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

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

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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,

46 respectively. Sterols confer important properties to the PM through their interactions with
47 phospholipids and sphingolipids and play an essential role in the stability of membranes, by
48 affecting rigidity, fluidity, and permeability (Folmer et al., 2008; Abe and Hiraki, 2009; Róg
49 et al., 2009). Sterols are proposed to maintain the lateral heterogeneity of the protein and lipid
50 distribution in the PM because of their putative role in inducing microdomains, so-called lipid
51 rafts (Simons and Ikonen, 1997). The nature of sterols influence the properties of membrane
52 models such as the tensile properties (Hsueh et al., 2007), phase separation and the curvature
53 of the liquid-ordered phase in membranes (Bacia et al., 2005). Despite the numerous effects of
54 the nature of sterols on lipid bilayer properties, few studies have investigated the impact of
55 sterol composition on the "in vivo" behavior of cell PM to environmental perturbations and its
56 possible implication in cell resistance.
57 The aim of the proposed study was to estimate the effect of the nature of PM sterols on the
58 membrane response and the cell survival to hydric perturbations. Hydric stress was chosen
59 because the structure of the PM is strongly challenged during this perturbation (lipid phase
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
62 it is exposed to hydric fluctuations in its natural environments (plant surfaces, soil…) and is
63 able to survive extreme dehydration. Moreover, several mutant strains with alterations in the
64 ergosterol biosynthetic pathway (*erg*Δ) are available. These mutants accumulate different
65 sterols in their PM (Barton et al., 1974). It seems plausible that the sterol biosynthetic
66 pathway parallels sterol evolution (Bloch, 1994; Weete et al., 2010) and that these mutants are
67 evolutionarily precursors of the wild type strain (WT). In this work, we performed a
68 comparative study of the responses to osmotic dehydration (viability, ultrastructure, cell
69 volume, and permeability) between *S. cerevisiae* WT and the ergosterol mutant *erg6*Δ. The
70 *erg6* gene encoding the sterol C-24 methyltransferase, which catalyzes the first of the five

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

83

84 Results

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

86 To compare the sensitivity to osmotic dehydration of the WT and *erg6*Δ strains, yeasts were
87 treated with osmotic shocks at 30 and 166 MPa, maintained under hyperosmotic conditions
88 for 60 min and rehydrated to 1.4 MPa (Table 1). For the mild treatment (30 MPa
89 corresponding to a water activity of 0.8), the viability of the WT strain remained high (about
90 80%) whereas it was strongly decreased for the *erg6*Δ strain (3%). The survival of the WT
91 strain is in agreement with previous studies which reported that the magnitude of 30 MPa,
92 slightly higher than the one allowing osmoregulation (15 MPa) (Marechal et al., 1995),
93 affected weakly the yeast viability (Simonin et al., 2007). The difference in sensitivity to
94 osmotic shock between the two strains was also observed for the 166 MPa treatments
95 (corresponding to a water activity of 0.3): yeast survivals reached 0.3% and 0.01% for the WT

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

102

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

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

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

112 Without osmotic perturbation, the proportion of permeabilized cells was very low, reaching a
113 value close to 1% for the two strains. This proportion did not change after dehydration to 30
114 MPa for the WT strain and increased slightly to 7% for the *erg6*Δ strain (Table 1).
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*Δ strain occurred during
118 rehydration and could be the cause of the high sensitivity of the mutant to hyperosmotic
119 stress.

120 To estimate more accurately the mechanism of the loss of membrane integrity of *erg6*Δ
121 during rehydration, we characterized the changes in membrane permeability after rehydration
122 to different osmotic pressures between 1.4 and 22.5 MPa after hyperosmotic shock to 30 MPa
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*Δ strain, the proportion of permeabilized cells was about 40%
125 after rehydration to 22.5 MPa and increased with the amplitude of rehydration to reach 90%
126 after rehydration to 1.4 MPa. This result shows that the permeabilization of the PM during
127 rehydration step depends on the level of rehydration and that the loss of membrane integrity
128 already occurs for low magnitude rehydration.

