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Running title: **Ergosterol and cell survival to dehydration**

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Keywords: Dehydration – Yeast – Cell survival – Plasma membrane – Ergosterol – Sterol evolution

Introduction

Understanding the mechanisms involved in microorganism survival to environmental perturbations is a major issue in microbial ecology and biotechnology. Due to its interfacial position between the extracellular medium and the cytosol, plasma membrane (PM) is a primary target for damage during environmental stresses. Modifications of the environmental conditions affect the organization and the dynamic of membrane components highlighted by changes in PM fluidity and by the phase transition of membrane phospholipids. Such structural changes of the PM were observed during temperature and hydrostatic pressure shifts (Beney and Gervais, 2001; Denich et al., 2003). In addition to these changes, hydric perturbations lead to cell volume decrease causing an increase in the cell surface-to-volume ratio (s/v) (Gervais and Beney, 2001). This event conducts to PM deformations such as ruffles, wrinkles, and surface roughness (Guilak et al., 2002; Adya et al., 2006). Such deformations, when associated with lipid structural modifications, can lead to the PM internalization (Dupont et al., 2010). All these modifications can lead to permeabilization of the PM and to cell death during severe dehydration-rehydration cycles (Crowe et al., 1989; Rapoport et al., 1995; Simonin et al., 2007; Ragoonanan et al., 2008).

One of the parameters conditioning cell tolerance and survival to environmental perturbations is the lipid composition of the PM: the nature of polar phospholipids influences the resistance of microorganisms to stresses such as ethanol, freezing, salt, and cold treatments (Calcott and Rose, 1982; You et al., 2003; Rodriguez-Vargas et al., 2007). Changes in fatty acid unsaturation levels are involved in the modification of the cell sensitivity by changing physical PM properties, such as membrane fluidity. PM properties are also susceptible to the quantity and the nature of sterols. Sterols are one of the most abundant membrane constituents and are found in a wide range of species. For mammalian cells, the major sterol present in the PM is cholesterol whereas ergosterol and phytosterol predominate in the fungi and plant cells,

respectively. Sterols confer important properties to the PM through their interactions with phospholipids and sphingolipids and play an essential role in the stability of membranes, by affecting rigidity, fluidity, and permeability (Folmer et al., 2008; Abe and Hiraki, 2009; Róg et al., 2009). Sterols are proposed to maintain the lateral heterogeneity of the protein and lipid distribution in the PM because of their putative role in inducing microdomains, so-called lipid rafts (Simons and Ikonen, 1997). The nature of sterols influence the properties of membrane models such as the tensile properties (Hsueh et al., 2007), phase separation and the curvature of the liquid-ordered phase in membranes (Bacia et al., 2005). Despite the numerous effects of the nature of sterols on lipid bilayer properties, few studies have investigated the impact of sterol composition on the “in vivo” behavior of cell PM to environmental perturbations and its possible implication in cell resistance.

The aim of the proposed study was to estimate the effect of the nature of PM sterols on the membrane response and the cell survival to hydric perturbations. Hydric stress was chosen because the structure of the PM is strongly challenged during this perturbation (lipid phase transitions and membrane deformations caused by cell volume decrease) (Laroche et al., 2005; Dupont et al., 2010). The yeast *Saccharomyces cerevisiae* was used as a model because it is exposed to hydric fluctuations in its natural environments (plant surfaces, soil…) and is able to survive extreme dehydration. Moreover, several mutant strains with alterations in the ergosterol biosynthetic pathway (*erg*Δ) are available. These mutants accumulate different sterols in their PM (Barton et al., 1974). It seems plausible that the sterol biosynthetic pathway parallels sterol evolution (Bloch, 1994; Weete et al., 2010) and that these mutants are evolutionarily precursors of the wild type strain (WT). In this work, we performed a comparative study of the responses to osmotic dehydration (viability, ultrastructure, cell volume, and permeability) between *S. cerevisiae* WT and the ergosterol mutant *erg6*Δ. The *erg6* gene encoding the sterol C-24 methyltransferase, which catalyzes the first of the five

