a haem reactive group buried inside the enzyme structure. S. cerevisiae contains a cytosolic catalase Ctt1, and a peroxisomal catalase Cta1**	**Important role in removal H_2O_2 during stationary phase, and in the adaptative response to oxidative stress**	**Ruis and Hamilton, 1992; Grant *et al.*, 1998**
Methallothioneins	**In yeast there are two metallothionein genes, *CUP1* and *CRS5.***	**Act as scavengers for metal ions, especially copper ions**	**Kagi and Schaffer, 1988; Culotta *et al.*, 1995**
Thioredoxins	**Low molecular mass proteins that have a redox active disulphide bond, *TRX1* and *TRX2* genes have been identified in *S. cerevisiae.***	**Reduction the disulfide bonds of many proteins, contributing with GSH to keep a reduced cytoplasmic thiol redox balance**	**Muller, 1991; Kuge and Jones, 1994**
Glutaredoxins	**Small proteins, which carry a thioltransferase activity similar to thioredoxin.**	**Act as glutathione-dependent disulphide oxidoreductases, catalyze reduction of protein disulphides GSH as a cofactor**	**Luikenhuis *et al.*, 1998**

Table 2 Defense mechanisms against oxidative stress. Ezymatic activity that is activated to protect cells against oxidante agents.

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Antioxidant is any substance that when it is present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate (Halliwell and Gutteridge, 1999).

The term 'oxidizable substrate' includes every type of molecule found *in vivo*. The most important defense systems are glutathione, ubiquinol, ascorbic acid, superoxid dismutases, catalases, methallothionines, thioreducines and glutareduccines.

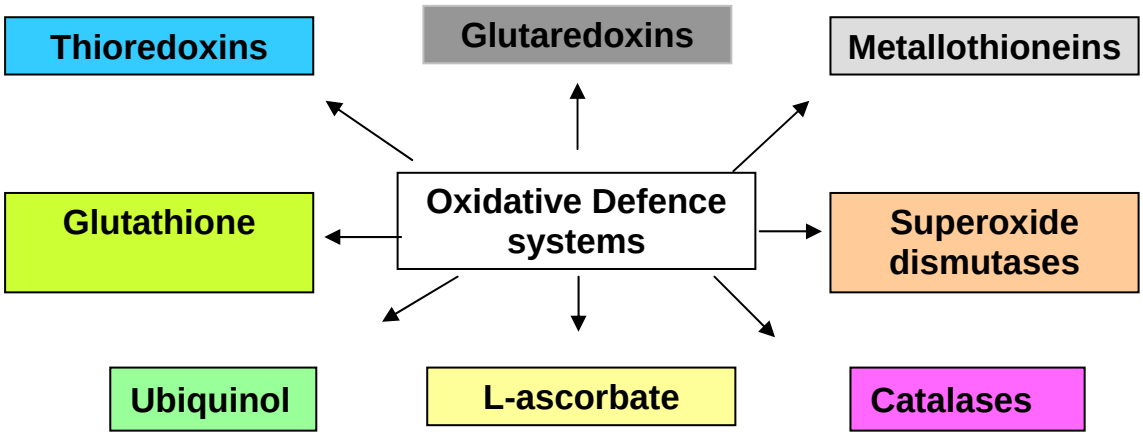

Fig. 6. Defense systems in response to oxidative stress. Defense systems include degrading or detoxifying ROS, maintaining metal ion homeostasis to prevent free metal ions from generating hydroxyl radicals, and repairing damaged molecules.

2.1.2.4 Intracellular probes for ROS detection

There are several probes, usually fluorescence based, that are used to detect the cellular production of ROS. We have focused our attention on two of them: the oxidation of Dihydrorhodamine 123 (DHR) and Dihydroethidium (DHE).

DHR is a fluorescent probe widely used to detect several ROS (OH^-, $ONOO^-$, NO_2^-, peroxidase derived species), but is poorly responsive to O_2^-, H_2O_2 and NO^- (Buxser *et al.,* 1999). DHR is oxidized to rhodamine, which is highly fluorescent around 536 nm. DHR is lipophilic and positively charged, and tends to accumulate in mitochondria, held there by the membrane potential (Buxser *et al*., 1999).

DHE, oxidized to a fluorescent product, is frequently used as a probe for O_2^-. This is usually thought to be ethidium, which tends to intercalate into nuclear DNA. Ethidium fluoresces strongly at around 600 nm.

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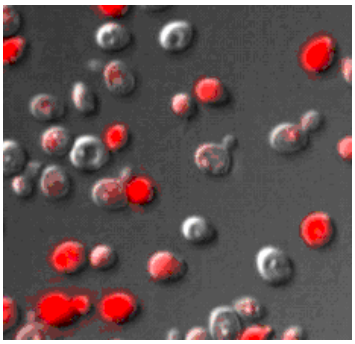

Fig. 7 Fluorescence microscopy of DHE→Ethidium conversion. Yeast cells of the BY4742 strain correspond to a stationary state culture in minimal media for 48 hours. The image shows the merging of red cells (DHE positive) and unstained cells.

2.1.2.5 Control of *S. cerevisiae* oxidative stress responses

Oxidative stress responses are activated by sophisticated redox sensory mechanisms that detect changes in the intracellular concentration of oxidants (Zehng and Storz, 2000). In *S. cerevisiae* the hunt for redox sensors has led to the discovery of the Yap1p transcription factor that co-regulates, in association with Skn7p, an important H_2O_2 – inducible oxidative stress response regulon (Brown and Bussey, 1993). Other transcription factors are involved in the oxidative stress response, in particular the Msn2/4 complex (Estruch, 2000; Boy Marcotte *et al.,* 1998).

Yap1p plays an essential role in regulating the oxidative stress response. Initially observed by (Schnell, 1992) based on the hypersensitivity of Δ*yap1* to H_2O_2 and other oxidants and drugs, this role was established by identifying the first Yap1p target gene, *TRX2* (Kuge and Jones, 1994).

Under non-stress conditions, Yap1p is restricted to the cytoplasm (Kuge *et al.,* 2001) by virtue of the rapid nuclear export receptor, Crm1/Xpo1, which recognizes and interacts with a non-canonical hydrophobic leucine-rich Nuclear Export Signal (NES) in the carboxyl terminal fraction of Yap1p (Kuge *et al*., 1998). This domain, approximately fifty amino acids, has been named the C-terminal cysteine rich domain (CRD) as it carries three repeats of the cysteine motif (Kuge *et al.,* 1997). Upon exposure to diamides, diethylmaleates (Kuge *et al.,* 1997) or peroxides (Coleman *et al*., 1999; Delaunay *et al*., 2000), Yap1p redistributes into the nucleus, due to the loss of interaction between Yap1p and Crm1p. Indeed, inactivation of Cmr1p, deletion of the C-terminal CRD, or substitution of leucine residues important for NES result in a constitutive nuclear localization of Yap1p (Kuge *et al.*, 1998). The kinetics of Yap1 activation/oxidation by H_2O_2 is very rapid, peaking at about 30 min and lasting for

about an hour. This suggests that Yap1p is somehow deactivated by reduction (Delaunay *et al*., 2000). Skn7p is another transcription factor that works, in cooperation with Yap1p, to activate genes in response to oxidative stress (Krems *et al*., 1996).

2.2.2 Osmotic stress

When cells are subjected to hyperosmotic shock, they lose cellular water because that water follows a concentration gradient by means of passive diffusion. Yeast cells frequently encounter dramatic and sometimes very rapid changes in osmolarity, for example, water availability in their natural habitat whose end effect is dehydration (Fig. 10). This phenomenon results in an increase in the inner concentration of molecules, which alters some cellular processes. To adjust to high external osmolarity, yeast cells have developed a battery of mechanisms to adapt to extracellular changes. Adaptation to these new conditions is a process that is divided in two phases: sensing osmotic changes and activating appropriate cellular responses. The accumulation of osmolytes plays a central role in osmoadaptation, and in *S. cerevisiae* this osmolyte is mainly glycerol (Brown, 1978; Yancey *et al*., 1982).

2.2.2.1 Hyperosmolarity

When *S. cerevisiae* is exposed to a higher osmotic pressure, the yeast instantly loses water from the cytoplasm to the medium. This leads to a reduction in cell volume, which is partially offset by the transfer of water from the vacuole to the cytoplasm. Nevertheless, this process leads to a decrease in or abolition of the turgor pressure of the membrane on the cell wall (Blomberg and Adler, 1992; Latterich and Watson, 1992).

In contrast to plant cells in which the plasma membrane shrinks away from the cell wall during plasmolysis, the entire cell envelope shrinks when yeast cells are placed in hypertonic solutions. The cell wall increases in thickness, buckling on the inner face to form irregular projections into the cytoplasm (Morris *et al.,* 1983).

The yeast cells that survive respond in the longer term to osmotic stress by means of the enhanced production and accumulation of a compatible solute glycerol at concentrations in the molar range (Alberty *et al.,* 1994) and the extrusion of ions by membrane

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ATPases.

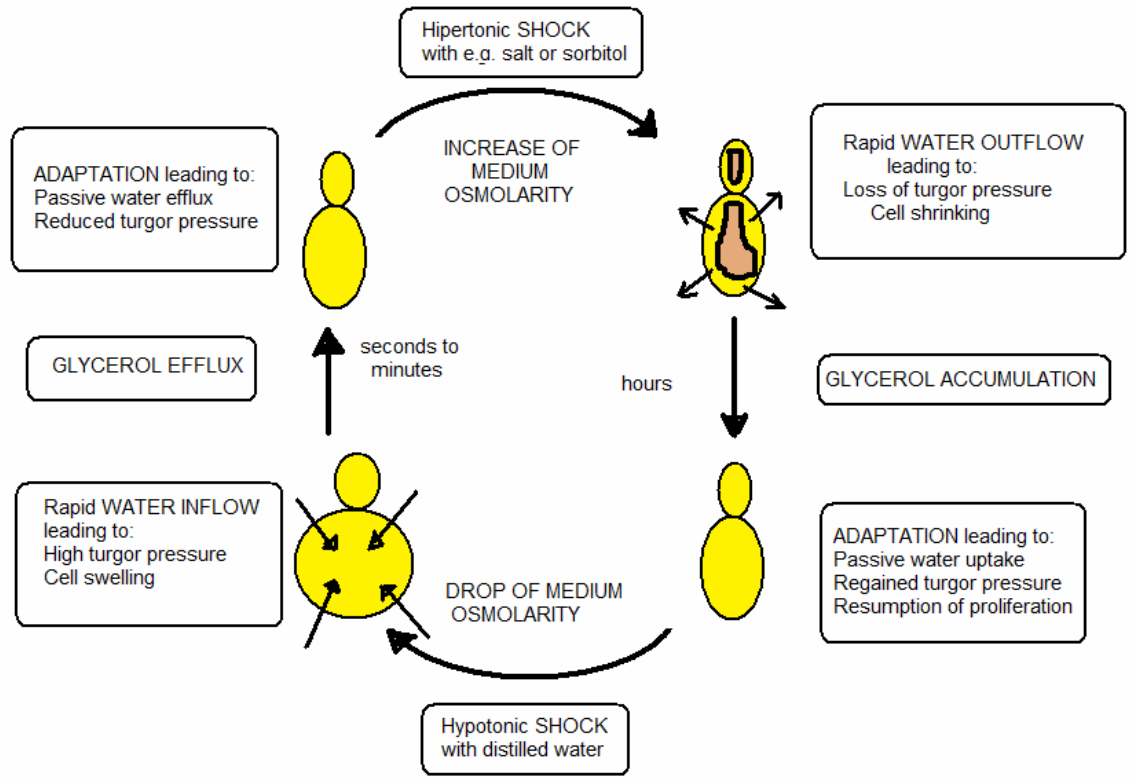

Fig. 8 Basic features of the response of *S. cerevisiae* to osmotic stress. Yeast exposed to an hypertonic shock (right side) suffer loss of turgor pressure and cell shrinking and upon hypotonic shock (left side) produce cell swelling with a glycerol efflux as a mode of response. Adapted from (Hohmann S. and Tamás M., 2003).

Compatible solutes are ions or compounds that can be accumulated in cells to balance internal and external osmolarity without seriously affecting physical and molecular processes. For other organisms, these can include sugars, sugar alcohols, potassium anion K^+, amino acids and glycine betaine. For yeast, glycerol appears to be the sole compatible solute (Brown, 1978; Hohmann, 1997). Under high salt conditions, other solutes such as trehalose may be important, possibly due to an overlap between the physiological effects of salt stress and other damaging conditions such as heat shock and oxidative stress, and the putative ability of trehalose to protect cellular structures such as membranes (Wiemke, 1990; Andre *et al*., 1991; Iwahashi *et al*., 1995). The involvement of a mitogen-activated protein kinase cascade, a conserved eukaryotic signal transduction module, in osmoadaptation has been reported (Brewster *et al.,* 1993; Gustin *et al.,* 1998). A consensus idea of osmoadaptation is conserved across eukaryotes, and thus yeasts are an ideal model system for studying these processes in order to better understand higher eukaryotes.

2.2.2.2 Signaling Pathways

Variability in medium osmolarity affects several different signaling pathways in *S. cerevisiae*: the cAMP-dependent protein kinase pathway, the phosphatidylinositol-3,5-biphosphate (IP3) pathway and, most importantly, the HOG pathway. Protein kinase A (cAMP-dependent protein kinase, PKA) has been shown to modify the expression of genes upon an osmotic shock (Norbeck *et al.,* 2000). PKA mediates a general stress response essentially under all stress conditions (e.g. oxidative stress, heat shock, high ethanol levels, nutrient starvation, and osmotic stress) (Marchler *et al.,* 1993; Ruis *et al.,* 1995). Thus, PKA probably does not respond directly to osmotic changes. PKA activity is controlled by stress, but it is not clear how it is regulated. An osmotic shock has been reported to stimulate the production of IP3, which could act as a second messenger in an osmotic signaling system (Dove *et al.,* 1997). However its actions have not yet been elucidated. Finally, there is the HOG pathway. The HOG pathway is the best-characterized system involved in osmostress. The HOG pathway is activated within seconds of osmotic shock (Brewster *et al.*, 1993) and cells lacking the pathway cannot survive in a high osmolarity medium. Therefore, the HOG pathway coordinates a significant part of the cellular response of yeast cells to high osmolarity.

2.2.2.3 MAPK Signaling Pathway

MAPK cascades share a tier of three consecutively activated kinases: a MAP kinase kinase kinase (MAPKKK or MEKK), a MAP kinase kinase (MAPKK or MEK), and a MAP kinase (MAPK). The MAPKKK activates the MAPKK, phosphorylating it on serine and threonine within a conserved N-terminal lobe of the kinase domain. After this activation, the MAPKK phosphorylates the MAP kinase on a tyrosine and threonine (sometimes serine), which are located adjacent to each other separated by a single amino acid. These residues are located at the activation loop of the catalytic domain. This double phosphorylation is a key step for the complete activation of the MAP kinase.

MAP kinase pathways are negatively controlled by protein phosphatases acting on two levels: the MAPKK (serine-threonine phosphatases) or on the MAP kinase (serine-threonine phosphatases and tyrosine phosphatases) (Keyse, 2000). Once MAPK is phosphorylated, this phosphorylation leads to a transfer of the MAPK from the cytosol to the nucleus, where it can phosphorylate its protein targets. MAPK targets can be divided

into two major groups, transcription factors and other proteins (Alepuz *et al*., 2003; Alepuz *et al.*, 1997 ; Andrews *et al*., 2000; Rouse *et al.*, 1994).

2.2.3 Desiccation tolerance mechanisms

Recently several studies have focused their attention on understanding mechanisms that allow anhydrobiotes survive after dehydration. These mechanisms include:

the replacement of water with sugars to form glasses,

proteins that stabilize macromolecules and membranes,

anti-oxidants that counter damage by ROS.

Knowledge of these mechanisms forms the basis for developing new methods for preserving biological materials that do not normally survive drying.

The plasma membrane is one of damaged targets during drying and rehydration. These damages must be repaired to restore viability. The main components of cell membrane are lipids and proteins. Thus, lipids synthesis is necessary to form new organelles and membranes, and consequently to proliferate and grow.

2.2.3.1 Plasma membrane structure

Biological membranes consist of lipids, proteins, and carbohydrates. Lipids include glycerolphospholipids (also called phospholipids), sphingolipids, and sterols. A membrane is a bilayer formed by phospholipids and sphingolipids organized in two single layers, with polar headgroups along the two surfaces and acyl chains forming the nonpolar domain in between. One of the most popular plasma membrane models is the Singer and Nicholson model, which considers the plasma membrane as a fluid-like phospholipid bilayer into which freely diffusing globular proteins are embedded to varying degrees (Singer, 1974; Singer. and Nicholson, 1972).

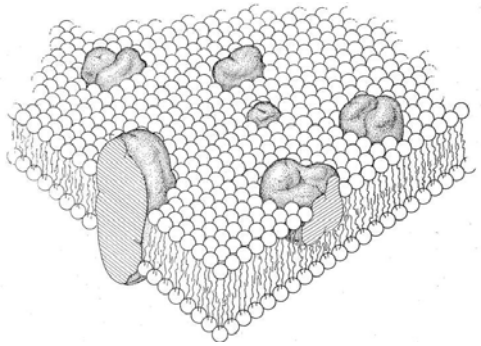

Fig. 9 Singer and Nicolson fluid mosaic model for membrane structure. Membrane as a fluid-like phospholipid bilayer into which freely diffusing globular proteins are embedded to varying degrees (Dr. Singer Drawing adapted from (Gennis R., 1989)

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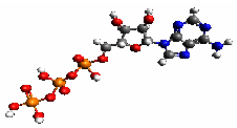
Lipid composition and the role of lipids in the plasma membrane

Fatty Acyl Chains

Fatty acid composition can greatly influence the physical state of phospholipids and thereby affect membrane fluidity and permeability. Oleic acid (18:1) and palmitoleic acid (16:1), together with trace amounts of palmitic acid (16:0) and stearic acid (18:0), are the principal fatty acyl chains in *S. cerevisiae* (Cottrell *et al.,* 1986; Rattray, 1988). The fatty acyl packing of these chains determines membrane fluidity to a large extent. Packing increases as the length of the acyl chains increases and as fluidity decreases. Perturbations of the bilayer that decrease the area of a lipid molecule, such as increased hydrostatic pressure, lowering of the temperature, or addition of sterols to phospholipids, also result in a decrease in fluidity (Shinitzky**,** 1984).

Phospholipids

Glycerophospholipids, commonly known as simply phospholipids (PL), are built on a glycerol molecule, which becomes chiral when derived to glycerol-3-phosphate. The backbone of membrane PLs is the L isomer, called sn-glycerol-3-phosphate. With fatty acyl chains in ester linkage on carbons 1 and 2 it becomes phosphatidic acid (PA). Esterification of PA with another alcohol creates the following PLs: phosphatidiylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG) and phosphatidylinositol (PI). In addition, PG can link through its glycerol headgroup to PA to form diphosphatidylglycerol (CL for its composition name, cardiolpin) (Luckey, 2008). The anionic phospholipid PLs (PS, PI, PG, and CL) have a net negative charge at physiological pH, while PE and PC are neutral.



Sphingolipids

Sphingolipids are ubiquitous components of eukaryotic plasma membranes. Studies on sphingolipids started with the discovery of these molecules in the human brain in 1884 (Thudichum, 1884). Current research on sphingolipids is focused primarily on their possible role in signal transduction across the plasma membrane (Ghosh, Bian and Gill, 1990; Hakomori, 1990; Hannun and Bell, 1989; Hannun *et al.,* 1986).

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Sterols

The primary sterol of yeast is ergosterol which shares many structural similarities with cholesterol, an important component of animal plasma membrane and which to a lesser extent is also found in their organellar membranes (Paltauf *et al*., 1992; Park and Casey, 1995; Zinser and Daum, 1995). One of the main functions of sterol is to maintain membrane fluidity in the face of environmental changes such as culture medium, temperature, etc. Since sterols are metabolically expensive cellular components, they may have other critical roles in the cell (Parks and Casey, 1995).

2.2.3.2 Lipid Phase Transitions

The most studied lipid phase transition is between a lamellar gel to a liquid crystalline phases. Phase transition can be induced by several ways, including changes in pressure (Chong, P. and Weber, 1983), temperature (Tenchov, B. G. *et al*., 1984), ionic strength, or pH (Cevc, G. 1987). Phase transition temperature is almost exclusivelly dependent on the fatty acyl chain which determines a relative stability of gel and liquid crystalline phases.

- Longer acyl chain lenghts result in higher Tm values, because the increase of the van der Waals interaction for longer chains.
- A *trans* double bond reduces the Tm as it will disrupt the ability of chains to interac optimally in the gel state.
- A *cis* double bond has an even larger effect than a *trans* double bond. The magnitude of the effect is dependent on the position of the double bond in the chain, with the maximal effect when the cis double bond is in the middle of the chain. (Russell, N.J., 1984).

2.2.3.3 The role of sugars in anhydrobiosis

A common feature in anhydrobiotes is the storage of large amounts of disaccharides in the dry state, basically trehalose or sucrose. In general, sucrose appears to be favored by seeds and higher plants, whereas microorganisms and small animals prefer trehalose (Crowe, 2006). In *S. cerevisiae* endogenous high levels of trehalose have been associated with dehydration resistance (Gaad, Chalmers and Reed, 1987).

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Trehalose is a non-reducing sugar formed from two glucose units. This sugar is a storage carbohydrate that can either be synthesized or obtained from the external environment. Trehalose can be used as a carbon source although enzymatic activity is necessary. A trehalase hydrolyses a trehalose into two molecules of glucose (Francois and Parrou, 2001). *S. cerevisiae* has two trehalase enzymes, an acid trehalase encoded by *ATH1* (Alizadeh, 1996) and a neutral trehalase encoded by *NTH1* (Kopp, 1993). Each trehalase is active in a different subcellular location. Thus Nth1p is a cytoplasmatic homodimer and it is required for the hydrolysis of intracellular trehalose (Jules, 2004). Intracellular trehalose either results from "*de novo*" biosynthesis, or to a lesser degree, from environment uptake via the Mal11p transporter (Jules, 2004). Ath1p was originally predicted to be a vacuolar protein, but experimental studies have shown it mainly localized in the periplasmic space, where extracellular trehalose is hydrolyzed (Jules, 2004).

2.2.3.3 The protective effect of trehalose

Initial studies about the interaction of trehalose with lipids were performed using phospholipid vesicles, or liposomes (Crowe *et al*., 1985). Trehalose lowers the gel to liquid crystalline transition temperature of dry lipids to below the hydrated transition of the same lipid (Crowe *et al*., 1985; Crowe and Crowe, 1988). A lipid or lipid mixture has a characteristic phase transition temperature; trehalose can lower the transition temperature of dry lipids between phases. Consequently lipids are in a liquid crystalline phase at the same temperature. Therefore, when lipids are rehydrated at room temperature, they do not undergo a phase transition, which are known to cause leakage in biological systems. This decrease is a consequence of the formation of hydrogen bonds between the sugar hydroxyls and phosphates of the phospholipid in the dry state (Crowe *et al.,* 1986, 1989 a, b; Drobnis *et al.,* 1993).

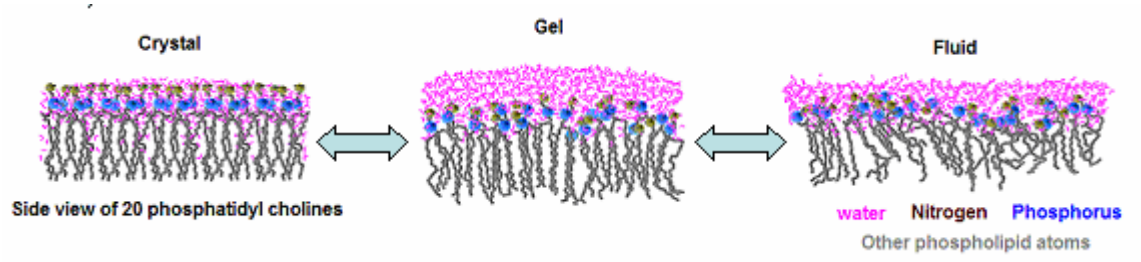

Fig. 10 Phase transition of phospholipids. Moving from left to right, the images show a crystalline to gel state transition. As a result of temperature transition, phosphatydilcoline layer in the left side (Crystal phase) suffer loss of packing due to a disorder in the lipid chains (fliud phase)

The size and shape of the sugar molecule affects the interaction and expansion of the phospholipid headgroup area. Trehalose is a disaccharide and provides more spacing than a monosaccharide such as glucose. Trisaccharides such as raffinose are even more effective than trehalose at lowering *T*m, but the effect is transitory, as phospholipids rapidly repack when held below *T*m and force raffinose out of the bilayer (Crowe *et al.,* 1996).

Like other sugars, trehalose is a good glass former. Glasses are amorphous solids with high viscosity in which the mobility of components is highly restricted. In the glassy state, deleterious interactions between components can occur, such as free radical formation (Heckly, 1978) and lipid peroxidation (Mouradian *et al.,* 1984, 1985) Glass transition temperature (*T*g) is the temperature above which the high viscosity state becomes a much more fluid, so-called 'rubbery' state in which mobility, and the potential for adverse reactions, increases (Sun and Leopold,1997).

2.2.3.4 Hydrophilins

Many transcripts and proteins are accumulated during drying in resurrection plants. Some of them have been cloned and sequenced (Galau *et al*. 1986), including gene products that may protect cytoplasmic structures during dehydration such as Late Embryogenesis Abundant (LEA) proteins. LEA proteins include a large group of proteins that accumulate in mature embryos during the onset of desiccation (Galau *et al*. 1986). This group of proteins, even if quite heterogeneous, is usually rich in hydrophilic amino acids, is water soluble and can be divided into different groups based on sequence similarities and properties (Goyal, 2005).

Hydrophilins are a group of proteins that share some characteristics with LEA proteins characteristics which they probably acquired as they evolved to respond to water-deficit conditions. Their defining characteristics are a high hydrophilic index and glycine content. Transcripts of most hydrophilins are accumulated in response to water deficit in organisms such as plants, fungi and bacteria (Garay Arroyo *et al*., 2000; Yale and Bohnert, 2001). Recent data suggests that LEA-like proteins are also found in fungi, and they also seem to be involved in pathways that respond to water deficit. In *S. cerevisiae,* Hsp12p (Sales *et al*., 1999) and Gre1p are LEA-like proteins (Singh *et al.,* 2005) because their amino acid composition shows a high content of hydrophilic amino acids that are distributed throughout their primary structure.

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STRESS RESPONSE

Based on the literature, *in vitro* studies and most recently complete genome data bases of *S. cerevisiae* and *Escherichia coli,* a high proportion of LEA-like proteins, chosen by physicochemical criterion, have been found to be induced at the mRNA level under water-deficit conditions. Therefore, hydrophilins exist in several kingdoms, including plant, bacteria, and fungi, and they have been recruited from diverse protein families to pathways involved in responses to water-deficit conditions (Garay Arroyo *et al*., 2000)

3. CELL DEATH DURING DEHYDRATION / REHYDRATION

During yeast dehydration, the viability of the population is seriously compromised by the sum of different severe stresses. Dehydration causes a rapid water efflux through the membrane, resulting in cytoskeleton collapse (Walker and van Dijck, 2006). This dry state may affect yeast cell physiology, altering the structure and function of vacuole, and the integrity and functionality of nuclear and cell membranes (Walker and van Dijck, 2006). The desiccation of cells causes molecular changes that completely arrest cellular metabolism. This is followed by a period in a state of suspended animation, and subsequent recovery of metabolic functions when water is again available. The liquid-crystalline phase transition experienced by both dry membranes and lipid bi-layers during rehydration leads to changes in their permeability (Crowe *et al*., 1989, 1992). The drying and rehydration process increases the leakage of nucleotides, ions and other soluble cell components (Rapoport *et al*., 1995).

As a result of drying and rehydration, there is a high mortality in yeast populations, which is partially genetically dependent and partially dependent on the drying-rehydration conditions. Previous studies have shown that after rehydration yeast viability can fluctuate between 60-85% (Van Stevenick and Ledeboer, 1974; Becker *et al.,* 1984; Attfield *et al.,* 2000). However, cell viability can improve after freeze-drying and increase by 66-98% by blending yeast cells with skimmed milk and various sugars. Unfortunately, this technique is not cost effective for industrial applications (Berny and Hennebert, 1991). The response to drying largely depends on the metabolic status of the cell (Potts, 2001). Damages suffered during air-drying and long-term desiccation become detrimental protagonists of cell recovery during the rehydration process, at which time cells can undergo a dynamic change in protein and nucleic acid content (Shirkey *et al*., 2000).

3.1 Mechanisms of yeast cell death

Cell death can occur by one of two distinct mechanisms (Schwartzman and Cidlowski, 1993; Vermes and Haanan, 1994): necrosis or apoptosis. In addition, certain chemical compounds are said to be cytotoxic to the cell, which also causes its death.

The two mechanisms of cell death can briefly be defined as follows:

Necrosis (“accidental” cell death): a pathological process which occurs when cells are exposed to a serious physical or chemical aggression;

Apoptosis ("normal" or "programmed" cell death): the physiological process by which superfluous or useless cells are eliminated during development and other standard biological processes.

There are numerous recognizable morphological events and biochemical properties that differentiate between necrosis and apoptosis (Vermes and Haanan, 1994) (Fig.14).

3.1.1 Necrosis

Necrosis occurs when cells are exposed to an extreme variance in physiological conditions (*e.g.* hypothermia, hypoxia) which can result in damage to the plasma membrane. Under some physiological conditions direct damage to the plasma membrane is brought about by agents such as complement factors and lytic viruses.