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

130 PI staining was performed after dehydration to 166 MPa and after rehydration to assess the
131 effect of each stage of the dehydration-rehydration cycle on PM integrity for both strains
132 (Table 1). After the dehydration step to 166 MPa, the proportion of permeabilized cells
133 reached 33% and 91 % for WT and *erg6*Δ strains, respectively. After rehydration, this ratio
134 was high for both strains: 87% and 95%, respectively. These high proportions of
135 permeabilized cells after rehydration were correlated with the low survival rates observed
136 after treatment to 166 MPa (Table 1). However, the results suggest that the mechanisms of
137 PM permeabilization are different for the two strains. PM of *erg6*Δ strain is strongly altered
138 by the dehydration stage whereas the two steps of the dehydration-rehydration cycle are
139 involved in the loss of PM integrity for the WT strain as previously reported (Dupont et al.,
140 2010).

141

142 ***Variations in yeast volume lead to plasma membrane stress***

143 Hyperosmotic perturbations cause movement of large quantities of water across the PM,
144 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*Δ 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.

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

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.

174 To improve the comprehension of the mechanisms of PM permeabilization during mild
175 dehydration-rehydration cycle (30 MPa) for the mutant strain, a comparative analysis of yeast
176 volume between the *erg6*Δ and WT strains was performed after dehydration to different levels
177 to 30 MPa (Fig. 4 A) and after dehydration to 30 MPa followed by rehydration to different
178 amplitudes (Fig. 4 B). The profile of cell volumes, with increasing levels of osmotic pressure
179 ranging from 7 MPa to 30 MPa, was very similar for the two strains. Hyperosmotic treatments
180 led to exponentially cell volume decrease: the variation of the volume was strong for
181 amplitudes to 14.5 MPa and yeast volume reached 68% of the initial volume (at 1.4 MPa).
182 Under higher osmotic pressures, the volume slightly decreased to reach a value of 65% at 30
183 MPa. After hyperosmotic shock to 30 MPa followed by rehydration to different osmotic
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
186 (Fig. 4 A and B). Thus, volume changes induced by hyperosmotic treatment to 30 MPa were
187 reversible and the cell volume was recovered after rehydration to 1.4 MPa. For the *erg6*Δ
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
192 permeabilized cells for the *erg6*Δ strain during rehydration (Fig. 2): cell swelling during
193 rehydration is related to osmosis phenomenon which requires the integrity of the PM. A
194 possible hypothesis to explain PM permeabilization during the rehydration step could be a

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*Δ occurring during the rehydration step strain following the shock to the moderate
201 osmotic pressure of 30 MPa.
202

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

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

208 After shock to 30 MPa, WT and *erg6*Δ strains presented PM deformations (Fig. 5 A, B, C,
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
211 in the case of progressive osmotic dehydration to 166 MPa (Dupont et al., 2010). For the
212 *erg6*Δ strain, PM presented a more undulated aspect in comparison to WT. Furthermore,
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
215 under hyperosmotic conditions (Koch, 1998). These vesicles could be at the origin of PM
216 surface reduction in the mutant strain after hyperosmotic shock and could explain the
217 permeabilization of the mutant during the swelling induced by rehydration.
218

219 ***Exogenous ergosterol restores the resistance of the erg6Δ strain to hyperosmotic shock to***