final steps of the ergosterol biosynthetic pathway, is nonessential. The corresponding mutant strain mainly accumulates zymosterol and cholesta-5,7,24-trienol instead of ergosterol, the major sterol species encountered in the WT strain (Zinser et al., 1993; Munn et al., 1999) (Fig. 1). Our results show that the *erg6*Δ strain is more sensitive to hydric perturbations than the WT strain. This high sensitivity is related to different PM behaviors between the *erg6*Δ and the WT strains during the dehydration/rehydration cycles. The nature of PM sterols influences the kinds of deformation and the stretching resistance of the PM during cell volume variations caused by hydric treatments. *Erg6*Δ strain is characterized by a permeabilization of the plasma membrane during dehydration under severe osmotic treatments and during the rehydration stage under mild hydric treatments. This result highlights the effect of the sterol nature on the mechanical properties of the PM and their role in cell adaption to hydric fluctuations of the environment.

Results

The erg6Δ mutant strain is more sensitive to hyperosmotic shocks than WT

To compare the sensitivity to osmotic dehydration of the WT and *erg6*Δ strains, yeasts were treated with osmotic shocks at 30 and 166 MPa, maintained under hyperosmotic conditions for 60 min and rehydrated to 1.4 MPa (Table 1). For the mild treatment (30 MPa corresponding to a water activity of 0.8), the viability of the WT strain remained high (about 80%) whereas it was strongly decreased for the *erg6*Δ strain (3%). The survival of the WT strain is in agreement with previous studies which reported that the magnitude of 30 MPa, slightly higher than the one allowing osmoregulation (15 MPa) (Marechal et al., 1995), affected weakly the yeast viability (Simonin et al., 2007). The difference in sensitivity to osmotic shock between the two strains was also observed for the 166 MPa treatments (corresponding to a water activity of 0.3): yeast survivals reached 0.3% and 0.01% for the WT

and the *erg6*Δ, respectively. Even if the decrease in viability was high for the two strains at this dehydration level, *erg6*Δ was also more sensible than the WT. This result suggests that mutating a protein involved in the ergosterol synthesis can directly affect the resistance of yeast to hyperosmotic perturbations. In the remainder of this study, we endeavored to understand the cause of the high sensitivity of the *erg6*Δ yeast strain to hyperosmotic treatments at 30 and 166 MPa.

High sensitivity of the erg6Δ strain to hyperosmotic shocks is linked to PM permeabilization

Leaking of cellular content induced by loss of PM integrity is often reported to explain cell death during hydric perturbations (Beker and Rapoport, 1987; Crowe et al., 1989). To understand the cause of the *erg6*Δ mutant strain sensitivity to hyperosmotic stress, we examined PM permeability by propidium iodide (PI) staining of the WT and *erg6*Δ strains before and after dehydration shock to 30 and 166 MPa, and after shock to 30 and 166 MPa followed by maintenance period of 60 min and by rehydration to 1.4 MPa (Table 1).

Rehydration step is critical for erg6Δ PM integrity during mild treatment (30 MPa)

Without osmotic perturbation, the proportion of permeabilized cells was very low, reaching a value close to 1% for the two strains. This proportion did not change after dehydration to 30 MPa for the WT strain and increased slightly to 7% for the *erg6*Δ strain (Table 1). Rehydration to 1.4 MPa raised dramatically the proportion of permeabilized cells to 90% for the mutant strain whereas this proportion remained at a low level (6%) for the WT strain. This result suggests that permeabilization of the PM of the *erg6*Δ strain occurred during rehydration and could be the cause of the high sensitivity of the mutant to hyperosmotic stress.

To estimate more accurately the mechanism of the loss of membrane integrity of *erg6*Δ during rehydration, we characterized the changes in membrane permeability after rehydration to different osmotic pressures between 1.4 and 22.5 MPa after hyperosmotic shock to 30 MPa (Fig. 2). For the WT strain, rehydration from 30 MPa to this range of osmotic pressures did not affect the PM. For the *erg6*Δ strain, the proportion of permeabilized cells was about 40% after rehydration to 22.5 MPa and increased with the amplitude of rehydration to reach 90% after rehydration to 1.4 MPa. This result shows that the permeabilization of the PM during rehydration step depends on the level of rehydration and that the loss of membrane integrity already occurs for low magnitude rehydration.

