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# Nature of sterols affects plasma membrane behavior and yeast survival to dehydration

4	Sebastien Dupont <sup>1</sup> , Laurent Beney <sup>1*</sup> , Thierry Ferreira <sup>2</sup> , Patrick Gervais <sup>1</sup>
5 6	<sup>1</sup> Laboratoire de Génie des Procédés Microbiologiques et Alimentaires, Université de
7	Bourgogne/AgroSup Dijon, 1, esplanade Erasme, 21000 Dijon, France
8	<sup>2</sup> Université de Poitiers, UMR-CNRS 6187, Institut de Physiologie et de Biologie Cellulaires,
9	1, rue Georges Bonnet, 86022 Poitiers, France
10	
11	
12	<b><u>Running title:</u></b> Ergosterol and cell survival to dehydration
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14	* For correspondence. E-mail <u>laurent.beney@u-bourgogne.fr</u> ; Tel. (+33) (0)3 80396666; Fax
15	(+33) (0)3 80772385.
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19	evolution

#### 21 Introduction

Understanding the mechanisms involved in microorganism survival to environmental 22 perturbations is a major issue in microbial ecology and biotechnology. Due to its interfacial 23 position between the extracellular medium and the cytosol, plasma membrane (PM) is a 24 25 primary target for damage during environmental stresses. Modifications of the environmental conditions affect the organization and the dynamic of membrane components highlighted by 26 changes in PM fluidity and by the phase transition of membrane phospholipids. Such 27 structural changes of the PM were observed during temperature and hydrostatic pressure 28 shifts (Beney and Gervais, 2001; Denich et al., 2003). In addition to these changes, hydric 29 perturbations lead to cell volume decrease causing an increase in the cell surface-to-volume 30 31 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 32 deformations, when associated with lipid structural modifications, can lead to the PM 33 internalization (Dupont et al., 2010). All these modifications can lead to permeabilization of 34 the PM and to cell death during severe dehydration-rehydration cycles (Crowe et al., 1989; 35 36 Rapoport et al., 1995; Simonin et al., 2007; Ragoonanan et al., 2008).

One of the parameters conditioning cell tolerance and survival to environmental perturbations 37 is the lipid composition of the PM: the nature of polar phospholipids influences the resistance 38 39 of microorganisms to stresses such as ethanol, freezing, salt, and cold treatments (Calcott and 40 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 41 42 physical PM properties, such as membrane fluidity. PM properties are also susceptible to the 43 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 44 PM is cholesterol whereas ergosterol and phytosterol predominate in the fungi and plant cells, 45

respectively. Sterols confer important properties to the PM through their interactions with 46 phospholipids and sphingolipids and play an essential role in the stability of membranes, by 47 affecting rigidity, fluidity, and permeability (Folmer et al., 2008; Abe and Hiraki, 2009; Róg 48 49 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 50 rafts (Simons and Ikonen, 1997). The nature of sterols influence the properties of membrane 51 models such as the tensile properties (Hsueh et al., 2007), phase separation and the curvature 52 53 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 54 sterol composition on the "in vivo" behavior of cell PM to environmental perturbations and its 55 possible implication in cell resistance. 56

The aim of the proposed study was to estimate the effect of the nature of PM sterols on the 57 58 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 59 60 transitions and membrane deformations caused by cell volume decrease) (Laroche et al., 61 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 62 able to survive extreme dehydration. Moreover, several mutant strains with alterations in the 63 ergosterol biosynthetic pathway ( $erg\Delta$ ) are available. These mutants accumulate different 64 sterols in their PM (Barton et al., 1974). It seems plausible that the sterol biosynthetic 65 pathway parallels sterol evolution (Bloch, 1994; Weete et al., 2010) and that these mutants are 66 evolutionarily precursors of the wild type strain (WT). In this work, we performed a 67 comparative study of the responses to osmotic dehydration (viability, ultrastructure, cell 68 volume, and permeability) between S. cerevisiae WT and the ergosterol mutant  $erg6\Delta$ . The 69 erg6 gene encoding the sterol C-24 methyltransferase, which catalyzes the first of the five 70

