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Salt-induced changes in lipid composition and membrane fluidity of halophilic yeast-like melanized fungi

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Abstract The halophilic melanized yeast-like fungi *Hortaea werneckii*, *Phaeotheca triangularis*, and the halotolerant *Aureobasidium pullulans*, isolated from salterns as their natural environment, were grown at different NaCl concentrations and their membrane lipid composition and fluidity were examined. Among sterols, besides ergosterol, which was the predominant one, 23 additional sterols were identified. Their total content did not change consistently or significantly in response to raised NaCl concentrations in studied melanized fungi. The major phospholipid classes were phosphatidylcholine and phosphatidylethanolamine, followed by anionic phospholipids. The most abundant fatty acids in phospholipids contained C_{16} and C_{18} chain lengths with a high percentage of C18:2$^{\Delta 9,12}$. Salt stress caused an increase in the fatty acid unsaturation in the halophilic *H. werneckii* and halotolerant *A. pullulans* but a slight decrease in halophilic *P. triangularis*. All the halophilic fungi maintained their sterol-to-phospholipid ratio at a significantly lower level than did the salt-sensitive *Saccharomyces cerevisiae* and halotolerant *A. pullulans*. Electron paramagnetic resonance (EPR) spectroscopy measurements showed that the membranes of all halophilic fungi were more fluid than those of the halotolerant *A. pullulans* and salt-sensitive *S. cerevisiae*, which is in good agreement with the lipid composition observed in this study.

Keywords *Aureobasidium pullulans* · Halophilic/halotolerant fungi · *Hortaea werneckii* · Lipid composition · Membrane fluidity · *Phaeotheca triangularis* · Salt stress

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Introduction

In environments with salinities ranging from 3% (w/v) to saturated solutions of NaCl, high fungal diversity has recently been identified (Gunde-Cimerman et al. 2000). Among the isolated fungal species, melanized yeast-like fungi dominated. The halophilic melanized yeast-like fungi *Hortaea werneckii* and *Phaeotheca triangularis*, and the halotolerant *Aureobasidium pullulans* (Ascomycota, Dothideales) were isolated from the hypersaline waters of marine salterns on the Adriatic coast of Slovenia (Zalar et al. 1999a, 1999b). The identification of these organisms in such an extreme environment prompted us to investigate the mechanisms by which they adapt to salt-stress conditions.

Changes in the membrane composition and properties represent an important factor in the adaptation to high salt concentrations (Russell et al. 1995). Membrane lipids are important in controlling membrane fluidity and phase, which are affected by changes in lipid composition. Alterations in lipid patterns might be induced by high salt concentrations, therefore, and in particular membrane lipid compositions may be expected to be related to halotolerance. Several factors are involved in the maintenance of proper membrane fluidity: the type of fatty acyl chains (their length and unsaturation), the amount of sterols and, to a lesser extent, the nature of the polar phospholipid head-groups (Russell 1989). In Bacteria, an increase in NaCl concentration caused an increase in anionic lipid content relative to zwitterionic lipids as well as in unsaturated and cyclopropane fatty

acids (Russell 1989). However, in eukaryotic microorganisms, the membrane lipid alterations are more complex due to the presence of an array of intracellular membranes and membrane organelles.

The effect of salt stress on lipid composition and membrane fluidity has been investigated in a restricted group of yeasts, including salt-sensitive *Saccharomyces cerevisiae* (Sharma et al. 1996). Membrane composition and properties were also examined in the halotolerant yeasts *Zygosaccharomyces rouxii*, *Debaryomyces hansenii*, *Candida membranefaciens*, and *Yarrowia lipolytica*. These fungi showed different responses under salt stress. *Z. rouxii* grown at 15% NaCl (w/v) showed increased amounts of free (non-esterified) ergosterol, decreased fatty acid unsaturation, and decreased membrane fluidity than when grown without NaCl (Hosono 1992). High salinity did not induce significant changes in the unsaturation of fatty acids in *Y. lipolitica* (Andreishcheva et al. 1999) but caused a decrease in phospholipid and sterol contents. In contrast, *C. membranefaciens* grown at high NaCl concentrations exhibited increases in fatty acid unsaturation and in the contents of phosphatidylinositol (PI) and phosphatidylethanolamine (PE), resulting in greater membrane fluidity (Khaware et al. 1995). In *D. hansenii*, 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). Meanwhile, relatively small alterations were observed in the fatty acid composition of the phospholipids.

All previous studies were performed on yeasts that we considered to be halotolerant, although they were not isolated from natural hypersaline environments. Therefore, we wanted to investigate how the extremely high salt concentrations in salterns affect the lipid composition and membrane fluidity of halophilic yeast-like fungi isolated from hypersaline waters of salterns as their ecological niche.

The present work focuses on changes in lipid composition and membrane fluidity in *H. werneckii*, *P. triangularis*, and *A. pullulans* induced by salinities ranging from 0 to 25% NaCl (w/v). The salt-sensitive *S. cerevisiae* was used as the reference organism.

Material and methods

Strains and growth conditions

Cultures of *H. werneckii* (MZKI B-763), *P. triangularis* (MZKI B-748), and *A. pullulans* (MZKI B-802) from the culture collection of the Slovenian National Institute of Chemistry (MZKI) were used in this study. The reference salt-sensitive strain was *S. cerevisiae* MZKI K-86.

Cells were grown in supplemented synthetic defined medium YNB (Qbiogene, Heidelberg, Germany) composed of 1.7 g of yeast nitrogen base, 0.8 g of complete supplement mixture, 5 g of $(NH_4)_2SO_4$, and 20 g of glucose per liter of deionized water. This solution was adjusted to pH 7.0 and to NaCl concentrations of 0, 5%, 10%, 17%, or 25% (w/v). Incubations were performed at 28°C using 500-ml Erlenmeyer flasks in a rotary shaker at 180 rpm.