Dehydration step affects PM integrity for high amplitude treatment (166 MPa)

PI staining was performed after dehydration to 166 MPa and after rehydration to assess the effect of each stage of the dehydration-rehydration cycle on PM integrity for both strains (Table 1). After the dehydration step to 166 MPa, the proportion of permeabilized cells reached 33% and 91 % for WT and *erg6*Δ strains, respectively. After rehydration, this ratio was high for both strains: 87% and 95%, respectively. These high proportions of permeabilized cells after rehydration were correlated with the low survival rates observed after treatment to 166 MPa (Table 1). However, the results suggest that the mechanisms of PM permeabilization are different for the two strains. PM of *erg6*Δ strain is strongly altered by the dehydration stage whereas the two steps of the dehydration-rehydration cycle are involved in the loss of PM integrity for the WT strain as previously reported (Dupont et al., 2010).

Variations in yeast volume lead to plasma membrane stress

Hyperosmotic perturbations cause movement of large quantities of water across the PM, decreasing cell volume (Morris et al., 1986; Gervais and Beney, 2001; Simonin et al., 2007).

Yeast membrane permeabilization could be related to changes in the cell surface-to-volume ratio (s/v) during the dehydration-rehydration cycle. Cell volume measurements were performed for WT and *erg6*Δ strains by optical microscopy. This method allows the observation of yeast surface delimited by the cell wall. Volume was then calculated by assimilating the yeast volume as a sphere.

Yeast volume was studied after hyperosmotic shocks from 1.4 MPa to osmotic pressure levels between 30 and 166 MPa for both strains (Fig. 3). The study of cell volume as a function of osmotic shock amplitudes showed that WT cells shrank under hyperosmotic conditions. With increasing osmotic pressure, cell volume, delimited by the cell wall, asymptotically reached a minimal value (50% of initial volume). This phenomenon, related to the semipermeability of the PM, results from the osmotic transfer of water from the cytoplasmic space to the external medium and allows equilibration of internal and external osmotic pressures (Gervais and Beney, 2001). The water outflow induces the volume decrease of the cytoplasm leading to the pull of the cell wall by PM. In contrast to vegetal cells where plasmolysis event is observed (Ferrando and Spiess, 2001), the cell wall of yeast remains in contact with the plasma membrane during dehydration because it presents many attachment sites with the PM and displays a great elasticity (Morris et al., 1986). The analysis of the volume of *erg6*Δ cells after increasing osmotic pressure revealed a behavior similar to WT cells for mild osmotic shocks (≤30MPa). This result indicates that sterol nature did not affect significantly the osmotic properties of the PM for such treatment. Beyond 30 MPa, e*rg6*Δ cells exhibited a different response than WT cells: volume decreased to a minimum value of 57.4% at 70 MPa but re-increased for higher osmotic levels and reached 64.2% after treatment to 166 MPa. Thus, for a range of osmotic pressures between 70 and 166 MPa, the final volumes of the mutant strain were higher than for the WT cells. Such volume behavior of the *erg6*Δ strain during dehydration to high amplitudes could be explained by relaxation of

cell wall due to the rupture of the PM for the most of the cells (Table 1). These results suggest that dehydration beyond 30 MPa involves high tensile strength in PM which could be at the origin of PM membrane rupture and permeabilization of the mutant yeast due to high membrane sensitivity to stretching for this strain.

To improve the comprehension of the mechanisms of PM permeabilization during mild dehydration-rehydration cycle (30 MPa) for the mutant strain, a comparative analysis of yeast volume between the *erg6*Δ and WT strains was performed after dehydration to different levels to 30 MPa (Fig. 4 A) and after dehydration to 30 MPa followed by rehydration to different amplitudes (Fig. 4 B). The profile of cell volumes, with increasing levels of osmotic pressure ranging from 7 MPa to 30 MPa, was very similar for the two strains. Hyperosmotic treatments led to exponentially cell volume decrease: the variation of the volume was strong for amplitudes to 14.5 MPa and yeast volume reached 68% of the initial volume (at 1.4 MPa). Under higher osmotic pressures, the volume slightly decreased to reach a value of 65% at 30 MPa. After hyperosmotic shock to 30 MPa followed by rehydration to different osmotic pressures, the profiles of cell volumes were different for the two strains. For the WT strain, cell volume after rehydration perfectly fitted with cell volume measured during dehydration (Fig. 4 A and B). Thus, volume changes induced by hyperosmotic treatment to 30 MPa were reversible and the cell volume was recovered after rehydration to 1.4 MPa. For the *erg6*Δ strain, rehydration from 30 MPa to osmotic pressures between 22.5 and 7 MPa conducted to an increase in cell volume to values slightly higher than the ones of the WT strain. However, these differences were not significant. Rehydration to 1.4 MPa conducted to a volume value of 82% of the initial volume. This phenomenon could be linked to the high proportion of permeabilized cells for the *erg6*Δ strain during rehydration (Fig. 2): cell swelling during rehydration is related to osmosis phenomenon which requires the integrity of the PM. A possible hypothesis to explain PM permeabilization during the rehydration step could be a

