Plasma membrane composition of *Debaryomyces hansenii* adapts to changes in pH and external salinity

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*Debaryomyces hansenii* is a marine yeast that has to cope with different stress situations. Since changes in membrane properties can play an important function in adaptation, we have examined the fluidity and lipid composition of purified plasma membranes of *D. hansenii* grown at different external pH values and salt concentrations. Growth at low pH caused an increase in the sterol-to-phospholipid ratio and a decrease in fatty acid unsaturation which was reflected in decreased fluidity of the plasma membrane. High levels of NaCl increased the sterol-to-phospholipid ratio and fatty acid unsaturation, but did not significantly affect fluidity. The sterol-to-phospholipid ratios obtained in *D. hansenii* grown under any of these conditions were similar to the ratios that have been reported for halophilic/halotolerant black yeasts, but much smaller than those observed in the model yeast *Saccharomyces cerevisiae*.

**INTRODUCTION**

Living organisms have to cope with different stress situations and they have to adapt to changes in salinity, temperature and pH. The cell comes into contact with its environment via the plasma membrane, and so membrane adaptability and flexibility is of vital importance for survival. The cell should be able to modify the lipid composition and, consequently, the properties of its membranes if the conditions in the environment change. The fluidity of biological membranes is largely influenced by the type of fatty acids (length, degree of unsaturation and branching), the amount of sterols and the nature of the phospholipids (Russell, 1989a, b).

The influence of salt stress on lipid composition and membrane fluidity has been studied predominantly in bacteria (Russell et al., 1995), but also in yeasts, including salt-sensitive *Saccharomyces cerevisiae* (Sharma et al., 1996; Tunblad-Johansson & Adler, 1987). Lipid composition and properties have also been examined in several halotolerant yeasts and in halophilic/halotolerant melanized yeast-like fungi. These organisms show different responses under salt stress. *Zygosaccharomyces rouxii* grown at 15 % NaCl (w/v) shows increased amounts of free (non-esterified) ergosterol, decreased fatty acid unsaturation and decreased membrane fluidity than when it is grown without NaCl (Hosono, 1992; Yoshikawa et al., 1995). High salinity does not induce significant changes in the unsaturation of fatty acids in *Yarrowia lipolytica* (Andreishcheva et al., 1999), but causes a decrease in phospholipid and sterol content. In contrast, *Candida membranefaciens* grown at high NaCl concentrations exhibits increases in fatty acid unsaturation and in the content of phosphatidylinositol (PI) and phosphatidylethanolamine (PE), resulting in higher membrane fluidity (Khaware et al., 1995). Meanwhile, relatively small alterations have been observed in the fatty acid composition of phospholipids. In *Hortaea werneckii*, *Phaeotheca triangularis* and the halotolerant *Aureobasidium pullulans*, salt stress does not significantly influence the total sterol content. In all three yeasts the most abundant fatty acids in phospholipids contain C16 and C18 chains with a high percentage of C18 : 2 \( \Delta 9,12 \). Salt stress causes an increase in fatty acid unsaturation in the halophilic *H. werneckii* and halotolerant *A. pullulans*, but a slight decrease in halophilic *P. triangularis*. The two halophilic fungi, *H. werneckii* and *P. triangularis*, maintain...

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*Abbreviations*: EPR, electron paramagnetic resonance spectroscopy; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SP, sphingolipid.

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a significantly lower sterol-to-phospholipid ratio than salt-sensitive *S. cerevisiae* and halotolerant *A. pullulans* do. Electron paramagnetic resonance spectroscopy (EPR) measurements have shown that the membranes of both these halophilic fungi are more fluid than the membrane of the halotolerant *A. pullulans* and salt-sensitive *S. cerevisiae* (Turk et al., 2004). More recently, the effect of salt on plasma membrane fluidity has been studied in some selected extremophilic yeasts and yeast-like fungi. When salinity exceeded their optimal range, the ubiquitous stress-selected extremophilic yeasts and yeast-like fungi. When plasma membrane fluidity has been studied in some inant extremophiles (*H. werneckii* increased plasma-membrane fluidity, while in the dominant extremophiles (*H. werneckii, Cryptococcus liquefaciens*), it decreased (Turk et al., 2007).