220 ***30 MPa***

221 The most probable hypothesis to explain the hypersensitivity of the *erg6*Δ to hyperosmotic
222 perturbations was the modification of the PM properties caused by the change in PM sterols.
223 However, a second explanation could be possible downstream effects induced by *erg6*Δ
224 mutation such as remodeling in transcription, translation, or cytoskeleton organization. These
225 effects could also induce changes in membrane properties. To determine if the first hypothesis
226 was correct, we inspected whether exogenous addition of ergosterol in the *erg6*Δ strain could
227 restore normal sensitivity to hyperosmotic perturbation at 30 MPa (Fig. 6). Addition of
228 ergosterol during cell growth under aerobic conditions did not change the survival to this
229 perturbation of the two strains: viabilities of the WT and *erg6*Δ remained unchanged at about
230 80% and 3%, respectively. Under aerobic growth conditions, yeasts synthesize sterols and do
231 not incorporate significant amounts of exogenous sterols, a phenomenon known as "aerobic
232 sterol exclusion" (Lorenz and Parks, 1991). In contrast, growth of yeasts performed without
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
235 ergosterol supplementation to the culture medium (250 µM), yielded to significant changes in
236 the yeast survival rates after hyperosmotic shock at 30 MPa. This rate reached 66% for the
237 WT and *erg6*Δ strains.

238

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

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

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

256

257 **Discussion**

258 The aim of this study focused on the effect of the effects of the sterol content on PM
259 properties in response to hyperosmotic perturbations, by comparing membrane behaviors
260 during dehydration-rehydration cycles between the WT strain (accumulating ergosterol in the
261 PM) and the *erg6*Δ mutant (accumulating zymosterol and cholesta-5,7,24-trienol) (Fig. 7).
262 The main finding is that nature of PM sterols influences the mechanical properties of the PM.
263 These changes in properties led to different osmotic behaviors of the PM and induced a high
264 sensitivity of the *erg6*Δ mutant to hyperosmotic perturbations, as compared to the WT strain.
265 Hyperosmotic perturbations induced an outflow of large quantities of water, causing a strong
266 decrease in cell volume (Figs. 3 and 4). Due to the low lateral compressibility of the
267 membrane (Evans et al., 1976), the cell s/v ratio increase conducted to compressive lateral
268 stress and to the deformation of the PM. TEM observation of WT and *erg6*Δ strains after

269 hyperosmotic shock at 30 MPa revealed that the kind of membrane deformations depended on
270 the sterol composition of the PM (Fig. 5). The link between membrane sterols and the kind of
271 membrane deformations observed could be related to the influence of the nature of sterols on
272 the physical properties of the lipid bilayer. Although the effect of zymosterol and cholesta-
273 5,7,24-trienol on model membrane properties have never been studied to our knowledge, it is
274 known that modifications of physical membrane properties can be directly related to the
275 structure of sterol molecule, including its planar structure, its size, and the properties of its
276 small polar 3-OH group (Xu and London, 2000). In particular, the double bond between C-7
277 and C-8 in the B ring is known to be involved in the packing and the overall rigidity of the
278 PM. Cholesta-5,7,24-trienol, like ergosterol, but not zymosterol displays a C-7,8 double bond
279 (Fig. 1). This induces the higher membrane fluidity of the mutant than the WT strain (Kaur
280 and Bachhawat, 1999; Abe and Hiraki, 2009). These differences in packing and fluidity of the
281 PM between both strains could account for the differences in membrane deformations during
282 osmotic cell volume contraction.
283 Changes in PM properties conducted to the difference in yeast strain sensitivity to hydric
284 perturbations. The high sensitivity of the *erg6*Δ strain is linked to the loss of PM integrity
285 which occurs during the two steps of the dehydration/rehydration cycle (Table 1). On the one
286 hand, dehydration stage conducted to the permeabilization of the PM: this phenomenon was
287 observed for the treatment to high hyperosmotic level (166 MPa). After this perturbation, over
288 90% of the mutant cells were permeabilized whereas only 35% of WT yeasts lost their
289 integrity (Table 1). Cell volume analysis on a range of osmotic treatments between 30 and
290 166 MPa (Fig. 3) revealed that PM pulled the cell wall and endured stretching stress during
291 severe dehydration. The influence of sterol structure on the stretching resistance of the
292 membrane (Hossack and Rose, 1976) may explain the higher fragility of the membrane in the
293 *erg6*Δ mutant, as compared to the WT, during the dehydration step. On the other hand, the