final steps of the ergosterol biosynthetic pathway, is nonessential. The corresponding mutant 71 72 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. 73 74 1). Our results show that the  $erg6\Delta$  strain is more sensitive to hydric perturbations than the WT strain. This high sensitivity is related to different PM behaviors between the  $erg \delta \Delta$  and 75 the WT strains during the dehydration/rehydration cycles. The nature of PM sterols influences 76 the kinds of deformation and the stretching resistance of the PM during cell volume variations 77 caused by hydric treatments.  $Erg6\Delta$  strain is characterized by a permeabilization of the 78 plasma membrane during dehydration under severe osmotic treatments and during the 79 rehydration stage under mild hydric treatments. This result highlights the effect of the sterol 80 nature on the mechanical properties of the PM and their role in cell adaption to hydric 81 82 fluctuations of the environment.

83

#### 84 **Results**

#### 85 The $erg6\Delta$ mutant strain is more sensitive to hyperosmotic shocks than WT

To compare the sensitivity to osmotic dehydration of the WT and  $erg6\Delta$  strains, yeasts were 86 treated with osmotic shocks at 30 and 166 MPa, maintained under hyperosmotic conditions 87 for 60 min and rehydrated to 1.4 MPa (Table 1). For the mild treatment (30 MPa 88 corresponding to a water activity of 0.8), the viability of the WT strain remained high (about 89 80%) whereas it was strongly decreased for the  $erg6\Delta$  strain (3%). The survival of the WT 90 91 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), 92 affected weakly the yeast viability (Simonin et al., 2007). The difference in sensitivity to 93 osmotic shock between the two strains was also observed for the 166 MPa treatments 94 95 (corresponding to a water activity of 0.3): yeast survivals reached 0.3% and 0.01% for the WT

and the  $erg6\Delta$ , respectively. Even if the decrease in viability was high for the two strains at this dehydration level,  $erg6\Delta$  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  $erg6\Delta$  yeast strain to hyperosmotic treatments at 30 and 166 MPa.

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# 103 High sensitivity of the erg6∆ strain to hyperosmotic shocks is linked to PM 104 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  $erg6\Delta$  mutant strain sensitivity to hyperosmotic stress, we examined PM permeability by propidium iodide (PI) staining of the WT and  $erg6\Delta$  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).

#### 111 Rehydration step is critical for $erg6\Delta$ PM integrity during mild treatment (30 MPa)

112 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 30 113 MPa for the WT strain and increased slightly to 7% for the  $erg6\Delta$  strain (Table 1). 114 115 Rehydration to 1.4 MPa raised dramatically the proportion of permeabilized cells to 90% for 116 the mutant strain whereas this proportion remained at a low level (6%) for the WT strain. This 117 result suggests that permeabilization of the PM of the  $erg6\Delta$  strain occurred during rehydration and could be the cause of the high sensitivity of the mutant to hyperosmotic 118 119 stress.

To estimate more accurately the mechanism of the loss of membrane integrity of  $erg \delta \Delta$ 120 121 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 122 123 (Fig. 2). For the WT strain, rehydration from 30 MPa to this range of osmotic pressures did 124 not affect the PM. For the  $erg6\Delta$  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% 125 after rehydration to 1.4 MPa. This result shows that the permeabilization of the PM during 126 rehydration step depends on the level of rehydration and that the loss of membrane integrity 127 already occurs for low magnitude rehydration. 128

129 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 130 131 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 132 133 reached 33% and 91 % for WT and  $erg6\Delta$  strains, respectively. After rehydration, this ratio was high for both strains: 87% and 95%, respectively. These high proportions of 134 permeabilized cells after rehydration were correlated with the low survival rates observed 135 136 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  $erg6\Delta$  strain is strongly altered 137 by the dehydration stage whereas the two steps of the dehydration-rehydration cycle are 138 139 involved in the loss of PM integrity for the WT strain as previously reported (Dupont et al., 2010). 140