*Debaryomyces hansenii* is one of the most abundant yeasts isolated from marine water, where the two important stress factors are salt and a slightly alkaline pH (Norkrans, 1966; Prista et al., 1997). Although several groups have reported the halotolerant character of *D. hansenii*, the molecular and physiological basis of that behaviour is not fully understood (Prista et al., 2005; Thomé, 2004; Thomé-Ortiz et al., 1998). Plasma membrane composition and fluidity must play an important role in the ability to thrive in sea water and salty environments. A previous study on *D. hansenii* revealed that salt stress caused a decrease in the relative contents of sterols, phosphatidylglycerol (PG), PI and PE, whereas the relative content of phosphatidylserine (PS) increased (Tunblad-Johansson et al., 1987). This study was performed with lipids extracted from whole cells and plasma membrane fluidity was not investigated.

Some information about the effect of salt on plasma membrane fluidity and lipid composition in different fungi is available, but almost nothing has been published in relation to the effect of external pH. Studies on adaptation to low pH in some oral bacteria have shown that aciduric strains alter their membrane composition by increasing the amount of long-chain, mono-unsaturated fatty acids (Fozo et al., 2004) and that certain Gram-positive bacteria increase the proportion of lysyl-PG relative to PG (Russell, 1989b). *Leuconostoc oenos*, a wine-processing bacterium, increases fatty acid unsaturation and the proportion of C19:0-cyclopropane at low pH (Drici-Cachon et al., 1996).

To our knowledge, this is the first paper reporting results on the adaptation of the marine yeast *D. hansenii* to different pH values and NaCl concentrations at the level of membrane fluidity and lipid composition of isolated plasma membranes. We show that both pH and salt affect lipid composition of the plasma membrane. However, while acidic pH, a stress factor not present in environments where *D. hansenii* can be isolated, greatly decreased membrane fluidity, high pH and/or high salt conditions, present in sea water, did not affect fluidity in this marine yeast very much.

### METHODS

**Yeast strain and growth conditions.** *Debaryomyces hansenii* strain IGC2968 (CBS767) was used in this study. Cells were grown in YPD medium (yeast extract, 1 %; peptone, 2 %; glucose, 2 %, w/v) adjusted to the required pH (4.0, 6.0 or 8.0) with HCl or arginine, and buffered with 10 mM tartaric acid (pH 4.0), MES (pH 6.0) or glycyglycine (pH 8.0). When required, NaCl was added to the medium at concentrations of 0.5, 1, 1.5 or 2 M. Incubations were performed at 28 °C using 500 ml Erlenmeyer flasks on a rotary shaker at 180 r.p.m. Cells were harvested during the mid-exponential phase by centrifugation at 4000 g for 10 min.

**Growth experiments.** To calculate the doubling time, cells were grown overnight in liquid YPD medium adjusted to pH 4.0, 6.0 or 8.0 and with or without NaCl. Cells were then washed twice with sterile cool distilled water, inoculated (10⁵ cells ml⁻¹) in a similar medium and incubated under the same conditions. The optical density of the cultures (OD₅₅₀) was measured at different times.

**Lipid analysis.**

**Plasma membrane preparation.** Harvested *D. hansenii* cells were frozen in liquid nitrogen and mechanically disintegrated. Plasma membranes were isolated on a sucrose gradient (Panaretou & Piper, 1996). The purity of the plasma membranes was checked by measuring the plasma membranes, mitochondrial and vacuolar ATPase activity (Serrano, 1988).

**Lipid extraction.** Lipids were extracted from enriched plasma membranes as described previously (Turk et al., 2004).

**Isolation and quantification of phospholipids by thin-layer chromatography (TLC).** Lipid extracts from the plasma membranes of cells grown in media with different pH values and NaCl concentrations were isolated by one-dimensional TLC and quantified as described previously (Kates, 1986; Turk et al., 2004).