294 rehydration step was critical for mutant cell integrity under mild treatments (30 MPa) (Table
295 1). After this hyperosmotic treatment, membrane permeabilization increased with the level of
296 rehydration (Fig. 2) and with cell volume swelling (Fig. 4). A possible explanation is a lack of
297 membrane surface conducting to PM lysis during the cell volume increase caused by
298 rehydration. This phenomenon has been observed for WT yeasts only in the case of severe
299 hyperosmotic shocks: decrease of PM surface was caused by membrane internalization
300 (Dupont et al., 2010) and led to the loss of membrane integrity during the rehydration. This
301 event was also shown for protoplasts (Wolfe et al., 1986) during osmotic perturbations.
302 Decrease of membrane surface seems to be related to the formation of extracellular vesicles
303 between cell wall and PM observed only with the mutant strain by TEM microscopy (Fig. 5).
304 All these events indicate that the nature of the sterol molecules in the membrane influences
305 the physical behavior of the PM during hydric perturbations. This parameter is clearly
306 involved in cell survival during this environmental stress. This conclusion is reinforced by the
307 fact that *erg6*Δ resistance to dehydration is restored when cultivated under conditions of
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
311 shows that a non lethal (in the classic genetic sense) ergosterol mutant, accumulating
312 ergosterol precursors, is highly sensitive to hydric perturbations compared to the WT strain. It
313 is surmised that membrane sterols have been selected over a very long time period by
314 Darwinian evolution for their ability to optimize certain physical properties of the membranes.
315 As argued by Konrad Bloch, the temporal sequence of the sterol biosynthetic pathway can be
316 taken to represent the evolutionary sequence of sterol (Bloch, 1994). In this view, the *erg6*Δ
317 yeast strain is an evolutionary precursor of the WT strain. These results could provide some
318 response elements on the nature of a driving force, which led to the evolution of the ergosterol

319 biosynthetic pathway in members of fungi kingdom, which encounter hydric fluctuations in
320 their natural environment.
321

322 Experimental procedures

323 *Yeast strains and culture conditions*

324 The *Saccharomyces cerevisiae* strain BY 4742 Wild Type (WT) (*MAT*α *his3*Δ*1 leu2*Δ*0*
325 *lys2*Δ*0 ura3*Δ*0*) and the *erg6*Δ mutant (*MAT*α *his3*Δ*1 leu2*Δ*0 lys2*Δ*0 YMR008c::kanMX4*)
326 (Euroscarf, Frankfurt, Germany) were used in this study. Cells were grown aerobically at
327 25°C in 250 mL conical flasks containing 100 mL of Malt Wickerham modified medium
328 (MW). The MW medium contained 10 g glucose, 3 g pancreatic peptone, 3 g yeast extract,
329 and 1.5 g NaH_2PO_4 (Sigma-Aldrich, Saint Quentin Fallavier, France) in 1 L of water–
330 glycerol, which had an osmotic pressure of 1.4 MPa. This osmotic pressure has been
331 recommended for the optimal growth of *S. cerevisiae* (Anand and Brown, 1968). For aerobic
332 growth, a subculture (1 mL) was transferred into a conical flask containing MW medium, and
333 cultures were placed on a rotary shaker (New Brunswick Scientific, Edison, NY, USA) at 250
334 rpm for 24 h and allowed to grow to the early stationary phase. The final population was
335 nearly 10^8 cells mL^{-1}. The media used for anaerobic growth were supplemented with 2% (v/v)
336 Tween 80 (polyethylene sorbitan mono-oleate) and 250 µM ergosterol and was degassed of
337 oxygen by nitrogen bubbling for 12 h before inoculation with 1 mL of subculture. Culture was
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).