Cells were harvested in the mid-exponential phase by centrifugation at 4,000 *g* for 10 min and washed twice with the appropriate NaCl solutions.

Protoplast preparation

Cells (1 g wet weight) grown to the mid-exponential phase in YNB medium at various NaCl concentrations were washed twice with the appropriate NaCl solution and resuspended in 7 ml of osmotic buffer (0.05 M succinic acid, 1 M NaCl, pH 5.5). Then 1 ml of Glucanex lytic enzyme solution (Novo Nordisk, Denmark) was added and the cells were incubated at room temperature with gentle stirring until more than 80% of the cells had converted to protoplasts (1 h for *S. cerevisiae* and *A. pullulans*, 3 h for *H. werneckii*, and 4 h for *P. triangularis*). The protoplasts were centrifuged at 2,000 *g* for 5 min at room temperature, washed once with osmotic buffer, and resuspended in 1 ml of osmotic buffer.

Isolation of lipids

Cells were frozen in liquid nitrogen and mechanically disintegrated (Micro-Dismembrator II, B. Braun, Germany). Lipids were extracted according to a modified procedure of Kates (1986). Disintegrated cells (150 mg dry weight) were placed in a 10×150 mm Pyrex tube and lipids were extracted ultrasonically twice, with 5 ml methanol for 15 min of extraction at room temperature. The procedure was repeated twice with chloroform/methanol (2:1, v/v) and twice with chloroform. The organic phases were combined, washed once with deionized water, and the solvents were evaporated to dryness. Total lipids were then quantified gravimetrically.

Lipid extracts were hydrolyzed by the addition of 50 ml of 10% (w/v) methanolic KOH. The mixture was flushed with nitrogen and kept in the dark for 12–15 h at room temperature. Neutral lipids, mostly composed of squalene and sterols, were extracted three times with 30 ml of *n*-hexane. These neutral-lipid extracts were vacuum dried and the sterols converted to their trimethylsilyl (TMS) ethers by reaction with bis-(trimethylsilyl)trifluoroacetamide (70°C, 1 h).

After neutral lipid removal, the remaining methanolic phase was acidified to pH 1–2 with 6 M HCl. Fatty acids were extracted with 30 ml *n*-hexane three times. The solvent was removed under a stream of nitrogen and the isolated fatty acids were methylated (Kates 1986).

Double-bond positions were determined from gas chromatography–mass spectrometry (GC-MS) analysis of 4,4-dimethyloxazoline (DMOX) derivatives of fatty acids. These products were prepared by reaction of fatty acid methyl esters with 2-amino-2-methylpropanol at 180°C for 12–15 h under nitrogen, as described by Zhang et al. (1988).

Isolation and quantification of phospholipids by thin-layer chromatography (TLC)

Lipid extracts from the cells grown in media with different NaCl concentrations were subjected to one-dimensional TLC on activated silica gel 60 F_{254} TLC plates, 20×20 cm, 0.25 mm gel thickness (Merck, Darmstadt, Germany). A solvent system consisting of chloroform/methanol/acetic acid/deionized water (120/23/10/4.5, by volume) was used for the separation of phospholipids (Tunblad-Johansson et al. 1987). They were located by brief exposure to iodine vapor. Identification was achieved by co-chromatography with appropriate reference phospholipid homologues. Individual phospholipid classes were scraped off the TLC plates and eluted with chloroform/methanol (2:1). Phospholipid quantification was performed by assaying the phosphorus content of the extract (Kates 1986) and the values were multiplied by 25 to give the total amounts of phospholipids (Hossack and Rose 1976) except for the cardiolipin, where a standard of known concentration was

co-chromatographed and its phosphorus content assayed. To determine the fatty acid composition of individual classes of phospholipids, the extracts were subjected to methanolysis (Kates 1986). Methylated fatty acids were then analyzed by gas chromatography (GC) and GC coupled to mass spectrometry (GC-MS).

GC and GC-MS analysis

Sterols and fatty acids were dissolved in *n*-hexane and analyzed by GC using a Varian 3400 chromatograph (Varian, Sugar Land, Tex., USA) equipped with a splitless injector heated to 300°C and a HP-5 capillary column (25 m × 0.25 mm i.d., coated with a 0.25 mm thick stationary phase of 5% phenyl–methyl polysiloxane; Hewlett Packard, Palo Alto, Calif., USA) using hydrogen as the carrier gas. The oven temperature program for sterol analysis started at 70°C with 1 min holding time, then the oven was heated to 150°C at 15°C min^{-1}, to 310°C at 4°C min^{-1}, with a final holding time of 10 min. The temperature of the flame ionization detector (FID) was 320°C. The flame was fed with air at 300 ml min^{-1} and hydrogen at 30 ml min^{-1}. Nitrogen was used as the make-up gas at 30 ml min^{-1}. The oven temperature program for fatty acids started at 70°C with 1 min holding time, heating to 310°C at 2°C min^{-1}. The detector response was digitized by a Nelson 900 interface and processed with a Nelson 2600 software package (Perkin Elmer, Norwalk, Conn., USA). Relative concentrations of sterols and fatty acids were calculated from the GC-FID response areas of the compounds and of the internal standards; 5β-cholestan-3α-ol (Sigma-Aldrich Chemie, Taufkirchen, Germany) for sterols or methyl-nonadecanoate (Sigma-Aldrich Chemie) for fatty acids.