141

#### 142 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).

145 Yeast membrane permeabilization could be related to changes in the cell surface-to-volume 146 ratio (s/v) during the dehydration-rehydration cycle. Cell volume measurements were 147 performed for WT and  $erg6\Delta$  strains by optical microscopy. This method allows the 148 observation of yeast surface delimited by the cell wall. Volume was then calculated by 149 assimilating the yeast volume as a sphere.

Yeast volume was studied after hyperosmotic shocks from 1.4 MPa to osmotic 150 pressure levels between 30 and 166 MPa for both strains (Fig. 3). The study of cell volume as 151 152 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, 153 154 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 155 cytoplasmic space to the external medium and allows equilibration of internal and external 156 157 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 158 159 plasmolysis event is observed (Ferrando and Spiess, 2001), the cell wall of yeast remains in 160 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 161 162 volume of  $erg6\Delta$  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 163 significantly the osmotic properties of the PM for such treatment. Beyond 30 MPa,  $erg6\Delta$ 164 cells exhibited a different response than WT cells: volume decreased to a minimum value of 165 57.4% at 70 MPa but re-increased for higher osmotic levels and reached 64.2% after 166 treatment to 166 MPa. Thus, for a range of osmotic pressures between 70 and 166 MPa, the 167 final volumes of the mutant strain were higher than for the WT cells. Such volume behavior 168 of the  $erg6\Delta$  strain during dehydration to high amplitudes could be explained by relaxation of 169

170 cell wall due to the rupture of the PM for the most of the cells (Table 1). These results suggest 171 that dehydration beyond 30 MPa involves high tensile strength in PM which could be at the 172 origin of PM membrane rupture and permeabilization of the mutant yeast due to high 173 membrane sensitivity to stretching for this strain.

To improve the comprehension of the mechanisms of PM permeabilization during mild 174 dehydration-rehydration cycle (30 MPa) for the mutant strain, a comparative analysis of yeast 175 volume between the  $erg6\Delta$  and WT strains was performed after dehydration to different levels 176 177 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 178 179 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 180 amplitudes to 14.5 MPa and yeast volume reached 68% of the initial volume (at 1.4 MPa). 181 182 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 183 184 pressures, the profiles of cell volumes were different for the two strains. For the WT strain, 185 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 186 reversible and the cell volume was recovered after rehydration to 1.4 MPa. For the  $erg \delta \Delta$ 187 188 strain, rehydration from 30 MPa to osmotic pressures between 22.5 and 7 MPa conducted to 189 an increase in cell volume to values slightly higher than the ones of the WT strain. However, 190 these differences were not significant. Rehydration to 1.4 MPa conducted to a volume value 191 of 82% of the initial volume. This phenomenon could be linked to the high proportion of permeabilized cells for the  $erg6\Delta$  strain during rehydration (Fig. 2): cell swelling during 192 193 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 194

195 lack of membrane surface causing membrane rupture during cell swelling. Indeed, we 196 reported, in a previous study, that PM permeabilization occurred during rehydration of WT 197 yeasts after a shock to 166 MPa. This event was caused by a decrease in the PM surface 198 induced by membrane internalization after this perturbation (Dupont et al., 2010). Reduction 199 of PM surface after shock to 30 MPa could explain the membrane permeabilization of the 200  $erg6\Delta$  occurring during the rehydration step strain following the shock to the moderate 201 osmotic pressure of 30 MPa.