343 The mass of solute to be added to 1000 g of distilled water to obtain the desired water activity

344 (a_w) was calculated using the Norrish equation (Norrish, 1966):

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

346 where X_s is the molar fraction of the solute and K is the Norrish coefficient of the solute used

347 to increase the osmotic pressure. For glycerol, $K = 1.16$. Osmotic pressure (π) is related to the

348 water activity by the following equation:

349 $$\pi = -\frac{RT \ln a_w}{\overline{V_w}},$$

350 where R is the universal gas constant (J.mol^{-1}.K^{-1}), T is the temperature (K) and $\overline{V_w}$ is the

351 partial molar volume of water (m^3.mol^{-1}). Table 2 presents the quantity of glycerol used to

352 prepare the solutions of different osmotic pressures.

353 The osmotic pressure of all solutions was checked with a dew-point osmometer (Decagon

354 Devices Inc., Pullman, WA, USA).

355

356 ***Osmotic treatments***

357 Two levels of osmotic treatments were used: moderate (30 MPa), which is slightly higher than

358 that allowing osmoregulation, and severe (166 MPa).

359 Samples (20 mL) of culture were centrifuged (5 min, 2200 *g*), washed twice in the binary

360 water–glycerol mixture (1.4 MPa), and the pellets were resuspended in 10 mL of the same

361 medium. 1 mL aliquots of this suspension were placed in microsample tubes, which were then

362 centrifuged (10 min, 5100 *g*) and the supernatant was removed. Hyperosmotic shock was

363 induced by quickly introducing 1 mL of a binary water–glycerol solution (final osmotic

364 pressure of 30, or 166 MPa) to the pellets. The cells were maintained 1 h under hyperosmotic

365 conditions before rehydration. Rapid rehydration was performed by removing the

366 hyperosmotic solution from the microsample tube after centrifugation (10 min, 5100 *g*) and

367 by quickly introducing 1 mL of the binary water–glycerol solution (1.4 MPa) to the cell pellet.

368 Cell suspensions, rehydration solutions, and shock solutions were kept in an air-conditioned
369 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***
378 Cell volume variations were measured after dehydration to different osmotic pressures (7, 14,
379 22.5, and 30 MPa) and after dehydration to 30 MPa followed by rehydration to 14, 7, and 1.4
380 MPa. These variations were estimated on images acquired with a Nikon Eclipse TE 2000 E
381 microscope (Nikon, Tokyo, Japan) with spectral camera Nuance CRI. Cells were individually
382 analyzed with the software ImageJ 1.42q to determine their projected areas and volumes.
383
384 ***Assessment of plasma membrane permeability***
385 Propidium iodide (PI) (Invitrogen, Carlsbad, CA, USA) was used to assess PM integrity. This
386 probe stains nucleic acids after permeabilization of the PM. PI was dissolved in distilled water
387 (10 mg mL^{-1}) to prepare the stock solution, and 200 µg of PI was used to stain 10^8 cells.
388 Proportion of permeabilized cells was estimated by flow cytometric analysis for treatments to
389 30 MPa using a FACSCalibur flow cytometer (BD Biosciences). For each sample, 10 000
390 events were collected. Above this amplitude, it was not possible to use the flow cytometer due
391 to the high viscosity of the cell suspensions and the proportion of permeabilized cells was
392 assessed by fluorescence microscopy for treatments to 166 MPa. A Nikon Eclipse TE 2000 E