Analyses by GC-MS were performed with a Fisons 8000 gas chromatograph coupled to a Fisons MD-800 quadrupole mass analyzer (Thermoquest, Manchester, UK). Samples were injected in a splitless mode at 300°C onto a HP-5 capillary column (25 m × 0.25 mm i.d., coated with an 0.25 mm thick stationary phase of 5% phenyl–methyl polysiloxane; Hewlett Packard). Helium was used as the carrier gas; the temperature program was the same as for GC analysis. Mass spectra were recorded in an electron impact mode at 70 eV by scanning between m/z 50 and 500 every second. Ion source and transfer line were kept at 300°C. Data were processed with the Masslab software (THERMO Instruments, Finningan, San José, Calif., USA). Sterol and fatty acid identification were based on mass spectral interpretation and retention time data.

Electron paramagnetic resonance (EPR) spectroscopy measurements

Freshly-prepared protoplasts of the fungi were used for the EPR measurements which allowed us to study the membranes in situ. A lipophilic compound, 5-doxyl-methyl hexadecanoic acid [MeFASL(10,3)] was selected as the spin probe owing to its moderate stability in the membrane and to its relatively high resolution capability for local membrane ordering and dynamics (Schara et al. 1990). A 3 µl volume of 5 mM ethanolic solution of MeFASL(10,3) was added to the glass tube and the ethanol evaporated on a rotary evaporator to obtain a uniform distribution of MeFASL(10,3) on the walls of the tube. Protoplasts (10–30 mg) were placed in a glass tube with the spin probe. After 10 min of incubation at room temperature with gentle shaking, they were centrifuged at 2,000 *g* for 3 min, introduced into a glass capillary with an inner diameter of 1 mm, and subjected to EPR measurement on a Bruker ESP 300 X-band spectrometer at room temperature (Bruker Analytische Messtechnik, Rheinstetten, Germany). EPR measurements were as follows: center field 0.341 T, sweep width 0.01 T, microwave power 10 mW, microwave frequency 9.6 GHz, modulation frequency 100 KHz, modulation amplitude 0.1 mT. Spectra of two replicates were determined for each sample and the experiments were repeated three times at each NaCl concentration.

We took into account that the membrane was composed of several domains with different fluidity characteristics. The major fluidity parameters characterizing the lipid organization and mobility of the domains were the order parameter (S) and correlation time [τ_c (ns)]. The former describes the orientational order of phospholipid alkyl chains in the membrane with $S=1$ for perfectly ordered chains and $S=0$ for isotropic alignment of the chains; the latter describes the rotational motion of these chains. More fluid membranes are characterized by a smaller S and shorter τ_c with faster chain rotation. Overall membrane fluidity is determined by the fraction of the coexisting domains (d) and fluidity parameters of each domain. These parameters can be obtained by computer simulation of the line-shape of the experimental spectra using the software package EPRSIM 4.9 (Štrancar et al. 2000). In the calculation, besides the fraction (d), S, and τ_c for each domain, other parameters were also taken into account: the line width correction (W) due to the relaxation mechanisms of the spin probe and the polarity correction of the magnetic tensors g and A (pg and pa, respectively) due to the polarity of the spin probe environment.

Results

The *H. werneckii* and *P. triangularis* strains used in this study were able to grow in synthetic defined medium containing up to 25% NaCl (w/v) while *A. pullulans* was able to grow only in medium containing up to 10% NaCl (w/v), which make this organism similar to the reference salt-sensitive *S. cerevisiae*. The effect of exogenous lipids on lipid composition was eliminated by using the synthetic defined medium YNB.

In studying lipid composition, we focused on the changes in sterols and phospholipids as a response to the variations in environmental salinity.

Sterol composition

Total sterols for cells growing at various NaCl concentrations are shown in Fig. 1. When compared to *S. cerevisiae*, in which a considerable increase in total sterol amount was detected when cells were exposed to salt stress, the total sterol content in the halophilic/halotolerant fungi was much lower and did not change

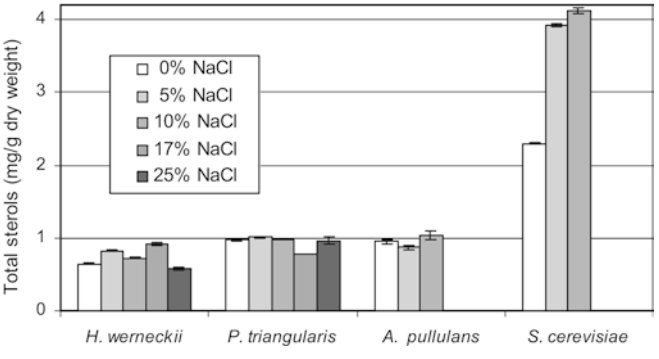

Fig. 1 Total sterol content (mg/g dry weight) of halophilic and halotolerant melanized yeast-like fungi grown at various NaCl concentrations. Results for *S. cerevisiae* are given for reference. Sterol amounts were calculated from the GC-FID response areas of the compounds and of the internal standard 5β-cholestan-3α-ol, and values are averages of two replicate analyses at each NaCl concentration

significantly with increased NaCl concentrations in the medium.

Identification of individual sterols obtained by GC-MS analysis showed that the predominant sterol in all the fungi was ergosterol, representing between 25% and 61% of total sterols. Besides ergosterol, 23 other sterols were also identified, most of them containing a methyl substituent at C-24. In the present study we confirmed the occurrence of C_{29} sterols in the halophilic/halotolerant melanized yeast-like fungi, but not in salt-sensitive *S. cerevisiae*, together with 4α,24-dimethylcholesta-7,24(28)-dien-3β-ol, 4α,24-dimethylcholesta-5,7-dien-3β-ol, 4α,24-dimethylcholest-5-en-3β-ol, and 4α,24-dimethylcholest-7-dien-3β-ol, which were first described in *H. werneckii* and *A. pullulans* as intermediates of ergosterol biosynthesis by Méjanelle et al. (2000). The content of these sterols and ergosterol did not vary much in *H. werneckii*, *P. triangularis*, and *A. pullulans* in response to the different NaCl concentrations.