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# 203 Plasma membrane deformations after hyperosmotic shock to 30 MPa depend on the nature 204 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  $erg6\Delta$  strains presented PM deformations (Fig. 5 A, B, C, 208 209 and D). For the WT strain, PM showed narrow and deep (~400-500 nm) invaginations which 210 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 211  $erg6\Delta$  strain, PM presented a more undulated aspect in comparison to WT. Furthermore, 212 213 small vesicles were observed between the plasma membrane and the cell wall (Fig. 5 D). The 214 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 215 surface reduction in the mutant strain after hyperosmotic shock and could explain the 216 217 permeabilization of the mutant during the swelling induced by rehydration.

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#### 219 Exogenous ergosterol restores the resistance of the $erg6\Delta$ strain to hyperosmotic shock to

#### 220 **30 MPa**

The most probable hypothesis to explain the hypersensitivity of the  $erg6\Delta$  to hyperosmotic 221 222 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  $erg6\Delta$ 223 mutation such as remodeling in transcription, translation, or cytoskeleton organization. These 224 effects could also induce changes in membrane properties. To determine if the first hypothesis 225 was correct, we inspected whether exogenous addition of ergosterol in the  $erg6\Delta$  strain could 226 restore normal sensitivity to hyperosmotic perturbation at 30 MPa (Fig. 6). Addition of 227 228 ergosterol during cell growth under aerobic conditions did not change the survival to this perturbation of the two strains: viabilities of the WT and  $erg6\Delta$  remained unchanged at about 229 80% and 3%, respectively. Under aerobic growth conditions, yeasts synthesize sterols and do 230 231 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 232 233 oxygen allowed exogenous ergosterol to incorporate the PM of the two strains because yeasts 234 become auxotrophic for sterols under anaerobic conditions. Anaerobic growth with an ergosterol supplementation to the culture medium (250 µM), yielded to significant changes in 235 the yeast survival rates after hyperosmotic shock at 30 MPa. This rate reached 66% for the 236 237 WT and  $erg6\Delta$  strains.

238

#### 239 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  $erg6\Delta$  strain accumulated mainly zymosterol and 244 cholesta-5,7,24-trienol, as previously described (Munn et al., 1999). In contrast, under 245 anaerobic growth condition with ergosterol supplementation, the sterol profiles of the two 246 247 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 248 anaerobiosis is not surprising because squalene epoxidation, which is the next step after 249 squalene in the ergosterol biosynthesis, requires oxygen (Jahnke and Klein, 1983). Altogether, 250 251 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 252 growth for  $erg6\Delta$ ; aerobic and anaerobic growth for WT) increase yeast resistance to 253 dehydration. Therefore, yeast survival to dehydration is directly linked to the nature of 254 255 membrane sterols.

256

#### 257 **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  $erg6\Delta$  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  $erg6\Delta$  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  $erg6\Delta$  strains after

hyperosmotic shock at 30 MPa revealed that the kind of membrane deformations depended on 269 270 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 271 272 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 273 known that modifications of physical membrane properties can be directly related to the 274 structure of sterol molecule, including its planar structure, its size, and the properties of its 275 276 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 277 PM. Cholesta-5,7,24-trienol, like ergosterol, but not zymosterol displays a C-7,8 double bond 278 (Fig. 1). This induces the higher membrane fluidity of the mutant than the WT strain (Kaur 279 and Bachhawat, 1999; Abe and Hiraki, 2009). These differences in packing and fluidity of the 280 281 PM between both strains could account for the differences in membrane deformations during osmotic cell volume contraction. 282

283 Changes in PM properties conducted to the difference in yeast strain sensitivity to hydric 284 perturbations. The high sensitivity of the  $erg6\Delta$  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 285 hand, dehydration stage conducted to the permeabilization of the PM: this phenomenon was 286 287 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 288 integrity (Table 1). Cell volume analysis on a range of osmotic treatments between 30 and 289 290 166 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 291 292 membrane (Hossack and Rose, 1976) may explain the higher fragility of the membrane in the  $erg6\Delta$  mutant, as compared to the WT, during the dehydration step. On the other hand, the 293