**GC-MS analysis of sterols and fatty acids.** Trimethylsilyl ethers (TMS ethers) of sterols and methylated fatty acids were dissolved in 50 µl n-hexane and analysed by GC-MS using a Hewlett Packard HP5890 gas chromatograph (Hewlett Packard) coupled to an AutospecQ mass spectrometer (Micromass). The gas chromatograph was equipped with a splitless injector heated to 250 °C and an HP-5 MS capillary column (25 m x 0.25 mm i.d., coated with a 0.25 mm thick stationary phase of 5 % phenylmethylpolysiloxane; Hewlett Packard), using helium as the carrier gas. The oven temperature programme for analysis started at 50 °C with 1 min holding time, then the oven was heated to 220 °C at a rate of 20 °C min⁻¹, then to 300 °C at a rate of 100 °C min⁻¹ with a final holding time of 5 min. Relative concentrations of sterols and fatty acids were calculated from the total ion current response areas of the organic compounds and of the internal standards 5β-cholestan-3α-ol (Sigma-Aldrich) for sterols or methyl-nonadecanoate (Sigma-Aldrich) for fatty acids.

Mass spectra were recorded in electron impact mode at 70 eV by a scanning magnet in the range m/z 50–500 every second. The ion source and transfer line were kept at 250 °C. Data were processed with OPUS software. Sterol and fatty acid identification were based on mass spectral interpretation and retention time data. Standard interpretation of the mass spectra was mostly realized by comparison of the mass spectrum of the unknown substance with those listed in available databases, e.g. the NIST Library (NIST/EPA/NIH Mass Spectral Library, 2002, National Institute of Standards and Technology and Advanced Chemistry Development, Washington, DC, USA) or the Wiley/NBS Registry (F. W. McLafferty & D. B.
EPR measurements. Freshly prepared plasma membranes were used for EPR measurements. The ester of 5-doxylmethyl hexadecanoic acid [MeFASL(10,3)] was selected as the spin probe (Kates, 1986). An 80 µl volume of a 100 mM ethanolic solution of MeFASL(10,3) was added to a glass tube and the ethanol was evaporated on a rotary evaporator to obtain a uniform distribution of MeFASL(10,3) on the walls of the tube. Fifty microlitres of plasma membranes (4 mg suspended in 0.4 M sucrose buffer (2 mM EDTA, 25 mM imidazole buffer, pH 7) was placed in a glass tube with the spin probe. After 10 min incubation at room temperature with gentle shaking, they were introduced into a glass capillary with an i.d. of 1 mm and subjected to EPR measurement on a Bruker ESP 300 X-band spectrometer at 28 °C (Bruker Analytische Messtechnik). The parameters for EPR measurements were as described by Turk et al. (2004). Spectra of two replicates were determined for each sample and three independent experiments were carried out for each condition under study. We took into account that the membrane is composed of several domains with different fluidity characteristics. The calculation of the different parameters for the three domains obtained by computer simulation of the line shape of the experimental spectra with the software package EPRSIM 4.9 (Strancar et al., 2000) was done as described previously (Turk et al., 2004).

EPR spectra are actually a superimposition of several spectra with different fluidity parameters. The relative portion of each domain in the membrane as well as their ordering and dynamics were determined from the best fit of calculated spectra with the experimental spectra. The calculated spectrum composed from three superimposed spectra fitted the experimental spectrum of studied yeasts grown under defined conditions. These spectra corresponded to the spin probe molecules in three different types of domains with different fluidity parameters.

For all conditions of growth under study, we used the same fluidity parameters for calculation of the line shape of the EPR spectra using the software package EPRSIM 4.9 for domain 1 [more fluid (less ordered) domain], S=0.13, σ=1.2 ns, W=1.1, pa=0.92, pg=1.00008; for the second less fluid domain (domain 2), S=0.24, σ=1.6 ns, W=0.2, pa=1.03, pg=0.99995; for domain 3 [more rigid (more ordered) domain], S=0.6, σ=0.4 ns, W=3, pa=1.05, pg=0.99976. When the cells were grown at various pH values and NaCl concentrations, the relative portions of the coexisting domains changed.