lack of membrane surface causing membrane rupture during cell swelling. Indeed, we reported, in a previous study, that PM permeabilization occurred during rehydration of WT yeasts after a shock to 166 MPa. This event was caused by a decrease in the PM surface induced by membrane internalization after this perturbation (Dupont et al., 2010). Reduction of PM surface after shock to 30 MPa could explain the membrane permeabilization of the *erg6*Δ occurring during the rehydration step strain following the shock to the moderate osmotic pressure of 30 MPa.

Plasma membrane deformations after hyperosmotic shock to 30 MPa depend on the nature of membrane sterols

TEM was used to characterize ultrastructural changes induced by hyperosmotic shock to 30 MPa on the two strains. Fixation of cells was performed just after the treatment by using a chemical fixation protocol.

After shock to 30 MPa, WT and *erg6*Δ strains presented PM deformations (Fig. 5 A, B, C, and D). For the WT strain, PM showed narrow and deep (~400-500 nm) invaginations which appeared curled back toward (Fig. 5 B). This kind of deformations has already been observed in the case of progressive osmotic dehydration to 166 MPa (Dupont et al., 2010). For the *erg6*Δ strain, PM presented a more undulated aspect in comparison to WT. Furthermore, small vesicles were observed between the plasma membrane and the cell wall (Fig. 5 D). The formation of vesicles out of the cytoplasm has ever been reported for gram-negative bacteria under hyperosmotic conditions (Koch, 1998). These vesicles could be at the origin of PM surface reduction in the mutant strain after hyperosmotic shock and could explain the permeabilization of the mutant during the swelling induced by rehydration.

Exogenous ergosterol restores the resistance of the erg6Δ strain to hyperosmotic shock to 30 MPa

The most probable hypothesis to explain the hypersensitivity of the *erg6*Δ to hyperosmotic perturbations was the modification of the PM properties caused by the change in PM sterols. However, a second explanation could be possible downstream effects induced by *erg6*Δ mutation such as remodeling in transcription, translation, or cytoskeleton organization. These effects could also induce changes in membrane properties. To determine if the first hypothesis was correct, we inspected whether exogenous addition of ergosterol in the *erg6*Δ strain could restore normal sensitivity to hyperosmotic perturbation at 30 MPa (Fig. 6). Addition of ergosterol during cell growth under aerobic conditions did not change the survival to this perturbation of the two strains: viabilities of the WT and *erg6*Δ remained unchanged at about 80% and 3%, respectively. Under aerobic growth conditions, yeasts synthesize sterols and do not incorporate significant amounts of exogenous sterols, a phenomenon known as “aerobic sterol exclusion” (Lorenz and Parks, 1991). In contrast, growth of yeasts performed without oxygen allowed exogenous ergosterol to incorporate the PM of the two strains because yeasts become auxotrophic for sterols under anaerobic conditions. Anaerobic growth with an ergosterol supplementation to the culture medium (250 µM), yielded to significant changes in the yeast survival rates after hyperosmotic shock at 30 MPa. This rate reached 66% for the WT and *erg6*Δ strains.