The sterol composition of *S. cerevisiae* differed from the sterol composition of melanized yeast-like fungi due to the presence of a different ergosterol biosynthesis pathway in the salt-sensitive yeast (Mercer 1984). As shown previously, the sterol concentration in this yeast increased with salt stress (Fig. 1), due to an accumulation of ergosterol intermediates rather than of ergosterol itself. In addition, *S. cerevisiae* grown in elevated NaCl concentration showed an unusual percentage of squalene, the isoprenoid precursor of sterols, corresponding to concentrations of about 0.5 mg/g dry weight.

Phospholipids

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were the predominant phospholipids in all fungi, followed by the anionic phospholipids phosphatidylinositol (PI), phosphatidylserine (PS), cardiolipin (CL), phosphatidic acid (PA), and phosphatidylglycerol (PG), listed in decreasing order of occurrence.

As shown in Fig. 2, there were no consistent changes in phospholipid composition in the melanized yeast-like fungi as a response to NaCl concentration variations when compared to *S. cerevisiae*. At higher salinity, PC and PE increased in *H. werneckii*, decreased in *P. triangularis*, and changed only slightly in *A. pullulans*. In *S. cerevisiae* the PE percentage was twice as high at 10% (w/v) than at 0% NaCl (w/v).

The sterol-to-phospholipid ratios are often the major determinant feature of membrane properties in eukaryotic organisms. Halophilic melanized yeast-like fungi maintained this ratio at a considerably lower level than the salt-sensitive *S. cerevisiae*, whereas in halotolerant *A. pullulans* this ratio was closer to the ratio of *S. cerevisiae* (Table 1). In *H. werneckii* this ratio was maintained between 0.4 and 0.36 for NaCl concentrations from 0% to 17% NaCl (w/v), and slightly decreased at 25% NaCl (w/v). In *P. triangularis* the sterol-to-phospholipid ratio was lowest between 5% and

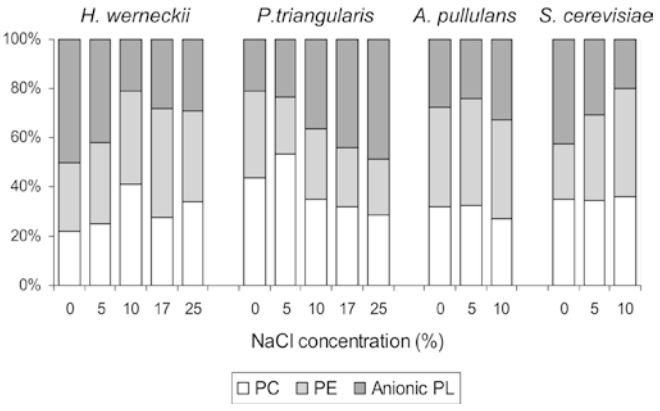

Fig. 2 Relative percentages of phospholipid classes of halophilic and halotolerant melanized yeast-like fungi grown at various concentrations of NaCl. Data for *S. cerevisiae* are given for reference. *PC* phosphatidylcholin, *PE* phosphatidylethanolamine, *anionic PL* phosphatidylserine + phosphatidylglycerol + phosphatidylinositol + cardiolipin + phosphatidic acid. Phospholipid amounts were determined by assaying the phosphorus content. Values (%) are averages of two replicate analyses at each NaCl concentration

Table 1 Sterol-to-phospholipid ratio (mg/mg) of halophilic and halotolerant melanized yeast-like fungi grown at various concentrations of NaCl. Data for *S. cerevisiae* are given for reference. The phospholipid value was determined by assaying the phosphorus content. Values for phosphorus were multiplied by 25 to give the total amount of phospholipids from which the sterol-to-phospholipid ratio was calculated. The total amount of sterols was calculated from the GC-FID response areas of all compounds and of the internal standard 5β-cholestan-3α-ol of known concentration. All values are averages of two replicate analyses at each NaCl concentration

Yeast	NaCl concentration in growth medium (%, w/v)				
	0	5	10	17	25
H. werneckii	1:2.54 (±0.21)	1:2.48 (±0.12)	1:2.34 (±0.02)	1:2.74 (±0.43)	1:3.21 (±0.36)
P. triangularis	1:1.30 (±0.01)	1:2.13 (±0.11)	1:2.09 (±0.00)	1:1.38 (±0.30)	1:1.27 (±0.24)
A. pullulans	1:0.82 (±0.18)	1:1.42 (±0.00)	1:1.25 (±0.03)		
S. cerevisiae	1:0.87 (±0.20)	1:0.73 (±0.04)	1:0.27 (±0.10)		

10% NaCl (w/v) and increased with a further rise in salinity. A similar trend was observed in *A. pullulans*, in which the lowest relative content of sterols was detected at 5% NaCl (w/v). In *S. cerevisiae* the sterol-to-phospholipid ratio increased with higher salinity.