RESULTS

Growth rates under the different growth conditions

To analyse the effect of pH on plasma membrane fluidity and lipid composition, D. hansenii cells were grown in complex YPD medium adjusted to different pH values. Under such conditions, the doubling time of the cells was 9.2 h at pH 4.0, 2 h at pH 6.0 and 6 h at pH 8.0 (Fig. 1a). These results indicated that D. hansenii is not an acidophilic yeast. Consequently, a significantly longer lag phase was observed when the cells were pregrown at pH 4.0 in comparison to pH 6.0 or 8.0. We also included NaCl in the analysis at high pH, since D. hansenii is a marine yeast. The doubling times observed confirmed the results of Almagro et al. (2000) which showed an improvement in growth with relatively low concentrations of NaCl (at pH 8.0 and in the presence of 0.5, 1, 1.5 or 2 M NaCl, doubling times were 4, 4.75, 6.4 and 12 h, respectively) (Fig. 1b). It may be relevant to note that pH determinations at the end of the experiments showed that under our growth conditions yeast cells decreased the pH of the medium very slightly (0.2–0.3 units).

Plasma membrane fluidity

We isolated and purified plasma membranes from cells grown under the conditions mentioned above. The purity of the isolated plasma membranes was confirmed by...
measuring plasma membrane ATPase activity. The contamination of isolated plasma membranes with mitochondrial and vacuolar membranes was quite small, around 5–7% of the isolated membranes (data not shown).

As revealed by EPR measurements, the membranes are heterogeneous, composed of several coexisting domains. The smaller probes used in EPR are expected to produce less pronounced perturbation of the membranes than other techniques used to measure membrane fluidity and the data provided is of high resolution (in the order of nanodomains).

Results in Fig. 2(a) show the relative proportions of all three domains at different pH values with the corresponding experimental EPR spectra of *D. hansenii*. The yeast maintained the relative proportions of fluid domains (domains 1 and 2) at pH 6 and 8, while at pH 4 there was an important decrease in the fluidity. Since the results at pH 6 and 8 are comparable, and since sea water has a pH close to 8, we chose this value to study the effect of the addition of NaCl at different concentrations on membrane fluidity. As shown in Fig. 2(b), increased concentrations of NaCl did not greatly affect membrane fluidity. The proportion of fluid domains decreased at 0.5 M NaCl. This effect was reversed at higher NaCl concentrations, where a gradual increase in fluidity was observed with the increase in NaCl concentration, reaching almost the same value at 1.5 and 2 M NaCl as with no NaCl.

**Lipids**

Changes in fluidity are the consequence of changes in lipid structure and/or the composition of the membrane. Therefore, we decided to focus on the changes in phospholipids, sterols and fatty acids as a response to variations in environmental pH and salinity.

**Phospholipids.** Phospholipids were separated by TLC and quantified by assaying the phosphorus content of the extract. In plasma membranes of *D. hansenii*, phosphatidylcholine (PC) and PE were the predominant phospholipids under all growth conditions, followed by the anionic phospholipids PI, PS, PG and sphingolipid (SP), listed by decreasing occurrence (Fig. 3, Table 1).

![Fig. 2.](http://mic.sgmjournals.org) Relative proportions of all three domains with different fluidity parameters (area charts on the left) and the corresponding experimental EPR spectra (line charts on the right) of *D. hansenii* plasma membranes purified from cells grown at different external pHs (0 M NaCl) (a) and different NaCl concentrations (pH 8.0) (b). Experimental EPR spectra (line charts) correspond to the spin probe molecules in the three different types of domains with different fluidity parameters. Overall membrane fluidity is determined by the relative proportions of these three domains (area charts) and the fluidity parameters of each domain (see Methods). These parameters can be obtained by computer simulation of the line shape of the experimental spectra by using the software package EPRSIM 4.9. The results were determined from EPR spectra of two replicates for each sample. Data represent means ± SD of three independent experiments under each condition. B, Magnetic field strength.
Some small amounts of additional anionic phospholipids were also detected (not shown). The pH of the growth medium had a significant effect on the content of anionic phospholipids, which increased, and PE, which decreased, with increased pH. On the other hand, pH hardly affected the PC content. NaCl did not have an important effect on PC, PE or anionic phospholipids, and the slight changes observed did not follow a defined pattern (Fig. 3). Interestingly, 1.5 and 2 M NaCl caused a substantial increase in PG and a decrease in PI and PS (Table 1).