Necrosis begins with an impairment of the cell's ability to maintain homeostasis, leading to an influx of water and extracellular ions. Intracellular organelles (most notably the mitochondria) and the entire cell swell and rupture, causing cell lysis. Due to the ultimate breakdown of the plasma membrane, cytoplasmic contents including lysosomal enzymes are released into the extracellular fluid. Therefore, *in vivo* necrotic cell death is often associated with extensive tissue damage resulting in an intense inflammatory response (Van Furth and Van Zwet, 1988).

3.1.2 Apoptosis

Apoptosis, in contrast, is a mode of cell death that occurs under normal physiological conditions and cells are an active participant in their own demise ("cellular suicide"). It frequently occurs in several different processes: normal cell turnover and tissue homeostasis, embryogenesis, induction and maintenance of immune tolerance, development of the nervous system and endocrine-dependent tissue atrophy.

Cells undergoing apoptosis show characteristic morphological and biochemical features (Cohen, 1993). These features include chromatin aggregation, nuclear and cytoplasmic condensation, partition of cytoplasm and nucleus into membrane bound-vesicles (apoptotic bodies) which contain ribosomes, morphologically intact mitochondria and nuclear material.

In vivo, these apoptotic bodies are rapidly recognized and phagocytized by either macrophages or adjacent epithelial cells (Savill *et al*., 1989). Due to this efficient mechanism for the removal of apoptotic cells *in vivo,* no inflammatory response is elicited. *In vitro*, apoptotic bodies as well as the remaining cell fragments ultimately

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swell and finally lyse. This terminal phase of *in vitro* cell death has been termed "secondary necrosis" **(Fig. 11)**.

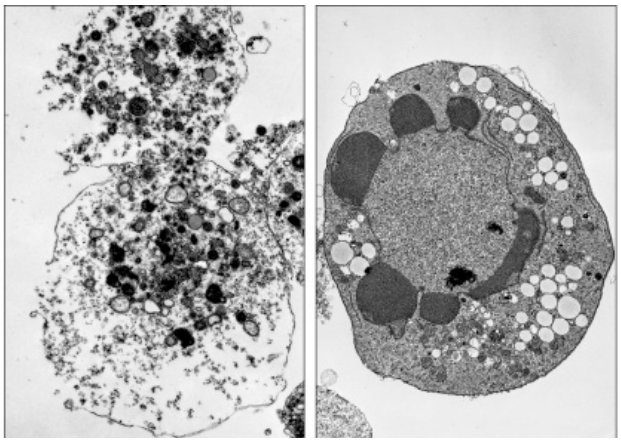

Fig. 11 Necrosis and Apoptosis are two mechanisms of death with observable morphological and biochemical differences. In the left panel a necrotic cell swell with rupture of the plasma membrane, in contrast in the right an apoptotic one shrinks showing apoptotic bodies.

Routes leading to death in yeast are multiple and include exogenous and endogenous triggers. Many different drugs, the heterologous expression of pro-apoptotic human genes, defects in cellular processes, aging and mating stress all lead to an apoptotic phenotype in yeast.

3.2 Apoptosis markers

3.2.1 Phosphatidylserine externalization

In the early stages of apoptosis, changes occur in the cell surface and plasma membrane. One of these plasma membrane alterations is the translocation of phosphatidylserine (PS) from the inner to the outer side of plasma membrane, by which PS becomes exposed on the external surface of the cell.

3.2.2 DNA fragmentation

A hallmark of apoptosis is genomic DNA fragmentation, an irreversible event that commits a cell to die and occurs before changes in plasma membrane permeability (prelytic DNA fragmentation). In many systems, DNA fragmentation has been shown to result from the activation of an endogenous Ca^{2+} and Mg^{2+}- dependent nuclear endonuclease. This enzyme selectively cleaves DNA at sites located between nucleosomal units (linker DNA) generating mono- and oligonucleosomal DNA

fragments.

3.2.3 Mitochondrial changes

Mitochondrial physiology is disrupted in cells undergoing either apoptosis or necrosis. During apoptosis mitochondrial permeability is altered and apoptosis specific protease activators are released from mitochondria. Specifically, the discontinuity of the outer mitochondrial membrane results in the redistribution of cytochrome C towards cytosol followed by the subsequent depolarization of the inner mitochondrial membrane. Aif1p (Apoptosis Inducing Factor), is released in the cytoplasm and has proteolytic activity and is by itself sufficient to induce apoptosis.

3.4 Exogenous and endogenous ways to elicit yeast Apoptosis

Numerous stimuli can induce yeast cell apoptosis. Such stimuli can be provided externally in the form of chemical or physical stress or via a heterologous expression of human pro-apoptotic proteins (exogenous triggers) or can be generated by the yeast cells themselves, as part of lethal signal transduction pathways (endogenous triggers) (Carmona-Gutierrez *et al*., 2010).

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Apoptosis triggers	Effects	References
	Exogenous triggers	
H_2O_2	High doses lead to a necrosis / low doses induce apoptosis	Madeo F. *et al*., 1999
Acetic Acid	Cytochrome c release associated with enhanced mitochondrial membrane potential and the loss of COX activity / apoptosis induction	Ludovico P. *et al.*, 2000
Heat and osmotic stress, UV, HOCl and ethanol	Induction the apoptotic phenotype	Silva RD *et al.,* 2005; King DA, *et al.,* 2004; Kitagaki H, *et al.,* 2007
Copper and Manganese	Lead to apoptosis at moderately toxic levels .	Liang Q *et al.*, 2007
Excess of Iron	Leads to yeast death probably in a programmed fashion	Cabiscol E *et al.*, 2002; Desmyter L *et al.*, 2004; Almeida T *et al.*, 2007
Calcium	Calcineurin/calmodulin system is proposed to inhibit apoptosis.	Bonilla M *et al.*, 2002
Plant toxins ricin and osmotin	Trigger apoptosis	Gourlay CW *et al.*, 2006; Li XP *et al*., 2007
Amphibian-derived peptides from the dermaseptin family	Induce Aif1p-dependent but Yca1p-independent yeast apoptosis	Morton C. *et al.,* 2007
Heterologous expression of the human apoptotic inducer Bax	Leads to cell death in yeast, cytochrome c release, ROS accumulation and apoptotic features	Madeo F *et al.*, 1999; Ligr M *et al.*, 1998, Matsuyama S. *et al.*, 1998
	Endogenous triggers	
Defects in several cellular processes	N-glycosylation, chromatid cohesion and ubiquitination cause apoptosis	Hauptmann P *et al.*, 2006; Ren Q *et al.*, 2005
DNA damage and replication failure	Trigger cell death in yeast	Weinberger M. *et al.*, 2005; Qiu J *et al.*, 2005
Replicative aging	Produce apoptotic features	Laun P. *et al.*, 2001
Chronological aging	Cell death showing markers of apoptosis	Wissing S. *et al.*, 2004; Herker E. *et al.*, 2004

Table 4 Exogenous and endogenous ways to elicit yeast Apoptosis.

3.2.2 Proteasome and proteases

The first molecular evidence for yeast apoptosis arose from experiments conducted in a *CDC48* mutant (*cdc48S565G*) which exhibited an apoptotic phenotype (Madeo *et al.*, 1997*)*. This protein is described as an ATPase in endoplasmic reticulum with a nuclear membrane and cytosol homologous to mammalian p97, in a complex with Npl4p and Ufd1p which participates in the retrotranslocation of ubiquitinated proteins from the ER into the cytosol for degradation by the proteasome **(**Ye *et al.*, 2003**).**

This death has recently been linked to mitochondria: *cdc48S565G* mutants show proteomic alterations in mitochondria, the release of cytochrome c to the cytosol and increased ROS production (Braun *et al.*, 2006).

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CELL DEATH

As signals leading to the activation of a family of intracellular cysteine proteases, caspases (Cysteinyl-aspartate-specific proteinases) play a pivotal role in the initiation and execution of apoptosis induced by various stimuli.

The yca1p or mca1 (MetaCAspase) is a putative cysteine protease similar to mammalian caspases involved in the regulation of apoptosis upon hydrogen peroxide treatment and in cell cycle progression (Madeo *et al.*, 2002; Lee *et al.,* 2008). In conditions of oxygen stress, the disruption of *YCA1* leads to decreased cell death and the reduced formation of apoptotic markers **(**Madeo *et al.*, 2002**).**

Furthermore, *YCA1*-dependency has been implicated in numerous cell death scenarios including valproic acid induced cell death (Mitsui *et al*., 2005) as well as for apoptosis following defects in different cellular processes including loss of ubiquitination control (Bettiga *et al*., 2004), altered mRNA stability (Mazzoni *et al.*, 2005), and defective initiation of DNA replication (Weinberger *et al.,* 2005). Interestingly, regulated cell death during the long-term development of multicellular yeast colonies is independent of *YCA1* (Vachova *et al.*, 2005). This example emphasizes the fact that Yca1p involvement is not necessary for all apoptotic scenarios.

Moreover, apoptotic death mediated by Nuc1p, the yeast homologue of Endonuclease G, or the yeast apoptosis inducing factor Aif1p is *YCA1*-independent (Wissing *et al.*, 2004; Morton *et al.*, 2007; Büttner *et al.*, 2007).

Another protease involved in yeast apoptosis is Nma111p (nuclear mediator of apoptosis), the yeast homologue of the pro-apoptotic mammalian HtrA2/Omi. The deletion of *NMA111* reduces apoptotic markers while the overexpression of Nma111p increases cell death upon elevated temperature or H_2O_2 treatment (Fahrenkrog *et al.,* 2004).

Recently, it was shown that Bir1p, the only known inhibitor-of-apoptosis (IAP) protein in yeast which is localized to the cytoplasm and the nucleus, is a substrate for Nma111p. When challenged with oxidative stress, *BIR1* disruptants show enhanced apoptosis.

Consistently, overexpression of Bir1p reduces cell death, an effect that can be antagonized by the simultaneous overexpression of Nma111p. The overexpression of Bir1p also affects chronological aging by delaying cell death, whereas in this scenario *BIR1* disruption has no significant effect (Walter *et al.*, 2006).

Another important nuclear process is epigenetic cell death control. As described for mammals (Cheung *et al*., 2003), histone H2B phosphorylation is also necessary for cell death induction upon oxidative stress in yeast (Ahn *et al.*, 2005). Therefore, H2B

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epigenetics may be cross-linked to replicative life-span control and/or the MAPK cascade in a manner yet to be defined (Carmona-Gutierrez and Madeo, 2006).

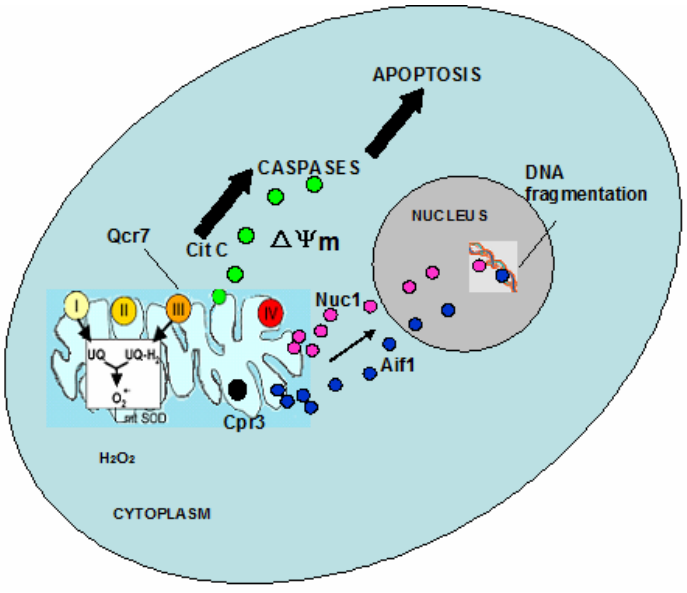
3.4.3 Mitochondrial factors: toxic when released

One caspase-independent manner of death is the apoptosis inducing factor (AIF). With the identification of an AIF homologue in yeast (Wissing *et al.*, 2004) it has become clear that the roots of cell death may harbor at least in part different Programmed Cell Death (PCD) types beyond caspase-dependent cell death pathways. As in its mammalian counterpart, yeast Aif1p translocates from the mitochondria to the nucleus when apoptosis is induced (by H_2O_2, acetate, or aging) leading to chromatin condensation and DNA degradation (Wissing *et al.*, 2004).

Recently, a yeast orthologue of mammalian endonuclease G (EndoG), *NUC1*, was identified and its apoptotic function characterized (Büttner *et al.*, 2007). Mammalian EndoG is mitochondrially located and translocates to the nucleus upon the induction of apoptosis (Li *et al.,* 2001; Parrish *et al*., 2001).

The disruption of *NUC1* inhibits apoptotic cell death when mitochondrial respiration is increased but enhances necrotic death when oxidative phosphorylation is repressed, discriminating between vital and lethal functions of *NUC1*. Finally, *NUC1* mediated death is *YCA1* and *AIF1* independent but requires yeast homologues of the mammalian permeability transition pore, the karyopherin Kap123p, and histone H2B (Büttner *et al.*, 2007).

A further mitochondrial protein, cytochrome c, is released when apoptosis is triggered via heterologous Bax or acetic acid induction (Ludovico *et al.*, 2002).



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Vitality enhancement of the rehydrated active dry wine yeast

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Abstract

In winemaking, spontaneous grape must fermentations have been replaced by inoculation of commercial Active Dry Wine Yeast (ADWY). Yeast rehydration is key to avoiding stuck and sluggish fermentations. Despite the importance of this step, not enough is known about what this process implies for winemaking as a whole or about what kind of practices could help to improve it. The main aim of this study is to determine the best yeast rehydration conditions for ensuring good cell viability and vitality before inoculation into the must.

The experimental rehydration media in this study can be divided into four groups: carbon and nitrogen compounds (glucose, raffinose, trehalose, proline and glutamate), metallic ions (Ca, Mg and Fe), oxidant and antioxidant agents (SO_2, ascorbic acid and H_2O_2), and membrane fluidity agents (DMSO, benzyl alcohol, 2-phenyl 1-butanol, 2-phenyl 1-propanol, ethyl hexanol, p-cresol and ethyl-phenol). We studied the biochemical and biophysical behaviour of ADWY after rehydration in the various media under oenological conditions, i.e. incubation at 37ºC for 30 minutes. The viability of rehydrated yeast cells was evaluated by plating, and vitality was assessed by fluorescence microscopy and flow cytometry. The vitality of rehydrated cells was estimated by indirect impedance. The rehydrating solution complemented with magnesium provided the best vitality rate because the time taken to reach the activity threshold was cut by two thirds. This improvement was also illustrated by the less time needed to stop the leakage of intracellular compounds during the rehydration process.

Introduction

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DEHYDRATION TOLERANCE IN YEAST
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RESULTS

CHAPTER I

In the last few decades, spontaneous grape must fermentations, which have traditionally been used in winemaking, have been replaced by the inoculation of commercial Active Dry Wine Yeast (ADWY). When selected ADWY are used, they must be rehydrated before they can be added to musts in the fermentation tanks. Cells are recovered beforehand in water at 37-43ºC for 30 minutes (Ribéreau-Gayon *et al*., 2004). This water is required by the cell for the biochemical reactions and for the interaction between structural macromolecules and components of the plasmatic membrane (Rapoport *et al*., 1995). Despite the importance of this step, in traditional winemaking practices this process has not been sufficiently questioned scientifically. The scarce molecular information about the impact of yeast rehydration process in wine fermentation is puzzling and we still await a breakthrough that would enable its optimisation. Attempts have recently been made to describe the early transcriptional response of wine yeasts during and after rehydration, which have shown changes on the genes involved in lipid binding and synthesis, protein synthesis, and metabolism (Singh *et al*., 2005; Novo *et al*., 2007). The main parameters that impact on the physiological stability and fitness of ADWY, and therefore on the fermentation process, are yeast viability and vitality. Differentiation between yeast viability and vitality is based on these cells, which irreversibly lose their ability to reproduce even though they remain metabolically active. Viability is the cell's capacity to develop a colony and vitality is the measure of metabolic activity in relation to the robustness of a starter culture. Predicting these parameters is the main way ADWY producers and winemakers can avoid stuck and sluggish fermentations (Reed and Nagodawithana, 1991).Traditionally, the most popular methods for evaluating viability are the observation of colony-forming units on plate and direct counting of dyed-dead cells under the microscope. More recently, flow cytometry in combination with fluorescent biological dyes have been used to evaluate cell viability in several physiological conditions (Deere *et al*., 1998; Attfield *et al*., 2000). Previous studies have shown that values of cell viability after rehydration can fluctuate between 60 and 85% (Van Stevenick and Ledeboer, 1974; Becker *et al*., 1984; Attfield *et al*., 2000). However, viability after freeze-drying increased from 66% to 98% by blending yeast cells with skimmed milk and various sugars before treatment. Unfortunately, this technique is not cost effective for industrial applications (Berny and Hennebert, 1991). The mode of response to drying any cell population largely depends on its metabolic status when water is removed (Potts, 2001). Damages suffered during air-drying and long-term desiccation become detrimental

protagonists of cell recovery during the rehydration process. At that moment the cell could undergo a dynamic change in protein and nucleic acid content (Shirkey *et al*., 2000). Dehydration causes a rapid efflux of water through the cell membrane, resulting in the collapse of the cytoskeleton. This dry state may deleteriously affect yeast cell physiology by altering the structure and function of vacuole, and the integrity and functionality of nuclear and cell membranes (Walter and van Dijck, 2006). The liquid-crystalline phase transition experienced by both dry membranes and lipid bi-layers during rehydration leads to changes in their permeability (Crowe *et al*., 1989; 1992). Drying and rehydration processes increase the leakage of nucleotides, ions and other soluble cell components (Rapoport *et al.*, 1995). Cellular membrane is rapidly repaired during rehydration, and a fast mobilization of lipid storage within the cells has been observed (Becker *et al*., 1984). It has been shown that ADWY rehydration in the presence of a high concentration of inactive dry yeast (150 $g \cdot l^{-1}$ dry weight) increases the fermentation rate and thus diminishes the duration of fermentation, which suggests the transfer of sterols between inactive and active dry yeasts (Dulau *et al*. 2002; Soubeyrand *et al*., 2005).

In the present study, we analyse ADWY biochemical and biophysical features during the rehydration process in several physiological solutions in order to determine whether rehydrating could enhance the fitness of the recovered cells and improve early yeast adaptation to the grape must after inoculation (which could allow a shorter lag phase performance). We therefore report the determination of cell viability by plating and the determination of cell vitality by impedance and fluorescent methods together with the kinetic leakage of intracellular compounds.

Materials and Methods

Yeast strain and re-hydration conditions

The active dry wine yeast strain *Saccharomyces cerevisiae* QA23 (Lallemand S.A., Canada) was used throughout this study. The effect of the rehydration solutions on ADWY after rehydration was studied by adding each compound individually to the pure water basal condition. Cell viability and vitality was determined for the following compounds: 1% proline; 1% glutamate; 1% raffinose; 50, 2 and 0.5% glucose; 1 and 0.25% trehalose; 3 mM calcium; 5, 10 and 50 mM Mg; 1 and 2 mM $FeSO_4$; 1 and 2 mM $FeCL_3$; 5, 10, 20 and 50 $mg \cdot l^{-1}$ sulphur dioxide; 1, 5, and 10 mM ascorbic acid; 0.1

and 0.5 mM hydrogen peroxide; 125 mM dimethyl sulfoxide (DMSO); 5 mM benzyl alcohol; 5 mM 2-phenyl 1-butanol; 5 mM 2-phenyl 1-propanol; 5 mM ethyl hexanol; 5 mM *p*-cresol; 5 mM *o*-cresol; 5 mM *m*-cresol; and 3, 5 and 7 mM 2-ethyl-phenol. In all cases, 1 g of ADWY was re-hydrated into 10 ml final volume at 37ºC for 30 minutes in accordance with the manufacturer's specifications.

Determination of yeast viability

The viable cell count was calculated by spreading cell dilutions, after the re-hydrating process, using a Whitley Automatic Spiral Plating (AES *Laboratoire*, France) on YPD agar medium. The plates were incubated at 28°C for 48 hours and the CFU were quantified using the ProtoCOL SR/HR counting system software version 1.27, supplied by Symbiosis (Cambridge, UK). The commercially available kit LIVE/DEAD *Bac*Light (Molecular Probes, USA) was also used to determine cell viability after rehydration in plain water. Although this kit was primarily developed to detect bacterial viability, it can also be used to determine yeast viability (Zhang and Fang, 2004) and comprises two nucleic acid dyes: SYTO9, which emits green fluorescence and propidium iodide (PI), which emits yellow fluorescence. SYTO9 stains all cells, regardless of their viability, whereas PI stains only cells with damaged membrane integrity, and masks SYTO9 stain fluorescence. Samples were stained according to the manufacturer's specifications by mixing approximately $1.5 \cdot 10^6$ cells with 1.5 µl of SYTO9 and 0.7 µl of PI. The stained samples were examined by fluorescence microscopy (Leica DM4000 B) and both the total cell number and the differential stained cells were counted with a Neubauer chamber.

Determination of yeast vitality

To test cell vitality (fermentation activity), we used the *Bac*Trac 4300 microbiological analyzer (SY-LAB Instruments, Austria). This device is based on the measurement of variations in electrical impedance experimented by a chemical solution (Owens *et al.*, 1989; Ribeiro *et al*., 2003). The typical measured impedance curve can be divided into three phases: the adaptation phase, the exponential phase and the stationary phase. The central range defined by the point of inflection after the adaptation phase and the earlier exponential phase is the most relevant for this measuring technology (Ribeiro *et al*. 2003), which was defined for rehydrated yeast in pure water from 5 to 15 % of impedance variation. The CO_2 produced by the metabolic activity of yeast is absorbed

by a KOH solution remaining in the impedance cell. The 0.2% KOH solution was prepared fresh with extra pure water before each experiment. After rehydration, the cells were immediately inoculated into a glass vial containing 5 ml YPD at a reason of 10^7 cells·ml^{-1}. Using a separate container, the open vial with the sample was immediately introduced into the measuring cell containing 1 ml of KOH solution. The measuring cell was then immediately tightly sealed and incubated. BacTrac monitored the impedance level every 10 minutes, drawing a curve to represent the percentage decrease of impedance over time. The time taken to reach the 10% decrease of total impedance at 15ºC was selected as threshold value. The M10% discrepancy between water condition and the complemented rehydration conditions was calculated using the formula $dM10\% = (M_{H20}10\% - M_n10\%) \cdot M_{H20}10\%^{-1}$ (Fig. 2). That low temperature was helpful for showing the evolution of vitality by recording a significant number of impedance values, taking into account the unique 10 min span allowed between two successive measurements by the BacTrac device. The results were statistically analysed by one-way ANOVA and the Scheffé test from the statistical software package SPSS 13.0. Statistical significance was set at $P < 0.05$.

Measurement of intracellular nucleotide leakage

The re-hydrated ADWY cells were harvested by centrifugation at 5000 rpm for 3 minutes at 4°C. The supernatant's absorbance values at 260 and 280 nm were used to calculate the nucleotide equivalents in mg·ml^{-1} = $(0.063 \cdot A_{260}) - (0.036 \cdot A_{280})$ (Herbert *et al*., 1971). The whole intracellular nucleotides per gram of ADWY was calculated in 3.01 mg·ml^{-1}. These analyses were done at least in triplicate and standard deviations were <10%.

Determination of analytical trehalose

Both the extracellular and intracellular trehalose contents of the ADWY QA23 strain were determined following the protocol described by Parrou and François, 1997.

Flow cytometry analysis

Flow cytometry was carried out using a *Cell Lab Quanta™ SC* instrument (Beckman Coulter, USA) fitted with a 22 mW ion laser for excitation (488 nm) while monitoring with a single emission channel (575-nm band-pass filter). *Cell Lab Quanta SC* software (Beckman Coulter, USA) was used for instrument control, data acquisition and data

analysis. We took, as control of full viability (99% by PI stain), an overnight YPD culture of the QA23 strain.

Results and discussion

Evaluation of metabolic activity of re-hydrated yeast

Because cell mortality and loss of vitality upon rehydration is one of the main problems of this process, several methods have been used to obtain a rapid and consistent evaluation of the yeast metabolic activity at this specific time-point. The estimation of the cell vitality by extrapolation of counting CFU (colony-forming units) on plates does not correlate with the average metabolic performance of the cell population after the rehydration process. We therefore used several methods to evaluate the QA23 vitality of commercial wine yeasts after they had been rehydrated for 30 min in warm water (37°C), which is the protocol widely used by winemakers. Fluorescent dyes enable us to analyse functional cell parameters, including structural properties, biological activities and viability indicators (Deere *et al.,* 1998; Attfield *et al*., 2000; Malacrino *et al*., 2001). The similarity in the number of cells treated with both fluorescent methods was assessed by microscope cell counting ($1.5 \cdot 10^6$ cells per staining reaction), and the full vitality control was an overnight culture grown in YPD that showed approximately 96% of live cells regardless of the indicator (data not shown). The determination of viability by differential staining patterns for live and dead yeast cells was scored with the indicators SYTO9 and PI. Fig. 1A shows the wide-field epifluorescence microscopy of re-hydrated cell yeasts stained with SYTO9 (LIVE/DEAD® Yeast Viability Kit*,* Molecular Probes, USA). The relative proportions of unstained and stained cells by PI were quantified by flow cytometry (see Fig 1C). Approximately 74% of the cell yeast showed membrane integrity after re-hydration. Fluorescence microscopy of the rehydrated yeast treated with SYTO9/PI showed around 72% live and 28% dead cells. These data agree with the values of live and dead yeast cells shown by the PI stain. These distributions enabled us to choose the best method for discriminating between live and dead cells in our study. For further evaluations, we continued to use the flow cytometry quantification method because of its high repeatability and cost effectiveness. Moreover, the shorter yeast PI treatment time (30 s) compared with the 30 min required by SYTO9/PI guaranteed that there was no interference by any of the yeast's early responses after rehydration, as had been reported by Novo *et al*. 2007. For both stain evaluation experiments we also determined the number of $CFU \cdot ml^{-1}$ of re-hydrated

yeast. A similar mean of $1.05 \cdot 10^6$ CFU·ml^{-1} for the duplicate experiments was found for the rehydrated cells before they were subjected to cell staining treatment. However, the same cells counted at the microscope of the overnight culture grown control showed $1.44 \cdot 10^6$ CFU·ml^{-1}. The approximately 4% difference in mortality between plating and fluorescence techniques suggests that after rehydration a number of metabolic active cells lose their ability to form a colony. Similar or higher levels of mortality after rehydration, depending en the technique used, have previously been reported (Poirier *et al*., 1999).

It is interesting, therefore, to further study the rehydration process and to test alternative rehydration media in order to improve yeast viability and vitality during rehydration.

Evaluation of cell vitality under several rehydration conditions

We have tested several rehydration media to try to overcome the drop in yeast vitality and viability during this process. The rehydration media used in this study can be divided into four groups: carbon and nitrogen compounds; metallic ions; oxidant and antioxidants; and membrane fluidity (Fig. 2). In this experiment, all additives were tested using deionised water as a suspending medium, and pure water as the reference condition for the evaluation of yeast cell viability and vitality. After rehydration, cell vitality was assessed by the impedance technique. The CFU·ml^{-1} mean values of rehydrated samples before and after the vitality test were similar for all tested substrates. The vitality capacity of the cells rehydrated in the presence of carbon and nitrogen was tested and tabulated (Fig. 2A). Trehalose present during yeast drying has been shown to act as a membrane protector that reduces the membrane phase transition temperature during the rehydration process (Leslie *et al.* 1994). Rehydration with extra trehalose already present in the commercial QA23 strain needs roughly one more hour to reach M10% than the reference condition. The raffinose solution, which gave the worst performance of all carbon sources, showed a similar pattern. For 2% glucose, the preferred *S. cerevisiae* sugar, there was a slight reduction in the time needed to reach M10%. These results agree with those of Novo *et al.* (2007), who suggest that yeast's transcriptional switch after rehydration is led by the presence of fermentable sugar. Also, the other glucose concentrations delay the metabolic reactivation of the rehydrated cells by 30 min. Impedance graphs show that these slower performances reflect the extra adaptation time required by the cells to overcome the differences in glucose concentration between the rehydrating media and the YPD media used with the

impedance technique (data not shown). Increasing the intracellular proline of desiccated *S. cerevisiae* by two-fold led to a five-times-higher survival rate (Takagi *et al.* 2000). Proline rehydration shows the same vitality pattern as both the control and the monosodium glutamate. Moreover, the similar mean values of $CFU \cdot ml^{-1}$ for these three conditions illustrate that proline supplementation during the rehydration process does not enhance yeast survival rate.

The availability of certain metal ions in the fermentable media becomes a key factor for *S. cerevisiae* to develop an accurate fermentation performance (Jones and Greenfield 1984, Dedyukhina and Eroshin 1991, Walker 2003). We therefore evaluated the availability of several ions during the rehydration process to assess any impact on yeast vitality (Fig. 2B). The calcium, ferrous (Fe^{+2}) and ferric (Fe^{+3}) ion complementation had a deleterious effect on yeast vitality. However, the beneficial effect of 5 and 10 mM Mg complementation was shown by the 2/3 reduction in threshold time. The higher magnesium level led to lower yeast vitality. On the other hand, rehydrating conditions co-complemented with 10 mM magnesium and 3, or 5 mM calcium profiled broadly similar than 3 mM calcium condition (data not shown). These results suggest that calcium exerts a suppressive effect on yeast vitality through an antagonism of magnesium. Magnesium is involved in numerous functions essential to yeast physiology, such as the respirofermentative metabolism and the responses to environmental stress (Birch and Walker, 2000). It is also interesting to note that when magnesium in the growth medium becomes limiting, cells not only cease to grow but actually die. This contrasts with starvation for most other nutrients whose cells have mechanisms that allow them to enter G_o, where they can survive for extended periods. Cells that enter G_o due to lack of glucose remain viable in magnesium-free medium, which indicates that it is the dilution of cellular magnesium by growth that leads to cell death in the magnesium-free medium (Beeler, *et al.* 1996).

