Effect of salt stress on lipid composition and membrane fluidity of the salt-tolerant yeast *Zygosaccharomyces rouxii*

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When the salt-tolerant yeast *Zygosaccharomyces rouxii* was grown in YPD medium containing 15% (w/v) NaCl, the relative amounts of C_{16:1} and C_{18:1} fatty acids in acyl lipids increased and those of C_{18:0} and C_{18:2} acids decreased both in whole cells and in crude plasma membrane preparations, as compared with cells grown in YPD medium alone. The proportions of C_{12:0} and C_{14:0} acids, which are minor components of yeast lipids, decreased in whole cells and markedly increased in plasma membranes when 15% NaCl was included in the growth medium. The degree of unsaturation of fatty acids in the membranes and in whole cells decreased in the presence of 15% NaCl in the culture medium. The amount of free ergosterol in the membranes of cells grown in 15% NaCl increased to 2.9 times that of control cells. The ratio of ergosterol to phospholipid increased to 5 times that of control cells, whereas the ratio of phospholipid to protein in the membranes of cells grown in 15% NaCl decreased to less than half that of control cells. The fluorescence polarization value of DPH (1,6-diphenyl-1,3,5-hexatriene) in membranes of cells grown in 15% NaCl was 1.2 times higher than that for membranes from control cells, indicating a decrease in membrane fluidity in the presence of a high concentration of NaCl.

Introduction

In most organisms that can grow at high osmotic pressure, low molecular mass substances are accumulated internally to equilibrate the cytoplasm osmotically with the surrounding medium (Yancey *et al.*, 1982). Adjustment of the internal osmotic pressure with organic solutes has been reported for a wide range of microorganisms, including bacteria (Csonka, 1989), cyanobacteria (Reed *et al.*, 1986; Hagemann *et al.*, 1987), fungi (Gadd *et al.*, 1984; Jennings, 1984; Hocking, 1986), algae (Ben-Amotz & Avron, 1983) and yeasts (Adler *et al.*, 1985; Meikle *et al.*, 1988). Osmotically stressed yeasts accumulate osmoregulatory solutes such as glycerol and arabitol (Onishi, 1963). These neutral, low molecular mass compounds were thought to protect enzymes and structural proteins against inactivation, inhibition and denaturation, which would otherwise be brought about by low water activity (Brown, 1978).

Zygosaccharomyces rouxii is one of a small number of yeasts which can grow well in media of high osmolarity. The strain of *Z. rouxii* used in the present study can

tolerate a high concentration of NaCl (ca. 20%, w/v; 3.4 M) in the growth medium whereas *Saccharomyces cerevisiae* cannot. This distinguishing property of osmotolerant yeasts has long been recognized (Edgley & Brown, 1983; Reed *et al.*, 1987), but the mechanism of osmotolerance has not been fully elucidated. However, several aspects of osmoregulation in bacteria have been reported. Le Rudulier *et al.* (1984) discussed the molecular biology of the osmotic tolerance genes that protect *Escherichia coli* against osmotic stress and which might work in a similar manner in plants and animals. Higgins *et al.* (1987) reviewed the transcription of the genes encoding the high-affinity K⁺ and betaine transport systems of *E. coli* and *Salmonella typhimurium*, which are tightly regulated in response to osmotic stress.

The plasma membrane controls the entry of nutrients and the exit of waste products, and generates differences in their concentrations between the interior and exterior of the cell. It also acts as a sensor of external signals, allowing the cell to change in response to environmental cues. Biological membranes are generally thought of as a kind of two-dimensional solution of globular integral proteins dispersed in the fluid lipid matrix (Singer & Nicolson, 1972). Membrane fluidity influences various important functions that take place in biological membranes. It is membrane lipids especially that play a

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Abbreviation: DPH, 1,6-diphenyl 1,3,5-hexatriene.

crucial role in controlling membrane fluidity. Several factors are involved in the maintenance of proper fluidity: the type of fatty acyl chains, the amount of sterols and, to a lesser extent, the nature of the polar head-groups of phospholipids (Russell, 1989).

This paper describes a study of the changes in lipid composition of whole cells and crude plasma membranes when *Z. rouxii* was grown in medium with or without 15% NaCl. To determine changes in membrane fluidity, crude plasma membranes were labelled with a fluorescent probe and the fluorescence polarization was measured. From both chemical analyses and spectroscopic analysis, membrane fluidity was found to decrease when cells were grown in the presence of a high concentration of salt.