Sterols. Analysis of isolated plasma membrane sterol composition by GC-MS showed that the main sterol present was ergosterol with traces of lanosterol, zymosterol and 4,4-dimethylcholesta-4,24-dien-3-ol (Fig. 4). The amount of ergosterol in the membranes slightly decreased with increased pH, while the presence of NaCl caused a notable increase in ergosterol content.

In eukaryotic organisms, the sterol-to-phospholipid ratio is one of the major determinant features of membrane properties. In D. hansenii, both a decrease in pH and an increase in salt concentration enhanced this ratio (Fig. 5).

Fatty acids. The fatty acid profile of D. hansenii plasma membranes is shown in Fig. 6. The most abundant were oleic (cis-9-octadecenoic acid, C18:1 Δ9), palmitic (hexadecanoic acid, C16:0) and stearic (octadecanoic acid, C18:0) acids, with linoleic acid (cis-9,12-octadecadienoic acid, C18:2 Δ9,12) present in small amounts. Low pH was accompanied by an increase in palmitic acid and a decrease in oleic acid. On the other hand, the presence of salt resulted in an increase in palmitic acid at the expense of stearic acid, and the level of unsaturation increased significantly at high salt concentrations (Fig. 6).

### DISCUSSION

In this study we present data which demonstrate the effect of salt and pH stress on lipid composition and membrane fluidity in the marine yeast D. hansenii. Sterols and phospholipids, together with fatty acids, the major lipid constituents of eukaryotic biological membranes, were studied in detail in conjunction with the determination of plasma membrane fluidity.

We demonstrated that pH affects growth (Fig. 1a), membrane fluidity and lipid composition of D. hansenii. Whereas in the range of pH 6–8 plasma membrane fluidity was hardly affected, low pH (pH 4) caused a significant decrease in membrane fluidity (Fig. 2a). It seems that the decreased membrane fluidity at low pH reflects changes in the lipid composition: the increase in ergosterol content (Fig. 4) and sterol-to-phospholipid ratio (Fig. 5), together with the decrease in anionic phospholipids and an increase in PE (Fig. 3, Table 1). Changes in membrane lipid composition and the corresponding decrease in fluidity may indicate that at pH 4 cells are struggling to survive. D. hansenii is not an acidophilic yeast and one of its natural environments is sea water with pH higher than 7.0; therefore, low pH is probably quite stressful for this yeast.

It was found previously that the presence of Na⁺ improved growth under different stress conditions, which implies that D. hansenii is halotolerant (Almagro et al., 2000). Salt did not have a significant effect on the membrane fluidity and the relative proportions of domains were almost the same in cells growing without salt or at high NaCl concentrations. Interestingly, at 0.5 M NaCl, which is close to the salt concentration in sea water, a decrease in membrane fluidity was observed (Fig. 2b) which correlates...
with the changes in lipid composition. The sterol analysis of the plasma membrane showed that the main sterol identified is ergosterol (Fig. 4). The enhancement of ergosterol content was dramatic with an increase in NaCl concentration. Such an effect of NaCl on the amount of the plasma membrane ergosterol was previously reported in *Z. rouxii* (Hosono, 1992).

We also demonstrated that NaCl only caused minor changes in phospholipids, which is in agreement with the results reported for total cellular lipids of *D. hansenii* (Tunblad-Johansson et al., 1987).

