Fatty acid composition and molecular order of phospholipids from *Eurotium chevalieri* in response to changes in water activity

LAURENCE LESAGE,¹* CLAUDE GENOT,² ERIC RECORD,¹ CHRISTIANE POULIQUEN¹
and DANIEL RICHARD-MOLARD¹

¹Laboratoire de Microbiologie et Technologie Céréalières, INRA, BP 527, 44026 Nantes Cédex 03, France

²Laboratoire d'Etude des Interactions des Molécules Alimentaires, INRA, BP 527, 44026 Nantes Cédex 03, France

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The mycelial growth of *Eurotium chevalieri* was examined at different water activities (a_w) using glycerol as the osmoticum. Growth was optimal at 0.90 a_w and restricted at 0.995 a_w , highlighting the xerophilic behaviour of *E. chevalieri*. Decreased a_w produced an increase in the proportion of oleic acid ($C_{18:1}$) at the expense of the proportion of linoleic acid ($C_{18:2}$) of cellular phospholipids. The degree of unsaturation of phospholipid fatty acids showed a 20% decrease between 0.995 and 0.80 a_w of growth. Steady-state fluorescence anisotropy (r_s) and fluorescence lifetime (τ) measurements for liposomes prepared from cellular phospholipids of *E. chevalieri* and labelled with DPH (1,6-diphenyl-1,3,5-hexatriene) were made at 25 °C. The lipid order parameter (S , describing molecular order) and the rotational correlation time (ρ , describing molecular dynamics) were calculated from r_s and τ data. Except at 0.995 a_w , a decrease in a_w was accompanied by increasing r_s and S values, indicating a rigidification of membranes, while ρ values were not significantly different. Plots of order parameters and their first derivatives as a function of temperature exhibited break areas in the temperature range 20–48 °C. These large temperature ranges for lipid transitions could correspond to chain melting of complex lipid systems which made up the liposomes prepared from phospholipids of *E. chevalieri*. However, as a_w decreased, the transition temperatures increased globally, between 0.97 and 0.90 a_w .

Introduction

The importance of fungi in food microbiology is now widely recognized. Spoilage of food and stored products involves a wide range of fungi that differ greatly in their water requirements (Richard-Molard *et al.*, 1985; Lacey, 1989). Pitt & Hocking (1977) divided the spoilage of foods by fungi into two broad classes: the spoilage of fresh or perishable foods, and the spoilage of dry or processed and stored foods. In this second class of foods, water activity (a_w) is the main environmental factor which governs the growth of spoiling fungi and thus the shelf life of foods.

Xerophilic fungi are classified according to their ability to grow at reduced a_w , below 0.85 a_w according to Pitt (1975). Compared with the published data on osmophilic

yeasts, there is still little information on the osmoregulatory mechanisms of xerophilic filamentous fungi (Hocking, 1988). Glycerol accumulation by xerophilic fungi in response to reduced a_w was reported in the xerophilic species *Chrysosporium fastidium* and xerotolerant species *Penicillium chrysogenum* (Luard, 1982). Glycerol has since been reported as the major internal osmolyte in four xerophilic fungi, *Penicillium janczewskii*, *Eurotium chevalieri*, *Wallemia sebi* and *Polypaecilum pisce* and one non-xerophilic species, *Penicillium digitatum* (Hocking, 1986). As shown by Brown (1978) for yeasts, the non-xerophilic species *P. digitatum* leaked glycerol into the growth medium while xerophilic species such as *P. janczewskii* were able to retain glycerol against a strong concentration gradient. These observations led authors such as Biondi *et al.* (1991) to conclude that the permeability properties of plasma membrane are involved in the adaptation of fungal species to low a_w . Cell membranes constrain the free diffusion of solutes and catalyse specific exchange reactions, which together combine to determine the unique chemical compositions of both the cellular and subcellular compartments. Membrane lipids play a central role in mediating

* Author for correspondence. Present address: Laboratoire de Biotechnologie des Champignons Filamenteux, INRA, Faculté des Sciences de Luminy, Case Postale 929, 13288 Marseille Cedex 09, France. Tel. (33) 91 41 62 85; fax (33) 91 41 67 07.

Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; THF, tetrahydrofuran; SUV, small unilamellar vesicles.

membrane functions by acting as physical barriers to diffusion of electrolytes, and are solvents for a variety of constituents and conformational stabilizers of membrane proteins (Hazel & Williams, 1990).