393 epifluorescence microscope (Nikon, Tokyo, Japan) with spectral camera Nuance CRI was
394 used to observe cells. Images were acquired with a ×40 (NA: 0.95) Plan Apo objective
395 (Nikon) and collected with Nuance software (Nikon). Black and white images were captured
396 to observe the total cell population (at least 300 cells). A monochromatic epifilter (540-580
397 nm and 600-660 nm excitation and emission wavelengths, respectively) was used to observe
398 cells stained with PI.
399
400 ***Electron microscopy***
401 TEM was used to assess the yeast ultrastructure just after shock to 30 MPa. Concentrated
402 yeast samples were fixed for 12 h at 4°C with 3% glutaraldehyde and 2% paraformaldehyde
403 in water–glycerol solution at osmotic pressure of 30 MPa. Treated cells were fixed just after
404 the end of the osmotic treatment. After washing, cells were postfixed with 0.5% OsO_4–0.1 M
405 phosphate buffer, pH 7.2, for 1 h at 4°C. Cells were dehydrated progressively in 30%, 50%,
406 70%, 90%, and 100% ethanol, 30 min for each step, impregnated with Epon, and polymerized
407 at 60°C for 48 h. Ultrathin sections (90 nm) were obtained using an Ultracut E ultramicrotome
408 (Reichert, Depew, NY, USA) and contrasted with uranyl acetate and lead citrate.
409 Observations were performed on a Hitachi 7500 transmission electron microscope (operating
410 at 80 kV) equipped with an AMT camera driven by AMT software (AMT, Danvers, MA,
411 USA).
412
413 ***Membrane sterol identification and quantification***
414 Lipid extracts were obtained from ≈5 10^8 shock-frozen yeast cells grown as indicated. Cells
415 were harvested, washed with distilled water and taken up to 1 mL with cold water. Cells were
416 then broken by vigorous shaking with a mini-beadbeaterTM (Biospec Products) for 1 min at
417 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 µL of hexane.
421 The different sterol species were then separated by Gas Chromatography using a 25 m×0.32
422 mm AT-1 capillary column (Alltech) and identified by the means of their retention times
423 relative to cholesterol, used as a standard. The results are expressed as nmol of sterol/10^9
424 cells.
425

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432

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534

535 Figure legends

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

539

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

542 MPa and were stained with PI after rehydration to different osmotic pressures: 1.4, 7, 14.5,
543 and 22.5 MPa. Measurement of the proportion of permeabilized cells was performed by flow
544 cytometry. Error bars correspond to the SD calculated from three repeat experiments.
545 Asterisks denote statistical significance with respect to wild type strain (*, P<0.05; **,
546 P<0.01; ***, P<0.001).
547
548 **Fig. 3.** Evolution of yeast volume as a function of the magnitude of hyperosmotic shocks.
549 Volume estimation was performed by microscopic image analyzing after shocks to 30, 70,
550 110, and 166 MPa. Error bars correspond to the SD calculated from three repeat experiments.
551 Asterisks denote statistical significance with respect to wild type strain (*, P<0.05; **,
552 P<0.01; ***, P<0.001).
553
554 **Fig. 4.** Comparison of cell volumes between WT and *erg6*Δ during dehydration to 30 MPa
555 followed by rehydration to 1.4 MPa. Volume estimation was performed by microscopic image
556 analyzing. For the cell volume profile during dehydration (A), yeasts were observed after
557 hyperosmotic treatments to 7, 14.5, 22.5, and 30 MPa. For the profile during rehydration (B),
558 yeasts were observed after rehydration to 1.4, 7, 14.5, and 22.5 MPa from cells dehydrated to
559 30 MPa. Error bars correspond to the SD calculated from three repeat experiments.
560
561 **Fig. 5.** Ultrastructure of WT and *erg6*Δ yeasts after hyperosmotic shock to 30 MPa. TEM
562 micrographs of representative yeast sections. Cells after shock to 30 MPa for WT (A and B)
563 and *erg6*Δ (C and D). Micrographs were taken of 2% paraformaldehyde/3% glutaraldehyde-
564 fixed, metaperiodate-treated, reduced osmium post-fixed and EPON embedded cells. Cells
565 were fixed just after the hyperosmotic shock to 30 MPa. (A), and (C): bar scale = 500 nm.
566 (B), and (D): bar scale = 100 nm.

567

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

574

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