The AWDY biomass propagation process occurs in high concentration of molasses, which requires high aeration to achieve the optimum fully oxidative growth. The most important adaptive responses developed by *S. cerevisiae* during biomass production, are related to osmotic and oxidative stress (Pérez-Torrado, *et al.* 2005). Compressed yeast is then extruded to form strands and generally dried using a continuous tunnel dryer. This drying process produces highly porous particles, which allow access for, among other things, environmental oxygen (Beker and Rapoport, 1987). We designed several rehydrating solutions complemented with oxidant or antioxidant agents to evaluate their

effect on cell vitality during the rehydration process (Fig. 2C). Ascorbic acid, sulphur dioxide and hydrogen peroxide showed no significant detrimental or beneficial consequence for cell vitality. Our results indicate that antioxidant agents do not act as yeast protectants and may therefore directly influence its early fermentative performance. Several studies have reported a general correlation between growth rate and stress sensitivity in which cells in the exponential phase are more sensitive to stressing agents than those in the stationary phase (Elliot and Futcher, 1993; Osorio, *et al.* 2003). This could explain the high tolerance of non-growing yeast cells during rehydration treatments in the presence of hydrogen peroxide. After these rehydration processes, comparable survival values were obtained. However, a rehydrating mix containing 0.5 mM hydrogen peroxide and 2% glucose was highly detrimental, increasing cell death to 50% compared with the pure water reference condition (data not shown). This high sensitivity towards hydrogen peroxide of cells metabolising a fermentable carbon source but not on oxidative substrates such as carbon sources has already been reported (Godon, *et al* 1998; Cabiscol *et al* 2000). Our results indicate that oxidant and antioxidant agents do not act as yeast vitality enhancers and may therefore not directly influence the early fermentative performance as over-active starter yeast.

A direct, and perhaps the first, effect of cell rehydration is its change in membrane fluidity. Also, cell adaptation to the new physiological conditions after the rehydration process is initiated by the perception of environmental signals and the transduction of these signals into biochemical processes that finally led adapted cells to resume proliferation (Hohmann and Mager, 2003). The faster achievement of the membrane optimum dynamic permits to the stress sensors in the membrane and the cytoplasm to act so that the cell will adapt to the new physiological condition. These observations led us to explore whether changes in membrane fluidity have an effect on rehydrated yeast vitality (Fig. 2D). Dimethyl sulfoxide and benzyl alcohol have routinely been used as a membrane rigidifier and fluidizer, respectively (Panadero, *et al*. 2006; Sangwan, *et al*. 2002). We also evaluated other membrane-fluidizer agents with hydrophobic properties, such as *p*-cresol, ethyl-phenol, 2-phenyl 1-propanol, 2-phenyl 1-butanol, and ethyl-hexanol. For 3 and 5 mM ethyl-phenol, a minor reduction in time needed to reach threshold activity was observed. Our results suggest that altering membrane fluidity during yeast rehydration does not enhance cell vitality. For the experiments mentioned above, the viability values obtained by plating on YPD before vitality testing generally showed no significant mortality. Moreover, 2% glucose to co-complement the

mentioned re-hydration conditions was somewhat detrimental to cell vitality in comparison with the single solutions (data not shown).

Cell leakage during cell rehydration

Dehydrated yeast can lose up to 30% of soluble cell compounds when rehydrated, which proves the non-functionality of the cell membrane (Beker, *et al*. 1984; Rapoport, *et al*. 1995). A faster reduction in leakage may therefore be beneficial for the vitality of rehydrated yeast cells. The degree of intracellular compound leakage was assessed by evaluating 260 nm light-absorbing substances. We also quantified the trehalose content in the supernatant at each point in time and evaluated the time course of nucleotide concentration in the various rehydrating ADWY supernatants (Fig. 3). For all experimental rehydrating conditions, the nucleotide concentration time course in the supernatant appears to exhibit two periods that are delimited at the inflection point where cell leakage rate is inhibited (e.g. in pure water, 25 min). The experiments reveal two trends for the permeability change in the first period: glucose and magnesium at around 15 min, and the fluidity agents at more or less 25 min, like water. Also, for water rehydrating conditions we observed a 33% relative leakage in the total nucleotide cell content at the end of the re-hydration process and found the fastest leakage kinetic of 30% after 20 min. We also monitored the change in trehalose content during ADWY QA23 strain rehydration by determining both intra- and extra-cellular trehalose contents. Before starting the rehydration process, the intra-cellular trehalose content increased dry weight by 14.5 %. At time zero, a rapid efflux of trehalose was observed, which agrees with previous observations (Becker and Rapoport, 1987; Roustan and Sablayrolles, 2002). We also noticed a decrease of around 2% at 30 min but, after 5 min rehydration, there was no trehalose release and intracellular levels remained at 12% of dry weight, thus confirming our previous data (Novo *et al*., 2003).

Evaluation of yeast viability using flow cytometry

The wine industry has long been interested in applying flow cytometry to the microbiology of the fermentation process. This technique has already been used to quantify the viability of wine yeast (Breeuwer, *et al*. 1994; Bruetschy, *et al.* 1994) and bacteria (Diaper, *et al*. 1992), and to classify the wine yeast population (Deere, *et al.* 1998). In this section, we use flow cytometry with DNA-reactive dye propidium iodide (PI) to quantify the yeast cell with compromised membranes under several conditions of

cell rehydration. The ADWY QA23 cells were rehydrated for 30 min at 37°C in the presence of metallic ions (Mg^{2+}, Ca^{2+}), membrane fluidity agents (*p*-cresol, dimethyl sulfoxide), glucose and pure water. Later we stained them separately with PI (Fig. 4). In general, the data illustrate two trends for cells with a compromised membrane permeability barrier, roughly 53% for the fluidizer agents and around 24% for the other conditions. These data support even further those obtained for leakage of intracellular compounds (Fig. 3), which suggests that the high leakage values result from the change in architectural membrane during rehydration. A similar correlation has been observed for cold rehydration of yeast cells (Peña, *et al.* 1992; Attfield, *et al*. 2000). On the other hand, similar permeability values from the rehydration process in water, glucose or ions do not support the idea that a single interaction between any of these compounds with the damaged phospholipid bilayer of injured or dead cells could reduce membrane influx permeability towards PI.

In conclusion, we have characterized the cell vitality of the active dry wine yeast strain QA23 after the rehydration process for a set of physiological conditions. The various methods used in this study enabled us to evaluate the effect of several rehydrating conditions by uncoupling cell vitality and viability. The leakage of intracellular compounds is one of the main negative features of rehydration. Also, in oenological conditions leakage leads to a critical point that is detrimental for yeast vitality. However, most of the rehydrated cells do not lose their ability to reproduce after rehydration stress if, after rehydration, they are subjected to optimal growth conditions. We have demonstrated that the presence of magnesium during the rehydration process has a synergistic effect on the recovery of cell activity. Our results suggest that the acquired re-hydrating vitality was not strictly correlated with a shorter time for the kinetic leakage of cellular compounds. We believe that the discrepancy between cytometry evaluation and the kinetics of leakage for the rehydrated cells in the presence of magnesium might facilitate the recovering cell metabolic activity coupled to a reduction in impedance threshold time. We are currently examining these primary factors in order to better understand the rehydration process in *S. cerevisiae* cells. The magnesium rehydrating condition reported here, which is able to enhance the cell vitality of the worldwide ADWY, may have a broad industrial application.

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Figure Legends

Fig. 1 Evaluation of the viability of *S. cerevisiae* strain QA23 after rehydration. Images of the same cells depict fluorescence (A), and bright field (B). In fluorescence assays, live cells above the Neubauer chamber fluoresce green and dead cells fluoresce yellowish. The cells were stained with propidium iodide and analysed by flow cytometry (C). Both the picture and the graph are a representative example of rehydration experiments carried out in duplicate.

Fig. 2 Effect of re-hydration treatments on cell vitality. Yeast cells were incubated at 37°C for 30 min in pure water or in the presence of carbon or nitrogen compounds (A), metallic ions (B), oxidant and antioxidant agents (C), and membrane fluidity agents (D). Time zero represents the time taken by the cells to reach the 10% decrease of total impedance in water condition. The values are means of three independent experiments. *Results with statistically significant differences (p-value < 0.05).

Fig. 3 Time course of extracellular nucleotide concentration in cell suspension. Yeast cells were incubated at 37°C in pure water (□) or in the presence of: 2% glucose (■), 5mM Mg^{2+} (■), 125 mM DMSO (▨), and 5mM p-cresol (▩). The data represented are the means of triplicate re-hydration experiments.

Fig. 4 Frequency distribution histograms and flow cytometry analysis of re-hydrated cells as detected by the fluorescence of propidium iodide. Dry *S. cerevisiae* strain QA23 was incubated at 37°C for 30 min in pure water (A) or in the presence of 2% glucose (B), 3 mM Ca^{2+} (C), 0.1 M dimethyl sulfoxide (D), 5 mM *p*-cresol (E), 5mM Mg^{2+} (F).

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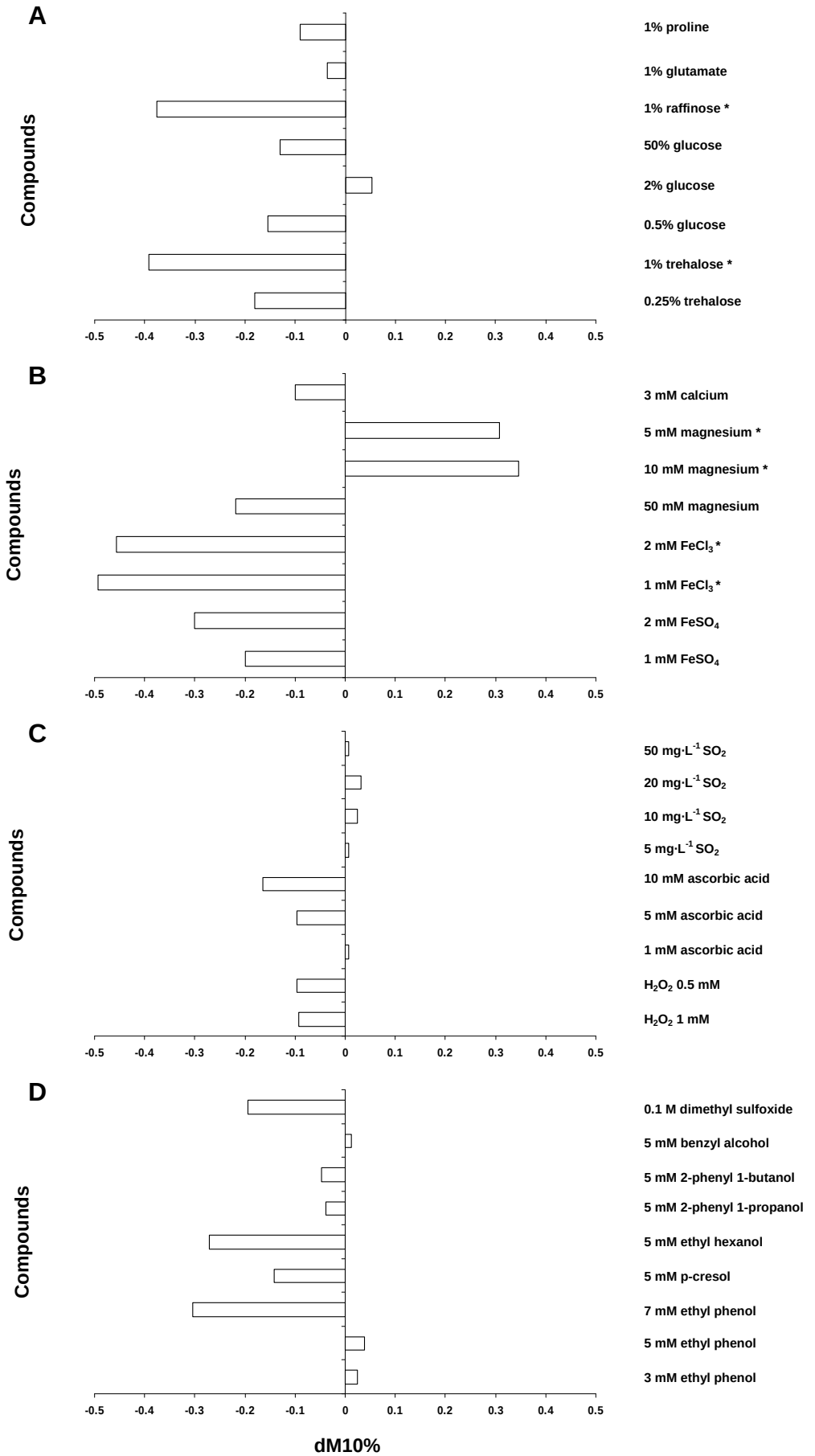

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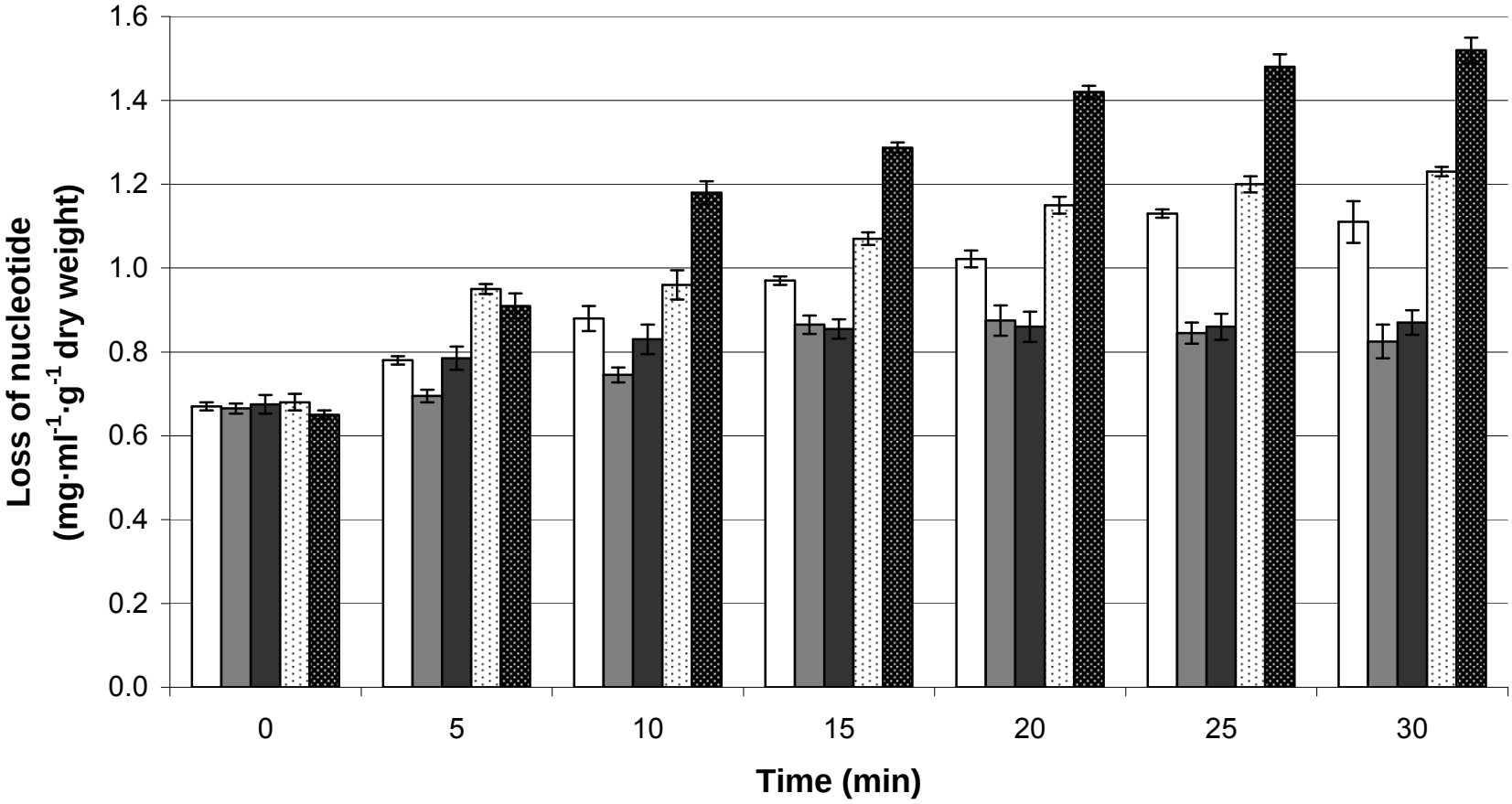

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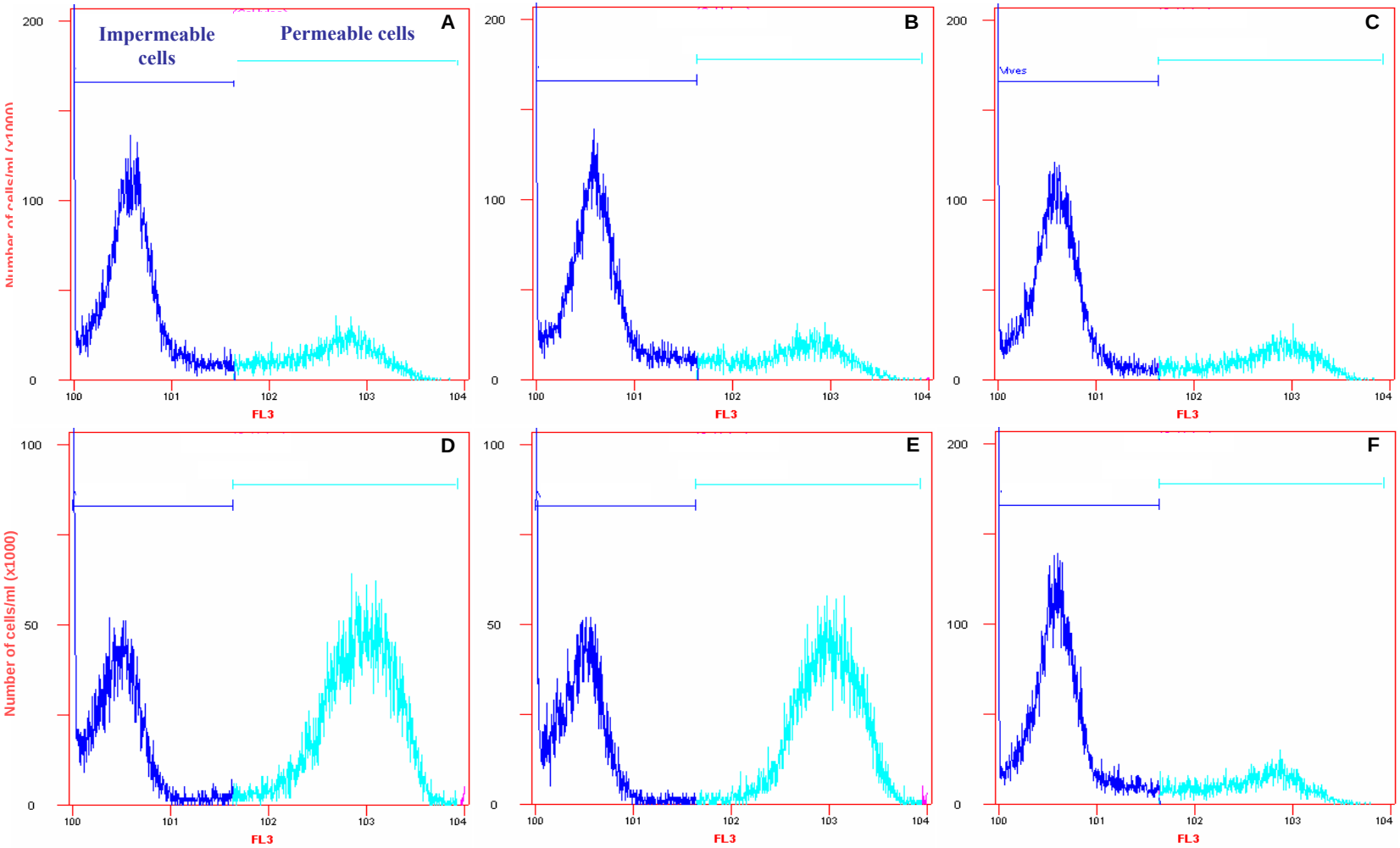

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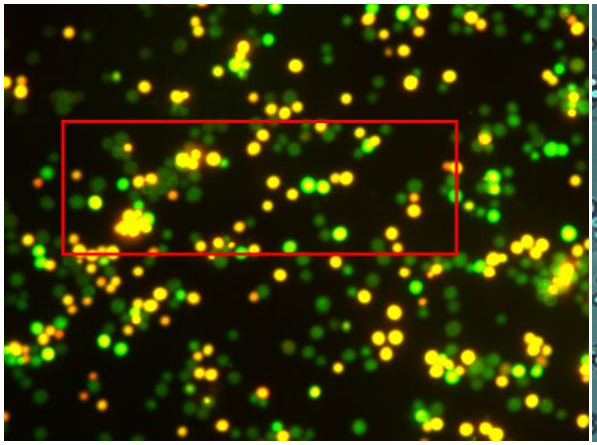

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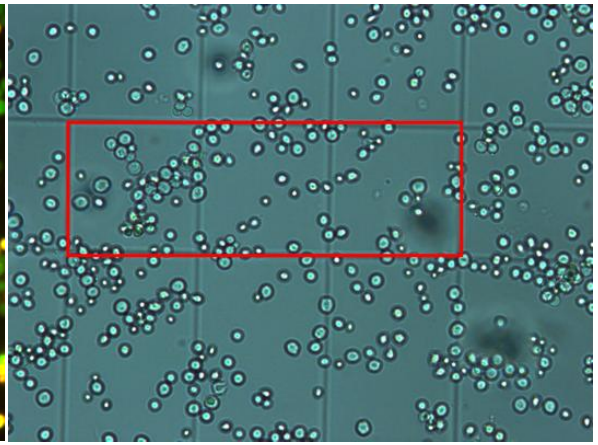

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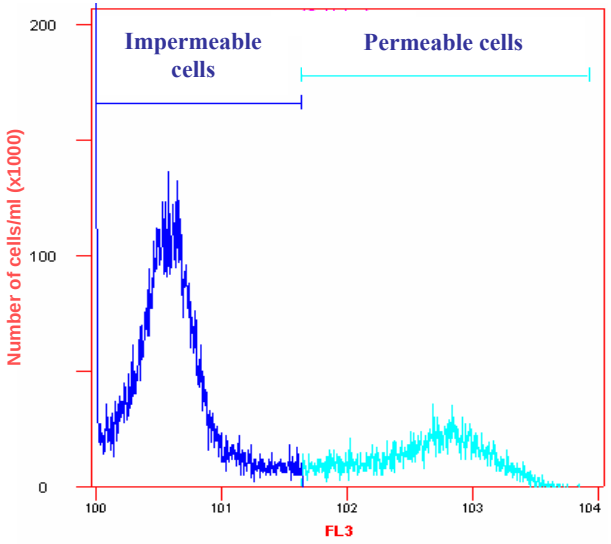

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Effect of lipids on the desiccation tolerance of yeasts

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Abstract

The ability of yeast cells to survive dehydration and be reactivated after the rehydration process is of fundamental importance to food technology and biotechnology. Unfortunately, not all wild yeast strains with improved biotechnological capacities for the food industry exhibit similar patterns in the process of overcoming dehydration. In spite of the importance of this step in yeast production, little is known about its importance to whole yeast metabolites and others metabolites that could be essential to improving cell viability. In the present study, we analyze metabolite features during the dehydration-rehydration process for different yeast species, which are genetically closely related to *S. cerevisiae* in order to determine whether metabolites might play a roll in cell viability. We rank the species *S. cerevisiae, S. paradoxus, S. kudriavzevii, L. kluyveri, N. castellii, S. mikatae, S. bayanus,* and *S. servazzii* according to their viability rate after the dehydration-rehydration process, and we show that desiccation tolerance across the species does not correlate with the intracellular content of trehalose or glycogen. We also investigate the changes in cell lipid composition during this process, where the content of triacylglycerols and phosphatidylcholine show significant variations across the species. The increase in intracellular phosphatidylcholine content before dehydration in higher sensitive yeasts enhances cell viability after stress imposition.

Keywords: viability, dehydration, fatty acids, phospholipids, trehalose, *Saccharomyces* sp.

Introduction

The *Saccharomyces* genus is divided into two subgroups: (i) The sensu stricto group comprised of yeast species closely related to *S. cerevisiae* (*S. cerevisiae*, *S. paradoxus*, *S. bayanus*, *S. kudriavzevii*, and *S. mikatae*), and (ii) The sensu lato group, which involves heterogeneous yeast species that diverge more from *S. cerevisiae* (*Naumovia castellii*, and *Lachancea kluyveri*) (Muller and McCusker, 2009). These species may differ from each other in a very limited number of physiologic characteristics, some of which may be controlled by single mutable genes (Montrocher *et al.* 1998). From an applied point of view, many of the species lead to specific fermentations such as: *S. bayanus*, lager beer (Naumov, 2000); *S. uvarum*, wine and cider (Nguyen and Gaillardin, 2005); *S. paradoxus* Croatian wines (Redzepovic *et al.* 2002). In addition, other *Saccharomyces* species isolated from non-biotechnological environments (*S. mikatae*, *S. kudriavzevii*, etc.) have been used to develop hybrids, which show appropriate biotechnological properties (Belloch *et al.* 2008). Almost all yeast-based food industries have gradually embraced the use of Active Dry Yeast (ADY) because of its greater genetic stability at room temperature and, therefore, the resulting savings in transport and storage costs. In contrast, its lower activity requires higher consumption to obtain high-quality inoculums (Rodríguez-Porrata *et al.* 2008). Most laboratory developed wine strains or those isolated from oenological environments have the biotechnological handicap of cell viability loss during industrial drying processes. Therefore, such strains are excluded from the commercial catalogues of yeast manufacturers, awaiting the breakthrough that would allow their optimization. Most previous physiological studies on yeast dehydration tolerance have been performed on intracellular glutathione concentration (de Souza Espindola *et al.* 2003); the role of cytoplasmic catalase (França *et al.* 2005); cell viability at different drying kinetics (Beney *et al.* 2000); drying in the presence of chemic-protecting drugs (Beker and Rapoport, 1987); osmotic pressure during the cell-dehydration and -rehydration process (Simonin *et al.* 2007); the effect of magnesium complementation during yeast rehydration process (Rodríguez-Porrata *et al.* 2008); and when nitrogen catabolite repression is active during the rehydration phase (Vaudano *et al.* 2009). In apparent contradiction with the prevailing intracellular trehalose-based model for *S. cerevisiae* to tolerate desiccation, Ratnakumar and Tunnacliffe (2006) demonstrated that desiccation

tolerance in a mutant strain unrelated to the production of trehalose (deleted trehalose-6-phosphate synthase gene, *tps1*□), and also that an increasing degree of tolerance exists after diauxic shift or heat stress, albeit slightly less than in the wild type. These findings show that there is no consistent relationship between intracellular trehalose levels and desiccation tolerance in *S. cerevisiae*; this observation coinciding with those previously observed for two different *S. cerevisiae* strains (Krallish *et al.* 1997). Most recently published studies aiming to ascertain whether the response to drying involves regulation at the level of transcription and/or translation find changes in the genes involved in lipid binding and synthesis, protein synthesis, and metabolism (Singh *et al.* 2005; Rossignol *et al.* 2006; Novo *et al.* 2007).

Dehydration causes a rapid efflux of water through the cell membrane, resulting in the collapse of cell structures and membranes. The cellular membrane is rapidly repaired during rehydration, and a fast mobilization of lipid storage within the cells has been observed to occur (Beker *et al.* 1984). The bulk of lipids in yeast membranes are constituted by fatty acids (FA), phospholipids (PL), sterols and sphingolipids (Daum *et al.* 1998). In all types of cells, neutral lipids are stored as an energy reserve and a source of the building blocks needed for membrane formation. This is also true for the yeast *S. cerevisiae* which synthesizes triacylglycerols and steryl esters as the most prominent storage lipids. These are stored in phospholipid-protein monolayer particles (Czabany *et al*. 2007). PC is the major lipid constituent of the *S. cerevisiae* endomembranous system, comprising up to ~33% of total PL (Tuller *et al*. 1999).

In the present study, we analyze metabolite features during the dehydration-rehydration process for different yeast of *Saccharomyces* genus in order to determine whether metabolite might have a major impact on the cell viability of these yeasts. We rank the species *S. cerevisiae, S. paradoxus, S. kudriavzevii, L. kluyveri, N. castellii, S. mikatae, S. bayanus,* and *S. servazzii* according to their viability after the dehydration-rehydration process, and we show that desiccation tolerance between the species is not in correlation with the intracellular content of trehalose or glycogen. We also investigate the changes of cell lipid composition during that process, where the content of triacylglycerols and PL shows significant variations among the species. In addition, we analyze the influence on cell viability by supplementation of these lipids in the yeasts with higher sensibility before cell desiccation.

Material and Methods

Yeast strains

Table 1 summarizes the yeast species used in this study.