PM ergosterol is a key factor in the yeast resistance to hydric perturbations

Sterol composition of the two strains was analyzed as a function of the growth conditions (aerobic or anaerobic) to confirm that the effects observed on yeast resistance to dehydration was correlated with the nature of sterols in the PM (Fig. 7). Under aerobic growth condition, WT strain accumulated mainly ergosterol and some of its precursors (squalene, lanosterol,

zymosterol, and ergosta-5,7) whereas *erg6*Δ strain accumulated mainly zymosterol and cholesta-5,7,24-trienol, as previously described (Munn et al., 1999). In contrast, under anaerobic growth condition with ergosterol supplementation, the sterol profiles of the two strains were very similar: both strains accumulated mainly ergosterol, at a level close to that observed for the WT strain grown under aerobiosis. Squalene accumulation under anaerobiosis is not surprising because squalene epoxidation, which is the next step after squalene in the ergosterol biosynthesis, requires oxygen (Jahnke and Klein, 1983). Altogether, these results suggest that resistance to dehydration is related to the presence of ergosterol in the PM, since growth conditions that allow ergosterol accumulation in the PM (anaerobic growth for *erg6*Δ; aerobic and anaerobic growth for WT) increase yeast resistance to dehydration. Therefore, yeast survival to dehydration is directly linked to the nature of membrane sterols.

Discussion

The aim of this study focused on the effect of the effects of the sterol content on PM properties in response to hyperosmotic perturbations, by comparing membrane behaviors during dehydration-rehydration cycles between the WT strain (accumulating ergosterol in the PM) and the *erg6*Δ mutant (accumulating zymosterol and cholesta-5,7,24-trienol) (Fig. 7). The main finding is that nature of PM sterols influences the mechanical properties of the PM. These changes in properties led to different osmotic behaviors of the PM and induced a high sensitivity of the *erg6*Δ mutant to hyperosmotic perturbations, as compared to the WT strain.

Hyperosmotic perturbations induced an outflow of large quantities of water, causing a strong decrease in cell volume (Figs. 3 and 4). Due to the low lateral compressibility of the membrane (Evans et al., 1976), the cell s/v ratio increase conducted to compressive lateral stress and to the deformation of the PM. TEM observation of WT and *erg6*Δ strains after

hyperosmotic shock at 30 MPa revealed that the kind of membrane deformations depended on the sterol composition of the PM (Fig. 5). The link between membrane sterols and the kind of membrane deformations observed could be related to the influence of the nature of sterols on the physical properties of the lipid bilayer. Although the effect of zymosterol and cholesta-5,7,24-trienol on model membrane properties have never been studied to our knowledge, it is known that modifications of physical membrane properties can be directly related to the structure of sterol molecule, including its planar structure, its size, and the properties of its small polar 3-OH group (Xu and London, 2000). In particular, the double bond between C-7 and C-8 in the B ring is known to be involved in the packing and the overall rigidity of the PM. Cholesta-5,7,24-trienol, like ergosterol, but not zymosterol displays a C-7,8 double bond (Fig. 1). This induces the higher membrane fluidity of the mutant than the WT strain (Kaur and Bachhawat, 1999; Abe and Hiraki, 2009). These differences in packing and fluidity of the PM between both strains could account for the differences in membrane deformations during osmotic cell volume contraction.

Changes in PM properties conducted to the difference in yeast strain sensitivity to hydric perturbations. The high sensitivity of the *erg6*Δ strain is linked to the loss of PM integrity which occurs during the two steps of the dehydration/rehydration cycle (Table 1). On the one hand, dehydration stage conducted to the permeabilization of the PM: this phenomenon was observed for the treatment to high hyperosmotic level (166 MPa). After this perturbation, over 90% of the mutant cells were permeabilized whereas only 35% of WT yeasts lost their integrity (Table 1). Cell volume analysis on a range of osmotic treatments between 30 and 166 MPa (Fig. 3) revealed that PM pulled the cell wall and endured stretching stress during severe dehydration. The influence of sterol structure on the stretching resistance of the membrane (Hossack and Rose, 1976) may explain the higher fragility of the membrane in the *erg6*Δ mutant, as compared to the WT, during the dehydration step. On the other hand, the

rehydration step was critical for mutant cell integrity under mild treatments (30 MPa) (Table 1). After this hyperosmotic treatment, membrane permeabilization increased with the level of rehydration (Fig. 2) and with cell volume swelling (Fig. 4). A possible explanation is a lack of membrane surface conducting to PM lysis during the cell volume increase caused by rehydration. This phenomenon has been observed for WT yeasts only in the case of severe hyperosmotic shocks: decrease of PM surface was caused by membrane internalization (Dupont et al., 2010) and led to the loss of membrane integrity during the rehydration. This event was also shown for protoplasts (Wolfe et al., 1986) during osmotic perturbations. Decrease of membrane surface seems to be related to the formation of extracellular vesicles between cell wall and PM observed only with the mutant strain by TEM microscopy (Fig. 5). All these events indicate that the nature of the sterol molecules in the membrane influences the physical behavior of the PM during hydric perturbations. This parameter is clearly involved in cell survival during this environmental stress. This conclusion is reinforced by the fact that *erg6*Δ resistance to dehydration is restored when cultivated under conditions of ergosterol accumulation (Figs 6 and 7).