The active restructuring of membrane lipid composition in response to environmental changes preserves a suitable dynamic state of the bilayer (Singer & Nicholson, 1972) and restores membrane function (Hazel & Williams, 1990). Among the numerous parameters which describe the dynamic state of lipids in biological membranes, the concept of membrane fluidity is often used to describe the relative motional freedom of membrane lipids (Russell, 1989). The phospholipid composition, both head-group and fatty acyl chains, and the presence of various sterols are reported as important fluidity-modulating factors in membranes. Generally, higher unsaturations of fatty acids fluidize a membrane and decrease the gel to the liquid-crystalline transition temperature (T_c) for phospholipids (Russell, 1989). Adler & Liljenberg (1981) and Hosono (1992) showed that salt-tolerant yeasts responded to salt stress by decreasing the acyl chain unsaturation of their phospholipids and increasing the sterol to phospholipid ratio, leading to a decrease in membrane fluidity. Evaluation of membrane fluidity is often achieved by measuring the movement of hydrophobic fluorescent probes using fluorescence polarization spectroscopy, the most widely used probe being 1,6-diphenyl-1,3,5-hexatriene (DPH) (Shinitzky & Barenholz, 1978). Initially, steady-state fluorescence anisotropy data were interpreted exclusively in terms of microviscosity (associated to membrane fluidity) using the Perrin equation (Shinitzky & Inbar, 1976). However, it was later shown that these data contain not only information on the dynamics (viscosity) but also on the statics (order) of the membrane, which led to a new form of the Perrin equation including the structural order parameter (Van Blitterswijk *et al.*, 1981). Structural parameters such as the lipid order parameter describe molecular order, whereas motion parameters such as membrane fluidity or microviscosity describe molecular dynamics (Heyn, 1979; Van Ginkel *et al.*, 1989).

This paper describes a study of changes in the fatty acid composition of cellular phospholipids and in the structural and dynamic properties of membrane lipids in relation to the xerophilic behaviour of the filamentous fungus *E. chevalieri*. To determine order and dynamics in membranes, liposomes labelled with a fluorescent probe (DPH) were used and the DPH steady-state fluorescence anisotropy and fluorescence lifetime were measured.

Methods

Strain. Strain LMTC 2 of *Eurotium chevalieri*, holomorph form described by Raper & Fennell (1965), was isolated from mouldy cereal

grains and grown on maize grains at 25 °C for routine maintenance and production of conidia.

Media. The basal medium used was 3% (w/v) glucose/1% (w/v) yeast extract, pH 6.4. This basal medium was supplemented with glycerol to obtain cultures with different water activities. The appropriate glycerol concentrations were obtained from Chen (1987). The six media were of a_w 0.995 (maximal without glycerol), 0.97, 0.93, 0.90, 0.86 and 0.80. The a_w of these media, measured with an electric hygrometer (Rotronic), did not deviate from the calculated a_w by more than 0.005.

Cultivation. Conidia from *E. chevalieri* grown on maize grains at 25 °C for 10 d were suspended in distilled water containing 0.0033% Tween 80 and glass beads. After shaking, the suspension of spores was filtered through glass wool and counted with a haemocytometer.

Mycelia were grown in one litre Roux flasks containing 100 ml of each medium inoculated with spore suspension to a final concentration of 10^4 conidia ml⁻¹. Cultures were incubated at 25 °C in non-hermetically sealed containers in which constant relative humidities of 97, 93, 90, 86 and 80% were maintained with saturated potassium sulphate, ammonium phosphate, barium chloride, sodium sulphate and ammonium sulphate solutions, respectively (Bizot *et al.*, 1978). For 0.995 a_w , salt solutions were replaced by distilled water. At least six samples were taken at intervals according to the growth rate of *E. chevalieri* at each a_w . Incubation was for a maximum of 20 d at the highest a_w of 0.995, and 86 d at the lowest a_w of 0.80. Experiments were repeated twice.

Growth measurement. Biomass from cultures grown in media of different a_w was washed with 3 l distilled water and filtered rapidly under vacuum in a Buchner funnel. Moist mycelia were lyophilized and immediately weighed.