Growth conditions and desiccation-rehydration process

Yeast species were grown in shake flasks (150 rpm) in YPD (1% yeast extract, 2% bacto-peptone and 2% glucose) at 28°C by inoculating with overnight liquid culture at an initial OD_{600} of 0.5. Growth curves were determined and 5 x 10^7 yeast cell suspensions, measured by microscope cell counting, from the stationary phase were desiccated by exposure to dry-air at 28°C for ~20h. In all cases, 5 x 10^7 cells were re-hydrated into 1 ml final volume of pure water at 37ºC for 30 min. Various rehydration temperatures using pure water were tested: 25°C, 30°C, 37°C and 40°C. Rehydrating with water at 25°C and 40°C led to around 20% less viability than the other two rehydration temperatures, both of which gave similar results. Therefore, water at 37°C was used during the re-hydration process. In addition, 5 x 10^8 yeast cells from the stationary phase were exposed for 2 hours on a rotary shaker to different lipid molecules to a final concentration of: oleic acid (3.5 mg/l), monoolein, diolein and triolein (50 □M), PC (50 □M), cholesteryl oleate (5 mg/l). These concentrations were based on previous studies (Avery *et al.* 1996; Deytieux *et al.* 2005; Watson and Rose, 1980) to ensure lipid incorporation without affecting the yeast growth. The dry weight of cells was calculated after maintaining the cell-pellets at 100°C for 2 days.

Determining the yeast viability

After the re-hydrating process, the viable cell count was calculated by spreading cell dilutions using a Whitley Automatic Spiral Plating (AES *Laboratoire*, France) on YPD agar medium. The plates were incubated at 28°C for 48 h and the CFU were quantified using the ProtoCOL SR/HR counting system software version 1.27, supplied by Symbiosis (Cambridge, UK).

Determining the glycogen and trehalose

Glycogen and trehalose were determined following the protocol described by Parrou and François, 1997. The measurements were performed before and after dehydration/rehydration process.

Determining the total yeast fatty acids

Sedimented cells (5 x 10^8 cells/ml) were placed in sealed tubes with a Teflon-lined screw cap and saponified using 1ml of 5% NaOH in 50% methanol/water (Rozès *et al.* 1992). The tubes were placed in a dry bath (100ºC) for 30 minutes. Then the saponified material was cooled at room temperature and 2 ml HCl 6M was added. Free fatty acids were extracted by adding 0.5 ml hexane:methyl tert-butyl ether (MTBE) (1:1, v/v). Each tube was vortexed twice for 30 seconds. The organic phase was collected after centrifugation at 1000 x g for 3 min. Analytical gas chromatography was performed using a Hewlett-Packard 6850 (Agilent Technologies). One µl of cellular extract was injected (splitless, 1 min) into an FFAP-HP column (30 m x 0.25 mm x 0.25 µm, from Agilent Technologies) with an HP 6850 automatic injector. The initial temperature was set at 140ºC and increased by 4ºC/min up to 240ºC. Injector and detector temperatures were 250ºC and 280ºC, respectively. The carrier gas was helium at a flow rate of 1.7 ml/min. Heptanoic and heptadecanoic acids (10 and 40 mg/ml, respectively) were added as internal standards before cell saponification. Relative amounts of fatty acids were calculated from their respective chromatographic peak areas. These values were related to the dry weight of cells and expressed as a percentage of the total fatty acid extracted.

Lipid extraction from yeast cells

Prior to lipid extraction, a solution of 100 µl of cold methanol and 10 µl of EDTA 0.1 mM was added to the yeast cells (15-20 mg dry mass) with 1 g of glass beads (0.5 mm, Biospec Products) in an Eppendorf tube, and then mixed for 5 minutes in a mini-bead-beater-8 (Biospec Products, Qiagen). Lipid extraction was performed in five steps: four steps with 300 µl chloroform/methanol (2:1, v/v, for 1 hour) and the fifth with 300 µl of chloroform/methanol 1:2 (v/v) overnight. Both organic phases were transferred to a 15 ml glass screw-cap tube and cleaned twice by adding KCl 0.88% (one fourth of the total volume of the extract). After vortexing and cooling at 4ºC for 10 min, the samples were centrifuged at 1300 x g for 5 min. The organic phase was collected and finally concentrated to dryness under a nitrogen stream. The residue was dissolved in chloroform/methanol (2:1, v/v) and stored at -80ºC until analysis.

Yeast neutral lipid (NL) composition by thin-layer chromatography (TLC)

The neutral lipid composition of the yeasts was separated by one-dimensional TLC on silica gel $60F_{254}$ plates (10 x 20 cm, 250 µm) (Merck, Germany). The plate was developed in two steps with (i) hexane, methyl tert-butyl ether (MTBE), glacial acetic

acid (70:30:3, v/v/v) applied to the half of the plate and (ii) hexane applied to 9.5 cm (Redón *et al.* 2009). The standard lipids lanosterol, ergosterol, squalene, cholesteryl oleate, ethyl oleate, diolein, triolein were purchased from Sigma and applied to every plate in the range of 1-4 μg/μl. After TLC, lipids were charred with 10% $CuSO_4$ in 8% H_3PO_4 and heated at 180ºC for 4 min on a TLC Plate Heater (CAMAG). An image of the plate was acquired with an Image Scanner (Amersham Biosciences). Each spot of the image was quantified as an integrated optical density (IOD) with Quantity One software (Bio-Rad) and the calibration curves were constructed by plotting the IOD of the lipid standard *versus* the amount of lipid loaded.

Yeast phospholipid (PL) composition by HPTLC

The yeast extract phospholipids were separated by one-dimensional HPTLC on silica gel $60F_{254}$ plates (10 x 20 cm, 200 μm) with chloroform:acetone:methanol:glacial acetic acid:water (50:15:10:10:5, v/v/v/v/v). After charring the plate with 10% $CuSO_4$ in 8% H_3PO_4 and heating it at 180ºC for 4 min (Redón *et al.* 2009). PL were identified using the following known standards purchased from Sigma: phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), cardiolipin (CL) and phosphatidic acid (PA). To quantify PL, calibration curves were constructed by applying standards to every plate in the range of 1-4 μg/μl.

Statistical analysis

The results were statistically analysed by one-way ANOVA and the Scheffé test from the statistical software package SPSS 15.1. Statistical significance was set at $P < 0.05$.

Results and discussion

Evaluation of yeast viability after stress imposition

In this experiment, the different capacities of desiccation tolerance of species closely related to *S. cerevisiae* were assessed by a colony-counting assay. The CFU/ml mean value of survival after rehydration was calculated after taking into account viability before drying. Before drying the samples, the cells for each species were re-suspended in both 10% trehalose and in pure water, as the reference condition for the evaluation of yeast cell viability. The survival rate for deionized water condition of *S. mikatae*, *S. servazzii*, *S. paradoxus*, and *S. bayanus* after rehydration was very low, none of the species having more than 30% viability. The *S. cerevisiae* used in this study presented

the highest viability rate, viz. 81%. The other three species (*S. kudriavzevii*, *N. castellii*, and *L. kluyveri*) yielded an average of 50% viability. However, cells from all species dried in the presence of trehalose increased their cell viability around 16% (Fig. 1). These findings support the idea that the presence of trehalose during yeast-drying acts as a membrane protector, reducing the membrane phase transition temperature during the rehydration process (Leslie *et al.* 1994; Eleutherio *et al.* 1993). We therefore investigated whether the changes in intracellular carbohydrate reserve content during cell rehydration might be correlated with desiccation tolerance between the species. Among the beneficial aspects associated with trehalose with yeast cell protection is the prevention of cell dehydration (Thevelein, 1996), and glycogen could be recognized as a readily mobilizable energy source required by the yeasts to adapt to a new growth condition (Pretorius, 2000). Glycogen is also linked to yeast viability (Silljé *et al.* 1999, Pérez-Torrado *et al.* 2002), suggesting that it too has a function during the yeast stress response (Gietz *et al.* 1995). Furthermore, there is a close functional interrelation between the glycogen and trehalose metabolism and cell growth (Parrou *et al*. 1999). We observed a 2% decrease in trehalose for all the species between time zero and 30 min during rehydration (data not shown), which is concurrent with previous data developed by us for a wine *S. cerevisiae* strain (Novo *et al.* 2003). Table 2 shows the values for the carbohydrate reserve at time zero, where *S. paradoxus*, *S. servazzii*, *S. mikatae*, *L. kluyveri*, *S. kudriavzevii*, and *S. cerevisiae* have broadly similar values of intracellular trehalose: around 20 mg/g dry weight. The *N. castellii* used in this study showed the highest trehalose content (76 mg/g dry weight), and the lowest was found for *S. bayanus,* with only 5 mg/g dry weight. However, the intracellular trehalose content does not correlate gradually with the related viability values obtained after stress imposition. In addition, the capacity for glycogen production in different yeasts shows miscorrelation with the desiccation tolerance capacity between the species. Taking the storage carbohydrates results as a whole, it seems that there is no simple relation between intracellular levels and survival of desiccation within the yeast group.

Evaluation of yeast lipid composition during cell-drying and -rehydration process

A direct, and perhaps the main, effect of cell-drying and -rehydration is the change in membrane fluidity. An asymmetric lipid composition of the membranes determines to a large extent the membrane fluidity (Shinitzky, 1984). Therefore, we explored whether changes in cell lipids composition have an effect on rehydrated yeast viability. For *S.*

cerevisiae, *N. castellii,* and *S. bayanus*, with different desiccation tolerance capabilities, we evaluated the total lipid composition at the time points zero of desiccation (BD), and after 30 min rehydration (AR). The values obtained for fatty acids and neutral lipids at both time-points did not show significant differences. Table 3 and 4 are representative examples of values obtained at BD time. The fatty acid composition profile shows similar values for palmitic acid, palmitoleic acid, and oleic acid, which are the more abundant fatty acids for all species (Table 3). In *N. castellii* myristic, myristoleic, and stearic acid contents were around 3 fold higher than in the other two *Saccharomyces* sp. Table 3 also shows that *N. castellii* has 10% lower content of unsaturated fatty acids (UFA) which is mainly due to the low synthesis of C16:1 and C18:1. The UFA was reported, in cooperation with phosphatidylserine (PS) and phosphatidylethanolamine (PE), as lipids that allow cells to conserve adequate fluidity during dehydration condition (Redón *et al.* 2008). Interestingly enough is the relation between C16:1 and C18:1. We observe that the ratio increases with the low cell viability found after rehydration according to the *Saccharomyces* sp. It increased from 1.10 to 1.41 in *S. cerevisiae* and *S. bayanus* while the viability after rehydration was 81 and 30%, respectively. Once again, no significant differences were observed in the total fatty acid contents for the evaluated species. We also analyzed the neutral lipid composition of yeast cells (Table 4). The total sterol level for *S. cerevisiae* is two fold higher than the other yeasts due to a significantly higher content of sterol esters. In contrast, in *S. bayanus* total triacylglycerols (TAG) content was 3 fold lower than the other species. This difference does not correlate with the diacylglycerols (DAG) values. It has been observed in *S. cerevisiae* that oxygen is necessary to convert cyclic squalene into lanosterol, and this leads to an increase in ergosterol synthesis with the concurrent enhancement in growth of the yeast (Aries *et al.* 1978). Nevertheless, *S. bayanus* yielded the lowest lanosterol:squalene ratio of the yeasts' growth in similar aerobic conditions, suggesting a disparity in the squalene metabolism. Hayashida and Ohta (1980) reported that ergosterol and oleic acid accumulation enhance yeast stress tolerance by providing rigidity to the membrane. Moreover, a decrease in ergosterol content has been directly related to a decrease in cell viability after stress imposition (Larue *et al.* 1980; Lees *et al.* 1980). However, we could not observe any significant variations of ergosterol (Table 4) or oleic acid (Table 3) accumulation between the evaluated yeasts. Sterol ester (STE) and TAG are normally considered energy stored molecules, which also play an essential role in the metabolism of acetyl-CoA and fatty

acids (Czabany *et al.* 2007). Furthermore, the rise of the synthesis of these neutral lipids allows cells to neutralize excessive amounts of membrane-perturbing (sterols and FA) and signal-transducing (DAG) molecules. Interestingly, a gradually positive correlation was observed between the relative viability values obtained after stress imposition and the sum of STE and TAG stored by the cell. The storage of these neutral lipids could contribute to ensuring the yeast's needs for the metabolic reactivation of the cell after the rehydration process.

The change in phospholipid contents was investigated during the imposed stress process by determining the total phospholipid composition at both BD and AR times (Table 5). In *S. bayanus*, and particularly at BD time, the more significant difference observed was in PE and PS contents with 50% less than in the other two yeasts. In *S. cerevisiae* the PE biosynthesis is accomplished through either of two pathways: via the CDP-ethanolamine branch of the Kennedy pathway, or by the major route via decarboxylation of PS, which is the major route for PE production (Daum *et al*. 1998). PS is the end product of phosphatidic acid (PA) conversion to CDP-DAG by the phosphatidate cytidylyltransferase CDS1, and then CDP-DAG is modified by CHO1 to produce PS. The CDP-DAG is also a common precursor for the synthesis of phosphatidylinositol (PI) and cardiolipin (CL). We did not observe any significant difference in the stored PI, suggesting a similar PI metabolism for all three species. Compared to other yeasts, the highest content of CL in *S. bayanus* and its lowest PS level allow us to speculate that this yeast has a different balance from *S. cerevisiae* in the CDP-DAG partition between the metabolic pathways of CL and PS formation. Therefore the lowest PE level in *S. bayanus* could be the result of the lowest available substrate for the PS synthase (Psd1/2p), other than a putative differential in the kinetic proprieties of Psd1/2p between the yeasts.

It is worthy of note that the ratio of total phospholipid composition lost remained the same in all cases between BD and AR times, around 10%, except for *S. bayanus,* which decreased by 30%. The data clearly illustrates that the main difference between the yeast phospholipid compositions is given by the significant drop of phosphatidylcholine (PC) contained in *S. bayanus* at AR time, with a 60% decrease. The turnover of PC in yeast occurred through either of two better known phospholipase D (Spo14p), and two phospholipases B (Plb1p, Nte1p). Spo14p - which yields phosphatidic acid and choline - is situated in at the plasma membrane. Pbl1p and Nte1p give GPC and fatty acids, and are situated in the plasma membrane and the ER, respectively. Gde1p metabolises the

GPC yielding glycerolphosphate and choline (reviewed by de Kroon, 2007). When choline is absent from the culture medium, turnover of PE-derived PC by phospholipases D and/or B yields choline for use in the CDP-choline pathway, thus contributing to the species composition of PC (McMaster and Bell, 1994). Therefore, it is tempting to speculate that the loss of phospholipids after cell rehydration in pure water supports phospholipase activity, yielding in a reduction of phospholipid contents. Recently, Bouman *et al*. (2006) suggested that PC was involved in a regulatory mechanism for balancing the membrane contents of bilayer and non-bilayer lipids in order to maintain membrane intrinsic curvature in an optimal range. Therefore, the reduction of intracellular PC after dehydration stress could participate in the viability reduction of the rehydrated cells.

Effect of PC supplementation upon yeast cell viability

We wanted to ascertain whether the low viability rate of *S. bayanus* after the dehydration process could be due to the low content in TAG (Table 4) or to the significant PC lost (Table 5). Therefore, *S. cerevisiae* and two other yeasts with similar low viability rate (*S. bayanus* and *S. paradoxus*, Fig1) were grown in YPD medium supplemented with 10 mM of PC or TAG to determine the best time of incorporation of these lipids. The cells were harvested at several time periods after supplementation (5 min, 1 h, 2 h, and 4 h), and their intracellular concentration was determined as described in Materials and Methods. For the three evaluated species, the 2 h time presented the highest intracellular PC accumulation (data not shown). Then, desiccation tolerance of these three yeasts was evaluated after 2 h with simple supplementation of oleic acid, three neutral lipids (monoolein, diolein and triolein), PC, and choline (Chl), (Fig. 2). In this trial, we considered the oleic acid and the choline as putative products from the intracellular hydrolysis of 1,2-dioleoyl-sn-glycero-phosphocholine (PC) used in this study; as the mono- and diacylglycerol versions of the triacylglycerol 1,2,3-tri(*cis*-9-octadecenoyl)glycerol (TAG). The values obtained for the fatty acid and the neutral lipids did not show significant differences in the cell viability of the treated cells after stress imposition (data not shown). In contrast, the increasing of intracellular PC of both *S. paradoxus* and *S. bayanus* led to a better viability performance after desiccation (60% and 80%, respectively), (Table 6). Nevertheless, a raise of cell survival driven by choline supplementation was observed only in *S. bayanus,* with an improvement of 20% viability. No statistically significant differences were observed for *S. cerevisiae*'s

viability rate between supplementations. Moreover, the similar values for both PC composition and cell viability when oleic acid was supplemented to these species rejects the hypotheses of a viability increase as result of the oleic acid release from the PC (data not shown). PC in wild type yeast is a major determinant of the physical properties of the membrane (Brügger *et al.* 1997). Moreover, yeast cells under stress conditions (H_2O_2, UV irradiation) lose membrane asymmetry as a result of programmed cell death activation (Del Carratore *et al.* 2002). The loss of PC is important not only because of its role as membrane constituent to maintain membrane integrity, but also because the change in ergosterol/PC ratios acts in detriment of membrane endurability (Hayashida and Ohta, 1980). Therefore, the PC level increase in both *S. paradoxus* and *S. bayanus* cells grown in supplemented media means not only an improvement in cell viability but also ensures lipid storage to provide for the yeast's needs after recovery from dehydration stress.

In conclusion, we have characterized the cell viability after the dehydration-rehydration process of the yeast species, which are genetically closely related to *S. cerevisiae*. Our results agree with early publications were desiccation tolerance among different yeast species does not correlate with the intracellular trehalose or/and glycogen contents (reviewed by Pretorius, 2000; Walker and Dijck, 2006). However, the complementation with trehalose during the drying process increases cell viability by 16% for all the species, acting as a membrane protector by reduction of temperature at the transition phase of membranes during the dehydration-rehydration process. We have demonstrated for *S. paradoxus* and *S. bayanus* that most rehydrated cells lose their ability to reproduce after desiccation stress, even if they are subjected to optimal growth conditions. Also, these yeasts have shown a significant loss of the initial PC content after the rehydration process. We have demonstrated that the presence of PC during the biomass production, and therefore its intracellular accumulation, has a synergistic positive effect on cell viability after rehydration. We are currently examining related metabolites in order to better understand the rehydration process in *Saccharomyces* cells.

The enhancement of PC content in dehydration sensitive yeast reported here is of practical interest to producers who wish to enhance the cell viability of worldwide active dry yeast, and it may, therefore, have a broad industrial application.

Acknowledgements

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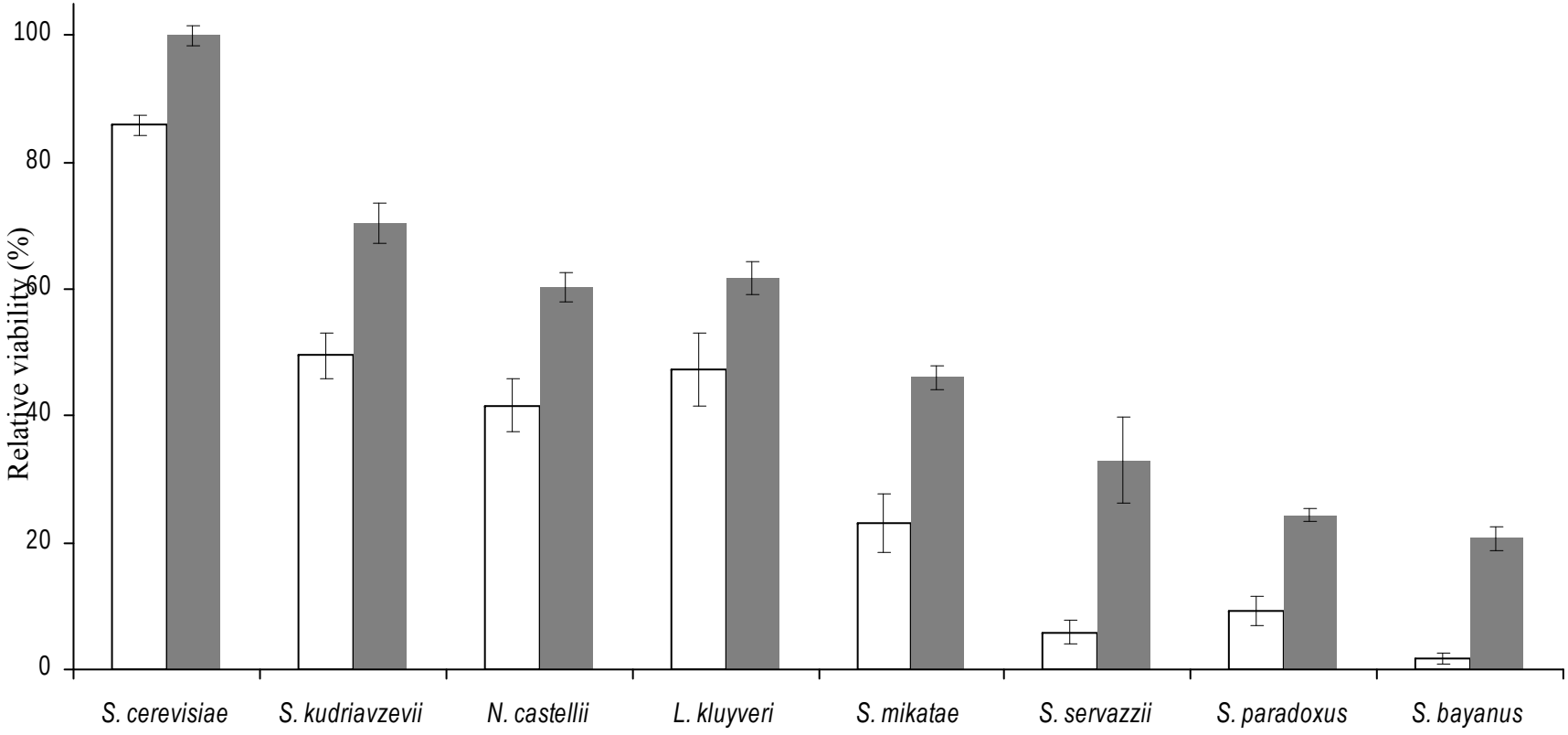

Fig. 1 Effect of trehalose treatment on yeasts cell viability following air-drying and rehydration. The cells were dried at 28°C until 5% relative humidity content. The scale of relative viability (%) indicates the percentage of experimental values for the different yeasts relative to the highest viability for *S. cerevisiae*. Values shown are means of n=3 independent samples ±SD. Cell viability with previous trehalosa treatment (black bars) and control condition (white bars), [§]mg/g dry weight.

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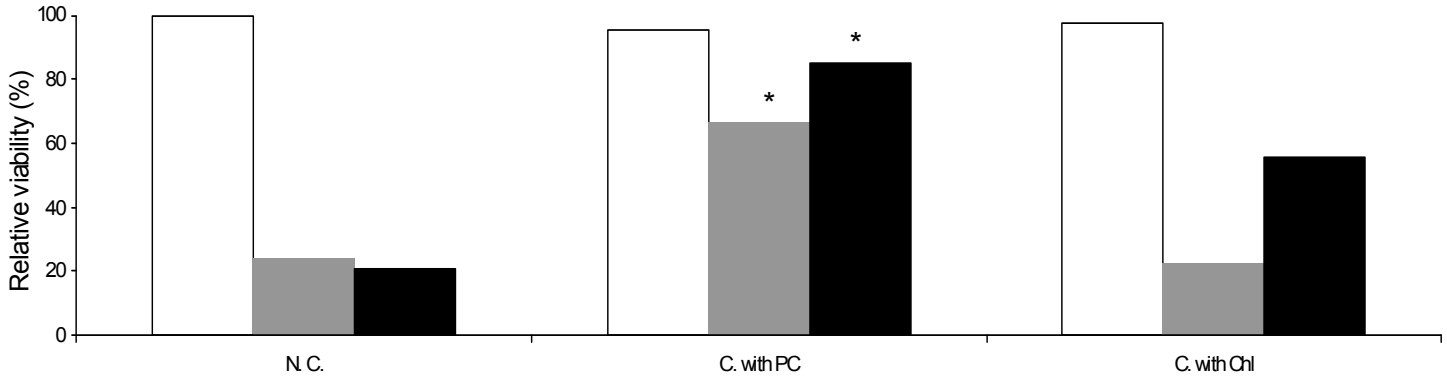

Fig. 2 Effect in yeast cell viability after treatment with phosphatidylcholine and choline. The scale of relative viability (%) indicates the percentage of experimental values for the different yeasts relative to the highest value for *S. cerevisiae* in the non-complemented condition. Cell viability of *S. cerevisiae* (white bars), *S. paradoxus* (grey bars) and, *S. bayanus* (black bars). N. C. cells growth without either phosphatidylcholine or choline; C. with PC, cell growth complemented with phosphatidylcholine; C. with ChL, cells growth complemented with choline. Experiments were performed in quadruplicate and the standard deviation was less than 10%. * Significant differences ($p \leq 0.05$) to the N.C. condition.

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Table 1: Yeast strains studied.

Species	Source	Origin
S. cerevisiae	[a]CBS 1171 = CECT1942	Beer
S. paradoxus	[b]CECT 1939 = CBS 432	Tree sap
S. bayanus	CECT 1969 = CBS 395	Beer
L. kluyveri	CECT 11039 = CBS 3082	*Drosophila pinicola*
S. mikatae	CECT 11823	Soil
S. kudriavzevii	CECT 11825	Decayed leaf
S. servazzii	CECT 11353 = CBS 4311	Soil
N. castellii	CECT 11356 = CBS 4309	Soil

[a]Centraalbureau voor Schimmelcultures (http://www.cbs.knaw.nl/)

[b]Spanish Type Culture Collection (http://www.cect.org/)

Table 2: Carbohydrate reserve before drying treatment in yeast species

Species	Trehalose[§]	Glycogen[§]
S. cerevisiae	12.9±0.19	126.9±16.3
S. kudriavzevii	20.8±0.26	126.2±14.5
N. castellii	76.5±3.12	325.6±33.8
L. kluyveri	20.7±1.35	5.1±0.78
S. mikatae	25.3±0.29	3.1±0.46
S. servazzii	20.6±0.17	72.4±9.78
S. paradoxus	38.6±0.59	0.5±0.03
S. bayanus	4.9±0.03	0.4±0.06

Values shown are means of n=3 independent samples ±SD
[§]mg/g dry weightM@joba86M

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Table 3 Total yeast fatty acid composition

Fatty Acids	*S. cerevisiae*	*N. castellii*	*S. bayanus*
Capric (C10)	0.05±0.01	ND	0.06±0.01
Lauric (C12)	0.12±0.02	0.07±0.01	0.22±0.03
Myristic (C14)	0.14±0.03	2.07±0.05	0.31±0.03
Myristoleic (C14:1)	0.15±0.04	2.81±0.02	0.29±0.05
Palmitic (C16)	15.74±2.02	19.63±0.32	18.76±2.63
Palmitoleic (C16:1)	43.21±4.08	38.39±2.15	46.38±3.59
Stearic (C18)	1.29±0.15	3.55±0.95	0.98±0.02
Oleic (C18:1)	39.29±0.51	33.52±1.13	33.01±0.33
ChL	16.79±0.01	16.27±0.59	16.65±0.04
SFA (%)	17.03±2.19	20.47±1.27	16.92±1.67
UFA (%)	83.04±4.61	74.72±3.28	79.39±3.93
Total FA[§]	99.99±4.88	113.22±5.22	116.66±5.73

Values are means of *n*=3 ±SD
[§]mg/mg^{-1} dry weight
SFA, Saturated Fatty Acids; UFA, Unsaturated Fatty Acids; Chl, Chain Length; ND, not determined

Table 4 Total yeast neutral lipids composition

Neutral lipids	*S. cerevisiae*[§]	*N. castellii*[§]	*S. bayanus*[§]
Squalene	0.81±0.05	0.51±0.02	0.65±0.06
Lanosterol	0.15±0.01	0.13±0.01	0.08±0.03
Ergosterol	0.64±0.07	0.87±0.04	0.77±0.01
Sterol esters	7.13±0.55	3.17±0.12	2.46±0.15
DAG	0.63±0.01	0.63±0.02	0.41±0.07
TAG	8.03±0.82	9.68±0.74	2.59±0.45

Values are means of *n*=3 ±SD
[§]mg/mg^{-1} dry weight
DAG, diacylglycerols; TAG, triacylglycerols

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Table 5 Total yeast phospholipid compositions before drying treatment and after yeast cell rehydration

	S. cerevisiae[§]		*N. castellii*[§]		*S. bayanus*[§]	
	BD	AR	BD	AR	BD	AR
PI	3.46±0.27	2.30±0.35	3.11±0.23	2.49±0.29	3.06±0.15	2.07±0.19
PS	1.34±0.12	1.03±0.19	1.01±0.19	1.19±0.22	0.45±0.29	0.39±0.12
PE	7.85±0.21	7.72±0.39	7.21±0.68	7.24±0.83	3.39±0.39	3.01±0.23
PC	9.16±0.14	9.47±0.26	9.09±0.73	9.19±0.78	7.45±0.23	3.02±0.46[a]
CL	3.51±0.35	2.45±0.21	1.94±0.13	1.76±0.54	4.44±0.53	3.32±0.79
PA	3.15±0.22	2.49±0.09	4.27±0.61	2.29±0.65[a]	2.23±0.59	2.18±0.65
Total	28.47	25.46	26.63	24.16	21.02	13.99

[a]Significant differences due to the process of drying and rehydration, $P \leq 0.05$
BD, before drying; AR, after rehydration; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; CL, cardiolipin; PA, phosphatidic acid
Values are mean of n=3 determinations ± SD
[§]mg/mg^{-1} dry weight

Table 6 Effect in yeast cell total phospholipid composition after treatment with phosphatidylcholine and choline

		Cells growth without complementation			Cells growth complemented with PC			Cells growth complemented with ChL		
		S. cerevisiae	*S. paradoxus*	*S. bayanus*	*S. cerevisiae*	*S. paradoxus*	*S. bayanus*	*S. cerevisiae*	*S. paradoxus*	*S. bayanus*
PI[§]	BD	3.46±0.27	0.90±0.05	3.06±0.15	7.10±0.52	1.73±0.01	4.03±0.18	10.74±0.11	1.39±0.19	2.87±0.01
	AR	2.30±0.35	0.33±0.09	2.07±0.19	6.92±0.19	1.33±0.03	3.18±0.32	9.11±0.17	1.15±0.06	2.38±0.04
PS[§]	BD	1.34±0.12	0.18±0.05	0.45±0.29	0.77±0.21	0.18±0.21	0.32±0.47	1.34±0.61	0.21±0.03	0.29±0.14
	AR	1.03±0.19	0.15±0.03	0.39±0.12	0.94±0.16	0.14±0.19	0.19±0.28	0.80±0.16	0.11±0.07	0.16±0.42
PE[§]	BD	7.85±0.21	1.30±0.27	3.39±0.40	8.65±0.24	1.69±0.01	3.57±0.27	8.65±0.12	1.40±0.05	3.59±0.13
	AR	7.72±0.39	2.08±0.31	3.01±0.12	7.89±0.21	1.11±0.21	2.38±0.28	8.91±0.23	1.53±0.01	2.38±0.17
PC[§]	BD	9.16±0.14	4.22±0.31	7.45±0.53	14.72±0.60	9.91±0.31	13.79±0.45	15.05±0.19	5.97±0.53	8.86±0.06
	AR	9.47±0.26	1.33±0.07	3.02±0.19	13.34±0.12	6.35±0.25	10.42±0.23	14.97±0.10	4.43±0.33	5.88±0.29

Values are means of *n*=4 ±SD
[§]µg/mg dry weight
BD, AR, PI, PS, PE and PC as in Table 5

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Yeast cell death during drying and rehydration process

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ABSTRACT

The ability of yeast cells to survive dehydration and to be reactivated after the rehydration process is of fundamental importance to food technology and biotechnology. Desiccation of tolerant cells implements molecular changes that completely arrest cellular metabolism. This is followed by a period in a state of suspended animation and the subsequent recovery of metabolic functions. To understand what yeasts do, we must address controversial issues such as cell age, longevity, the structural and biochemical properties of anhydrous cytoplasm, and metabolic stasis. The different desiccation tolerance capacities of a collection of 4,850 single mutated strains were assessed by means of a colony-counting assay. Of 4,850 mutants, 116 (~2.4%) were identified as having enhanced desiccation sensitivity and very few (~0.2%) were found to have improved tolerance.