The phospholipid fatty acid profile is shown in Fig. 3. The major fatty acids in the fungi were *cis*-hexadecanoic acid (C16:0), *cis*-octadecenoic acid (C18:0), *cis*-9-octadecenoic acid (C18:$1^{\Delta 9}$), *cis*-12-octadecenoic acid (C18:$1^{\Delta 12}$), *cis*-9,12-octadecandienoic acid (C18:$2^{\Delta 9,12}$), and *trans*-9,12-octadecandienoic acid (*trans*-C18:$2^{\Delta 9,12}$). Members of this series also occurred in the reference strain of *S. cerevisiae*, but in this yeast *cis*-C18:$2^{\Delta 9,12}$ was present only in trace level and no *trans*-C18:$2^{\Delta 9,12}$ was

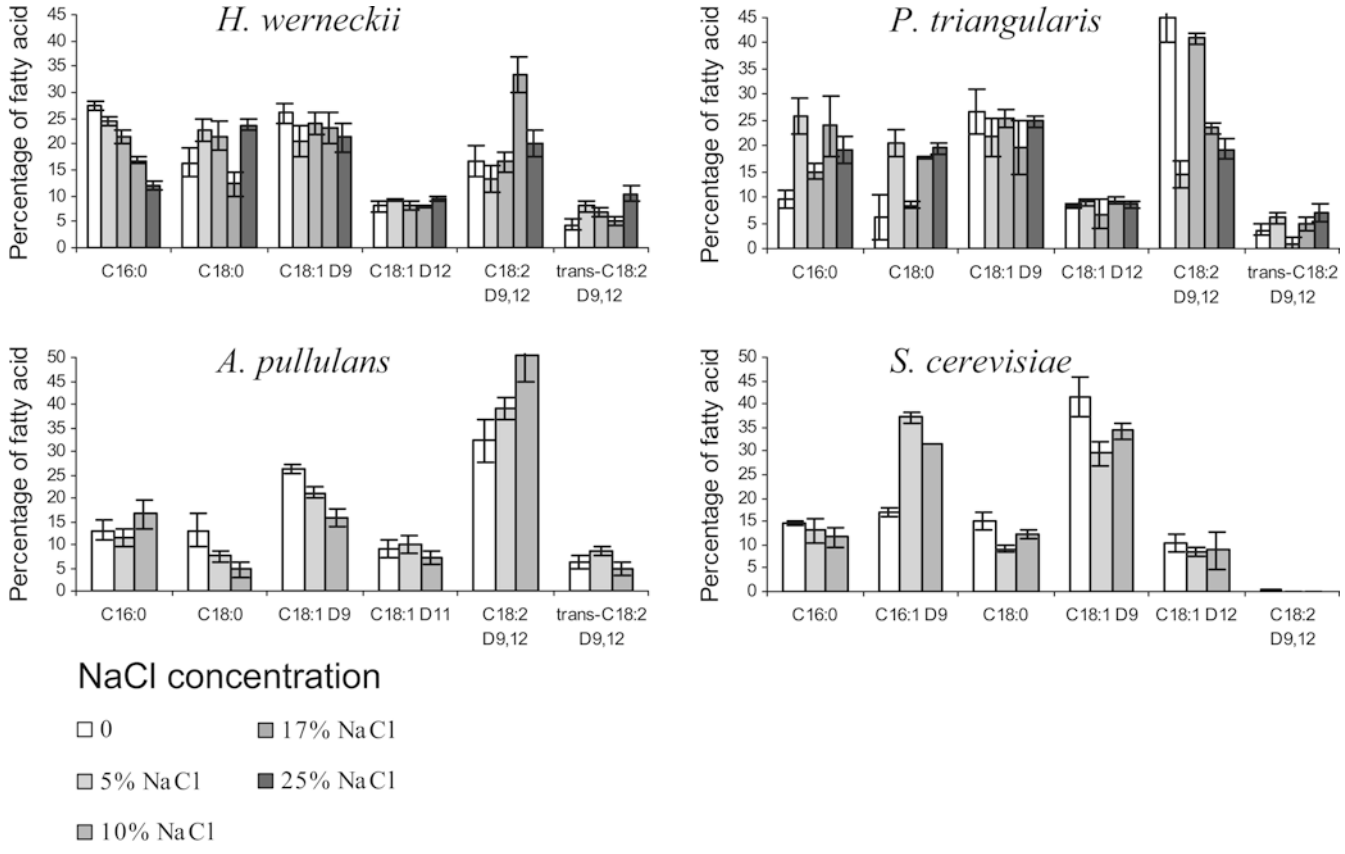

Fig. 3 Relative percentage of major fatty acids in halophilic and halotolerant melanized yeast-like fungi grown at various NaCl concentrations. Data for *S. cerevisiae* are given for reference. *C16:0* hexadecanoic acid, *C16:1*Δ9 cis-9-hexadecanoic acid, *C16:1*Δ12 cis-12-hexadecanoic acid, *C18:0* octadecenoic acid, *C18:1*Δ9 cis-9-octadecenoic acid, *C18:1*Δ12 cis-12-octadecenoic acid, *C18:2*Δ9,12 cis-9,12-octadecandienoic acid, *trans-C18:2*Δ9,12 trans-9,12-octadecandienoic acid

detected. Another major dissimilarity concerned the higher abundance of hexadecanoic acids in *S. cerevisiae*, with *cis*-9-hexadecanoic acid (C16:1$^{\Delta 9}$) accounting for 10%–30% of total fatty acids.

Salt stress affected the composition of phospholipid fatty acids, although changes were not uniform. In *H. werneckii*, increased salinity was accompanied by a decrease in C16:0 together with an increase in *cis*-C18:2$^{\Delta 9,12}$ (Fig. 3). In contrast, *P. triangularis* showed a relative decrease of *cis*-C18:2$^{\Delta 9,12}$ at higher salinities (Fig. 3). In *A. pullulans* *cis*-C18:2$^{\Delta 9,12}$ predominated among all fatty acids and increased with raised salinity, whereas C18:0 and *cis*-C18:1$^{\Delta 9}$ decreased. In *S. cerevisiae* salt stress induced a depletion of *cis*-C18:1$^{\Delta 9}$ and a concurrent enrichment in *cis*-C16:1$^{\Delta 9}$ (Fig. 3).