The sterol-to-phospholipid ratio is more informative than the total amount of sterols and/or phospholipids. This is a major determinant of membrane properties in eukaryotic organisms. The ratios we obtained from *D. hansenii* cells grown under the conditions used in this study were similar to the ratios of halophilic black yeasts (0.40–0.48 in salt) and much lower than ratios detected in salt-sensitive *S. cerevisiae* (1.15–3.7 in salt), moderately halotolerant *A. pullulans* (1.22–0.8 in salt) (Turk et al., 2004) and *Z. rouxii* (1.34–6.83 in salt) (Hosono, 1992). An increase in the sterol-to-phospholipid ratio was observed concomitant with the increase in NaCl concentration (Fig. 5). This correlates well with results obtained on whole cells of *D. hansenii* grown at moderate NaCl concentrations (Tunblad-Johansson et al., 1987). Similar results were also observed in halotolerant *Z. rouxii* (Hosono, 1992) and in salt-sensitive *S. cerevisiae*, while halophilic black yeasts maintained this ratio over a range of NaCl concentrations (Turk et al., 2004). The abilities of yeasts to thrive under salty conditions and to keep low sterol-to-phospholipid ratios are probably connected.

Besides the sterol to phospholipid ratio, fatty acids also affect the properties of the membrane. The major fatty acid in the plasma membrane of *D. hansenii* was oleic acid (cis-9-octadecenoic acid) under all conditions tested. A decrease in the level of fatty acid unsaturation and shortening of acyl chains was observed with lower pH. In contrast, the presence of higher amounts of salt led to an increase in the level of fatty acid unsaturation (Fig. 6). This observation is important and different from the results obtained for the whole cellular lipids of *D. hansenii* cells (Tunblad-Johansson et al., 1987), where only minor changes in fatty acid composition of the total phospholipids were reported. The increase in fatty acid unsaturation observed at higher NaCl concentrations (1.5 and 2 M) as compared to the level at 0.5 M NaCl could explain the slight increase in the plasma membrane fluidity, although the sterol-to-phospholipid ratio at high NaCl concentrations was higher than at low NaCl concentrations.

![Fig. 4. Sterol content of plasma membranes of *D. hansenii* grown at different pH values and NaCl concentrations. Data represent means ± SD of two independent experiments under each condition. White bars, lanosterol; mid-grey bars, 4,4-dimethylcholesta-4,24-dien-3β-ol; dark grey bars, zymosterol; black bars, ergosterol.](http://mic.sgmjournals.org)

![Fig. 5. Sterol-to-phospholipid ratio of plasma membranes of *D. hansenii* cells grown at different pHs and NaCl concentrations. Data represent means ± SD of two independent experiments under each condition.](http://mic.sgmjournals.org)

![Fig. 6. Relative percentages of major fatty acids of plasma membranes of *D. hansenii* cells grown at different pH values and NaCl concentrations. White bars, C16:0 palmitic (hexadecanoic acid); light grey bars, C18:0 stearic (octadecanoic acid); mid-grey bars, C18:1 cis-9 oleic (cis-9-octadecenoic acid); dark grey bars, C18:2 cis-9,12 linoleic (cis-9,12-octadecadienoic acid). Data represent means ± SD of two independent experiments under each condition.](http://mic.sgmjournals.org)
An increase in the degree of unsaturation of fatty acids has also been observed in *C. membranefaciens* (Khaware *et al.*, 1995) and in halophilic/halotolerant black yeasts (Turk *et al.*, 2004), while increased shortening and saturation has been reported in *S. cerevisiae* (Turk *et al.*, 2004) and *Z. rouxii* (Hosono, 1992). Once again, our results suggest that the behaviour of *D. hansenii* is intermediate between halophilic/halotolerant black yeasts and salt-sensitive *S. cerevisiae*.

Tolerance to pH and salt stress are very complex processes. Therefore, it does not seem reasonable to explain adaptation exclusively on the basis of changes in plasma membrane fluidity and lipid composition, since adjustments of specific growth rate and ion homeostasis also play fundamental roles in these stress responses. However, our results demonstrate that pH and salt affect the lipid composition of the plasma membrane in different ways. Both stresses have similar effects on the sterol-to-phospholipid ratio, but influence fatty acid and sterol metabolism differently. We propose that in an acid-sensitive yeast such as *D. hansenii* those changes in composition are reflected in important decreases in the fluidity of the plasma membranes of cells growing at low pH, but not in membranes obtained from cells grown at high pH or at high salt levels, since those factors are present in the natural environment of *D. hansenii*.

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