In their ecological niche, such as the plant surfaces and soil, yeasts are subjected to hydric fluctuations of the environment and can encounter high solute concentrations. This study shows that a non lethal (in the classic genetic sense) ergosterol mutant, accumulating ergosterol precursors, is highly sensitive to hydric perturbations compared to the WT strain. It is surmised that membrane sterols have been selected over a very long time period by Darwinian evolution for their ability to optimize certain physical properties of the membranes. As argued by Konrad Bloch, the temporal sequence of the sterol biosynthetic pathway can be taken to represent the evolutionary sequence of sterol (Bloch, 1994). In this view, the *erg6*Δ yeast strain is an evolutionary precursor of the WT strain. These results could provide some response elements on the nature of a driving force, which led to the evolution of the ergosterol

biosynthetic pathway in members of fungi kingdom, which encounter hydric fluctuations in their natural environment.

Experimental procedures

Yeast strains and culture conditions

The *Saccharomyces cerevisiae* strain BY 4742 Wild Type (WT) (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) and the *erg6*Δ mutant (*MATα his3Δ1 leu2Δ0 lys2Δ0 YMR008c::kanMX4*) (Euroscarf, Frankfurt, Germany) were used in this study. Cells were grown aerobically at 25°C in 250 mL conical flasks containing 100 mL of Malt Wickerham modified medium (MW). The MW medium contained 10 g glucose, 3 g pancreatic peptone, 3 g yeast extract, and 1.5 g NaH_2PO_4 (Sigma-Aldrich, Saint Quentin Fallavier, France) in 1 L of water–glycerol, which had an osmotic pressure of 1.4 MPa. This osmotic pressure has been recommended for the optimal growth of *S. cerevisiae* (Anand and Brown, 1968). For aerobic growth, a subculture (1 mL) was transferred into a conical flask containing MW medium, and cultures were placed on a rotary shaker (New Brunswick Scientific, Edison, NY, USA) at 250 rpm for 24 h and allowed to grow to the early stationary phase. The final population was nearly 10^8 cells mL^{-1}. The media used for anaerobic growth were supplemented with 2% (v/v) Tween 80 (polyethylene sorbitan mono-oleate) and 250 µM ergosterol and was degassed of oxygen by nitrogen bubbling for 12 h before inoculation with 1 mL of subculture. Culture was performed in an anaerobic chamber for 24 h at 25°C.

Preparation of binary water–glycerol solutions of different osmotic pressures

The solute used in all experiments to perform hyperosmotic treatments was glycerol (Sigma-Aldrich).

The mass of solute to be added to 1000 g of distilled water to obtain the desired water activity (a_w) was calculated using the Norrish equation (Norrish, 1966):

$$a_w = (1 - X_s)e^{-KXs^2},$$

where X_s is the molar fraction of the solute and K is the Norrish coefficient of the solute used to increase the osmotic pressure. For glycerol, K = 1.16. Osmotic pressure (π) is related to the water activity by the following equation:

$$\pi = -\frac{RT \ln a_w}{\overline{V_w}},$$

where R is the universal gas constant (J.mol^{-1}.K^{-1}), T is the temperature (K) and $\overline{V_w}$ is the partial molar volume of water (m^3.mol^{-1}). Table 2 presents the quantity of glycerol used to prepare the solutions of different osmotic pressures.

The osmotic pressure of all solutions was checked with a dew-point osmometer (Decagon Devices Inc., Pullman, WA, USA).

Osmotic treatments

Two levels of osmotic treatments were used: moderate (30 MPa), which is slightly higher than that allowing osmoregulation, and severe (166 MPa).