Total lipid extraction. Dry mycelia (from 200–300 ml culture media) were moistened with distilled water (15 ml) before lipid extraction. Lipids were then extracted three times successively with 72.5 ml chloroform/methanol/water (2:2:1.8, by vol.) following the method of Bligh & Dyer (1959). To remove non-lipid materials, the extracted lipids (evaporated to dryness) were washed with 93.5 ml chloroform/methanol/water containing 0.77% NaCl (8:4:3, by vol.) according to Folch *et al.* (1957). The total lipid extract was evaporated to dryness under nitrogen, weighed, redissolved in chloroform and stored at –20 °C.

Separation of phospholipids. Lipids (30 mg) dissolved in chloroform were separated in different classes on 1 g Silica Sep-Pak cartridge (Waters) as described by Christie (1982). After elution of neutral lipids with chloroform (10 ml) and elution of glycolipids and sphingolipids with acetone (40 ml), phospholipids were recovered by elution with methanol (10 ml). The phospholipid samples were then evaporated to dryness, resuspended in chloroform and stored at –20 °C. For quantitative phospholipid determination, phosphorus content was measured by the method of Bartlett (1959). The measured values were multiplied by 25 to give the total phospholipid content.

Fatty acid analysis. Fatty acid methyl esters of phospholipids were prepared by direct transmethylation with 14% (w/v) BF₃ in methanol according to the method of Morrison & Smith (1964). Fatty acid methyl esters were then extracted with hexane and analysed by GLC using a Hewlett Packard 5890 Series II chromatograph equipped with a flame ionization detector and a capillary column (60 m × 0.32 mm) coated with Carbowax 20 M. Oven temperature was 200 °C; detector and injector temperatures were 250 °C. Helium was the carrier gas, at a flow rate of 20 ml min⁻¹ with a split ratio of 1/20. The proportion of each fatty acid methyl ester was calculated from the integrated area of each peak and expressed as a percentage of the total area of all peaks.

Preparation of DPH-labelled vesicles. Liposomes, prepared from extracted phospholipids as small unilamellar vesicles (SUV), were labelled with the 'fluidity probe' DPH, as described by Lentz (1989). Only phospholipids from mycelia harvested in the early exponential growth phase (i.e. 7, 8, 10, 13, 17 and 55 d at a_w 0.995, 0.97, 0.93, 0.90, 0.86 and 0.80, respectively) were converted to vesicles. A stock solution of 1 mM-DPH (Sigma) in tetrahydrofuran (THF) was prepared and stored protected from light at -20°C . Seven microlitres of freshly prepared 0.2 mM-DPH in THF were added to each aliquot of phospholipid extract (equivalent to 1 mg). The molar DPH/phospholipid ratio used was 1:1000. Chloroform and THF were evaporated under a stream of nitrogen for 20 min. To prepare SUV, the lipid residue was hydrated overnight in 7.5 ml deoxygenated 0.2 M-phosphate buffer solution (pH 7.5), vortexed to suspend it as multilamellar vesicles and kept at 4°C overnight. This suspension was then sonicated (40% duty cycle) for 10 min under nitrogen in an ice-bath using a 500 W, 2 kHz pulse sonifier (Sons & Sonics) equipped with a titanium microtip. The sonicated mixture was then centrifuged at 1500 g for 15 min at 4°C . The upper three-quarters of the supernatant was kept for fluorescence experiments, and the pellet was removed. As preliminary experiments had shown no changes in anisotropy values during 24 h storage of samples at room temperature in the dark, probed vesicles were stored in these conditions and used within 24 h of their preparation. To avoid light scattering effects in the measurements of fluorescence anisotropy (Lentz *et al.*, 1979), the final concentration of vesicles was about 0.2 mM, which corresponded to $A_{352} < 0.1$, and quartz cuvettes with pathlength 0.4 cm in excitation were used.

Fluorescence measurements. Steady-state fluorescence anisotropy and fluorescence lifetime experiments were performed with a SLM 4800C spectrofluorimeter equipped with a two-chamber temperature-controlled cell holder. After excitation of samples at 352 nm (4 and 0.5 nm slits for steady-state fluorescence and lifetime experiments, respectively), DPH fluorescence was measured using Oriel 435 nm long pass filters. A Shott BG18 filter was placed in the excitation pathway to avoid high-order grating scattering. Shutters were closed at all times except during measurements, and gentle stirring of samples was maintained during measurements in order to reduce the possibility of DPH photobleaching. Spectrofluorimeter averaging value was set at 20 for all experiments.