Among the knockout mutants, Δ*Aif1*, Δ*Cpr3*, Δ*Nuc1*, and Δ*Qcr7* were identified as hyper tolerant to dehydration stress. Yeast cells were analysed for apoptotic hallmarks, such as an accumulation of reactive oxygen species (ROS), phosphatidylserine externalization (Annexin V/PI staining), and DNA-strand breaks (TUNEL assay), before desiccation and after rehydration. DHE staining showed that after the dehydration and rehydration process the wild type shows enhanced ROS production compared to the mutants. Additionally, Annexin V/PI double staining revealed that, after the imposition of stress, the wild type culture also holds an elevated percentage of necrotic and late apoptotic/secondary necrotic cells. Further evaluations using the strains Δ*oxa1*, Δ*mgm1*, and Δ*yac1* suggest that cell death during dehydration stress is neither caspase nor respiratory dependent.

INTRODUCTION

Dehydration and rehydration stress (DRS) is a serious problem affecting plants, animals and humans and has been the focus of much scientific

research. Attempts to enhance the desiccation tolerance of cells first focused on agriculturally significant plant cells and seeds, because the availability of water is one of the main parameters limiting plant productivity (Bartels D. *et al.,* 2001). More recently lyophilization and other dehydration-based technologies have been explored by a number of groups for the purpose of cell and tissue preservation (Liang Y.H. *et al*., 2002; Crowe J.H. *et al*., 2000; Guo N. *et al*., 2000; Gordon S.L. *et al*., 2001; Chen T. *et al*., 2001; Acker J.P. *et al*., 2002; Puhlev I. *et al*., 2001; Tunnacliffe A. *et al*., 2001; Wolkers W.F. *et al*., 2002; Wolkers W.F. *et al*., 2003; Elliott G.D. *et al.,* 2006). Because cell therapies advance from research laboratories to clinical applications, cells and tissues need to be transported across long distances while cell viability and function are maintained. Lyophilization is also commonly used in food preservation and for a variety of pharmaceutical applications, including protein-based drugs (Heller M.C. *et al*., 1997; Cleland J.L. *et al*., 2001).

Another important example in the industrial sector is the use of active dry yeast for the production of beer, wine and bread. *Saccharomyces cerevisiae*, in addition to being an excellent model for the study of eukaryotic cells due to its qualities of anhydrobiotes, is the starting point for deciphering the response mechanisms to drying and rehydration stress (DRS). DRS of yeasts, individual cell life within a population and the viability of the population itself are seriously compromised by the sum of a set of severe stresses, such as osmotic and oxidative stress. The change in yeast cells from a state of vital activity to a state of anhydrobiosis as a result of dehydration is accompanied by a number of structural and functional rearrangements in the cells (Beker, M.E. *et al*., 1981; Beker M.J. and Rapoport, A.J., 1987). The resulting damage can be classified into damage of different macromolecules, structures, organelles, and defensive intracellular reactions. Of special interest are the membrane changes (Rapoport A.I. *et al.*, 1994) – increased plasma permeability or rehydration has been suggested as the main cause of cell death during dehydration. In fact, an increase and decrease in osmotic pressure causes nucleotides, ions and other soluble cell components to leak into the surrounding medium (Attfield, P.V. *et al*., 2000).

The highly dynamic lipid bilayer of the plasma membrane is known to undergo phase transitions during dehydration (Laroche C., *et al.,* 2005) and rehydration (Crowe J.H. *et al.*, 1992). The phase transitions of some phospholipids in the membrane may be the cause of membrane rupture or changes in permeability (Laroche C. *et al*., 2003). Other

authors suggest that the formation of endovesicles during dehydration leads to plasma membrane lysis during osmotic expansion when the cells are rehydrated (Mille Y. *et al.,* 2003). Yeast cells recover at different rates depending on culture conditions (Anand, J.C. and Brown, A.D., 1968; Palmfeldt and Hahn-Hägerdal, 2000; Rodríguez-Porrata B. *et al*., 2009) and/or rehydration conditions (Poirier I.A. *et al*., 1999; Rodríguez-Porrata B. *et al.*, 2007).

Despite the accumulated knowledge about the structural changes and mechanical damage to cells during DRS, little is known about the molecular mechanisms involved in yeast cell death under these stressful conditions.

It has become clear in recent years that yeast can succumb to cell death, exhibiting typical apoptotic markers (Ludovico *et al.,* 2001; Madeo *et al*., 1997; Madeo *et al*., 1999). Moreover, the yeast genome codes for many proteins of the basic molecular machinery executing cell death, including caspase orthologs (Madeo *et al*., 2002), AIF (Wissing *et al*., 2004), and the yeast EndoG (NUC1) (Büttner S., *et al.*, 2007). In addition, programmed cell death in yeast has been linked to complex apoptotic scenarios such as mitochondrial fragmentation (Fannjiang *et al*., 2004), cytochrome C release (Ludovico *et al*., 2002), and aging (Fabrizio *et al*., 2004; Herker *et al*., 2004; Laun *et al*., 2001). Notably, histone H2B phosphorylation, which is considered to be a universal prerequisite for apoptosis execution (Cheung *et al*., 2003), was shown to be necessary for cell-death induction upon oxidative stress in yeast (Ahn *et al*., 2005).

Recently, yeast apoptosis research has begun to resolve the complex interplay of mitochondrial cell death mediators. It is becoming increasingly clear that the connection between mitochondrial respiration and apoptosis is intricate, as suppression of respiration can either be beneficial or detrimental to the cell, strongly depending on the apoptotic scenario (Eisenberg *et al*., 2007). It was probably not by chance that nature has coupled pro-apoptotic potential to many molecules that have a genuine function in the respiratory chain in healthy cells, such as cytochrome C, AIF and AMID. In this way, by simply changing the localisation from mitochondria, the daytime place of action, to the cytosol, cell death is executed in a redundant, highly effective manner. As a result, mitochondrial outer membrane permeabilisation is probably the point of no return in cell death execution and thus an excellent target for clinical manipulations of apoptosis (Galluzzi L. *et al*., 2006) and perhaps even necrosis (Golstein P, Kroemer G., 2006).

UNIVERSITAT ROVIRA I VIRGILI
DEHYDRATION TOLERANCE IN YEAST
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In this study we identified a group of mitochondrial knockout mutants, Δ*Aif1*, Δ*Cpr3*, Δ*Nuc1*, and ΔQcr7, as hyper tolerant to dehydration stress. Yeast cells were analysed for apoptotic hallmarks. DHE staining showed that during dehydration and rehydration, the wild type showed enhanced ROS production compared to the mutants. Additionally, Annexin V/PI double staining revealed that after the imposition of stress the wild type culture also holds an elevated percentage of necrotic and late apoptotic/secondary necrotic cells. Further evaluations using the strains Δ*oxa1*, Δ*mgm1*, and Δ*yac1* suggest that cell death during dehydration stress is neither caspase nor respiratory dependent.

MATERIALS AND METHODS

Yeast strains and media

Table 1. Yeasts strains used in this study.

Strain	Genotype	Source
BY4742	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0	EUROSCARF
Δ*yca1*	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 yca1::kanMX4	EUROSCARF
Δ*aif1*	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 aif1::kanMX4	EUROSCARF
Δ*qcr7*	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 qcr7 ::kanMX4	EUROSCARF
Δ*cpr3*	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 cpr3::kanMX4	EUROSCARF
Δ*nuc1*	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 nuc1::kanMX4	EUROSCARF
Δ*oxa1*	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 oxa1::kanMX4	EUROSCARF
Δ*mgm1*	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 mgm1::kanMX4	EUROSCARF

Yeast cells were grown in SC media containing 0.17% yeast nitrogen base (Difco), 0.5% $(NH_4)_2SO_4$ and 84 mg/l Leucine, 25 mg/l uracil and 42 mg/l lysine and histidine.

Determination of viability

Yeast cells were cultivated in SC medium until the stationary phase. Some of the culture was then transferred to a 12-well plate in the presence of trehalose at 10% W/V of the final concentration. Half of the cell suspension was transferred to another 12-well plate for drying. The cells in the second plate were dried with air at 28 °C over a period of 24 hours. They were subsequently rehydrated with sterile water at 37 °C for 30 minutes.

For survival platings, the cell cultures were diluted, the cell concentration was determined with a CASY cell counter, and aliquots containing 500 cells were plated on YPD plates before drying and after rehydration. The number of colonies was determined after two days at 28 ºC. The CFU were quantified using a Microbiology-Colony-Counter (Lemnatec) and processed using SAWmicrobio version 3.1. After the

colonies were counted the viability percentage was determined through a simple calculation.

Tests for apoptotic markers

DHE staining, Annexin V/PI costaining and TUNEL staining were performed as described in Büttner S. *et al.* (2007). The same samples were analysed by fluorescence microscopy. To determine frequencies of morphological phenotypes revealed by TUNEL, DHE- and Annexin V/PI double staining, at least 300 cells from three independent experiments were evaluated by using flow cytometry and BD FACSDiva software. A fluorescence microscope (model BH-2RFCA; Olympus), a digital camera (model c35AD-4; Olympus), and Metamorph® software (Soft Imaging System GmbH) were used for image acquisition.

RESULTS

Drying and Rehydration Stress compromises yeast survival

In order to study the molecular response to drying and rehydration stress (DRS) in yeast, we analysed the viability of the complete EUROSCARF collection of *Saccharomyces cerevisiae* under DRS. This collection comprises a total of 4,794 mutants, each deleted in one of the non-essential genes. While viability in the wild type reference strain BY4742 was approximately 40%, we detected a group of approximately 100 deletion mutants whose viabilities were lower than 10%. Figure 1 shows the functional distribution of the corresponding genes ranked according to their relative abundance. Pathways involving protein synthesis and biogenesis of cellular components occurred more frequently, while pathways connected to cell fate, cellular rescue and environmental interaction were less abundant.

Several mutants can rescue cell death upon rehydration stress

We also detected a group of 12 deletion mutants showing viability values higher than the reference strain (data not shown). Among the corresponding genes, some are directly connected to programmed cell death (PCD). Figure 2 represents the fold increase in viability (normalized to the wild-type By4742) of some mutants deleted in genes closely linked to PCD. Interestingly, the knockouts with viabilities higher than the wild type lack the mitochondrial genes *AIF1*, *CPR3* and *NUC1* (2-fold increase in viability) and *QCR7* (3-fold increase).

CPR3 encodes for a yeast homolog of cyclophilin D (Dolinski *et al*., 1997), which is a prolyl isomerase located within the mitochondrial matrix along with adenine nucleotide translocator (ANT) and the voltage-dependent anion transporter (VDAC), consist the mPTP. Cyclophilin D is thought to facilitate a calcium-triggered conformational change in ANT mitochondrial permeability transition and is associated with mitochondrial swelling, outer membrane rupture, and the release of apoptotic mediators (Halestrap, 2005). Cyclophilin D has previously been implicated in both necrosis and apoptosis programmes (Halestrap, 2005; Schneider, 2005). Cpr3p has been reported as central to the PCD process induced by Cu in *S. cerevisiae* (Liang Q and Zhou B., 2007). MPTP has been shown to be a key factor in necrotic cell death caused by calcium overload and oxidative stress (Crompton, M., Ellinger, H. & Costi, A., 1988) and does not usually have much of a role in apoptosis. The release of proteins from the compartment between the two mitochondrial membranes, of which citochrome C is the major payer, triggers the apoptosis in many cells through the caspase pathway. However many researchers argue that this is unlikely because it would disrupt ATP production, which is required for apoptosis. This is supported by results obtained with cyclophilin D knockout mice in which the extensive apoptosis involved in development was not affected by loss of cyclophilin D (Nakagawa *et al*, 2005). Crompton *et al* (2004), who examined a neuronal cell line (B50) synthesizing large amounts of cyclophilin D, found that the cells were hypersensitive to necrotic cell death induced by calcium and oxidative stress, but were more resistant than usual to apoptosis induced by nitric oxide or staurosporine.

AIF1 encodes for Aif1p, a homolog of the mammalian Apoptosis-Inducing Factor (AIF). Aif1p is a flavoprotein with NADH oxidase activity and contains a mitochondrial localization sequence in the NH_2 terminus and a nuclear localization sequence in the COOH terminus, as well as a putative DNA binding domain composed by positively charged amino acids (Wu *et al*., 2002). Upon the induction of apoptosis, Aif1p translocates to the nucleus, where it leads to chromatin condensation and DNA degradation (Susin *et al*., 1999). After treatment with 0.6 mM H_2O_2 for five hours, $Aif1p^{yEGFP}$ relocalises from mitochondria to the nucleus (Madeo *et al*., 1999). Recently, chronological aging has been shown to be a physiological trigger for apoptosis in yeast. In agreement with this, a predominantly nuclear localisation of $Aif1p^{yEGFP}$ has been observed in aged cells (Herker *et al.,* 2004). Although early reports describe human AIF as a mediator in a caspase-independent method of cellular suicide (Susin *et al*., 1999; Cregan *et al.,* 2002), the release of Aif1p from mitochondria may be subordinated to

earlier caspase activation (Arnoult *et al*., 2003), supporting the notion that caspases and Aif1p would be engaged in cooperative or redundant pathways and they are activated by the same apoptotic stimulus (Madeo *et al.,* 2002).

NUC1 encodes for the major mitochondrial nuclease and has RNAse and DNA endo- and exonucleolytic activities. Nuc1p plays a role in mitochondrial recombination, apoptosis and the maintenance of polyploidy. EndoG has been described as a mitochondrial nuclease that digests both DNA and RNA (Ruiz-Carrillo and Renaud, 1987; Schafer *et al.,* 2004). Upon the induction of apoptosis, the translocation of mammalian EndoG to the nucleus coincides with large-scale DNA fragmentation (Li *et al*., 2001; Parrish *et al.,* 2001). Nuclear translocation of EndoG has been associated with the progression of several degenerative disorders, such as cerebral ischemia (Lee *et al.,* 2005) and muscle atrophy (Leeuwenburgh *et al.,* 2005). Similar to other mitochondrially located cell-death regulators like cytochrome C or apoptosis inducing factor (AIF) (Cheng *et al*., 2006; Vahsen *et al*., 2004), EndoG may have a genuine vital function, in addition to its proapoptotic function. In fact, it has been suggested that EndoG plays a role in cell proliferation and replication (Huang *et al*., 2006). Nuc1p and mammalian EndoG share highly conserved residues in the catalytically active site, suggesting that both belong to the large family of DNA/RNA non-specific bba-Me-finger nucleases (Schafer *et al.,* 2004). Like mammalian EndoG, Nuc1p contains a potential mitochondrial localization sequence (MLS).

Qcr7p is the subunit 7 of the ubiquinol cytochrome-c reductase complex, which is a component of the mitochondrial inner membrane electron transport chain. It is oriented facing the mitochondrial matrix and its N-terminus appears to play a role in complex assembly.

Improved survival is associated with diminished apoptosis and necrosis

The next step in our study was to characterise the mode of death accompanying DRS by performing diverse assays using flow cytometry to quantify apoptotic and necrotic markers. The conversion of dihydroethidium (DHE) to fluorescent ethidium was used to visualize the accumulation of reactive oxygen species (ROS). DNA fragmentation was detected using TUNEL staining. Furthermore, Annexin V/propidium iodide (PI) costaining was used to quantify the externalization of phosphatidylserine, an early apoptotic event, and membrane permeabilisation, which is indicative for necrotic death. This staining allows a distinction to be made between early apoptotic (Annexin V

positive, PI negative), late apoptotic (Annexin V positive, PI positive), and necrotic (Annexin V negative, PI positive) death (Büttner 2008, Mol Cell). Figure 3 shows the results obtained for the reference strain BY4742, Δ*aif1,* Δ*nuc1,* Δ*cpr3* and Δ*qcr7* before dehydration and after rehydration, respectively. All strains depart with comparable ROS levels before dehydration (Fig. 3 A-C). However, after rehydration the mutants Δ*aif1,* Δ*cpr3,* Δ*nuc1* and Δ*qcr7* show a significant reduction in ROS accumulation compared to the wild type control. These reduced ROS levels are accompanied by an increase in the apoptotic/late apoptotic and necrotic populations (Fig. 3 D-E). We observed that Δ*aif1,* Δ*nuc1,*Δ*cpr3* prevent apoptosis in the range of approximately 15% and Δ*qcr7* in about 20% compared to wt. The four knockouts prevent necrosis by 10% compared to wt.

This result suggests that the aforementioned improved viability upon absence of these genes corresponds to a prevention of necrosis and apoptosis under these stress conditions.

Respiration deficiency is not responsible for a reduction in apoptosis and necrosis

All four genes whose knockout provide resistance upon DRS are mitochondrial, and one of them (QCR7) is essential for respiratory activity. We therefore evaluated two further respiratory deficient strains (Δ*mgm1* and Δ*oxa1*) in order to exclude an effect due to loss of respiratory capacity. Neither Δ*mgm1* nor Δ*oxa1* showed improved survival or reduced ROS levels compared to the wild-type (fig. 4A). The analysis of apoptotic and necrotic markers also showed no significant differences in DNA fragmentation, phosphatidylserine externalization or loss of cell integrity after rehydration (Fig. 4 B-D). Thus, the observed reduction in the apoptotic and necrotic populations after DRS is independent of respiration.

Reduction of apoptosis and necrosis is independent of caspase pathway

We also evaluated the Δ*yca1* knockout to determine if under our stress conditions cell death was associated with the caspase pathway. The YCA1 gene in *S. cerevisiae* encodes a metacaspase that is involved in yeast apoptosis in response to different stimuli (Madeo *et al*., 2002; Herker *et al*., 2004, Wadskog *et al*., 2004; Wissing *et al*., 2004). Δ*yca1* did not show improved survival or reduced ROS levels compared to the wild-type (fig. 4A). The analysis of apoptotic and necrotic markers further showed no significant differences in DNA fragmentation, phosphatidylserine externalization or loss

of cell integrity after rehydration (Fig. 4 B-D). Thus, after DRS the observed reduction in the apoptotic and necrotic populations is caspase independent.

DISCUSSION

Dehydration and rehydration stress (DRS) is an important issue that affects plants and animals. Along with the implications of DRS in human pathologies (Heller M.C. *et al*., 1997, Cleland J.L. *et al*., 2001), it is of great interest in the food industry, for example during wine production, where the rehydration of the active dry wine yeast is probably the most critical phase during the process. Only proper rehydration can ensure the viability of healthy cells, which retain good fermentation characteristics. Dehydration causes a rapid efflux of water through the cell membrane, resulting in the collapse of the cytoskeleton. This dry state has a deleterious effect on yeast cell physiology by altering the structure and function of the vacuole, and the integrity and functionality of nuclear and cell membranes (Walker and van Dijck, 2006). The liquid–crystalline phase transition experienced by both dry membranes and lipid bi-layers during rehydration leads to changes in their permeability (Crowe *et al*., 1989, 1992). In fact, dehydrated yeast has been shown to lose up to 30% of soluble cell compounds when rehydrated, thus proving the loss of cell membrane functionality (Beker *et al*., 1984; Rapoport *et al.,* 1995; Rodríguez-Porrata *et al.,* 2008). In agreement with this, in this study we found that only approximately 40% of wild type cells are able to generate a colony in rich medium after DRS.

We analysed the viability of the complete EUROSCARF collection of *Saccharomyces cerevisiae* upon DRS and detected a series of knockouts displaying increased viability (as compared to the wild type). Interestingly, among them were the deletion mutants of *AIF1*, *CPR3* and *NUC1*, all genes directly involved in yeast apoptosis and necrosis and coding for mitochondrial proteins (Büttner S., *et al.*, 2007; Madeo *et al*., 1999; Halestrap, 2005). Of note, the lack of a further mitochondrial protein, Qcr7p (the subunit 7 of the ubiquinol cytochrome-c reductase complex), also provided resistance upon DRS.

Beyond their importance in energy metabolism, mitochondria have emerged as crucial organelles in PCD control (Kroemer, G., 2002). Like mammals, yeast also bears mitochondrially dictated cell death pathways (Eisenberg *et al*., 2007). For instance, Aif1p and Nuc1p are caspase-independent pro-death mitochondrial factors that upon various stresses translocate from mitochondria to the nucleus to facilitate the

degradation of nuclear DNA (Wissing S, Ludovico P, Herker E *et al*., 2004; Qiuli Liang, Wei Li , Bing Zhou, 2008). Therefore, the lethal function of Aif1p has been shown to depend on the yeast homologue of cyclophilin A (Candé *et al*., 2004; Herker 2004). However, until now there is no direct mention of a link between Aif1p and Cpr3p. Our experiments suggest that Aif1p and Crp3p as well as Nuc1p are activated upon DRS. In addition, we show that they perform their lethal activity in a caspase-independent manner since metacaspase deficiency did not rescue DRS-mediated cell death.

Importantly, mitochondria is a major source of reactive oxygen species (ROS), which play a central role in mediating yeast cell death (Madeo *et al*., 1999; Laun *et al*., 2001; Ludovico *et al*., 2001; Mazzoni *et al*., 2003, Weinberger *et al*., 2003). The bulk of mitochondrial ROS generation occurs at the electron transport chain (ETC), as a by-product of respiration (Cadenas, E.; Davies, K. J., 2000; Turrens, J. F., 2003), as Q-cytochrome c oxireductase (Complex III) of the ETC is a well-documented source of mitochondrial ROS (Boveris, A *et al*., 1976; Cadenas, E., *et al*., 1977; Loschen, G.; *et al*., 1974; Dröse, S., *et al*., 2008; Kwong, L. K., *et al*., 1998; Turrens, J. F., *et al*., 1985). In keeping with this, the deletion of *QCR7*, which is derived from the disassembly of complex III, shows lower levels of ROS before and more marked levels after the imposition of DRS. This effect, however, does not seem to rely solely on respiratory disruption, as other respiration deficient mutants did not show any rescuing effect. The molecular significance of Qcr7p in cell death will need to be further clarified in future experiments.

Intriguingly, our results show that DRS mediates a type of death which combines both apoptosis and necrosis. The enhanced viability of the different deletion mutants is thereby accompanied by a reduction in both apoptotic and necrotic markers. In fact, death mediated by mammalian AIF, cyclophilin D and endonuclease G has been described as including both types of death depending on the scenario (Madeo *et al.,* 1999; Rana S. Moubarak, *et al*., 2007; Halestrap, 2005; Schneider, 2005; Büttner S. *et al*., 2006). It is therefore possible that DRS activates both types of death in yeast which the proteins presented here are able to execute in parallel or sequentially.

In conclusion, based on our results we suggest that under DRS cell death is closely linked to molecular pathways that induce death by apoptosis and necrosis in a caspase and respiratory independent way and that DRS is at least partially dependent on the execution of mitochondrial death. The study of yeast genes involved in PCD under these

stress conditions can provide new insights into the mechanistic pathways behind DRS in eucaryotic cells and the resulting pathologies in a bona fide PCD model organism. Additionally, it allows new cell death based strategies to be established in order to confront the difficulties arising form DRS in any industry using dry yeast.

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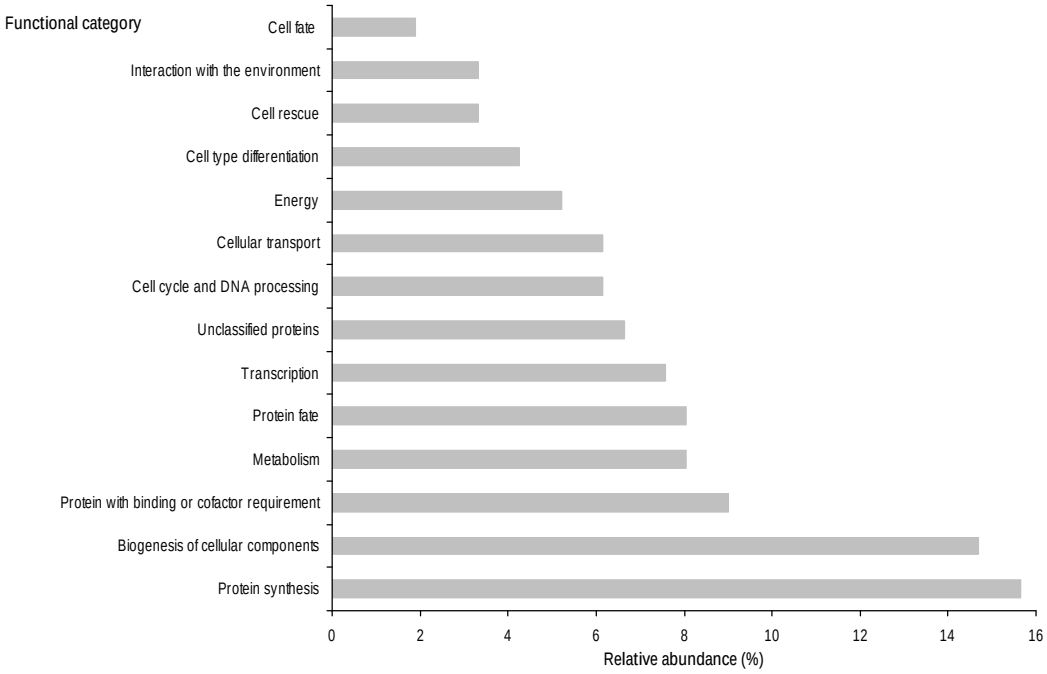

Figure 1. Distribution of functional classes of a group of 112 deletion mutants of the EUROSCARF collection showing less than 10% viability after drying and rehydration.

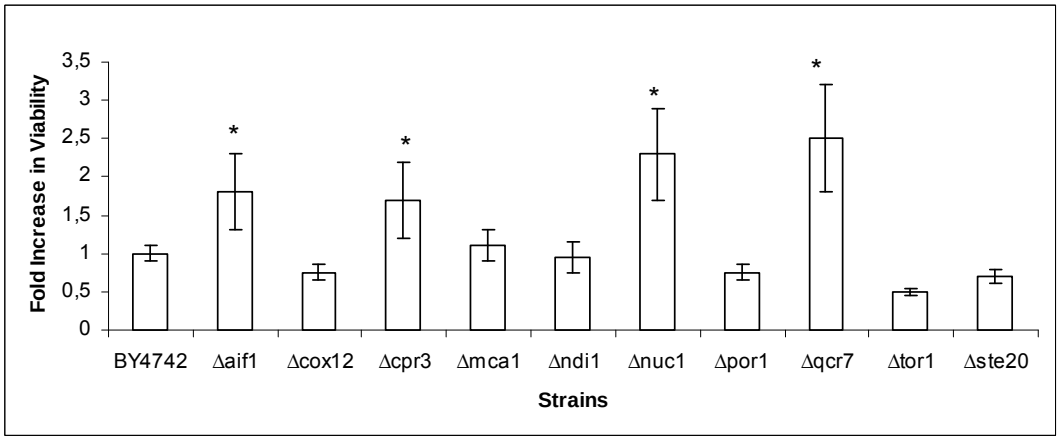

Figure 2. Relative viabilities of a group of deletion mutants closely linked with the cell death mechanisms after drying and rehydration. The viability of the wild type control (By4742) was set at 1. Data represent means +/- standard deviation ($n = 3$, *$p < 0.05$).