Samples (20 mL) of culture were centrifuged (5 min, 2200 *g*), washed twice in the binary water–glycerol mixture (1.4 MPa), and the pellets were resuspended in 10 mL of the same medium. 1 mL aliquots of this suspension were placed in microsample tubes, which were then centrifuged (10 min, 5100 *g*) and the supernatant was removed. Hyperosmotic shock was induced by quickly introducing 1 mL of a binary water–glycerol solution (final osmotic pressure of 30, or 166 MPa) to the pellets. The cells were maintained 1 h under hyperosmotic conditions before rehydration. Rapid rehydration was performed by removing the hyperosmotic solution from the microsample tube after centrifugation (10 min, 5100 *g*) and by quickly introducing 1 mL of the binary water–glycerol solution (1.4 MPa) to the cell pellet.

Cell suspensions, rehydration solutions, and shock solutions were kept in an air-conditioned room at 25°C. The temperature of the solutions was checked using a thermocouple.

Measurement of yeast viability

Yeast viability was estimated in triplicate by the CFU method. After osmotic treatment, fully rehydrated cells were diluted serially and the appropriate dilutions were plated in MW medium with 15 g L^{-1} of agar. CFU were counted after incubation for 36 h at 25°C. The initial cell suspension was used as the control.

Cell volume analysis

Cell volume variations were measured after dehydration to different osmotic pressures (7, 14, 22.5, and 30 MPa) and after dehydration to 30 MPa followed by rehydration to 14, 7, and 1.4 MPa. These variations were estimated on images acquired with a Nikon Eclipse TE 2000 E microscope (Nikon, Tokyo, Japan) with spectral camera Nuance CRI. Cells were individually analyzed with the software ImageJ 1.42q to determine their projected areas and volumes.

Assessment of plasma membrane permeability

Propidium iodide (PI) (Invitrogen, Carlsbad, CA, USA) was used to assess PM integrity. This probe stains nucleic acids after permeabilization of the PM. PI was dissolved in distilled water (10 mg mL^{-1}) to prepare the stock solution, and 200 μg of PI was used to stain 10^8 cells. Proportion of permeabilized cells was estimated by flow cytometric analysis for treatments to 30 MPa using a FACSCalibur flow cytometer (BD Biosciences). For each sample, 10 000 events were collected. Above this amplitude, it was not possible to use the flow cytometer due to the high viscosity of the cell suspensions and the proportion of permeabilized cells was assessed by fluorescence microscopy for treatments to 166 MPa. A Nikon Eclipse TE 2000 E

epifluorescence microscope (Nikon, Tokyo, Japan) with spectral camera Nuance CRI was used to observe cells. Images were acquired with a ×40 (NA: 0.95) Plan Apo objective (Nikon) and collected with Nuance software (Nikon). Black and white images were captured to observe the total cell population (at least 300 cells). A monochromatic epifilter (540-580 nm and 600-660 nm excitation and emission wavelengths, respectively) was used to observe cells stained with PI.

Electron microscopy

TEM was used to assess the yeast ultrastructure just after shock to 30 MPa. Concentrated yeast samples were fixed for 12 h at 4°C with 3% glutaraldehyde and 2% paraformaldehyde in water–glycerol solution at osmotic pressure of 30 MPa. Treated cells were fixed just after the end of the osmotic treatment. After washing, cells were postfixed with 0.5% OsO_4–0.1 M phosphate buffer, pH 7.2, for 1 h at 4°C. Cells were dehydrated progressively in 30%, 50%, 70%, 90%, and 100% ethanol, 30 min for each step, impregnated with Epon, and polymerized at 60°C for 48 h. Ultrathin sections (90 nm) were obtained using an Ultracut E ultramicrotome (Reichert, Depew, NY, USA) and contrasted with uranyl acetate and lead citrate. Observations were performed on a Hitachi 7500 transmission electron microscope (operating at 80 kV) equipped with an AMT camera driven by AMT software (AMT, Danvers, MA, USA).

Membrane sterol identification and quantification

Lipid extracts were obtained from ≈5 10^8 shock-frozen yeast cells grown as indicated. Cells were harvested, washed with distilled water and taken up to 1 mL with cold water. Cells were then broken by vigorous shaking with a mini-beadbeaterTM (Biospec Products) for 1 min at 5000 rev./min in the presence of 500 µL of glass beads (diameter 0.3–0.4 mm; Sigma).