rehydration step was critical for mutant cell integrity under mild treatments (30 MPa) (Table 294 1). After this hyperosmotic treatment, membrane permeabilization increased with the level of 295 rehydration (Fig. 2) and with cell volume swelling (Fig. 4). A possible explanation is a lack of 296 297 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 298 hyperosmotic shocks: decrease of PM surface was caused by membrane internalization 299 (Dupont et al., 2010) and led to the loss of membrane integrity during the rehydration. This 300 301 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 302 between cell wall and PM observed only with the mutant strain by TEM microscopy (Fig. 5). 303 All these events indicate that the nature of the sterol molecules in the membrane influences 304 the physical behavior of the PM during hydric perturbations. This parameter is clearly 305 306 involved in cell survival during this environmental stress. This conclusion is reinforced by the fact that  $erg6\Delta$  resistance to dehydration is restored when cultivated under conditions of 307 308 ergosterol accumulation (Figs 6 and 7).

309 In their ecological niche, such as the plant surfaces and soil, yeasts are subjected to hydric 310 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 311 312 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 313 314 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 315 316 taken to represent the evolutionary sequence of sterol (Bloch, 1994). In this view, the  $erg6\Delta$ 317 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 318

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

321

#### 322 Experimental procedures

#### 323 Yeast strains and culture conditions

The Saccharomyces cerevisiae strain BY 4742 Wild Type (WT) (MATa his $3\Delta 1$  leu $2\Delta 0$ 324  $lys2\Delta0 \ ura3\Delta0$ ) and the  $erg6\Delta$  mutant (MATa  $his3\Delta1 \ leu2\Delta0 \ lys2\Delta0 \ YMR008c::kanMX4$ ) 325 (Euroscarf, Frankfurt, Germany) were used in this study. Cells were grown aerobically at 326 25°C in 250 mL conical flasks containing 100 mL of Malt Wickerham modified medium 327 (MW). The MW medium contained 10 g glucose, 3 g pancreatic peptone, 3 g yeast extract, 328 and 1.5 g NaH<sub>2</sub>PO<sub>4</sub> (Sigma-Aldrich, Saint Quentin Fallavier, France) in 1 L of water-329 glycerol, which had an osmotic pressure of 1.4 MPa. This osmotic pressure has been 330 recommended for the optimal growth of S. cerevisiae (Anand and Brown, 1968). For aerobic 331 growth, a subculture (1 mL) was transferred into a conical flask containing MW medium, and 332 cultures were placed on a rotary shaker (New Brunswick Scientific, Edison, NY, USA) at 250 333 334 rpm for 24 h and allowed to grow to the early stationary phase. The final population was nearly  $10^8$  cells mL<sup>-1</sup>. The media used for anaerobic growth were supplemented with 2% (v/v) 335 Tween 80 (polyethylene sorbitan mono-oleate) and 250 µM ergosterol and was degassed of 336 oxygen by nitrogen bubbling for 12 h before inoculation with 1 mL of subculture. Culture was 337 338 performed in an anaerobic chamber for 24 h at 25°C.

339

#### 340 Preparation of binary water-glycerol solutions of different osmotic pressures

341 The solute used in all experiments to perform hyperosmotic treatments was glycerol (Sigma-342 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):

345 
$$a_w = (1 - X_s)e^{-KXs^2}$$
,

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 ( $\pi$ ) 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<sup>-1</sup>.K<sup>-1</sup>), *T* is the temperature (K) and  $\overline{V_w}$  is the partial molar volume of water (m<sup>3</sup>.mol<sup>-1</sup>). 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 (DecagonDevices Inc., Pullman, WA, USA).

355

#### 356 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 359 360 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 361 centrifuged (10 min, 5100 g) and the supernatant was removed. Hyperosmotic shock was 362 induced by quickly introducing 1 mL of a binary water-glycerol solution (final osmotic 363 pressure of 30, or 166 MPa) to the pellets. The cells were maintained 1 h under hyperosmotic 364 365 conditions before rehydration. Rapid rehydration was performed by removing the hyperosmotic solution from the microsample tube after centrifugation (10 min, 5100 g) and 366 by quickly introducing 1 mL of the binary water-glycerol solution (1.4 MPa) to the cell pellet. 367

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.