Steady-state fluorescence anisotropy was measured in the T-format with the light modulation and frequency electronics turned off. Excitation light was vertically polarized through a Glan-Thompson polarizer. Emission light was simultaneously analysed using two Polaroid polarizers in vertical ($I_{\parallel}$) and horizontal ($I_{\perp}$) orientations, respectively. The correcting factor (G) for polarization inherent in the instrument was measured using horizontally polarized excitation light. Control samples of DPH suspension alone and of phospholipids alone were examined, but these readings could be neglected since they contributed less than 3% to the fluorescence of the complete system.

Steady-state fluorescence anisotropy (r_s) was calculated using the relationship

$$r_s = (I_{\parallel} - GI_{\perp}) / (I_{\parallel} + 2GI_{\perp}) \quad (1)$$

The r_s values were means of 10 measurements taken on the same sample. Anisotropy measurements were performed from $5-60 \pm 0.2^\circ\text{C}$ every 2.5°C , the actual temperature of the samples being monitored using a PT100 platinum thermocouple. Experiments were done in triplicate. The mean standard deviation of the r_s values was about ± 0.009 .

The interpretation of anisotropy data in terms of membrane fluidity has been discussed extensively by Lakowicz (1983) and Marangoni (1992). Initially, steady-state anisotropy measurements of membrane probes were converted to microviscosity units with reference to isotropic oils. However, after the development of time-resolved

fluorescence anisotropy, more information on the complex motions of the fluorophore in the lipid bilayer became obtainable. Accordingly, r_s is resolved into a static part (r_{∞}) and a dynamic part (r_f) as shown in equation 2.

$$r_s = r_f + r_{\infty} \quad (2)$$

$$r_f = (r_0 - r_{\infty}) / (1 + \tau/\rho) \quad (3)$$

The r_f term is related to the rotational relaxation time of the fluorophore (ρ) and depends on the fluorescence lifetime (τ) (equation 3), while the r_{∞} part describes the restriction to rotation of the fluorophore and is proportional to the square of the order parameter (S) (equation 4). r_0 is the maximal fluorescence anisotropy value in the absence of any rotational motion of the fluorophore (0.362 for DPH). For DPH, r_{∞} can be calculated from the steady-state fluorescence anisotropy data (r_s) using the relationship (equation 5) proposed by Van der Meer *et al.* (1986) where $m = 1.71$.

$$S^2 = r_{\infty}/r_0 \quad (4)$$

$$r_{\infty} = r_0 r_s^2 / [r_0 r_s + (r_0 - r_s)^2 / m] \quad (5)$$

Thereafter, S can be deduced from the calculated r_{∞} value and equation (4).

Accordingly, the temperature dependence of the lipid order parameter of DPH was estimated from r_s data obtained at the range of temperatures studied ($5-60^\circ\text{C}$). Using the Kaleida Graph software program (Macintosh), curves were smoothed and first order derivatives were calculated, smoothed and plotted as a function of temperature to visualize the inflection points, indicative of phase transitions in liposomal membranes. When artificial bilayers of phospholipids or membranes are cooled below a critical temperature, called the transition temperature, they undergo a transition from a fluid state (liquid-crystalline phase) to an ordered and condensed state (gel phase); this temperature is particularly sensitive to phospholipid fatty acid and polar head composition (Brenner, 1984).

Lifetime measurements. Even though r_s for DPH is determined mainly by the static component r_{∞} in membranes, a strict interpretation of r_s requires fluorescence lifetime measurements on which the dynamic component r_f depends. DPH fluorescence lifetime measurements were done at 25°C with unpolarized light (excitation and emission polarizers being taken out), at modulation frequency of 30 MHz (Debye-Sears ultrasonic modulator). The sample and the reference solution (1 mg glycogen ml^{-1} ; lifetime = 0 ns) were placed in the two-chamber turret of the spectrofluorimeter. The results were averaged (10 measurements taken on the same sample) and analysed by an interfaced calculator using commercial SLM software based on the routines described by Lakowicz (1983). Each experiment was performed in duplicate. The contribution of lifetime changes to steady-state anisotropy was measured by calculating the rotational correlation time of the fluorophore (ρ) from equations 2, 3 and 5.

$$\rho = \tau(r_s - r_{\infty}) / (r_0 - r_s) \quad (6)$$

Results

Growth

The effect of a_w and culture age on the mycelial growth of *Eurotium chevalieri* is shown in Fig. 1. The fungus was able to grow significantly at all a_w tested. As a_w decreased, the lag phase increased, up to 50 d at 0.80 a_w . Growth of *E. chevalieri* was drastically restricted at 0.80 a_w , indicating the minimal a_w requirement of this species.