Methods

Yeast and growth conditions. *Zygosaccharomyces rouxii* IFO 1876 was maintained at 4 °C on slopes of YPD (1%, w/v, yeast extract, 2%, w/v, peptone and 2%, w/v, glucose, pH 5.0) agar with 15% (w/v) NaCl. The yeast was grown aerobically in YPD medium with or without 15% NaCl at 30 °C.

Preparation of protoplasts and crude plasma membranes. Yeast cells were converted to protoplasts by a modified enzymic digestion of the cell wall as described previously (Hosono & Hahn-Hägerdal, 1986). Cells grown in YPD medium with or without 15% NaCl were harvested in the exponential phase by centrifugation and washed twice with 15% NaCl or distilled water, respectively. Washed cells were resuspended in 1.0 M-sorbitol containing 25 mM-EDTA and 2.5% 2-mercaptoethanol and incubated with slow stirring for 30 min at room temperature. The cells were then collected by centrifugation and washed twice with 1.0 M-sorbitol. Pelleted cells were resuspended at 5×10^9 cells ml⁻¹ in protoplasting buffer (1.0 M-sorbitol and 10 mM-EDTA in 20 mM-Tris/HCl buffer, pH 7.5). After addition of Zymolyase 20T (ca. 0.5–1.0 mg ml⁻¹; Seikagaku Kogyo Co., Japan), the mixture was incubated with stirring at 30 °C and formation of protoplasts was followed by phase contrast microscopy (ca. 30–60 min). Cells grown in 15% NaCl were more easily converted to protoplasts than cells grown in YPD medium. After more than 90% of cells had been converted to protoplasts, the mixture was centrifuged at 2200 g for 10 min at room temperature. The pellet was resuspended in a small volume of 1.0 M-sorbitol and the viscous suspension was added to a 10-fold volume of distilled water to osmotically lyse the protoplasts. To sediment plasma membranes the suspension was centrifuged at low speed (2200 g, 10 min). The membranes were washed by resuspension in distilled water and recentrifugation until the supernatant reached an optical density of less than 0.01 at 660 nm. Cross-contamination of plasma membrane preparations was monitored by measuring the specific activity of the mitochondrial marker enzyme cytochrome oxidase (Wharton & Tzagoloff, 1967): the activity of this enzyme in the membrane preparations was approximately one-fifth that in protoplast lysates. Finally, the membranes were collected by centrifugation at 5300 g for 10 min at 4 °C before being freeze-dried. Freeze-dried membranes were stored at 4 °C in a desiccator and used for lipid analyses within 2–3 d. Freshly prepared membranes were used immediately for measurements of fluorescence polarization.

Extraction and analysis of fatty acids. Fatty acyl residues of acyl lipids in whole cells and crude plasma membranes were transmethylylated to give fatty acid methyl esters by a modification of the method of Stoffel

et al. (1959). Freeze-dried cells (20 mg) or membranes (15 mg) in a Pyrex tube closed with a screw cap were suspended in 1 ml methanol containing 5% (w/v) HCl. The suspensions were maintained under an atmosphere of nitrogen gas and heated for 3 h in boiling water. After cooling to room temperature, distilled water (1 ml) was added to each suspension and fatty acid methyl esters were extracted three times with light petroleum (b.p. 30–60 °C). Pooled extracts were dried overnight with anhydrous Na₂SO₄. The dehydrated extract containing fatty acid methyl esters was evaporated to dryness and the residue was dissolved in a small volume of diethyl ether. Fatty acid methyl esters were analysed by gas chromatography in a glass column (3 mm × 160 cm) packed with Chromosorb WAW DMCS (80/100 mesh) coated with 15% (w/w) diethyleneglycol succinate at 165 °C. The injection port temperature was 210 °C and the flow of nitrogen gas was 30 ml min⁻¹. Fatty acid methyl esters were detected with a flame ionization detector at 200 °C. The relative amount 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.

Extraction and estimation of ergosterol. Freeze-dried cells (100 mg) or membranes (30 mg) were suspended in distilled water (1 ml), and ethanol (9 ml) was added to the suspension to extract lipids (Letters, 1968). The suspension was mixed well, left for 24 h at 30 °C and then centrifuged to separate the supernatant liquid. The pellet was subsequently treated twice with 10 ml chloroform/methanol (2:1, v/v) to extract remaining lipids. The combined extracts (chloroform/methanol extracts plus ethanol extract) were evaporated to dryness. This residue and the lipid-extracted residue were saponified by refluxing for 2 h in 35% (w/v) aqueous KOH (10 ml) (Shaw & Jefferies, 1953). After cooling to room temperature, liberated sterols were extracted with diethyl ether. The extracts were evaporated to dryness and the residues were dissolved in chloroform for determination of the amount of free sterols and bound sterols by the Libermann–Burchard reaction as described by Hosono & Aida (1974). Chloroform suspensions (5 ml) containing sterols were mixed with 2 ml acetic anhydride/sulphuric acid (4:1, v/v) and kept for 15 min at 18 °C. The sterol content was determined immediately by measuring the absorbance at 625 nm using ergosterol as the standard.