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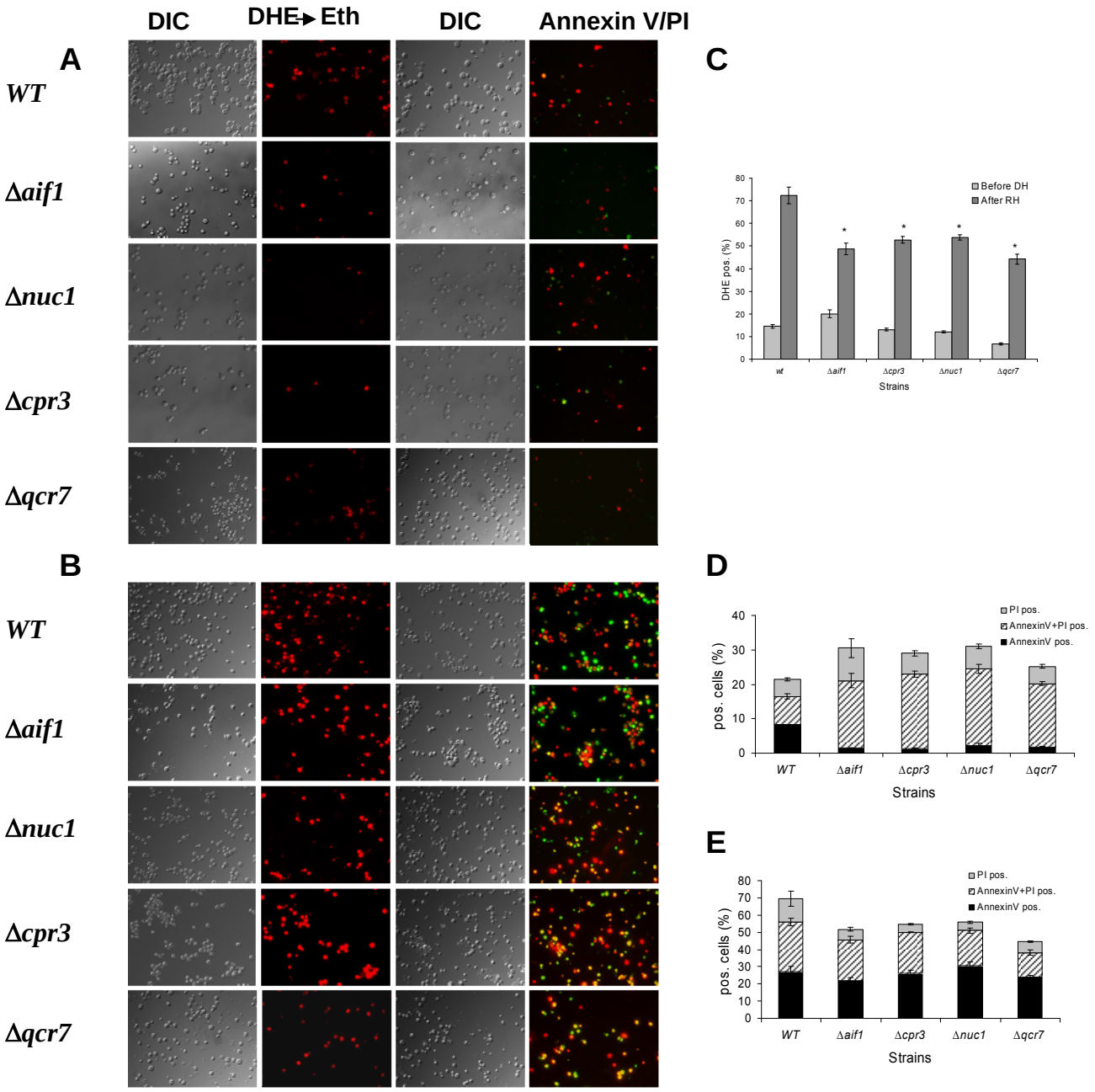

Figure 3. Deletion mutants of four mitochondrial genes showing a higher viability than the reference strain display reduced cell death markers. (A) and (B) Fluorescence microscopy of DHE→Ethidium conversion and Annexin V (green)/ PI (red) costaining of the reference strain BY4742, Δ*aif1,* Δ*nuc1,* Δ*cpr3* and Δ*qcr7* before dehydration and after rehydration, respectively. (C) ROS accumulation in cells of the reference strain BY4742, Δ*aif1,* Δ*nuc1,* Δ*cpr3 and* Δ*qcr7* before drying and after rehydration determined via DHE→Ethidium conversion. In each experiment, 30,000 cells were evaluated using flow cytometry. Data represent means +/- standard deviation (n = 3, *p < 0.05). (D) and (E) Quantification of Annexin V/PI costaining of the reference strain BY4742, Δ*aif1,* Δ*nuc1,*. Δ*cpr3 and* Δ*qcr7* before dehydration and after rehydration. Annexin V and PI positive cells were quantified using flow cytometry. In each experiment, 30,000 cells were evaluated.

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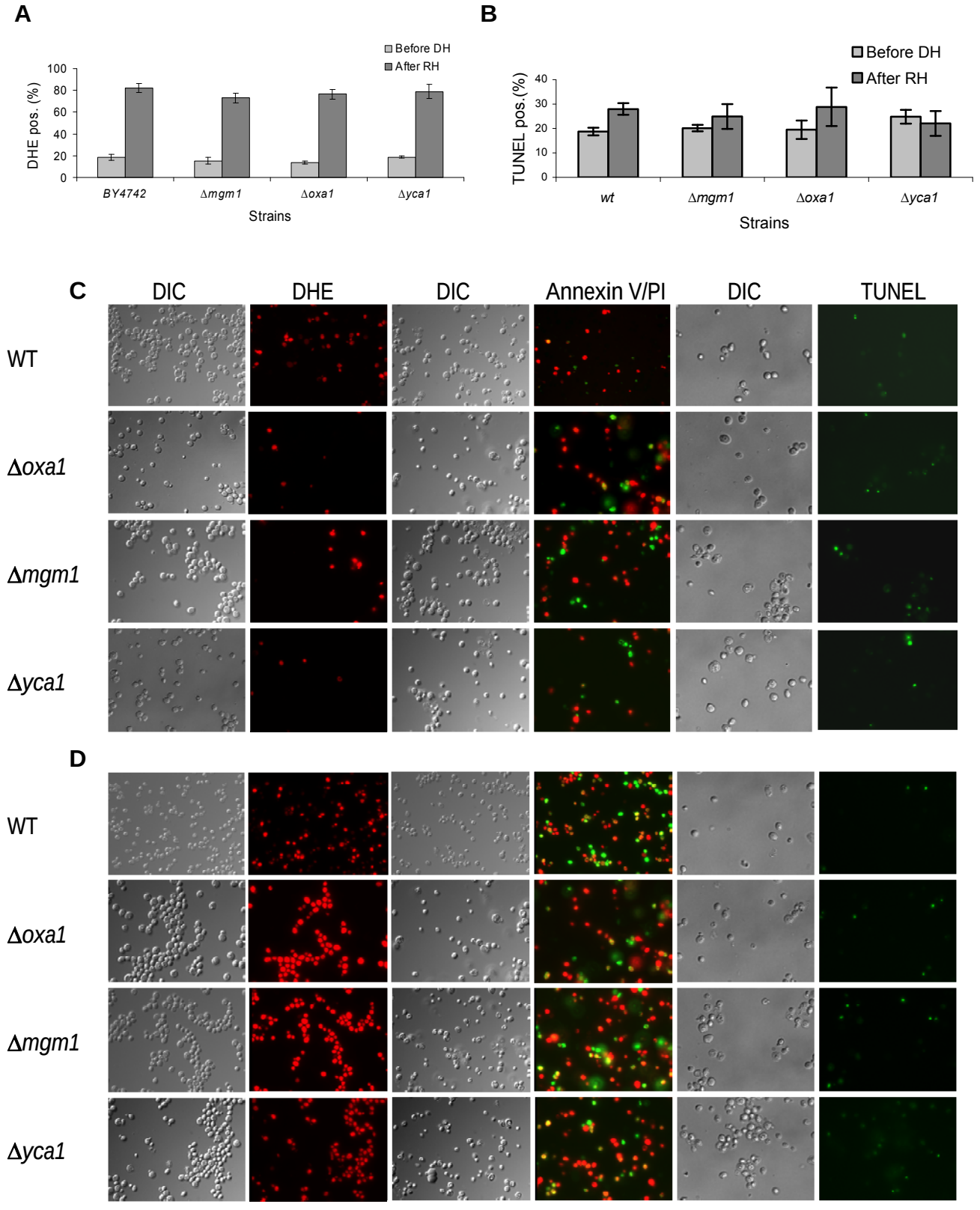

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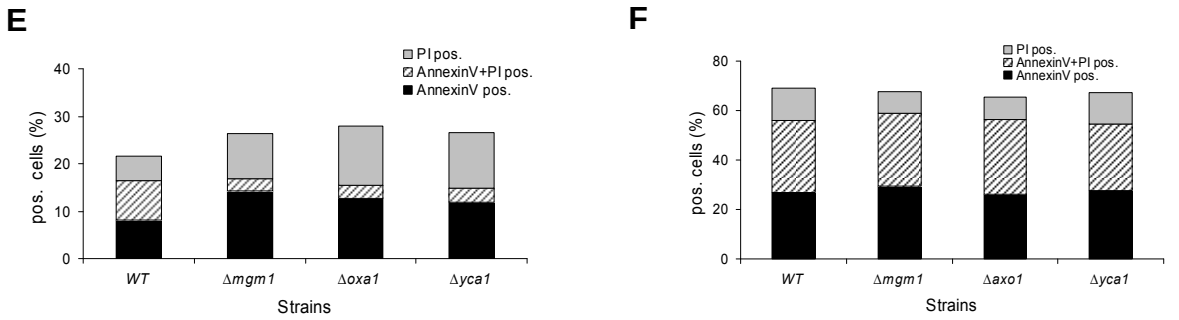

Figure 4. Cell death markers of two respiratory deficient strains (Δ*mgm1*, Δ*oxa1*), Δ*yca1* and the wild type BY4742 after drying and rehydration (A). ROS accumulation in cells of the reference strain BY4742, Δ*aif1,* Δ*nuc1,* Δ*cpr3 and* Δ*qcr7* before drying and after rehydration determined via DHE→Ethidium conversion. In each experiment, 30,000 cells were evaluated using flow cytometry. Data represent means +/- standard deviation (n = 3). (B) TUNEL positive quantification of the reference strain BY4742, Δ*mgm1,* Δ*oxa1,* and Δ*yca1* before dehydration and after rehydration respectively. (C) and (D) Fluorescence microscopy of DHE→Ethidium conversion, Annexin V (green)/ PI (red) costaining and TUNEL staining of the reference strain BY4742, Δ*mgm1,* Δ*oxa1,* and Δ*yca1* before dehydration and after rehydration, respectively. (E) and (F) Quantification of Annexin V/PI costaining of the reference strain BY4742 , Δ*mgm1,* Δ*oxa1,* and Δ*yca1* before dehydration and after rehydration respectively. In each experiment, 30,000 cells were evaluated using flow cytometry.

Sip18 hydrophilin prevents cell death during drying and rehydration process

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ABSTRACT

The yeast *Saccharomyces cerevisiae* is able to survive dehydration and long periods in a dry state; the metabolic activity of the cell is arrested during this time, but resumes after rehydration. We have used a yeast gene deletion set (YGDS) of 4850 viable mutant haploid strains to perform a genome-wide screen. Among the essential genes we characterized to overcome the cell-drying/rehydration process, six of them belong to the group of very hydrophilic proteins known as hydrophilins. Although the functional role of hydrophilins remains speculative, there is evidence to support their participation in acclimation and/or in the adaptive response to stress. The over-expression in *S. cerevisiae* of gene *SIP18*, which encodes the yeast's hydrophilin, increases cell viability during the dehydration process. The apoptotic hallmarks of over-expressing *SIP18* and Δ*sip18* strains, such as the accumulation of reactive oxygen species (ROS), phosphatidylserine externalization (Annexin V/PI staining) and DNA-strand breaks (Tunnel assay), were analysed before desiccation and after rehydration. DHE staining showed that the dehydration and rehydration process promotes a higher number of ROS-accumulating cells in the Δ*sip18* strain as compared to the over-expressed and reference strains. Furthermore, double staining Annexin V/PI reveals, for the Δ*sip18* strain, an elevated percentage of necrotic- and late apoptotic/early necrotic-cells after stress imposition. Further evaluations using the over-expressing strain suggest that SIP18 prevents cell death during dehydration stress by acting as an antioxidant.

Introduction

The assumption that "water is essential for life" is usually beyond contestation. However, since A. van Leeuwenhoek's revival of dried rotifers upon rehydration over

300 years ago, the phenomenon of desiccation tolerance has received, until now, little attention. Anhydrobiotic organisms are widespread in the plant kingdom, in fungi such as *Saccharomyces cerevisiae*, and in small animals such as rotifers, nematodes and tardigrades. Anhydrobiosis (desiccation tolerance) is considered in the context of a state of suspended metabolism (stasis) induced by the removal of cell water (Crowe *et al*. 1992). A cell that is sensitive to water deficit becomes consequently harmed at some point[s] during the phase[s] of drying, desiccation or rehydration. To understand what anhydrobiotics organisms do to overcome stress imposition, we must deal with controversial issues such as cell age, longevity, the structural and biochemical properties of anhydrous cytoplasm, and metabolic stasis (Potts 2001.

Recently, we performed a genome-wide screen in *S. cerevisiae* to identify genes that modify cell mortality after the dehydration stress. Among the essential genes characterized to overcome the cell-drying/rehydration process in this study, six belong to the group of very hydrophilic proteins known as hydrophilins. This group of proteins is defined as sharing a number of physicochemical characteristics: a Gly content greater than 6%, the presence of small amino acids such as Ala and Ser, and a high hydrophilicity index >1.0 (Battaglia *et al*. 2008). The fact that the transcripts of most genes encode hydrophilins in response to osmotic stress suggests that they represent an extensive adaptation to water deficit (Posas *et al.* 2000, Garay-Arroyo *et al*. 2000). *S. cerevisiae's* genome contains 12 genes which encode proteins with the characteristics of hydrophilins (Garay-Arroyo *et al*. 2000). Ectopic expression of some plant hydrophilins (Late Embryogenesis Abundant, LEA proteins) in yeast confers a tolerance to water-deficit conditions (Swire-Clark & Marcotte 1999, Zhang *et al*. 2000). Hydrophilin research in different organisms has provided us with significant advances towards understanding some of their biological properties. These include their roles as antioxidants and as membrane and protein stabilisers during water stress, either by direct interaction or by acting as a molecular shield (Reyes *et al*. 2005, Goyal *et al*. 2005, Tunnacliffe & Wise, 2007).

We report herein on a genetic screen of *S. cerevisiae*'s deletion library for mutants sensitive to dehydration stress, undertaken to discover cell dehydration-tolerant genes. We next characterize the effects of *SIP18* over-expression in yeast gene deletion strain, which is sensitive to stress imposition.

Materials and methods

Yeast strains and media

Yeasts strains used in this study (Table 1). Yeast cells were grown in SC media containing 0.17% yeast nitrogen base (Difco), 0.5% $(NH_4)_2SO_4$ and 84 mg/l Leucine, 25 mg/l uracil and 42 mg/l lysine and histidine.

Determination of viability

Yeast cells were cultivated in SC medium until the stationary phase. Some of the culture was then transferred to a 12-well plate in the presence of trehalose at 10% W/V of the final concentration. Half of the cell suspension was transferred to another 12-well plate for drying. The cells in the second plate were dried with air at 28 °C over a period of 24 hours. They were subsequently rehydrated with sterile water at 37 °C for 30 minutes.

For survival platings, the cell cultures were diluted, the cell concentration was determined with a CASY cell counter, and aliquots containing 500 cells were plated on YPD plates before drying and after rehydration. The number of colonies was determined after two days at 28 ºC. The CFU were quantified using a Microbiology-Colony-Counter (Lemnatec) and processed using SAWmicrobio version 3.1. After the colonies were counted the viability percentage was determined through a simple calculation.

Tests for apoptotic markers

DHE staining, Annexin V/PI costaining and TUNEL staining were performed as described in Büttner S. *et al.* (2007). The same samples were analysed by fluorescence microscopy. To determine frequencies of morphological phenotypes revealed by TUNEL, DHE- and Annexin V/PI double staining, at least 300 cells from three independent experiments were evaluated by using flow cytometry and BD FACSDiva software. A fluorescence microscope (model BH-2RFCA; Olympus), a digital camera (model c35AD-4; Olympus), and Metamorph® software (Soft Imaging System GmbH) were used for image acquisition.

Green fluorescent protein (GFP) fusions were used as biosensors for examining protein localization.

Results

Primary screening of deletion mutants to identify strains demonstrating lower viability after stress imposition

The different desiccation tolerance capacities of a collection of 4850 viable mutant haploid strains were assessed by performing a colony-counting assay. The CFU/ml mean value for survival after rehydration was calculated after taking into account viability before drying. Before screening the collection of mutants, cells from BY4742 were re-suspended in both 10% trehalose and in pure water, thus setting the reference condition for the evaluation of yeast cell viability prior to screening. The survival rate of BY4742 under a deionised water condition after rehydration was very low: 20% viability. However, the cells dried in the presence of trehalose showed an increase in viability of around 35%. The average viability rate presented by the reference *S. cerevisiae* strain used in this study was 55%. That result supports the idea that the use of trehalose for all mutants during yeast-drying is advantageous. It is known that trehalose acts as a membrane protector by reducing the membrane phase transition temperature during the rehydration process (Rodriguez-Porrata et al., 2009). Therefore, we ranked the 4850 mutants in order of their increasing viability rate; they were then polled into five arrays: <10%, 10-19%, 20-39%, 40-60%, and >60% of viability. Of the 4850 mutants, 116 (~2.4%) were identified as having enhanced desiccation sensitivity. These genes have been listed alphabetically by ORF in Table 1. 81% (94/116) of the mutants with <10% of viability corresponded to genes for which a function or genetic role has been determined experimentally or can be predicted (Table 1). Thirty percent (34/116) of these clustered in the functionally related categories of protein synthesis and biogenesis of cellular components, based on annotations in the Munich Information Centre for Protein Sequences functional catalogue. The remaining genes were dispersed among numerous and diverse functional categories (Fig. 1). Sixteen percent (16/116) of the identified genes are annotated as having human orthologs (Table 1), a value that is not significantly higher than the percent of genes in the yeast genome with mammalian orthologs (around 31%, $P < 1 \times 10^{-10}$) (Botstein *et al* 1997). Among the 23 knockout mutants identified as falling into the functional category of stress response with <10% of viability, seven of them normally show gene activation by different kinds of stress, such as: DNA damage response, *RDH54* (Klein 1997) and *RIF2* (Teixeira *et al.* 2004); osmotic stress response *SIP18* (Miralles & Serrano 2002); oxidative stress *GRX5* (Rodríguez-Manzaneque *et al.* 1999); and general stress response *RPB4* (Miyao *et al*

2001), *ORM2* (Hjelmqvist *et al*. 2002), and *HSP30* (Piper *et al*. 1997). We next characterized the effects of increasing Grx5p, Rpb4p, Sip18p, Rdh54p, Rif2p, Orm2p, and Hsp30p levels in stationary state cells, using a plasmid that allows expression of their encoding genes under the control of the *GAL1* promoter (*pGAL1*) in the corresponding yeast gene deleted strain. The transformants over-expressing *RPB4* (RNA Polymerase B), *RDH54* (Radiation sensitive Homolog to *RAD54*), *ORM2* (similar to *ORM1*), and *HSP30* (Heat Shock Protein) genes displayed wild-type viability or exhibited a slight decrease in cell viability. However, the *GRX5* (GlutaRedoXin), *SIP18* (Salt Induced Protein), and *RIF2* (Rap1p-Interacting Factor) transformants displayed at least a 30% higher viability than did the BY4742 strain (Fig. 2). No correlation between the kind of stress response genes and viability was observed in these over-expressing strains. Of these three genes, only *SIP18* has shown early transcriptional response in different genetic backgrounds during the process of drying and rehydration (Singh *et al*., 2005; Rossignol *et al*., 2006; Novo *et al*., 2007), therefore we prioritised the study of Sip18p to determine whether a correlation exists between intracellular Sip18p abundance and yeast dehydration tolerance.

GFP-SIP18 fusion protein is accumulated in the cytoplasm

With the aim of investigating Sip18p localization, a strain boarding a fusion between Sip18p and the green fluorescent protein (GFP) integrated in the *SIP18* locus (*GFP-SIP18*) was observed after 48 h growth in YPD at 28°C. This fusion was expressed, as is the case for the wild type protein, mainly due to its promoter induction at the stationary phase (Gash *et al*. 2000). The fluorescent signal from the fusion GFP-SIP18 protein was significant, giving a uniform labelling of the cells (Fig. 3 A), suggesting cytoplasmic localization, as was detected previously (Huh *et al.*, 2003), but in no case was labelling of the cell surface, nucleus or vacuole system observed. In order to increase the amount of protein within the cell to higher levels than in the wild type, the GFP-Sip18p fusion was placed under the control of the *GAL1* promoter (*pGAL*), which is less active than *pSIP18* at the stationary phase (Gash *et al*. 2000). The Δ*sip18* strain transformed with this plasmid was observed after 48 h cultivation in YPD and YPGal. This fusion was expressed at a very low level in the presence of glucose, giving a diffuse labelling of the cells mainly due to the low activity of *pGAL* even after glucose starvation. Although the cells grown in YPGal exhibited a higher fluorescent signal than the cells under glucose condition, they lacked a uniform labelling of cells (Fig. 3 A).

The transformants expressing the GFP fusion protein did not show significant statistical differences in cell viability when compared to the wild type after stress imposition (Fig 3 B). The cytoplasmic localization of GFP-Sip18p cells in stationary state were similar in the cells grown in the carbon sources glucose and galactose. These results allow us to propose that the addition of the GFP tag did not add/include any phenotypical defect in the viability of the BY4742 strain after the dehydration and rehydration process (Fig. 3 B).

Overexpression of GFP-Sip18p fusion does not result in its import into the nucleus

In order to further increase the amount of protein within the cell, the *pGAL-GFP-SIP18* transformants at stationary phase were complemented with 2% galactose. Two independent transformants expressing the GFP fusion protein in stationary phase did not show statistically significant differences in cell viability following stress imposition after 4 h of galactose induction (Fig. 4 C). Beyond 2 h of galactose exposure, the cells show a substantial fluorescent signal from the fusion GFP-Sip18p. The localization of this fluorescence changed from a uniform cell labelling at 0 h to a very significant nuclear localization after 2 and 4 h of incubation in the presence of galactose; at the same time, the control strain overexpressing GFPp did not show a cellular fluorescent pattern (Fig 5). In order to better observe the presence of the full protein in the nuclear compartment, cells of the overexpressing GFP-Sip18p fusion strain after 4 h of galactose induction were observed using a confocal microscope. The fluorescence was mainly revealed in a specific region of the cell, but also in the cytosol, with a pattern that suggested internal nucleus association (Fig. 4B). With the intention of clarifying whether Sip18p fusion results in its importation into the nucleus as a result of gene overexpression or osmotic stress (produced by the galactose, ~1.1M), or as a result of both acting synergistically, the 48 h stationary phase cells of transformant *pSIP18-GFP-SIP18* (*GFP-SIP18*) were submitted to different kinds of osmotic stress: 1M sorbitol, 1M glycerol, and 0.75 and 0.5M NaCl. These transformants exhibit similar localization in response to sorbitol and glycerol (Fig 4A); however, the cells exposed to NaCl did not reveal any change after up to 3 h of incubation, even when complemented with 10 mM or 30 mM ascorbic acid (data not shown). Therefore, the nuclear localization of SIP18p in stationary state cells is mainly driven by osmotic stress. On the other hand, these results confirm that the overexpression of SIP18p fusion did not exhibit any cytolocalization defect in the Δ*sip18*, *pGAL1-GFP-SIP18* strain but did raise the level

of the protein enough to produce a remarkable effect on the fluorescence, anti-GFP Western blot detection (data not shown), and dehydration tolerance phenotype.

Sip18p cytolocalizes at the nucleus during dehydration process

Since neither the fusion with GFP nor the overexpression seemed to affect the localization of the SIP18 protein, the fluorescent signal from the fusion GFP-Sip18p was followed during dehydration and rehydration stress. After GFP-Sip18p overexpression, the cells were exposed to a dehydration process. Samples analysed after 4 h, 8 h and 22 h of drying mainly showed a nuclear localization pattern for the GFP-Sip18p (Fig. 5). After 30 min of a rehydration process using pure water, we could not observe a clear cytolocalization of it in those cells that show fluorescence. Moreover, even after 3 h of inoculation into YPD media, the fluorescence profile of rehydrated cells was mainly nuclear (Fig. 5). For the same time-points, the reference strain pGAL-GFP shows fluorescence, but no pattern of cytolocalization was detected (data not shown). The characterised nuclear localization of the overexpressed Sip18p under the osmotic stress produced during cell dehydration process might permit the cell dehydration tolerance phenotype.

Dehydration tolerant strain shows reduction in apoptotic hallmarks during stress imposition

We wished to ascertain whether the higher viability rate of Sip18p overexpression after the dehydration and rehydration process could be due to differences in the apoptotic hallmarks profile, compared to that of the wild type. Yeast strains were grown in rich medium, and cells from stationary phase before desiccation and after rehydration were analysed for apoptotic hallmarks, such as the accumulation of reactive oxygen species (ROS), phosphatidylserine externalization (Annexin V/PI staining), and DNA-strand breaks (Tunnel assay). The intracellular accumulation of ROS is a warning sign of the onset of apoptosis. Yeast cells were incubated in the presence of Dihydroethidium (DHE); the intracellular ROS oxidizes it into the fluorescent ethidium bromide, permitting the quantification of ROS-accumulating cells (Fig. 6A). Before dehydration, around 20% of Δ*sip18* and Δ*sip18 pGAL-GFP* cells show fluorescence after DHE incubation, whereas only 15% of Δ*sip18 pGAL-GFP-SIP18* cells, of two independent transformants (I and II), show ROS-accumulation at this moment (Fig. 6B). After rehydration, 50% of the reference cells showed intense intracellular DHE staining, but

for both overexpressing Sip18p clones, DHE accumulation only rises to 30%. Therefore, we explored whether changes in cell viability for the strain with elevated dehydration tolerance correlates with another phenotypic marker of apoptosis. The co-staining with Annexin V and propidium iodide (PI) was used to quantify the early apoptotic event of phospahtidylserine externalization and the membrane permeabilization of necrotic cells, respectively (Fig. 7A). Consequently, using flow cytometry we were able to quantify apoptotic (Annexin V^+/PI^-), late apoptotic or early necrotic (Annexin V^+/PI^+), and necrotic (Annexin V^-/PI^+) cells (Fig 7B and 7C). Before dehydration, around 15% of Δ*sip18* and Δ*sip18 pGAL-GFP* cells are Annexin V^-/PI^+, whereas only 5% of *sΔip18 pGAL-GFP-SIP18* cells show necrotic fluorescent profiles at this moment. Additionally, the reference strains and the dehydration-tolerant clones have similar values of Annexin V^+/PI^- and Annexin V^+/PI^+ cells, ~12% and ~5% respectively (Fig. 7B). After rehydration, the Annexin V^+/PI^- and Annexin V^-/PI^+ cells for all the strains did not exhibit significant statistical differences, ~12% and ~11% respectively. Nevertheless, cells from the reference strains show 2-fold higher amounts of Annexin V^+/PI^+ cells as compared to the overexpressing Sip18p clones. Taking into collective consideration the cell viability results of overexpressing SIP18p strains (Fig. 4C) and the ROS accumulation values (Fig. 6), we can suggest that there is a correlation between the increase in the desiccation survival rate and the intracellular ROS levels, imparting a reduction of Annexin V^+/PI^+ cells after stress imposition. Therefore, we hypothesize that SIP18p plays a physiological role as an antioxidant agent.

Dehydration tolerant strain shows reduction in DHE cells after oxidative stress by H_2O_2

When 4 h galactose-induced cells from the Δ*sip18*, Δ*sip18 pGAL-GFP* and Δ*sip18 pGAL-GFP-SIP18* strains were subjected to 4 mM H_2O_2, the overexpressing SIP18p strain showed a significant reduction in the number of DHE cells. As shown in Fig. 8, after 10 min of exposure to H_2O_2, the number of ROS accumulating cells in the dehydrating tolerant strain was less than 50% of the value for the reference strains, supporting the hypothesis for the antioxidant capacity of SIP18p. However, at 20 and 40 min, the number of DHE positive cells was equally affected by H_2O_2 stress, suggesting that SIP18p did not show a positive, strong effect on the clearing of H_2O_2.

DISCUSSION

The desiccation of yeast tolerant cells causes a complete arrest of cellular metabolism, followed, for a period of time, by a state of suspended animation and a subsequent recovery of metabolic functions. A cell that is sensitive to water deficit is consequently harmed at some point[s] during the phase[s] of drying, desiccation or rehydration. There are not many organisms capable of achieving these complex phase changes which otherwise would allow them to overcome the simple stress of desiccation-rehydration process. We have systematically screened a haploid, single gene, deletion mutant library and identified 116 genes which are essential to desiccation tolerance. To our knowledge, this is the first systematic screen for the identification of putative genes essential to overcoming the cell dehydration process. The 116 genes identified in the screen cover a number of different biological processes based on the Gene Ontology annotations. The broad range of identified mutants indicates that these genes are involved directly or indirectly in dehydration tolerance. Attempts have recently been made to describe the early transcriptional response of both wine yeasts and the BY4742 haploid strain during the process of drying and rehydration, and they have shown changes in the expression of genes involved in lipid binding and synthesis, protein synthesis, and metabolism (Singh *et al*., 2005; Rossignol *et al*., 2006; Novo *et al*., 2007). The reported genes that show transcription activation early on in the rehydration or inoculation steps are consistent with the idea that some of them might be essential to the cell's ability to recover from dissecation stress. The hypersensitivity of the mutants to dehydration of the heat shock protein HSP30, the 6-phosphofructokinase alpha subunit PFK1, the epimerase peroxisomal FOX2, the ribosomal protein RPS1A, the RNA polymerase A chain RPA49, and the uncharacterized osmotic stress protein SIP18 allow us to draw a parallel with the relatively early gene activation previously observed. Based upon results presented here, we hypothesize that these genes are involved directly in the dehydration stress resistance pathways, which are present in different yeast strains.