Fig. 1. Effect of a_w and culture age on the mycelial growth of *E. chevalieri*. ■, 0.995 a_w ; □, 0.97 a_w ; ▲, 0.93 a_w ; △, 0.90 a_w ; ●, 0.86 a_w ; ○, 0.80 a_w .

Optimal growth occurred at 0.90 a_w while limited growth was observed at 0.995 a_w .

Fatty acid composition of phospholipids

Only results obtained from cultures harvested in the early stages of growth (from 7 d at 0.995 a_w to 55 d at 0.80 a_w) were reported. These cultures yielded mycelia in approximately similar physiological states. Moreover, glycerol accumulation, one of the main osmoregulatory mechanisms of the xerophilic fungi in response to reduced a_w , has been described predominantly for early stages of growth (Hocking, 1986). The fatty acid composition of phospholipids from mycelia of *E. chevalieri* grown at 0.995, 0.97, 0.93, 0.90, 0.86 and 0.80 a_w is summarized in Table 1. The major fatty acids were palmitic acid (C_{16}), stearic acid (C_{18}), oleic acid

($C_{18:1}$) and linoleic acid ($C_{18:2}$). Two odd-chain fatty acids, pentadecanoic acid ($C_{15:0}$) and margaric acid ($C_{17:0}$), were detected. As the a_w of the culture medium was reduced, the proportion of $C_{18:2}$ decreased substantially, and the proportion of $C_{18:1}$ and, less significantly, of $C_{18:0}$ increased correspondingly. The changes in distribution of $C_{18:2}$ and $C_{18:1}$, visualized by the $C_{18:2}/C_{18:1}$ ratio, were particularly marked at 0.90, 0.86 and 0.80 a_w . The degree of unsaturation of phospholipid fatty acids, calculated from the number of double bonds, showed a 20% decrease between cultures grown at 0.995 a_w and those grown at 0.8 a_w . The relative distributions of other fatty acids were more or less unchanged, and seemed independent of changes in a_w .

Anisotropy fluorescence and order parameter

In Table 2, the steady-state fluorescence anisotropy (r_s) and structural order parameter (S) values at 25 °C are shown for DPH-labelled liposomes from cellular phospholipids of 7, 8, 10, 13, 17 and 55-d-old *E. chevalieri* grown at 0.995, 0.97, 0.93, 0.90, 0.86 and 0.80 a_w , respectively. S values were calculated from r_s data according to equations 4 and 5 (see Methods). Except for the sample from mycelium grown at 0.995 a_w , lower a_w values corresponded to higher r_s and S values, the increase in values being particularly significant at lower a_w , i.e. 0.86 and 0.80 a_w , as shown by standard deviation.

Temperature-dependent changes in r_s values for DPH-labelled liposomal membranes derived from cellular phospholipids of *E. chevalieri* grown at various a_w are presented in Fig. 2. Plots of r_s vs temperature displayed

Table 1. Fatty acid composition of phospholipids from *E. chevalieri* mycelia harvested in the early stages of growth on media of 0.995, 0.97, 0.93, 0.90, 0.86 and 0.80 a_w

Values (expressed as percentages of total fatty acids) are means $\pm$ SD of two determinations made on mycelia of separate cultures. Values for unsaturation index (Δmol^{-1}) were calculated according to Kates & Hagen (1964).