Extraction of proteins from crude plasma membranes. Freeze-dried membranes (10 mg) in a glass-stoppered tube heated in boiling water were digested in 1.0 M-NaOH (2 ml) for 30 min to liberate proteins from the membranes. After cooling to room temperature, the suspension was centrifuged. The amount of protein in the supernatant was determined by the Lowry method with bovine serum albumin as the standard.

Extraction and analysis of lipids. Lipids were extracted successively with 10 ml ethanol and 10 ml chloroform/methanol (2:1, v/v) from freeze-dried membranes (100 mg). The combined extracts were evaporated to dryness and the residue was dissolved in 5 ml chloroform/methanol (2:1, v/v). In order to remove non-lipid materials the lipid solution was mixed with 1 ml 0.88% (w/v) KCl (Hunter & Rose, 1972). The mixture was separated into two phases by centrifugation at 2200 g for 5 min. The upper phase was removed and discarded. The lower phase was evaporated to dryness and the amount of total lipids was determined gravimetrically. Phospholipid was determined by assaying the phosphorus content of the extract, using the method of Chen *et al.* (1956), after digestion of the lipid extracts with perchloric acid (Allen, 1940). Values for phosphorus contents were multiplied by 25 to give the total amount of phospholipids (Hossack & Rose, 1976).

Fluorescence polarization measurements. To monitor the fluidity of lipid regions in the plasma membrane, 1,6-diphenyl 1,3,5-hexatriene (DPH) was used as a probe (Shinitzky & Inbar, 1976; Shinitzky & Barenholz, 1978). A solution of 2 mmol DPH in tetrahydrofuran was

Table 1. Fatty acid composition of whole cells and crude plasma membranes of *Z. rouxii* grown in YPD medium with or without 15% NaCl

Freeze-dried cells or membranes were transmethylated by heating in methanol containing 5% (w/v) HCl. Fatty acid methyl esters were analysed by gas chromatography as described in Methods. Values for unsaturation index were calculated as described by Kates & Hagen (1964). Values shown are means $\pm$ SD of at least four separate experiments.

Fatty acid	Fatty acid composition (percentage of total fatty acids)			
	Whole cells		Plasma membranes	
	YPD	YPD + 15% NaCl	YPD	YPD + 15% NaCl
12:0	0.45 $\pm$ 0.19	0.28 $\pm$ 0.06	0.16 $\pm$ 0.05	0.38 $\pm$ 0.10
14:0	1.15 $\pm$ 0.84	0.45 $\pm$ 0.16	0.32 $\pm$ 0.18	1.62 $\pm$ 0.43
16:0	7.88 $\pm$ 0.39	7.63 $\pm$ 0.26	8.05 $\pm$ 0.27	7.78 $\pm$ 0.38
16:1	11.53 $\pm$ 0.80	12.84 $\pm$ 1.46	11.59 $\pm$ 1.81	14.61 $\pm$ 0.53
18:0	1.96 $\pm$ 0.42	1.07 $\pm$ 0.39	1.47 $\pm$ 0.49	0.93 $\pm$ 0.27
18:1	31.06 $\pm$ 1.81	51.90 $\pm$ 2.24	32.63 $\pm$ 0.89	46.01 $\pm$ 1.16
18:2	45.99 $\pm$ 1.20	25.82 $\pm$ 1.39	46.27 $\pm$ 0.62	28.71 $\pm$ 1.05
Unsaturation index . . .	1.35	1.16	1.37	1.18

diluted 1000-fold with 50 mM-Tris/HCl buffer (pH 7.4) containing NaCl (0.9%, w/v). For labelling, freshly prepared membranes were incubated for 30 min at 30 °C with 3 ml of the diluted DPH. The final protein concentration of the membranes was 20–80 μ g ml⁻¹. All fluorescence measurements were carried out at 30 °C. Fluorescence polarization measurements were made in a Shimadzu RF-5000 spectrofluorophotometer. DPH was excited at 340 nm while the emission was measured at 440 nm. The measured fluorescence intensities were corrected for background fluorescence and light scattering from the unlabelled sample. The degree of fluorescence polarization, *P*, was calculated, according to Litman & Barenholz (1982), from the equation