370

#### 371 Measurement of yeast viability

372 Yeast viability was estimated in triplicate by the CFU method. After osmotic treatment, fully 373 rehydrated cells were diluted serially and the appropriate dilutions were plated in MW 374 medium with 15 g  $L^{-1}$  of agar. CFU were counted after incubation for 36 h at 25°C. The 375 initial cell suspension was used as the control.

376

#### 377 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.

383

#### 384 Assessment of plasma membrane permeability

Propidium iodide (PI) (Invitrogen, Carlsbad, CA, USA) was used to assess PM integrity. This 385 probe stains nucleic acids after permeabilization of the PM. PI was dissolved in distilled water 386 (10 mg mL<sup>-1</sup>) to prepare the stock solution, and 200 µg of PI was used to stain 10<sup>8</sup> cells. 387 Proportion of permeabilized cells was estimated by flow cytometric analysis for treatments to 388 30 MPa using a FACSCalibur flow cytometer (BD Biosciences). For each sample, 10 000 389 390 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 391 392 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.

399

#### 400 Electron microscopy

401 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 402 in water-glycerol solution at osmotic pressure of 30 MPa. Treated cells were fixed just after 403 404 the end of the osmotic treatment. After washing, cells were postfixed with 0.5% OsO<sub>4</sub>-0.1 M phosphate buffer, pH 7.2, for 1 h at 4°C. Cells were dehydrated progressively in 30%, 50%, 405 70%, 90%, and 100% ethanol, 30 min for each step, impregnated with Epon, and polymerized 406 407 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. 408 Observations were performed on a Hitachi 7500 transmission electron microscope (operating 409 at 80 kV) equipped with an AMT camera driven by AMT software (AMT, Danvers, MA, 410 411 USA).

412

#### 413 Membrane sterol identification and quantification

Lipid extracts were obtained from  $\approx 5 \ 10^8$  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). 418 Cellular lipids were extracted using chloroform/methanol (2:1, v/v) as described by Folch et 419 al. (Folch et al., 1957). The final organic phase was evaporated and sterols were dissolved in 420  $100 \mu$ L 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.

425

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- 533 534

#### 535 **Figure legends**

- Fig. 1. Ergosterol biosynthetic pathway in Saccharomyces cerevisiae. (A) The five final steps 536
- 537 in ergosterol biosynthesis. (B) Structure of the main sterols accumulated in the  $erg6\Delta$  mutant.
- (C) Structure of ergosterol, the major sterol accumulated in the WT strain. 538

539

- Fig. 2. Evolution of plasma membrane integrity as a function of the rehydration level after 540
- dehydration at 30 MPa. WT and  $erg6\Delta$  strains were treated with hyperosmotic shock to 30 541

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).

547

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; \*\*,</li>
P<0.01; \*\*\*, P<0.001).</li>

553

**Fig. 4.** Comparison of cell volumes between WT and  $erg6\Delta$  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.

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**Fig. 5.** Ultrastructure of WT and  $erg6\Delta$  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  $erg6\Delta$  (C and D). Micrographs were taken of 2% paraformaldehyde/3% glutaraldehydefixed, 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* $\Delta$  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).

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Fig. 7. Effects of growth conditions on the sterol composition of the WT and  $erg6\Delta$  strains. 575 Both strains were grown under aerobic, or anaerobic conditions with ergosterol 576 supplementation (250 µM). After lipid extraction, sterol amounts were determined by gas 577 chromatography using cholesterol as a standard, as described in Materials and Methods. 578 579 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 580 581 structure close to the one of ergosterol, is a by-product that accumulates in the  $erg6\Delta$  strain. 582 Error bars correspond to the SD calculated from three repeat experiments.