Fatty acid composition	a_w (growth time):					
	0.995 (7 d)	0.97 (8 d)	0.93 (10 d)	0.90 (13 d)	0.86 (17 d)	0.80 (55 d)
C_{12}	0.2 $\pm$ 0.1	—	1.5 $\pm$ 0.4	0.5 $\pm$ 0.1	0.1 $\pm$ 0.1	0.9 $\pm$ 0.4
C_{14}	0.1 $\pm$ 0.1	0.2 $\pm$ 0.1	—	0.2 $\pm$ 0.1	0.2 $\pm$ 0.1	0.6 $\pm$ 0.2
C_{15}	—	0.5 $\pm$ 0.1	—	0.6 $\pm$ 0.2	0.4 $\pm$ 0.1	—
C_{16}	20.0 $\pm$ 1.1	22.8 $\pm$ 1.2	20.1 $\pm$ 1.0	17.6 $\pm$ 0.8	20.1 $\pm$ 0.5	23.0 $\pm$ 0.7
$C_{16:1}$	0.3 $\pm$ 0.1	1.1 $\pm$ 0.5	3.3 $\pm$ 1.5	0.5 $\pm$ 0.1	0.4 $\pm$ 0.1	1.3 $\pm$ 0.4
C_{17}	0.6 $\pm$ 0.1	2.0 $\pm$ 0.5	1.1 $\pm$ 0.1	0.6 $\pm$ 0.1	1.2 $\pm$ 0.5	1.3 $\pm$ 0.2
C_{18}	3.1 $\pm$ 0.3	3.6 $\pm$ 0.5	4.8 $\pm$ 0.2	4.7 $\pm$ 0.3	8.0 $\pm$ 1.0	4.0 $\pm$ 0.5
$C_{18:1}$	3.3 $\pm$ 0.2	5.7 $\pm$ 0.5	7.9 $\pm$ 0.1	18.3 $\pm$ 0.8	18.6 $\pm$ 0.7	22.6 $\pm$ 0.5
$C_{18:2}$	72.3 $\pm$ 1.5	63.9 $\pm$ 1.1	61.0 $\pm$ 0.5	57.0 $\pm$ 0.7	50.9 $\pm$ 0.2	45.3 $\pm$ 0.2
$C_{18:3}$	0.1 $\pm$ 0.1	0.2 $\pm$ 0.1	0.3 $\pm$ 0.1	—	0.1 $\pm$ 0.1	—
Δmol^{-1}	1.48	1.35	1.33	1.34	1.21	1.15
$C_{18:2}/C_{18:1}$	21.9	11.2	7.7	3.1	2.7	2.0

Table 2. DPH steady-state fluorescence anisotropy (r_s), order parameter (S), lifetime (τ) and rotational correlation time (ρ) of liposomes from phospholipids of *E. chevalieri* grown at various a_w

Values for r_s and τ are means $\pm$ SD of at least two separate experiments. The measurements were taken at 25 °C. S and ρ values were calculated as described in Methods.

a_w (growth time)	r_s	S	τ (ns)	ρ (ns)
0.995 (7 d)	0.1427 $\pm$ 0.0011	0.5051 $\pm$ 0.0036	3.581 $\pm$ 0.151	0.822 $\pm$ 0.053
0.970 (8 d)	0.0946 $\pm$ 0.0010	0.3427 $\pm$ 0.0033	3.989 $\pm$ 0.191	0.777 $\pm$ 0.061
0.930 (10 d)	0.0955 $\pm$ 0.0009	0.3462 $\pm$ 0.0030	3.692 $\pm$ 0.200	0.722 $\pm$ 0.059
0.900 (13 d)	0.1033 $\pm$ 0.0007	0.3734 $\pm$ 0.0023	3.374 $\pm$ 0.180	0.689 $\pm$ 0.050
0.860 (17 d)	0.1570 $\pm$ 0.0009	0.5562 $\pm$ 0.0030	3.207 $\pm$ 0.060	0.704 $\pm$ 0.025
0.800 (55 d)	0.1823 $\pm$ 0.0011	0.6257 $\pm$ 0.0036	ND	ND

Fig. 2. Temperature dependence of steady-state fluorescence anisotropy in DPH-labelled liposomes derived from cellular phospholipids of *E. chevalieri* grown at various a_w . ■, 0.995 a_w ; □, 0.97 a_w ; ▲, 0.93 a_w ; △, 0.90 a_w ; ●, 0.86 a_w ; ○, 0.80 a_w .

a continuous decrease, with no marked discontinuity or inflection in slope, and consequently no liquid-crystalline to gel phase transition temperature in phospholipid

domains could be visualized. The temperature dependence of the structural order parameter of DPH in the various samples of liposomes was also investigated (Figs 3–5). Discrete discontinuities in slope were discernible in plots of S vs temperature. Corresponding slopes for the S data (smoothed first derivatives) vs temperature were plotted to confirm the existence and the location of breaks in the temperature profiles of S (Lynch *et al.*, 1987). Each liposomal membrane exhibited characteristic break points. For liposomal membranes obtained at 0.995 a_w , three successive breaks were visualized in the derivative plot of S (Fig. 3b). With 0.97 and 0.93 a_w liposomal membranes, two breaks were found at 27 and 44 °C (Fig. 3d) and 20 and 44 °C (Fig. 4b), respectively. For liposomal membranes from mycelia obtained at lower a_w (Figs 4 and 5), derivative plots exhibited continuous changes in slope, from 32 to 46 °C at 0.90 a_w , from 22 to 44 °C at 0.86 a_w , and from 22 to 50 °C at 0.80 a_w . The mid-points of the inflections in the derivative