Cellular lipids were extracted using chloroform/methanol (2:1, v/v) as described by Folch et al. (Folch et al., 1957). The final organic phase was evaporated and sterols were dissolved in 100 µL of hexane.

The different sterol species were then separated by Gas Chromatography using a 25 m×0.32 mm AT-1 capillary column (Alltech) and identified by the means of their retention times relative to cholesterol, used as a standard. The results are expressed as nmol of sterol/10^9 cells.

Acknowledgements

We thank the personnel of the Plateau Technique "Imagerie Spectroscopique" IFR 92 (University of Burgundy, Dijon, France) for technical support during the optical microscopy manipulations. The authors wish to thank the INRA/University of Burgundy microscopy center of Dijon for assistance during electron microscopy manipulations. This work was supported by French Ministry of Research and the Regional Council of Burgundy.

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Figure legends

Fig. 1. Ergosterol biosynthetic pathway in *Saccharomyces cerevisiae*. (A) The five final steps in ergosterol biosynthesis. (B) Structure of the main sterols accumulated in the *erg6*Δ mutant. (C) Structure of ergosterol, the major sterol accumulated in the WT strain.

Fig. 2. Evolution of plasma membrane integrity as a function of the rehydration level after dehydration at 30 MPa. WT and *erg6*Δ strains were treated with hyperosmotic shock to 30

MPa and were stained with PI after rehydration to different osmotic pressures: 1.4, 7, 14.5, and 22.5 MPa. Measurement of the proportion of permeabilized cells was performed by flow cytometry. Error bars correspond to the SD calculated from three repeat experiments. Asterisks denote statistical significance with respect to wild type strain (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

Fig. 3. Evolution of yeast volume as a function of the magnitude of hyperosmotic shocks. Volume estimation was performed by microscopic image analyzing after shocks to 30, 70, 110, and 166 MPa. Error bars correspond to the SD calculated from three repeat experiments. Asterisks denote statistical significance with respect to wild type strain (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

Fig. 4. Comparison of cell volumes between WT and *erg6*Δ during dehydration to 30 MPa followed by rehydration to 1.4 MPa. Volume estimation was performed by microscopic image analyzing. For the cell volume profile during dehydration (A), yeasts were observed after hyperosmotic treatments to 7, 14.5, 22.5, and 30 MPa. For the profile during rehydration (B), yeasts were observed after rehydration to 1.4, 7, 14.5, and 22.5 MPa from cells dehydrated to 30 MPa. Error bars correspond to the SD calculated from three repeat experiments.

Fig. 5. Ultrastructure of WT and *erg6*Δ yeasts after hyperosmotic shock to 30 MPa. TEM micrographs of representative yeast sections. Cells after shock to 30 MPa for WT (A and B) and *erg6*Δ (C and D). Micrographs were taken of 2% paraformaldehyde/3% glutaraldehyde-fixed, metaperiodate-treated, reduced osmium post-fixed and EPON embedded cells. Cells were fixed just after the hyperosmotic shock to 30 MPa. (A), and (C): bar scale = 500 nm. (B), and (D): bar scale = 100 nm.

Fig. 6. Effects of exogenous ergosterol addition during anaerobic culture on yeast survival to hyperosmotic shock. WT and *erg6*Δ strains were cultivated in aerobic and anaerobic conditions with a supplementation of ergosterol (250 µM). Hyperosmotic shock to 30 MPa were performed and viabilities were estimated by CFU method. Error bars correspond to the SD calculated from three repeat experiments. Asterisks denote statistical significance with respect to wild type strain (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

Fig. 7. Effects of growth conditions on the sterol composition of the WT and *erg6*Δ strains. Both strains were grown under aerobic, or anaerobic conditions with ergosterol supplementation (250 µM). After lipid extraction, sterol amounts were determined by gas chromatography using cholesterol as a standard, as described in Materials and Methods. Sterols are arranged along the x-axis according to their order of appearance in the biosynthetic pathway of ergosterol, excepting cholesta-5,7,24-trienol. This compound, which has a structure close to the one of ergosterol, is a by-product that accumulates in the *erg6*Δ strain. Error bars correspond to the SD calculated from three repeat experiments.