Plasma membrane fluidity

As membranes are heterogeneous, composed of several coexisting domains, EPR spectra are a superimposition of several spectra with different fluidity parameters. From the best fit of calculated spectra with the experimental spectra, the relative portion of each domain in the membrane as well as their ordering and dynamics were determined.

The calculated spectra which fitted the experimental spectra of the fungi grown at different NaCl concentrations, were a superimposition of three spectra. These spectra corresponded to the spin probe molecules in three different types of domains with different fluidity parameters.

For *H. werneckii* and *P. triangularis*, a background spectrum was present due to the presence of melanin in the cells. From the experimental EPR spectra of the protoplasts labeled with the spin probe MeFASL(10,3) the EPR spectra of protoplasts devoid of spin probe were subtracted.

Fluidity parameters used for calculation of the lineshape of the EPR spectra with the software EPRSIM 4.9 that best fitted the experimental spectra were the same for all studied yeasts: for domain 1, which is a more fluid (less ordered) domain, $S = 0.06$, $\tau = 2.3$ ns, $W = 1.27$, pa = 0.952, pg = 0.99989; for domain 2, which is an intermediate fluid domain, $S = 0.096$, $\tau = 1.1$ ns, $W = 1.0$, pa = 0.911, pg = 1.000088; for domain 3, which is more rigid (more ordered) domain, $S = 0.55$, $\tau = 2.0$, $W = 1.6$, pa = 1.18, pg = 0.99971. The fluidity parameters used for calculation of the background spectrum (melanin) were $S = 0.66$, $\tau = 0.1$ ns, $W = 2.96$, pa = 0.01, pg = 0.99865. Calculated spectra of all three domains and the

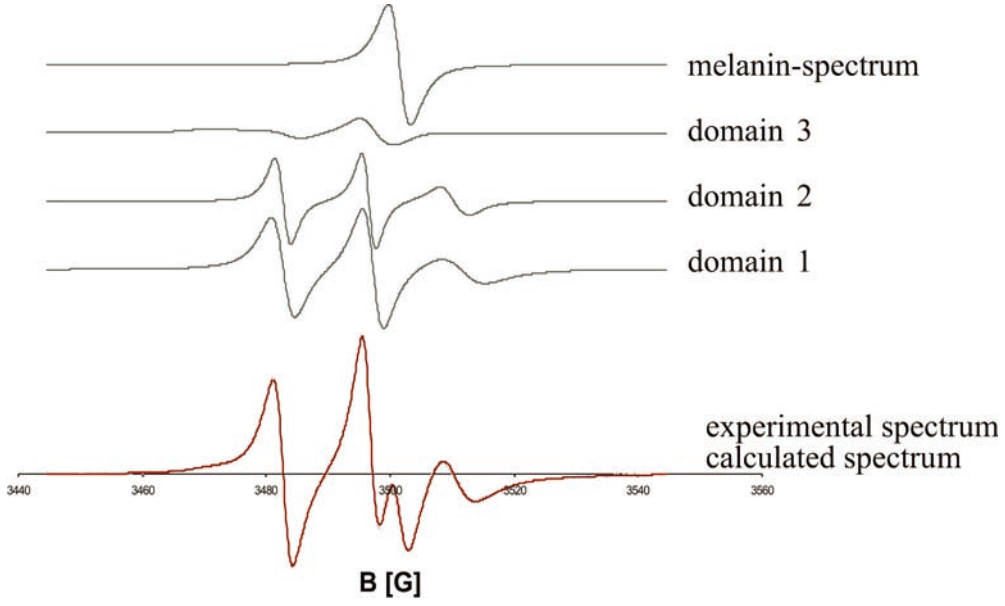

Fig. 4 The experimental EPR spectrum of spin probe MeFASL (10,3) incorporated into protoplast membranes of *H. werneckii*, grown without salt, recorded at room temperature (*bottom*). Its fitted calculated spectrum is superimposed. The calculated spectrum is a superimposition of spectra of all three domains with different fluidity parameters. The background EPR spectrum of melanin is also presented (*upper trace*). Fluidity parameters: *domain 1* (more fluid), $S=0.06$, $\tau=2.3$ ns, $W=1.27$, pa = 0.952, pg = 0.99989; *fluid domain 2* (intermediate fluidity), $S=0.096$, $\tau=1.1$ ns, $W=1.0$, pa = 0.911, pg = 1.000088; *domain 3* (more rigid), $S=0.55$, $\tau=2.0$, $W=1.6$, pa = 1.18, pg = 0.99971. Fluidity parameters used for calculation of the background spectrum (melanin), $S=0.66$, $\tau=0.1$ ns, $W=2.96$, pa = 0.01, pg = 0.99865

background spectrum together with the experimental spectrum for *H. werneckii*, grown without salt, are presented in Fig. 4.

When the yeast-like fungi were grown at various NaCl concentrations, the relative proportions of the coexisting domains changed, as shown in the EPR spectra presented in Fig. 5. All the halophilic melanized yeast-like fungi had higher relative proportions of fluid domains than did the halotolerant *A. pullulans* and salt-sensitive *S. cerevisiae*. As shown in Fig. 5, *H. werneckii* maintained relatively unchanged proportions of fluid domains (domains 1 and 2) from 0% to 17% NaCl (w/v), and then with further increase in salinity the proportions of fluid domains decreased. In *P. triangularis*, the fluid domains constituted almost the entire membrane between 0% and 5% NaCl (w/v), but with increased salinity an increase in the relative proportion of the more rigid domain (domain 3) was observed. In *A. pullulans* grown on 0% or 5% of NaCl (w/v), two-thirds of the membrane was constituted by the fluid domains, and this proportion rose with further increases in salinity. The proportion of fluid domains in the reference *S. cerevisiae* compared well with that of *A. pullulans* at 0% of NaCl (w/v), but with rising salinity a slight decrease was detected.