Fig. 3. Temperature dependence of the order parameter of DPH in liposomal membranes derived from cellular phospholipids of *E. chevalieri* grown at 0.995 a_w (a) and 0.97 a_w (c). The corresponding derivatives were calculated, smoothed and plotted vs temperature in (b) and (d) for 0.995 and 0.97 a_w , respectively. Break points are indicated by arrows.

Fig. 4. Temperature dependence of the order parameter of DPH in liposomal membranes derived from cellular phospholipids of *E. chevalieri* grown at 0.93 a_w (a) and 0.90 a_w (c). The corresponding derivatives were calculated, smoothed and plotted vs temperature in (b) and (d) at 0.93 and 0.90 a_w , respectively. Break points are indicated by arrows.

Fig. 5. Temperature dependence of the order parameter of DPH in liposomal membranes derived from cellular phospholipids of *E. chevalieri* grown at 0.86 a_w (a) and 0.80 a_w (c). The corresponding derivatives were calculated, smoothed and plotted vs temperature in (b) and (d) at 0.86 and 0.80 a_w , respectively. Break points are indicated by arrows.

plots coincided with discontinuities visualized in the temperature profile of S , with values of 40, 32 and 27 °C–45 °C, at 0.90, 0.86 and 0.80 a_w , respectively.

Lifetime and rotational relaxation time

DPH lifetime and calculated rotational correlation time at 25 °C in liposome membranes derived from *E. chevalieri* grown at various a_w are shown in Table 2.

Considering the standard deviations, the data for DPH lifetime and rotational correlation time were not significantly different for any of the analysed samples.

Discussion

This work confirms the considerable ability of the fungus *E. chevalieri* to grow at low a_w . The optimum for mycelial growth at a_w 0.90 and the restricted growth at very high

a_w (0.995 a_w) highlighted its xerophilic behaviour (Fig. 1). Studying the germination and growth of xerophilic fungi, Pitt and Hocking (1977) found that 0.94 a_w was the optimum for the growth of *E. chevalieri*. Differences in conditions of culture and growth measurements may explain this variation between the two studies.

In agreement with the data on Ascomycetes published by Wassef (1977), the fatty acid composition of *E. chevalieri* phospholipids showed a preponderance of fatty acids with chain lengths of 16 and 18 carbon atoms. Changes in a_w of the growth medium affected the fatty acid composition of these phospholipids, causing a decrease in C_{18:2} and an increase in C_{18:1}. Thus, one effect of growth of *E. chevalieri* at low a_w was a marked increase in the level of phospholipid fatty acid saturation as seen both by the decrease in unsaturation index and in the C_{18:2}/C_{18:1} ratio (Table 1). Modifications of fatty acid composition due to decreased a_w of the growth medium have been observed for other fungal systems. Tunblad-Johansson & Adler (1987) observed that increased salinity produced only minor changes in the fatty acid composition of phospholipids of the non-tolerant *Saccharomyces cerevisiae* species, whereas a marked decrease in C₁₈ polyenic acids with a concomitant increase in C_{18:1} was shown in the phospholipids of the osmotolerant yeast. Hosono (1992) described a decrease in C_{18:2} and a corresponding increase in C_{18:1} when the salt-tolerant yeast *Zygosaccharomyces rouxii* was grown in medium containing 15% (w/v) NaCl. Saad (1992) reported the accumulation of phospholipids and saturated fatty acids at low a_w . Such modifications of saturation of phospholipid fatty acids could be sufficient to affect the membrane permeability and explain, at least partially, cellular acclimation to decreased a_w . Indeed, xerophilic fungi have been reported to accumulate osmoregulator solutes (i.e. glycerol) in response to reduced a_w while the non-tolerant species leaked significant amounts of glycerol to the surrounding medium (Brown, 1978; Luard, 1982; Hocking, 1986, 1988). The response of the tolerant species to decreasing a_w is at the level of glycerol permeation and transport, whereas that of the non-tolerant one is only metabolic (Hocking, 1988).