Our results explain the fact that overexpressing *SIP18* impedes yeast mortality during the imposition of dehydration stress. The induction of *SIP18* transcription in response to osmotic stress suggests that this hydryphilin represents a widespread adaptation to water deficit (Garay-Arroyo *et al*. 2000; Posas *et al*. 2000; Yale and Bohnert 2001). Our observations showed a rapid nuclear accumulation of SIP18p in response to osmotic and cell dehydration stress. However, when the osmotic stress was induced by adding 0.5M NaCl to the culture medium, such nuclear accumulation was not observed. Hirasawa *et*

al. (2006) observed that the transcriptional regulation response to the addition of 0.5M NaCl is slightly different from that observed when 1M sorbitol is added, which corresponds to the same osmotic pressure for 0.5M NaCl. However, the difference becomes small under 0.75M NaCl and 1M sorbitol addition conditions when the dynamics of the changes in gene expression are similar. Yet, the difference in SIP18p cytolocalization when adding sorbitol as opposed to NaCl was very different, suggesting that this variation might not be driven by differential transcription regulation. The effects of osmotic pressure associated with sodium and chloride ion toxicity abolished the import of SIP18p into the nucleus. In this hypertonic condition (containing more than 0.8 M NaCl), yeast cells show an increase in both the generation of superoxide species and the oxidation of cellular low-molecular weight thiols and a decrease in the total antioxidant capacity of cellular extracts (Koziol *et al*. 2005). Another effect induced by exposure to this hypertonic medium is the modification of intracellular phosphotransfer rates, as was described within the SLN1-YPD1-SSK1 phosphorelay (Kaserer *et al.* 2009). If superoxide abolishes the export of SIP18p, one should expect antioxidant complementation to be able to scavenge superoxide. In that case, a correlation of SIP18p nuclear localization resulting from the reaction with superoxide and the antioxidant studied should be expected. However, the cells under ionic-hypertonic stress do not show any nuclear fluorescence in the presence of ascorbic acid.

The fact that SIP18p might therefore shuttle between the cytoplasm and the nucleus is an ability that might be related to its anti-apoptotic function. Here we have demonstrated that SIP18p acts as an inhibitor of necrosis in yeast under dehydration stress, suggesting its antioxidative capacity, demonstrated by the reduction of ROS accumulation after an H_2O_2 attack. Nevertheless, the molecular mechanism by which SIP18p can prevent yeast necrosis has remained elusive. *SIP18* was originally identified as an inducible gene by osmotic stress (Scheglmann *et al.* 2002; Marquez *et al.* 1998; Miralles and Serrano 1995), whereas the biological process remains unknown. We show here that SIP18p acts as an inhibitor of necrosis in yeast during the dehydration process, enhancing cell viability after stress imposition. The Gly content of SIP18p is greater than 6% and its hydrophilicity index higher than 1; these physicochemical characteristics allow SIP18p to be classified as a member of the widespread group of proteins called “hydrophilins” (Battaglia *et al.* 2008). Although the functional role of hydrophilins remains speculative, there is evidence to support their participation in acclimation and/or in the adaptive response to stress. Ectopic expression of some plant

hydrophilins (Late Embriogenesis Abundant, LEA proteins) in yeast confers tolerance to water-deficit conditions (Swire-Clark & Marcotte 1999, Zhang *et al*. 2000). On the other hand, in vitro dehydration tests in which the activities of malate dehydrogenase and lactate dehydrogenase were measured in the presence or absence of hydrophilins from plants (DSP16, ERD10, AtLEA76, AtD113, PvLEA18), bacteria (YCIG), and yeast (SIP18p), all except PvLEA128 proved to be efficient protectants of enzyme activities *in vitro* during partial water loss. Under similar conditions, trehalose was required in a 10^5-fold molar excess over hydrophilins to confer the same protective level, suggesting that they allow protection via different mechanisms (Reyes *et al*. 2005). A similar study was performed *in vitro* and *in vivo* with the group 3 LEA protein from the desiccation-tolerant nematode *Aphelenchus avenae* (AavLEA1), preventing enzyme aggregation in the yeast cell after desiccation or freezing stress (Chakrabortee *et al*. 2007). Hydrophilin research in different organisms has allowed us to make significant advances towards understanding some of their biological properties, including their roles as antioxidants and as membrane and protein stabilisers during water stress, either by direct interaction or by acting as a molecular shield. The hydrophilic proteins and compatible solutes might serve as "space fillers" to prevent cellular collapse at low water activity (Tunnacliffe & Wise, 2007). This recent data provides evidence that SIP18p allows the cell to survive probably by acting to stabilize other cellular proteins, as described for XIAP (Dohi *et al*. 2004) or BIR1p (Walter *et al.* 2006), rather than by directly interacting with apoptotic proteins in the nucleus, thereby inhibiting them.

With recent advances in tissue engineering, cell transplantation and genetic technology, successful long-term storage of living cells is of critical importance. The demand to meet even common needs, such as the storage of blood cells in blood banks, is still a major problem. The complex regulatory network and the often contradictory results associated with high eukaryotic cells make the application of a simpler model system desirable. A number of advantages have made yeast cells the model of choice for anhydrobiotic engineering, including ease of growth and modification, well characterised cell physiology, genetics and biochemistry. Yeast promises to provide a better understanding of desiccation-tolerant genetics for potential applications in biomedicine, plant biotechnology, and beverage and bio-ethanol technology.

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Table 1. Plasmids and yeast strains used in this study

Strain	Genotype	Source
BY4742	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0	EUROSCARF
Δ*sip18*	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 yca1::kanMX4	EUROSCARF
Δ*sip18* pGal-GFP	MATα his3Δ1 lys2Δ0 ura3Δ0 yca1::kanMX4	This study
Δ*sip18* pGal	MATα his3Δ1 lys2Δ0 ura3Δ0 yca1::kanMX4	This study
BY4742 GFP SIP18	MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0	EUROSCARF

Plasmids

Vector	Description
pGREG505	LEU2
pGREG575	N term GFP, LEU2

Both vectors contain a G418 resistence marker (KanMX)

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Table 2. Primers designed for PCR

Table 2. Primers PCR

Primer	**5'-sequence-3'**
rec1 Forward	GAATTCGATATCAAGCTTATCGATACCGTCGACA
rec2 reverse	GCGTGACATAACTAATTACATGACTCGAGGTCGAC
SIP18 forward	GAATTCGATATCAAGCTTATCGATACCGTCGACAATGTCTAACATGATGAATAA
SIP18 reverse	GCGTGACATAACTAATTACATGACTCGAGGTCGACTTATTTTTTCATGTTTTCGT

pGREG recombination primer sequences (rec1 y rec2) used to tail PCR fragments and used for targeting them to specific in vivo recombination sites in pGREG vectors.

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Table 3. Yeast genes which lose viability during dehydration stress

ORF	Gene	Function	ORF	Gene	Function
YBL025W	RRN10	RNA polymerase I-specific transcription initiation factor	YHR039C-B	VMA10	H+-transporting ATPase V0 domain 13 KD subunit, vacuolar
YBL033C	RIB1	GTP cyclohydrolase II	YHR079C-B	SAE3	DMC1-dependent meiotic recombination
YBL038W	MRPL16	Mitochondrial ribosomal protein, large subunit	YIL084C	SDS3	Transcriptional regulator
YBL045C	COR1	Ubiquinol--cytochrome-c reductase 44K core protein	YIR021W	MRS1	Protein involved in mitochondrial RNA splicing of COB mRNA
YBL053W		Unknown	YJL140W	RPB4	*DNA-directed RNA polymerase II, 32 kDa subunit
YBL057C	PTH2	*Aminoacyl-tRNA hydrolase	YKR006C	MRPL13	Mitochondrial ribosomal protein, large subunit
YBL080C	PET112	Protein required to maintain rho+ mitochondrial DNA	YKR009C	FOX2	*Hydratase-dehydrogenase-epimerase, peroxisomal
YBR073W	RDH54	Protein required for mitotic diploid and repair and meiosis	YKR033C		Unknown
YBR146W	MRPS9	Mitochondrial ribosomal protein, small subunit	YKR085C	MRPL20	Mitochondrial ribosomal protein, large subunit
YBR172C	SMY2	Kinesin-related protein	YKR089C	TGL4	Triacylglycerol lipase involved in TAG mobilization
YBR194W	SOY1	Synthetic with Old Yellow enzyme	YKR090W	PXL1	LIM domain-containing protein that localizes to sites of polarized growth
YBR267W	REI1	Required for Isotropic bud growth	YKR098C	UBP11	*Ubiquitin C-terminal hydrolase
YBR268W	MRPL37	Mitochondrial ribosomal protein, large subunit	YLL018C-A	COX19	Protein required for expression of mitochondrial cytochrome oxidase
YBR282W	MRPL27	Mitochondrial ribosomal protein, large subunit	YLR136C	TIS11	*tRNA-specific adenosine deaminase 3
YCL003W	PGS1	Phosphatidylglycerophosphate synthase	YLR138W	NHA1	Na+/H+ exchanger (also harbouring K+/H+ activity)
YCR021C	HSP30	Heat shock protein	YLR144C	ACF2	Endo-1,3-beta-glucanase
YCR045c		Unknown	YLR350W	ORM2	Protein required for resistance inducers of unfolded protein response
YCR050c		Unknown	YLR428C		Unknown
YCR068w	ATG15	Lipase, intravacuolar lysis of autophagic bodies	YLR429W	CRN1	Actin polymerization and crosslinking to microtubules
YCR071c	IMG2	Mitochondrial ribosomal protein, large subunit	YLR434C		Protein localized to mitochondria
YCR094w	CDC50	Cell division cycle mutant	YLR435W	TSR2	Twenty S rRNA accumulation protein
YDL020C	RPN4	Transcription factor stimulates proteasome genes	YLR437C		Conserved hypothetical protein
YDL039C	PRM7	Pheromone-regulated Membrane protein	YLR439W	MRPL4	Mitochondrial ribosomal protein, large subunit
YDL044C	MTF2	Mitochondrial protein involved in mRNA splicing	YLR441C	RPS1A	Ribosomal protein S3a.e
YDL045W-A	MRP10	Mitochondrial ribosomal protein, small subunit	YLR453C	RIF2	Rap1p interacting factor 2
YDL056W	MBP1	Transcription factor, subunit of the MBF factor	YLR454W	FMP27	Found in Mitochondrial Proteome
YDL068W		Unknown	YLR456W		Unknown
YDL091C	UBX3	UBX (ubiquitin regulatory X) domain-containing protein	YMR008C	PLB1	*Phospholipase B (lysophospholipase)
YDL093W	PMT5	Unknown	YMR009W	ADI1	Aci-reductone dioxygenease involved in the methionine salvage pathway
YDL107W	MSS2	COX2 pre-mRNA splicing factor	YMR158C-B		Unknown
YDL114W		Unknown	YMR158W	MRPS8	Mitochondrial ribosomal protein, small subunit
YDL133W		Unknown	YMR175W	SIP18	Osmotic stress protein
YDR010C		Unknown	YMR320W		Hypothetical protein
YDR175C	RSM24	Mitochondrial ribosomal protein, small subunit	YNL003C	PET8	Protein of the mitochondrial carrier family (MCF) - unknown function
YDR197W	CBS2	Cytochrome B translational activator protein	YNL073W	MSK1	*Lysyl-tRNA synthetase, mitochondrial
YDR204W	COQ4	Responsible for restoring ubiquinone biosynthesis	YNL081C	SWS2	Putative mitochondrial ribosomal protein of the small subunit
YDR337W	MRPS28	Mitochondrial ribosomal protein, small subunit	YNL248C	RPA49	DNA-directed RNA polymerase A (I) chain, 46 kDa
YDR350C	TCM10	Protein functions at a post-translational stage	YNL280C	ERG24	C-14 sterol reductase
YDR363W-A	SEM1	*Regulator of exocytosis and pseudohyphal differentiation	YNL294C	RIM21	Regulator of IME2
YDR405W	MRP20	Mitochondrial ribosomal protein, large subunit	YNR036C		Unknown
YDR432W	NPL3	*Nucleolar protein	YOR150W	MRPL23	Mitochondrial ribosomal protein, large subunit
YDR433W	KRE22	Unknown	YOR155C	ISN1	Catalyzes the breakdown of IMP to inosine
YDR511W	ACN9	Protein of gluconeogenesis in mitochondria	YOR158W	PET123	Mitochondrial ribosomal protein, small subunit
YEL024W	RIP1	Ubiquinol-cytochrome-c reductase iron-sulfur protein.	YOR305W		Unknown
YER087W		Putative prolyl-tRNA synthetase	YOR306C	MCH5	Transporter not monocarboxylate permease
YER145C	FTR1	High affinity iron transporter	YOR318C		Unknown
YER153C	PET122	Translational activator of cytochrome c oxidase subunit III	YOR333C		Unknown
YER154W	OXA1	Cytochrome oxidase biogenesis protein	YOR342C		Unknown
YFL036W	RPO41	DNA-directed RNA polymerase, mitochondrial	YPL029W	SUV3	ATP-dependent RNA helicase, mitochondrial
YFR032c		Weak similarity to S.pombe polyadenylate-binding protein	YPL059W	GRX5	*Glutaredoxin (subfamily Grx3, Grx4, and Grx5)
YFR032C-A	RPL29	60S large subunit ribosomal protein	YPL069C	BTS1	*Geranylgeranyl diphosphate synthase
YGR180C	RNR4	*Ribonucleotide reductase, small subunit	YPL104W	MSD1	*Aspartate--tRNA ligase, mitochondrial
YGR222W	PET54	Splicing protein and translational activator, mitochondrial	YPL183W-A		Unknown
YGR231c	PHB2	Prohibitin	YPR024W	YME1	*Protease of the SEC18/CDC48/PAS1 family of ATPases (AAA)
YGR240c	PFK1	6-phosphofructokinase, alpha subunit	YPR047W	MSF1	*Phenylalanine--tRNA ligase alpha chain, mitochondrial
YGR243w	FMP43	Unknown	YPR067W	ISA2	Mitochondrial protein required for iron metabolism
YHL007c	STE20	Cdc42p-activated signal transducing kinase			
YHR011W	DIA4	Probable mitochondrial seryl-tRNA synthetase	YPR099C		Unknown
YHR038W	RRF1	Mitochondrial ribosome recycling factor	YPR123C		Unknown

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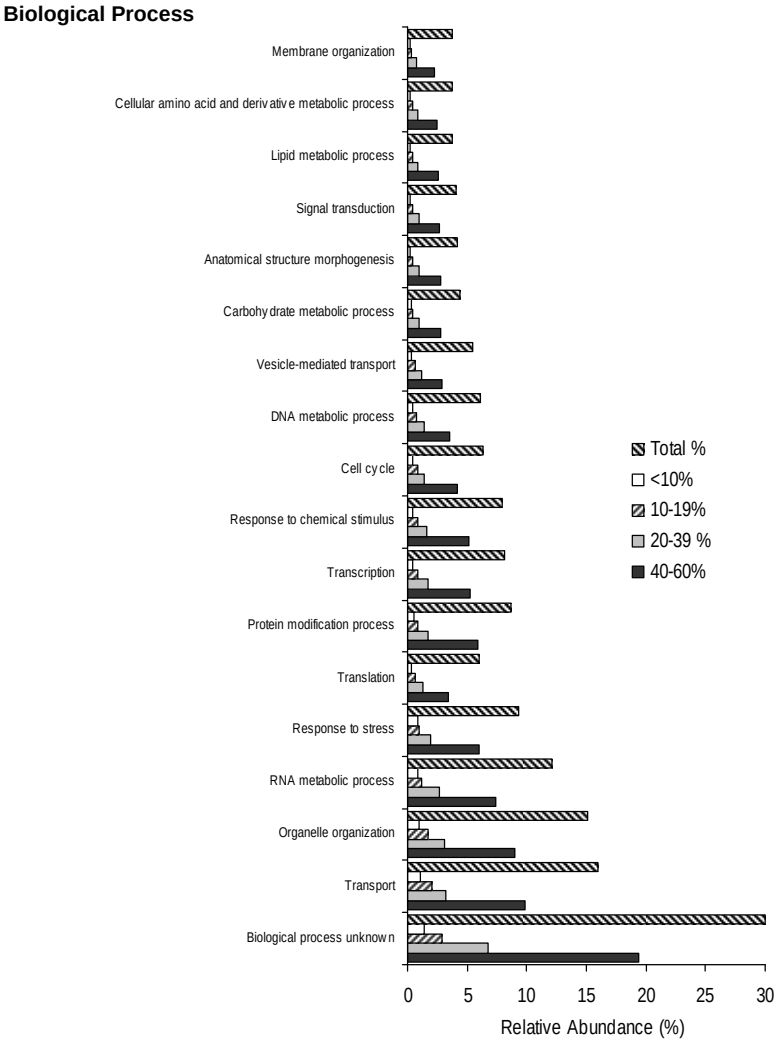

Fig. 1. Comparison of the relative percentage of genes in functional categories for the yeast gene deletion set (YGDS). Around 3 percent (116/4862) of genes that significantly enhance dehydration mortality (<10% of viability) clustered in the related categories of: response to stress, transport, organelle organization, and RNA metabolic process.

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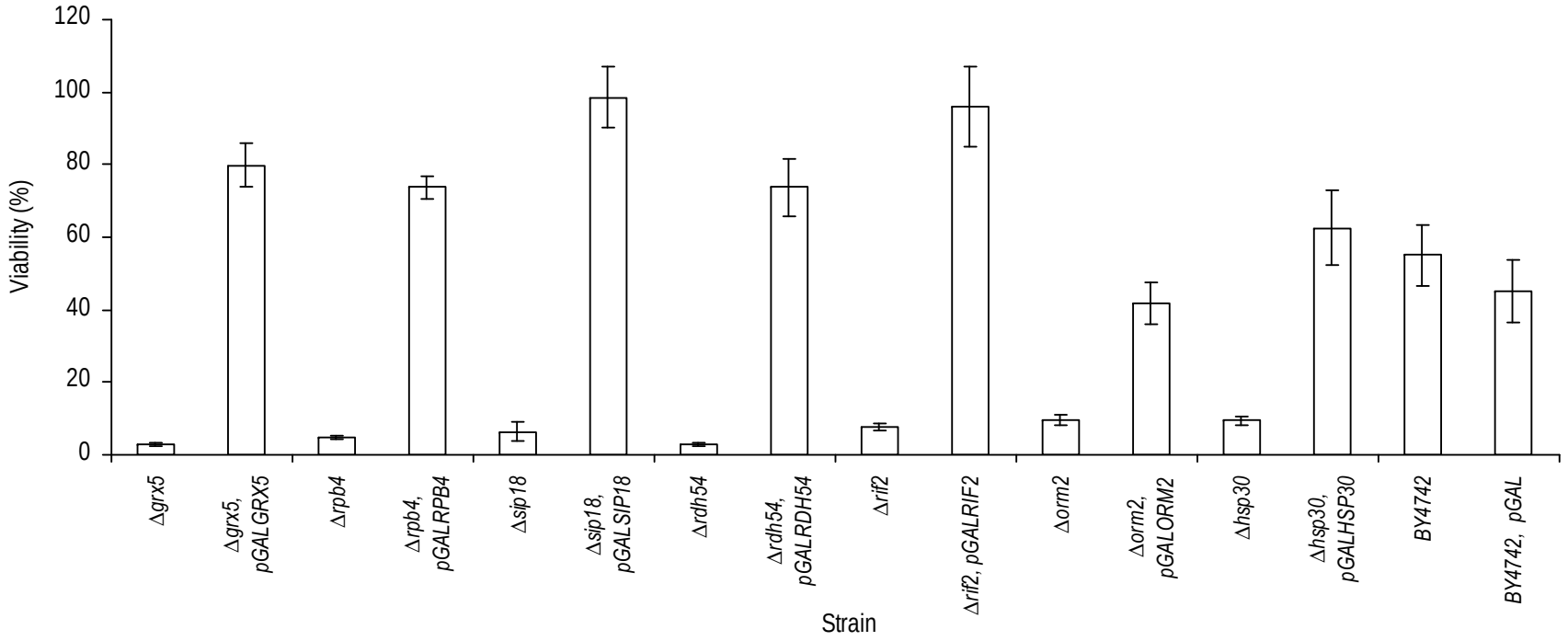

Fig. 2. Effect of overexpressing rescue cell genes in yeast viability after cell-drying and rehydration. The scale of viability (%) indicates the percentage of experimental values for the different strains. Values showed are means of n=3 independent samples ±SD. *Significant differences ($p \leq 0.05$) to the deleted strain.

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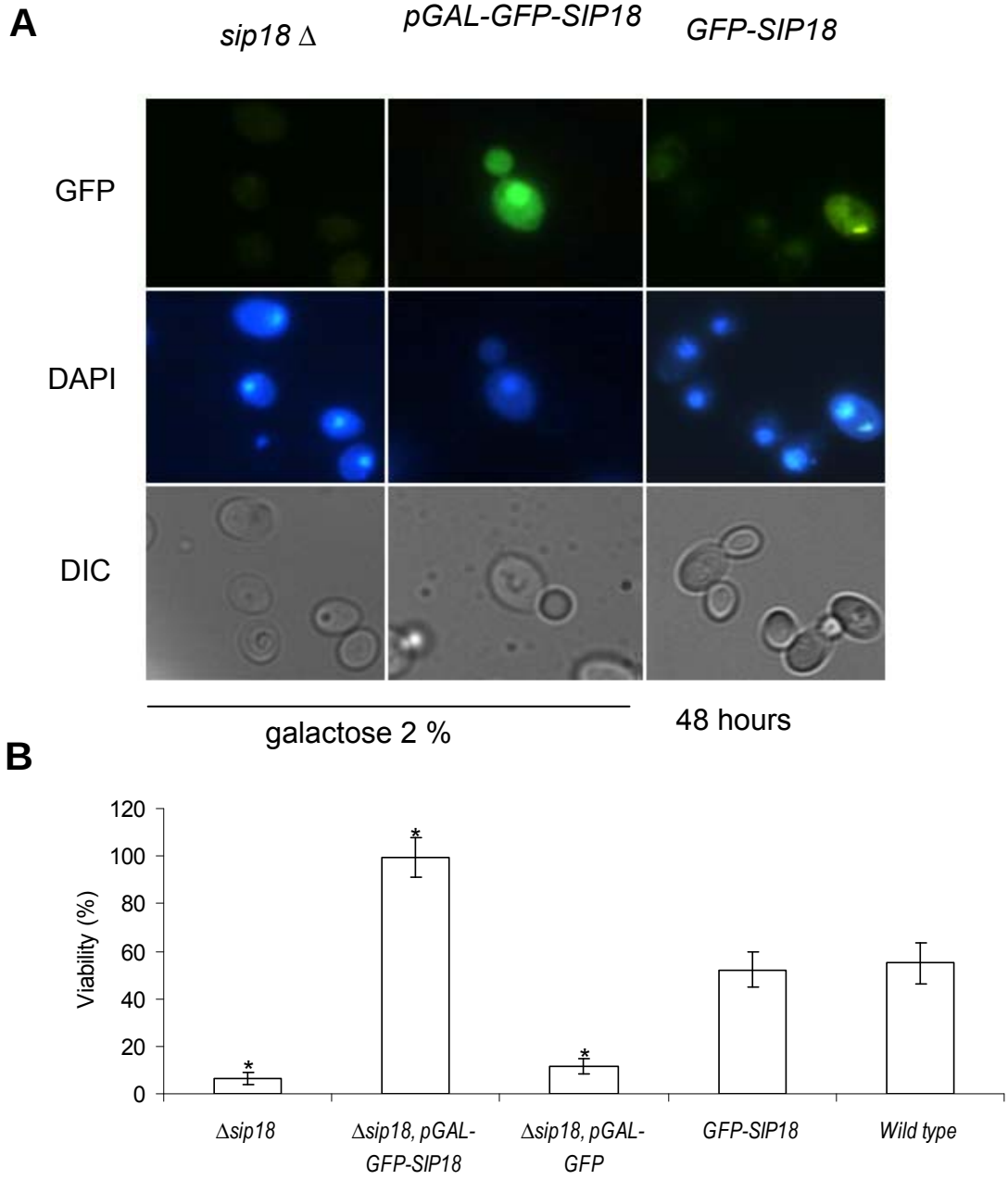

Fig. 3. *GFP-SIP18* fusion localize at the cytoplasm. A) Each column of pictures presents phase microscopy of the same field, which shows fluorescently-labeled green fluorescent protein (GFP), nuclear stained (DAPI), and differential interference contrast (DIC) images of cultured yeast cells. The Δ*sip18* cells transformed with the vector expressing *GFP-SIP18* under *GAL* promoter regulation were photographed after 4 h f galactose (YPGal) or glucose (YPD) supplementation. Cells expressing GFP-SIP18 fusion protein under *SIP18* promoter were photographed after 24 h in stationary phase. B) The scale of viability (%) indicates the percentage of experimental values for different strains, after the dehydration and rehydration process, relative to the highest value for the fresh cultures before stress imposition. Values are means of n=3 determinations ± standard deviation. *Significant differences ($p \leq 0.05$) to the wild type strain.

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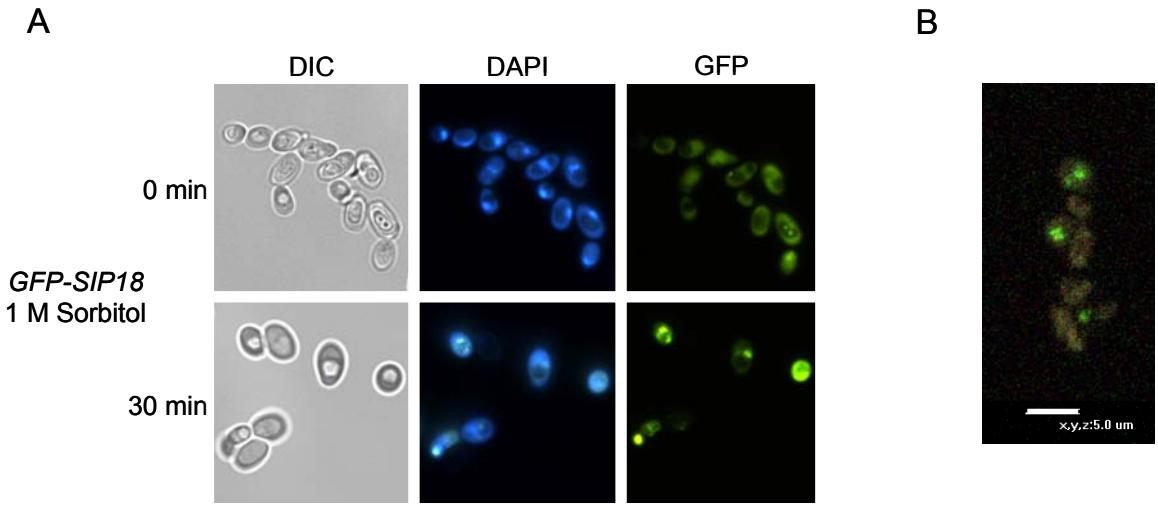

Fig. 4. GFP-Sip18p under osmotic stress moves into the nucleus. A) The cells expressing *GFP-SIP18* under native promoter were photographed before and 30 min after a hyperosmotic shock induced by 1 M sorbitol, and localization is shown with DAPI fluorescence to visualize the nuclear body. B) Analysis of hyperosmotic-shocked cells expressing GFP-SIP18p using the conofocal microscope. Image generated by the average of a pile of 3 optical sections.