$$P = (I_{VV} - I_{VH}G)/(I_{VV} + I_{VH}G) \quad [G = I_{HV}/I_{HH}]$$

where *I*_{VV} and *I*_{VH} are the fluorescence intensities determined at vertical and horizontal orientations of the emission polarizer, respectively, when the excitation polarizer is set in the vertical position, and *I*_{HV} and *I*_{HH} are the fluorescence intensities determined at vertical and horizontal positions of the emission polarizer when the excitation polarizer is set horizontally. *G* is a correction factor for the different response of the monochromators to vertically and horizontally polarized light.

Results and Discussion

Fatty acid composition of whole cells and crude plasma membranes

The fatty acid composition of whole cells and crude plasma membranes of *Z. rouxii* are shown in Table 1. In common with most higher eukaryotic cells, the fatty acid composition of yeast total lipids shows a preponderance of fatty acids with a chain length of 16 and 18 carbon atoms although a variety of other fatty acids has been observed (Ratray, 1988). Of these fatty acids, the proportion of C_{18:1} increased considerably and that of

C_{18:2} decreased by a corresponding amount both in whole cells and membranes when *Z. rouxii* was grown in medium with 15% NaCl. This is in agreement with a previous report that the percentages of cellular C_{18:1} acids increased and that of cellular C_{18:2} acids decreased when four strains of *Z. rouxii* were grown in medium containing 2 M (12%, w/v) NaCl (Watanabe & Takakuwa, 1984). The relative proportions of C_{16:1} and C_{18:0} acids increased and decreased, respectively, both in whole cells and in membranes of *Z. rouxii* grown in 15% NaCl. The proportion of C_{16:0} acids, both in whole cells and in membranes, was not influenced by the presence of 15% NaCl. There were also large changes in the proportions of the minor fatty acid components, C_{12:0} and C_{14:0} acids. When cells were grown in the presence of 15% NaCl, the proportions of C_{12:0} and C_{14:0} acids in whole cells decreased. In contrast, the relative amounts of C_{12:0} and C_{14:0} acids in membranes were approximately 2 and 5 times as high, respectively, as those of control cultures.

Changes in composition of acyl chains, such as unsaturation, length and branching, are thought to affect membrane fluidity. The degree of unsaturation can be expressed as the unsaturation index, defined by Kates & Hagen (1964) as: $\Delta\text{mol}^{-1} = 1 \times (\%\text{monoenes})/100 + 2 \times (\%\text{dienes})/100 + 3 \times (\%\text{trienes})/100$. The values calculated by using this equation are shown in Table 1. The unsaturation value of fatty acids in whole cells decreased from 1.35 to 1.16 and that of fatty acids in the membranes also decreased from 1.37 to 1.18 when cells were grown in the presence of 15% NaCl. This result might indicate that the plasma membrane is more rigid in the presence of a high concentration of salt in the

Table 2. Ergosterol content of whole cells and crude plasma membranes of *Z. rouxii* grown in YPD medium with or without 15% NaCl

Free ergosterol was released from whole cells or plasma membranes by a conventional lipid extract procedure and bound ergosterol was released by saponification of the lipid-extracted residue. Liberated sterol was extracted with diethyl ether after alkaline hydrolysis, and then colorimetrically determined by the Libermann-Burchard reaction using ergosterol as the standard. Ergosterol contents are expressed as the amount (mg) per g dry wt of plasma membranes. ND, Not determined. Values shown are means $\pm$ SD of at least four separate experiments.

Ergosterol	YPD	YPD + 15% NaCl
Whole cells		
Free ergosterol	6.1 $\pm$ 0.7	9.3 $\pm$ 1.2
Bound ergosterol	0.6 $\pm$ 0.1	0.2 $\pm$ 0.1
Plasma membranes		
Free ergosterol	5.5 $\pm$ 0.4	15.7 $\pm$ 1.2
Bound ergosterol	ND	ND

growth medium. Desaturation of fatty acids from C_{18:1} to C_{18:2} was inhibited by the presence of 15% NaCl in the growth medium.