The changes observed in the saturation of phospholipid C₁₈ fatty acids were expected to cause an increase in the membrane packing as indicated by Brenner (1984) and consequently to reduce the membrane permeability to osmoregulator solutes. More information about the membrane physical properties of *E. chevalieri* cultivated at reduced a_w was obtained from measurements of steady state fluorescence anisotropy (r_s) in DPH-labelled liposome systems prepared from cellular phospholipids of *E. chevalieri*. The lipid order parameter (S), determining the degree to which fluorophore movement in the membrane is restricted by the molecular

packing of surrounding lipids (Van Blitterswijk *et al.*, 1981; Van der Meer *et al.*, 1986), was calculated from r_s data.

Except at 0.995 a_w , the decreasing a_w values for mycelial growth were accompanied by increasing r_s and S values (Table 2). This result indicates that at lower a_w growth values *E. chevalieri* produces membranes in which the movements of lipid acyl chains are restricted. However, the r_s and S values never reached those of highly structurally ordered (rigid) membranes such as DPH-labelled liposomes of dipalmitoyl-phosphatidylcholine (gel phase) or eye lens membranes which at 25 °C, have values of 0.31 and 0.87 for r_s and S , respectively (Van Blitterswijk *et al.*, 1981). Values were rather close to these of fluid biological membranes such as leukaemic cell membranes. Consequently, liposomal membranes prepared from cellular phospholipids of *E. chevalieri* at 25 °C would be in the fluid liquid-crystalline state, whatever the a_w for growth. The rotational correlation time (ρ) values calculated from τ and r_s data of excited probe (DPH) (Table 2) indicated no significant variation as a function of a_w and allowed us to confirm that the static component r_∞ (proportional to the order parameter S) prevailed in the interpretation of r_s changes in our experiments (see Methods). Under these conditions, membrane fluidity, a parameter which describes membrane dynamics (Van Ginkel *et al.*, 1989) would be constant in our experiments, while the order parameter increased largely when a_w decreased.

This rigidification of the fungal membrane observed at low a_w is consistent with the increase in the level of phospholipid fatty acid saturation described previously. Similarly, Hosono (1992) reported increased rigidity of the plasma membrane (on the basis of fluorescence polarization values) associated with an increase in saturation of phospholipid fatty acids when the salt-tolerant yeast *Zygosaccharomyces rouxii* was grown under high salinity conditions.

By plotting the order parameter (S) and its first derivative as a function of temperature, phase transitions of liposomes prepared from cellular phospholipids of *E. chevalieri* grown at different a_w were visualized. Many break points, defined from inflections in slope and indicative of phase transitions, were observed overall in the temperature range 20–48 °C. These profiles were certainly due to the complex composition of phospholipids constituting the liposomes prepared at different a_w . Mixed lipid systems such as those found in cell membranes do not exhibit a well-defined transition from gel to liquid-crystalline state. Rather, chain melting occurs over a broad temperature range during which phase segregations of lipids occur (Chapman, 1975). Changes in slope of plots such as those observed in Figs 3–5 may indicate the start and end points of these

processes. However, chain melting seems to shift to higher temperatures with decreasing a_w , especially between 0.97 and 0.90 a_w . This observation is in agreement with Yamazaki *et al.* (1992), who reported an increase in phase transition temperatures of vesicles of dipalmitoyl-phosphatidylcholine associated with higher ethylene glycol concentrations.

As transition temperatures depend not only on the fatty acid composition of phospholipids but also on the polar head group (Brenner, 1984), further investigations are needed to determine the composition of different classes of phospholipids present in membranes of *E. chevalieri* grown at various a_w . It would be necessary to estimate the contribution of sterols and membrane proteins (especially in plasma membrane) to membrane physical properties by comparing DPH steady-state fluorescence anisotropy in isolated native membranes with that in liposomes prepared from membrane lipid extracts.

In conclusion, our results reveal that changes in water activity of growth leads to modifications in the phospholipid fatty acid saturation and in the membrane molecular order. These changes in the membrane structure are an adaptive response of the fungus to water stress, which could explain the differences of permeabilities observed for osmoregulator solutes in tolerant and non-tolerant species.

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