Discussion

In the present study several species of halophilic/halotolerant melanized yeast-like fungi isolated from a saltern were selected for membrane characterization under salt stress, and compared with a reference salt-sensitive strain of *S. cerevisiae*. Sterols and phospholipids, the two major lipid constituents of eukaryotic biological membranes, were studied in detail in conjunction with the determination of membrane fluidity. Our study showed that halophilic melanized yeast-like fungi behave differently from the salt-sensitive *S. cerevisiae*.

Different species of melanized yeast-like fungi respond to raised salinity by modifying different lipid classes, but in all of them sterol-to-phospholipid ratio, and consequently membrane fluidity, correlated well with their ecophysiology and ability to thrive in such an extreme saline environment.

Sterols generally decrease the fluidity of the lipid phase of natural membranes by reducing lipid acyl chain mobility (Demel and De Kruyff 1976). The major sterol identified in all the fungi we studied, including the reference yeast, was ergosterol, which is the principal sterol found in all Ascomycota and is the end-product of sterol biosynthesis in most fungi (Parks 1978; Weete 1989). The majority of other isolated sterols with a methyl substituent at C-24 have been documented previously as possible intermediates in ergosterol biosynthesis (Parks 1978; Mercer 1984; Lösel 1988; Weete and Gandhi 1996). The relative content of ergosterol and intermediates of its biosynthesis varied only slightly in response to high salinity in *H. werneckii*, *P. triangularis*, and *A. pullulans* (Fig. 1). Our data differ from earlier observations of the halotolerant yeasts *Debaryomyces hansenii* (Tunblad-Johansson et al. 1987) and *Yarrowia lipolytica* (Andreishcheva et al. 1999), in which an increase in NaCl caused a decrease in sterols. In contrast, *S. cerevisiae* showed an increase in total sterol

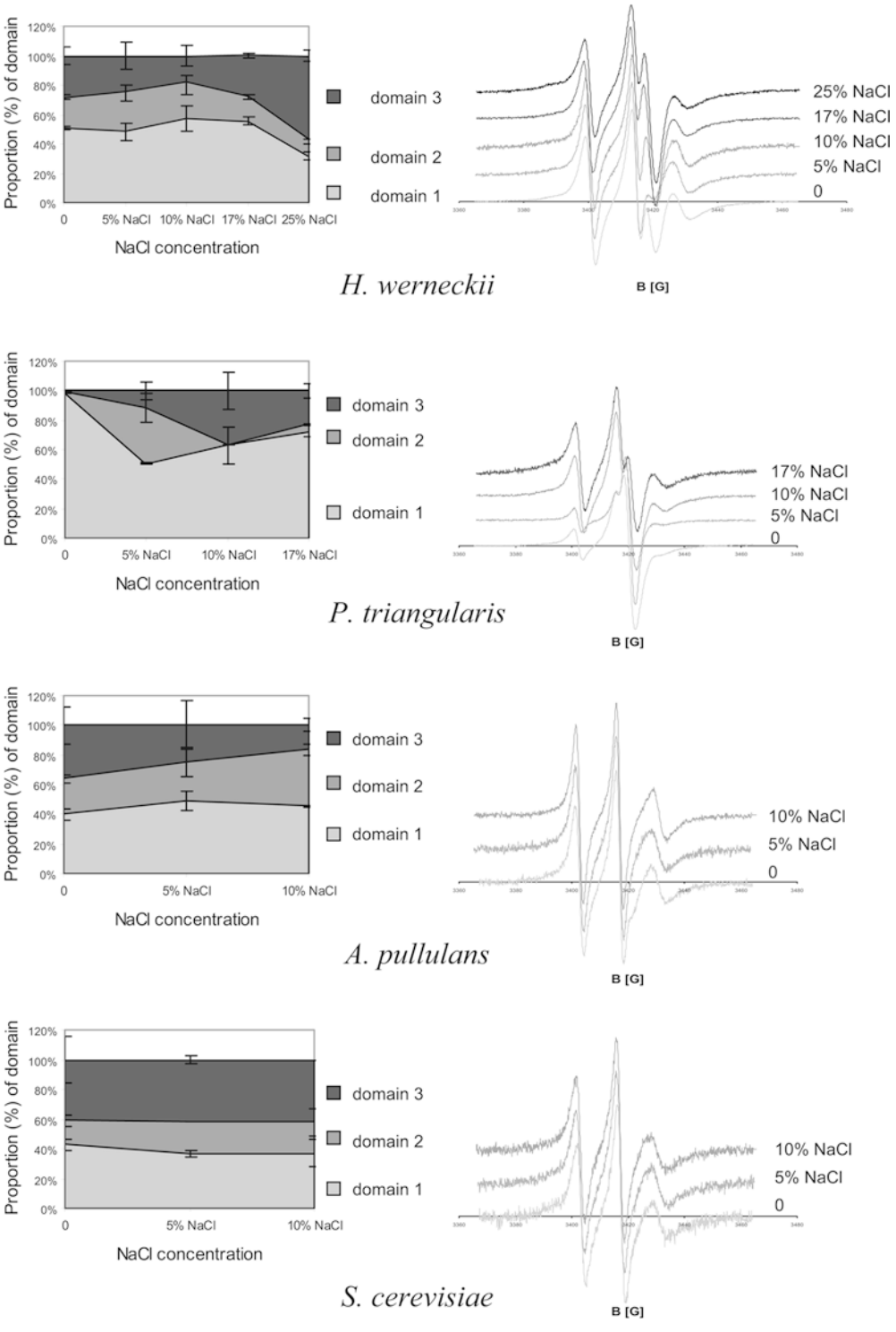

Fig. 5 Relative proportions of all three domains with different fluidity parameters (*left*) and the corresponding experimental EPR spectra (*right*) of the membranes prepared from halophilic and halotolerant melanized yeast-like fungi grown at different NaCl concentrations. Data for *S. cerevisiae* are given for reference. The results were determined from EPR spectra of two replicates for each sample. Experiments were performed three times at each NaCl concentration