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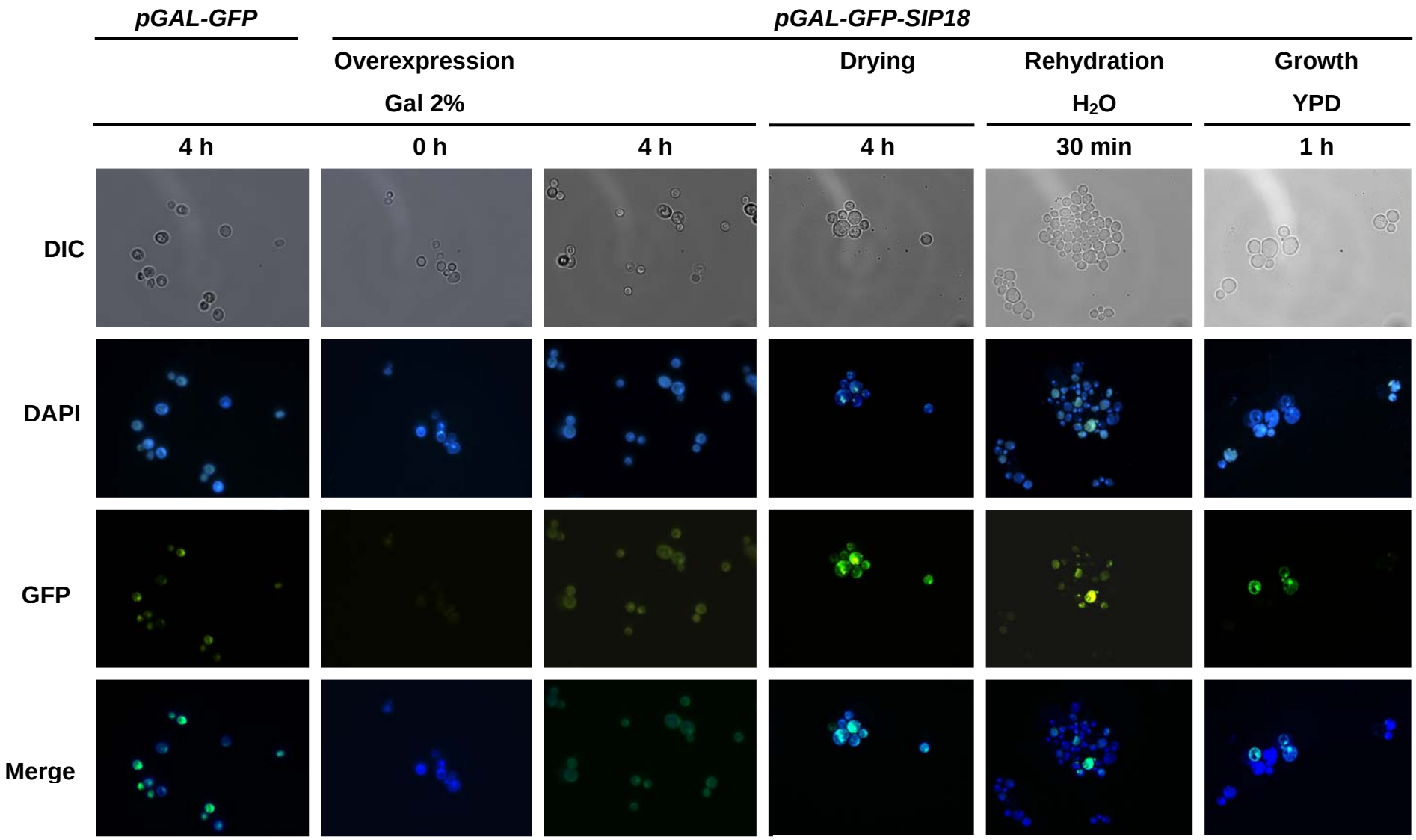

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Fig. 5. Yeast cells exhibiting SIP18p localization during dehydration and rehydration process. The *GFP-SIP18* overexpressing cells were photographed at the indicated times of galactose induction, after 4 h of dehydration, and 1 h in YPD after a 30 min rehydration treatment. Blue fluorescence shows nuclei counterstained with 4,6-diamidinophenylindole (DAPI). Cytolocalization of SIP18p expressed as a fusion protein with GFP. Overlaid images of blue (DAPI) and green (GFP) fluorescences are also shown. The control overexpressing *GFP* after 4 h of induction does not show a clear localization pattern.

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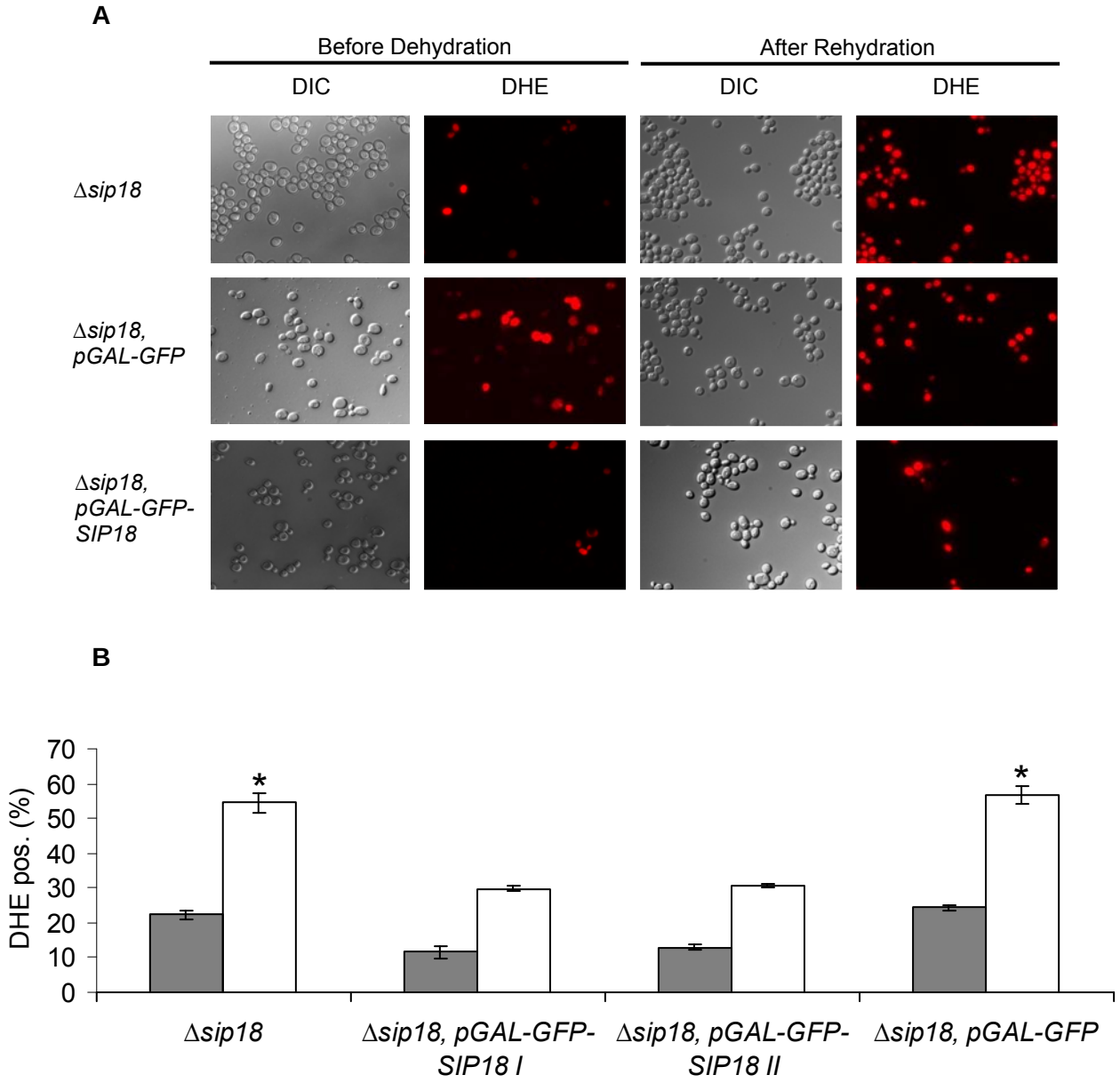

Fig. 6. Δ*sip18* cells undergo ROS accumulation during stress imposition. A) Differential interference contrast (first and third columns of board) and the coincident confocal red fluorescence of DHE staining (second and fourth columns) for different strains are shown. B) Quantification of ROS-accumulation using DHE-staining before drying (grey bars) and after rehydration (white bars). Two independent transformants overexpressing *GFP-SIP18* (I and II) were evaluated. Values are means of n=2 determinations ± standard deviation. In each experiment $5 \cdot 10^6$ cells were evaluated. DHE pos., DHE-positive cells. *Significant differences ($p \leq 0.05$) before dehydration step.

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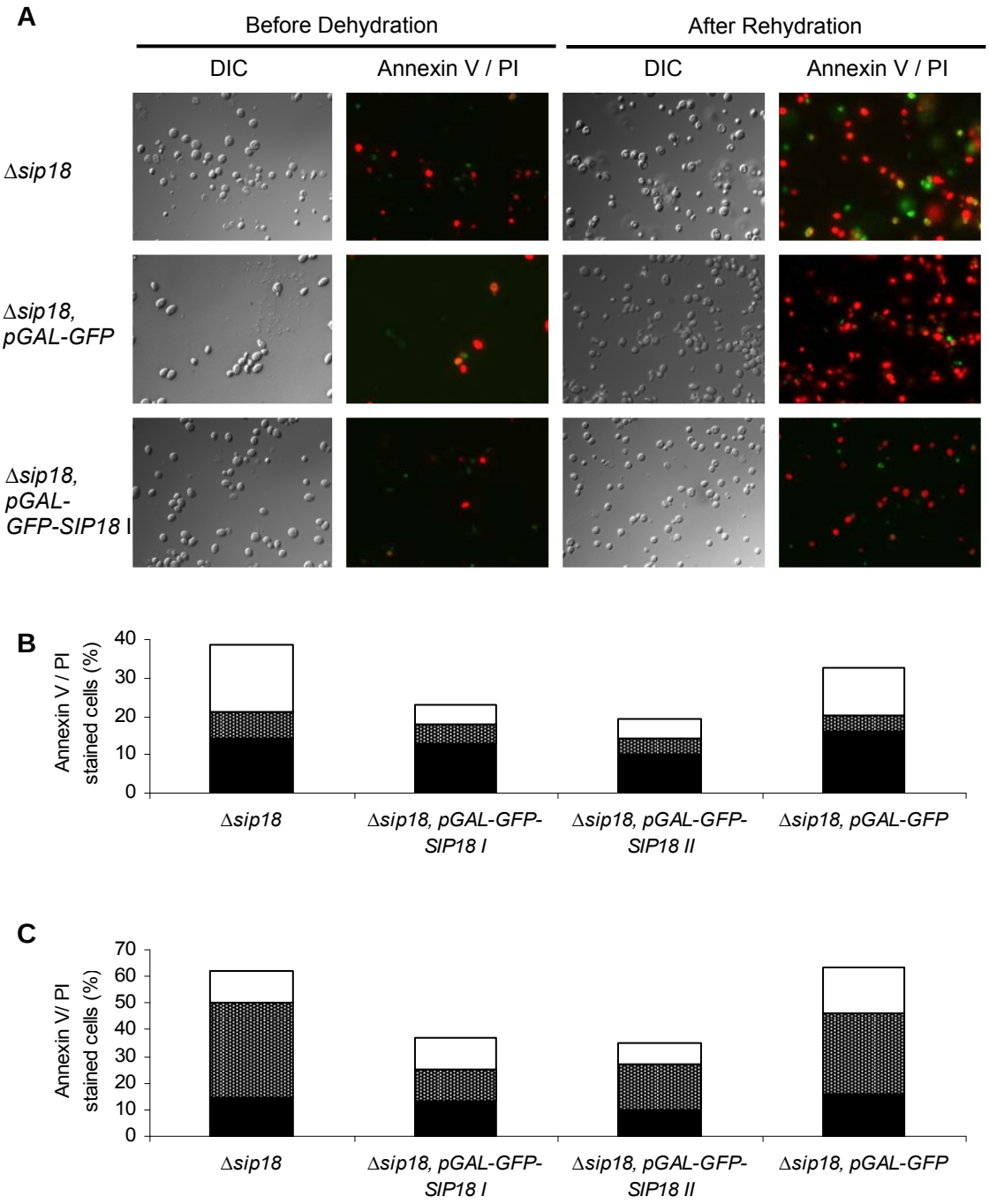

Fig. 7. Overexpression of SIP18p prevents necrotic cell death. A) Differential interference contrast (first and third columns of board) and the coincident confocal fluorescence (second and fourth columns) are shown for different strains before and after stress imposition. Stained cells: V^-/PI^+ (□), V^+/PI^+ ▒, and V^+/PI^- ■. The represented values are means of n=3 determinations and the standard deviation was less than 10%. In each experiment $5 \cdot 10^6$ cells were evaluated.

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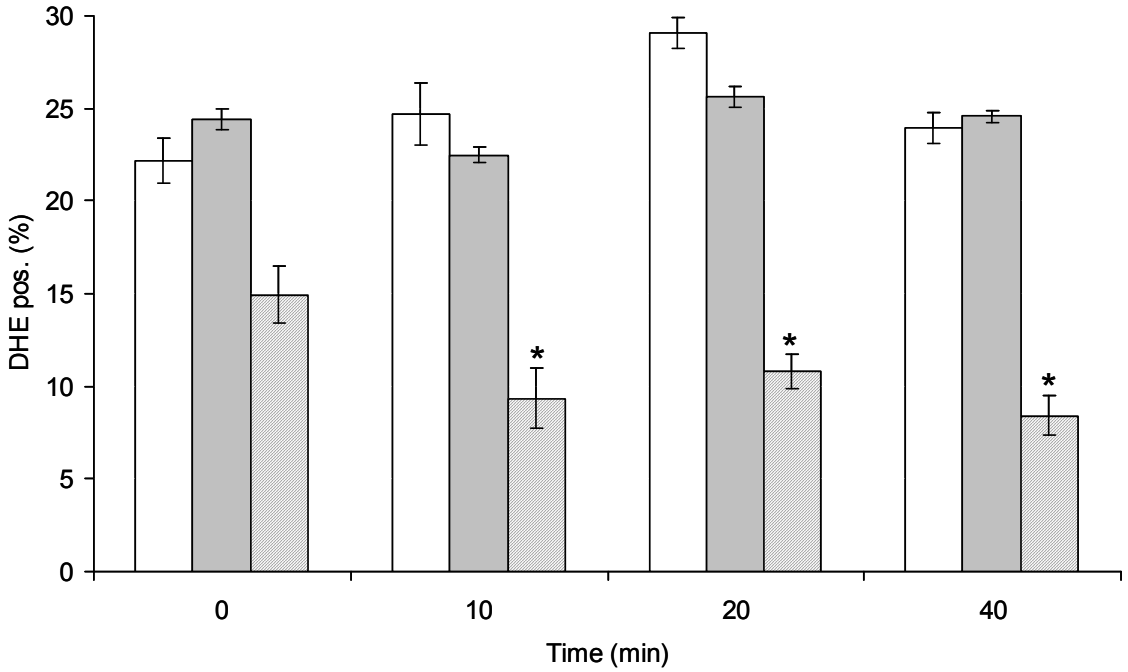

Fig. 8. Levels of DHE accumulation after oxidative stress by H_2O_2. Cells in stationary state from the Δ*sip18* (white bars), Δ*sip18, pGAL-GFP* (grey bars), and Δ*sip18, pGAL-GFP-SIP18* strains (striped bars) were exposed to 4mM H_2O_2 and at the indicated times, aliquots were taken either to evaluate DHE positive (DHE pos.) cells. The represented data are the mean values ±S.D. from two independent experiments. *Indicates $p<0.01$ compared to both reference strains at each time.

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DISCUSION AND PERSPECTIVES

Drying and Rehydration Stress (DRS) is a condition that few organisms can tolerate. Desiccation-tolerant organisms (*Anhydrobiotes*) can survive in dry state for years and restart metabolism in presence of water. *Anhydrobiotes* has been described in bacteria, plants, insects, yeasts, fungi and crustacea (Crowe *et al.*, 1993). DRS damage affects the cell physiology by altering the structure and function of several organelles and membranes (Walker *et al.*, 2006). According to the magnitude of the damage, cells may even die during these processes.

S.cerevisiae is an example of *anhydrobiotic* organism (Garay-Arroyo *et al.*, 2000) and at the same time is an excelent eukaryotic model. Moreover, this microorganism presents a decisive role in food industry, such as the use of Active Dry Yeast (ADY) to produce alcoholic beverages and bread. For this reason, we selected *S. cerevisiae,* as starting point to deepen in the molecular mechanisms and metabolites that allow the cells to overcome Drying and Rehydration Stress (DRS).

During wine production one of the main problems is cell mortality and loss of vitality after rehydration of Active Dry Wine Yeast (ADWY). Optimize the growing, drying and rehydration conditions are the ways to improve viability of yeast cells after rehydration. The ADWY production results in the acumulation of several stress situations for the cells (heat, osmotic and oxidative stress). During growing yeast requires high aeration to achieve an optimum oxidative growth. At the same time, providing all nutritional requirements during fermentation results in physiologically suitable cells able of overcome DRS. The most important adaptive responses developed by *S. cerevisiae* during growing and air-drying, are related to osmotic and oxidative stress (Pérez-Torrado *et al.*, 2005). This feature is connected with ROS accumulation that we observed in yeast after rehydration. (see chapter III)

Other important effects of cell rehydration are changes in membrane fluidity (Beker *et al.,* 1984; Rapoport *et al*., 1995; Hohmann and Mager, 2003). These observations led us to explore whether changes in fluidity of the plasmatic membrane have an effect on rehydrated yeast vitality. We evaluated these parameters using several rehydration situations to understand the drop in yeast vitality and viability. The rehydration media

was classified depending on additive compounds: carbon and nitrogen sources; metallic ions; oxidant and antioxidants; and membrane fluidity elements (see Chapter I).
Contrary to what we expected, neither oxidant and antioxidant agents nor compounds that enhance the rigidity or fluidity of the membrane act as yeast vitality enhancers. Therefore, these compounds did not influence directly the early fermentative performance. In contrast, in the group of carbon and nitrogen compounds, there was a slight reduction in the necessary time to recover from DRS for glucose. Previous results in our group suggests that yeast's transcriptional switch after rehydration is led by the presence of a fermentable sugar (Novo *et al.,* 2007) which can explain this slight increase in vitality.

Besides, calcium, ferrous, and ferric ion complementation had a deleterious effect on yeast vitality. However, the beneficial effect of magnesium complementation was shown by a reduction in the necessary time to recover from DRS, indicating an important role of magnesium in the functionality recovery. On the contrary, an increase in magnesium concentration over the optimun value led to lower yeast vitality. An increase in levels of free intracellular magnesium is evident in cells undergoing apoptosis. This increase is an early event in apoptosis, preceding DNA fragmentation and externalization of phosphatidylserine, and is likely due to a mobilization of magnesium from mitochondria. Therefore, the raise in intracellular free magnesium appears to serve as a "second messenger" for downstream events in apoptosis (Chien *et al.,* 1999).

Other important function of magnesium is to form complexes with phospholipids that stabilize the membranes (Planeéis *et al.,* 1993). However, in our experiments we observed that the increase in vitality in cells rehydrated in the presence of magnesium does not correspond with a reduction of leakage of intracellular soluble components. Our results do not support the idea that a single interaction between magnesium with the damaged phospholipid bilayer of injured or dead cells could reduce membrane influx permeability.

We do not know the causes of this increase in vitality so far, and they might be the combination of multiple events. Magnesium is an abundant element in cellular systems. It exerts a large variety of biological functions, ranging from structural to catalytic roles

in enzyme activation or inhibition, and regulatory roles by modulating cell proliferation, cell cycle progression, differentiation and apoptosis (Hartwig, 2000). An illustrative example is the desoxi-adenosine-triphosphate ATP complex, which magnesium provides stability making a complex that aids in the transfer of ATP phosphate (Hee *et al.*, 1999). Additionally, magnesium is involved in DNA repair and stabilization (Sirover, 1977; Eichhorn *et al.,* 1981). The properties of magnesium to enhance vitality of ADWY after rehydration may have a broad industrial application in wine production.

We characterized the cell viability after DRS for different yeast species. Our results agree with publications where desiccation tolerance does not correlate with the intracellular trehalose or/and glycogen contents (reviewed by Pretorius, 2000; Walker and Dijck, 2006). For example, in contrast to most anhydrobiontes, *bdelloids* rotifers have been shown to lack trehalose as protective chemical (Caprioli *et al.*, 2004). Other researchers showed that Δ*tps1* strain is unable to produce trehalose and able of desiccation tolerance, and the degree of tolerance to DRS also increases (Ratnakumar *et al.*, 2005). Our results, together with these listed above, suggest that adaptations in response to DRS are even more important than only accumulation of trehalose or/and glycogen for yeast dehydration tolerance (see chapter II).

Probably the main effect DRS is the change in membrane fluidity which is determined to a large extent by its asymetric lipid composition (Shinitzky, 1984). Therefore, we evaluated the total lipid composition during DRS for three species with different desiccation tolerance capabilities, *S. cerevisiae*, *N. castellii,* and *S. bayanus*. Fatty acids (FA) and neutral lipids (NL) did not show significant differences. But in *S. bayanus* which showed lowest viability, total triacylglycerols (TAG) content was lower than in the other species. TAG and Sterol ester (STE) are normally considered energy store molecules, which also play an essential role in the metabolism of acetyl-CoA and FA (Czabany *et al.*, 2007). The storage of these neutral lipids could contribute to ensuring the yeast's requirements for the metabolic reactivation after DRS.
On the other hand, in *Schizosaccharomyces pombe*, the terminal steps of TAG synthesis are controlled by Plh1p and Dga1p (Zhang *et al*., 2003). Cells with both *dga1*+ and *plh1*+ deleted lost viability upon entry into the stationary phase and demonstrated prominent apoptotic markers. Although TAG might be required for yeast cells to

survive at the stationary phase, failure to convert diacylglycerol and fatty acids to TAG could result in deleterious consequences.

In the same way, we investigated changes in phospholipids contents after DRS process. Our data showed that the main difference between phospholipid composition is given by a significant drop of phosphatidylcholine (PC) contained in *S. bayanus* (see Chapter II). We have demonstrated that *S. paradoxus* and *S. bayanus* lose their ability to grow after DRS. Also, these yeasts have shown a significant loss of the initial PC content after DRS. However, we observed that PC supplementation during growing and its intracellular accumulation have a positive effect on cell viability after DRS.
PC loss is important not only because plays critical roles in membrane structure and cellular signaling, but also because the change in ergosterol/PC ratios acts in detriment of membrane endurability (Hayashida *et al.*, 1980).

In addition to being a major structural component of mammalian and yeast membranes and lipoproteins, PC is also an important source for signaling molecules (*e.g.* phosphatidic acic, DAG, lysoPC) (White *et al*., 2001). Signalling events involving activation of lipases for PC are implicated in a very broad array of cellular events including cell death through ceramide generation, loss of mitochondrial membrane potential, cytochrome C release and caspase activation (Marchetti *et al.*, 2002).
Evidence that reduction of PC synthesis can trigger apoptosis was first found in a cell line of Chinese hamster ovary with a defect in cholinetransferase (CT) (Cui *et al*., 1996). Choline deficiency depletes the primary substrate for the CDP–choline pathway and can induce apoptosis *in vitro* and *in vivo* (Holmes-McNary *et al.*, 1997).

Yeast strains that are unable to synthesize PC have problems in maintaining their respiratory competence (Griac *et al.*, 1996), indicating that PC is important for mitochondrial function. An example is the mitochondrial glycerol-3-phosphate dehydrogenase Gut2p, that was shown to become preferentially labelled with photoactivatable PC, pointing to a functional relation between these molecules (Janssen *et al.*, 2002). PC depletion was found to reduce growth on glycerol and to increase glycerol excretion, both indicating that PC is needed for optimal Gut2p functioning *in vivo* (Rijken P. *et al.*, 2007).

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To sum up, our studies so far cannot answer exactly why PC presence during the growth stage prevents death after DRS. Perhaps studies of apoptotic hallmarks of knock-outs of the CDP-choline pathway could give us an explanation on the PC role in DRS.
What is the mechanism by which cells recognize PC and translate into intracellular signals perturbation for death? This may provide data about the causes of many human diseases associated with lipid. In relation to wine production could be interesting to study the effect of PC on viability and vitality of rehydrated yeast at production scale.

After studying the phenotypic response to DRS of a deletion mutants' collection of *S. cerevisiae*'s non essential genes, we detected two groups of *knockouts* of strains with significant differences in viability in comparison with the wild type. The first group of *knock outs* enhance dehydration mortality, and the second one formed only by k*nockouts* displaying increased viability. Within this last group some *knockouts* are directly connected with the Programed Cell Death (PCD). Interestingly, among them were the deletion mutants of *AIF1*, *CPR3* and *NUC1*, genes directly involved in yeast apoptosis and necrosis and coding for mitochondrial proteins (Büttner *et al.*, 2007; Halestrap, 2005). Of note, the lack of a further mitochondrial protein, Qcr7p (the subunit 7 of the ubiquinol cytochrome-C reductase complex), also provided resistance upon DRS. Mitochondria is a major source of reactive oxygen species (ROS), which play a central role in mediating yeast cell death (Madeo *et al*., 1999; Mazzoni *et al*., 2003, Weinberger *et al*., 2003). The bulk of mitochondrial ROS generation occurs at the electron transport chain (ETC), as a by-product of respiration (Cadenas, E. *et al*., 2003). In keeping with this, deletion of *QCR7*, which promotes the disassembly of complex III of ETC, shows lower levels of ROS after the imposition of DRS. This effect, however, does not seem to rely solely on respiratory disruption, as other respiration deficient mutants did not show any rescuing effect. The molecular significance of Qcr7p in cell death will need to be further clarified in future experiments.
These genes, whose *knockout* provides resistance upon DRS, are mitochondrial, Moreover, *QCR7* is essential for respiratory activity. Therefore we evaluated two further respiratory deficient strains (*mgm1*Δ and *oxa1*Δ) to exclude an effect due to loss of respiratory capacity. We also evaluated the *yca1*Δ *knockout* to determine whether under DRS conditions cell death was associated with apoptosis by the caspase pathway. In *S. cerevisiae YCA1* encodes for a metacaspase that is involved in yeast apoptosis in response to different stimuli (Madeo *et al*., 2002; Wissing *et al*., 2004). Unfortunately,

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we did not observe neither reduction of ROS, nor differences in apoptotic hallmarks in this group of *knockouts*. Therefore, we conclude that under DRS cell death involves apoptosis and necrosis in a caspase and respiratory independent ways. Moreover, DRS is, at least, partially dependent on the execution of mitochondrial death.

The study of yeast genes involved in PCD under DRS conditions can provide new insights into the mechanistic pathways in pathologies of higher eukaryotic cells. It might be interesting to study double deletions of some of these genes (*AIF1, NUC1, CPR3* and *QCR7*) and their effect on viability and cell death mechanisms. These hypertolerant strains could be used as positive controls of viability in wine industry for looking for new tolerant strains to DRS.

In our genome-wide screen we identified genes which are essential to desiccation tolerance. This set of genes covers a number of different biological processes based on the Gene Ontology annotations. To our knowledge, this is the first systematic screen for the identification of putative genes essential to overcoming the DRS (see chapter III).

Among these essential to desiccation tolerance genes are LEA-like proteins. Late embryogenesis abundant (LEA) are hydrophilic proteins that are accumulated under conditions of extreme desiccation in higher plants. These proteins are characterized by high hydrophilicity and high glycine percentage. *S. cerevisiae's* genome contains twelve putative genes which encode LEA-like proteins (Garay-Arroyo *et al*. 2000). Among the crucial genes we characterized to overcome the DRS, eleven of them belong to the group of very hydrophilic proteins known as hydrophilins.

In our study, the over-expression of *SIP18*, which encodes by an hydrophilin, increases markedly cell viability during DRS. Moreover, the apoptotic hallmarks in Δ*sip18* strain showed that the DRS promotes ROS accumulation and an increase in necrotic-cells. Other studies using the over-expressing strain under oxidative stress suggest that Sip18p prevents cell death during DRS acting as an antioxidant-like protein. Furthermore, our observations showed a nuclear accumulation of Sip18p in response to osmotic shock in no more than five minutes, as well as after immediate rehidration. Once osmotic presure is removed, Sip18p relocalized both nucleus and cytoplasm. However, the mechanisms through which Sip18p prevents cell death are still unknown and require further studies. The interaction of Sip18p with other molecules in the cytoplasm could be simplified by the expression of SIP18 in bacteria. It might be also interesting to isolate the nuclei of yeast cells fixed just after a hyperosmotic stress looking for interactions with DNA and other proteins. These data might indicate more about the Sip18p function.

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The induction of *SIP18* transcription in response to osmotic stress suggests that this hydryphilin represents a widespread adaptation to water deficit (Garay-Arroyo *et al.* 2000; Posas *et al.* 2000; Yale *et al.,* 2001). A possible application of Sip18p qualities could be the heterologous expresion of *SIP18* yeast gene in plants to achieve higher tolerance to desiccation. There are several examples of expresion of yeast gene in plants. One of them is the *trehalose-6-phosphate synthase* gene (*TPS1*) engineered under the control of the cauliflower mosaic virus regulatory sequences and constitutively expressed in *Nicotiana tabacum*. This genetic manipulation leads to an osmoprotectant effect, alteration of sugar metabolism and regulatory pathways affecting plant development and stress tolerance (Romero *et al.*,1996). Similarly, although functional Sip18p has no homologous in humans, it might be interesting to examine whether its overexpression in human cells prevents the PCD.

The use of Sip18p as a molecular marker in the production of ADWY could be a potential application to accept or reject lots of ADWY before their use in wine production. This could ensure high viability after rehydration and, therefore, the success of fermentation.

Our results about the protective role of Sip18p against DRS led us to study the effect of over-expression of a group of other five yeast genes (*HSP12, GRE1, STF2, NOP6* and *TIF11*) encoding proteins with high hydrofilic properties We observed a significant increase in viability after DRS in all of them. The case of Tif11p, which is the Translation Initiation Factor eIF1A (62 % identical to its functional homologous in human), is interesting. The over expression of other group of genes that codified hydrophilic proteins (*WWM1, GON7, RPL42A, YNL190W, YJL144W,*) identified in our screenig as essential for DRS is pending of further study. A deeper study of these proteins and their relationship to cell death open up an exciting area of research.

In conclusion, in this work we have demonstrated a positive effect on the viability of key metabolites such as magnesium and PC, suggesting a possible industrial application in the wine production. Moreover, we have detected molecular evidences that in DRS cell death occur by PCD linked to a mitochondrial scenario in caspase and respiration independent manner. We have identified some indispensable genes in DRS, which express hydrophilic proteins. In particular Sip18p, which accumulates in the nucleus under osmotic stress, and prevents cell death by necrosis. All together, these data propose new ways to optimize ADY production and a way to deep in the study of DRS response in eucaryotic systems.

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