content (Fig. 1) and a noticeable enrichment in squalene, the precursor of sterols, when the cells were exposed to salt stress. These different trends point to different regulatory responses in ergosterol biosynthesis in the several yeasts.

The distribution of phospholipid species in the melanized yeast-like fungi did not differ significantly from the known phospholipid content of other yeasts (Kaneko et al. 1976). The main phospholipids were PC and PE, followed by anionic phospholipids (PI, PS, CL, PA, and PG) (Fig. 2), which is in agreement with their generally accepted structural role in membranes (Lösel 1990).

Changes in phospholipid composition showed different responses to increased salinities depending on the fungal species. Whereas PC and PE were increased with raised salinity in *H. werneckzii*, anionic phospholipids were enriched in *P. triangularis* and were generally more abundant than in the reference yeast (Fig. 2). An

increase in the anionic phospholipids relative to the zwitterionic lipid content had been reported earlier for different microorganisms (Russell 1989). In the halotolerant *D. hansenii* the content of PS increased with the salinity (Tunblad-Johansson et al. 1987), as in the halotolerant *C. membranefaciens*, in which all anionic phospholipids increased while the proportion of PC decreased (Khaware et al. 1995). Our results only partly agreed with those studies and thus general conclusions cannot be drawn. It seems that changes in the nature of the phospholipid polar head-groups do not have any major influence on membrane fluidity (Fig. 5).

The physical properties of the membrane lipid matrix also depend on the fatty acyl composition of the phospholipids. Changes in the composition of their fatty acyl chains, such as unsaturation, length, and branching, are thought to affect membrane fluidity (Quinn 1981). Salt stress affected the composition of fatty acids although changes were not uniform (Fig. 3). The fatty acid unsaturation significantly increased in *H. werneckii* and *A. pullulans* due to an enrichment in $C18:2^{\Delta 9,12}$, but was less pronounced in *P. triangularis*. These results differ from those reported in other studies, in which a decrease in the proportion of polyunsaturated fatty acids versus NaCl concentrations was shown in the halotolerant yeasts *D. hansenii* (Tunblad-Johansson et al. 1987), *Z. rouxii* (Hosono 1992), and *Y. lipolytica* (Andreishcheva et al. 1999).

The sterol-to-phospholipid ratio is a determinant attribute of membrane fluidity in eukaryotic organisms. The halophilic melanized yeast-like fungi maintained this ratio at a level considerably lower than the halotolerant *A. pullulans* and salt-sensitive *S. cerevisiae* (Table 1). Low values have also been reported in the halotolerant *D. hansenii* (Tunblad-Johansson et al. 1987) and *Z. rouxii* (Hosono 1992). Thus, a low sterol-to-phospholipid ratio appears to be an important characteristic for halotolerance.

For the whole range of salinities, the value of this ratio was lower in *H. werneckii* and *P. triangularis* than in *A. pullulans*, even more so than in *S. cerevisiae*, and the membrane fluidity correlated with the ratio (Table 1, Fig. 5).

In *H. werneckii*, a low sterol-to-phospholipid ratio and increased unsaturated fatty acids resulted in high plasma membrane fluidity over a wide range of NaCl concentrations. These results indicate a high intrinsic salt stress tolerance and are in agreement with ecophysiological data and with the dominance of *H. werneckii* in hypersaline saltern waters (Gunde-Cimerman et al. 2000).

This concordance demonstrates the impact of this ratio on membrane fluidity and on the ability to survive in an extremely saline environment.

Amongst the other fungi studied here, *P. triangularis* showed the closest similarity to *H. werneckii*, although high membrane fluidity was maintained over a narrower range of salinity. This fungus is a true halophile, in the sense that it grows better in a medium with salt, but it is capable of adapting to narrower ranges of NaCl concentrations than is *H. werneckii*.

In *A. pullulans* the relative proportions of the fluid domains are similar to those in *S. cerevisiae*, but the fluidity increased with raised salinity whereas in *S. cerevisiae* the fluidity slightly decreased. This is in agreement with the fact that *A. pullulans* is somewhat halotolerant and can survive in an environment with increased salinity.

Our study on halophilic and halotolerant melanized yeast-like fungi isolated from salterns as their natural habitat confirmed that membrane fluidity is of crucial importance for tolerance against salt stress. Moreover, we demonstrated that higher salt tolerance correlates well with higher membrane fluidity, and that the ability of microorganisms to maintain their membrane fluidity over a wide range of salinities is linked directly with halophily.

This comparative study of halophilic *H. werneckii* and *P. triangularis*, halotolerant *A. pullulans*, and salt-sensitive *S. cerevisiae* reveals a correlation between halophily, membrane lipid composition, and consequent membrane properties. Further studies on a wider range of halophilic and halotolerant eukaryotic microorganisms are still needed in order to elucidate whether this is a phylogenetic trait of the studied halophilic fungi or perhaps a general characteristic of adaptation to high salinity.

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