Ergosterol content of whole cells and crude plasma membranes

Sterols play an important role in determining the structural organization of biological membranes, which in turn influences such functions as permeability of solutes and the activities of membrane-bound enzymes (Lees *et al.*, 1979; Yeagle, 1985). Sterols can be extracted from micro-organisms by two methods: (1) conventional lipid extraction procedures for sterols and/or sterol esters from cells, and (2) saponification to release bound sterols (Safe, 1973). In general, the major sterol of yeast has been reported to be ergosterol (Ratray, 1988). We examined the ergosterol content of whole cells and crude plasma membranes of *Z. rouxii* (Table 2). The amounts of free ergosterol increased both in whole cells and in membranes when cultures were grown in the presence of 15% NaCl. The amount of free ergosterol from cells grown in medium with 15% NaCl increased to approximately 1.5 times that of control cells, though the amount of bound ergosterol decreased to one-third of that from control cells. In the preparation of crude plasma membranes, only free ergosterol was found. The amount of free ergosterol in the membranes was more strikingly influenced by the presence of 15% NaCl in the growth medium, increasing to approximately 3 times that of control cells. The increase in the amount of free ergosterol in the membranes might cause a decrease in membrane fluidity.

Table 3. Total lipid, phospholipid and protein contents of crude plasma membranes of *Z. rouxii* grown in YPD medium with or without 15% NaCl

Total lipids were determined gravimetrically after drying the lipid extract. Phospholipids were determined by assaying the phosphorus content of the lipid extract. Proteins were determined by the Lowry method after digesting plasma membranes with hot alkali. The amount of ergosterol is taken from Table 2. Contents are expressed as the amount (mg) per g dry wt of plasma membranes. Values shown are means $\pm$ SD of at least four separate experiments.

	YPD	YPD + 15% NaCl
Total lipid	340 $\pm$ 95	283 $\pm$ 65
Phospholipid	4.1 $\pm$ 0.9	2.3 $\pm$ 0.3
Protein	84 $\pm$ 8	119 $\pm$ 7
Ergosterol	5.5 $\pm$ 0.4	15.7 $\pm$ 1.2
Phospholipid/protein ratio	0.05	0.02
Ergosterol/phospholipid ratio	1.34	6.83

Lipid, phospholipid and protein content of crude plasma membranes

The amounts of total lipid, phospholipid and protein in crude plasma membranes are shown in Table 3. The amount of phospholipids extracted from the membranes of cells grown in 15% NaCl was lower than that from the membranes of control cells. However, the protein content of membranes of cells grown in 15% NaCl was approximately 1.4 times higher than that of control cells. The ratio of phospholipid to protein in the membranes of cells grown in 15% NaCl was less than half that in the membranes of control cells.

Sterols and phospholipids are the two major lipid constituents of eukaryotic biological membranes. Sterol has been reported to decrease the fluidity of the lipid phase of membranes (Demel & de Kruffy, 1976). The microviscosity of biological membranes increases markedly with increases in the ratio of cholesterol to phospholipid (Russell, 1989). Changes in microviscosity might induce or alter various cellular functions that depend on the dynamics of the plasma membrane. The ratio of ergosterol to phospholipid in the membranes of cells grown in 15% NaCl was approximately 5 times greater than that of control cells. This change suggests that membrane fluidity might decrease due to the presence of a high concentration of salt in the growth medium.

When grown in medium of high osmolarity, *Z. rouxii* accumulates glycerol within the cells as a compatible solute (Onishi, 1963). Under high-osmolarity growth conditions, it is possible that the plasma membrane might have to become more rigid in order to keep glycerol within the cells for the purpose of increasing the internal osmotic pressure.

Measurement of fluorescence polarization

Chemical analyses of the lipid composition of the membranes indicated that they might be more rigid in 15% NaCl compared with control cells. In order to measure the fluidity of the plasma membrane, the membranes were labelled with a fluorescence probe. A crude plasma membrane fraction was prepared from protoplasts immediately prior to each experiment, and fluorescence polarization was measured at 30 °C, the temperature at which the cells were cultured. The concentrations of membranes in sample solutions varied from 20–80 µg protein ml⁻¹, but the degree of fluorescence polarization was not affected. The fluorescence polarization value, *P*, of DPH in the membranes prepared from cells grown in YPD medium with or without 15% NaCl was 0.308 ± 0.024 or 0.250 ± 0.009, respectively. The degree of fluorescence polarization, *P*, reflects the structural order of the membrane lipids, and the increased value of *P* indicates that the membrane fluidity of cells grown in 15% NaCl is less than that in control cultures. This is consistent with the results above, which suggested a decrease in membrane fluidity based on chemical analyses of lipids.

Under high-osmolarity growth conditions, *Z. rouxii* accumulates glycerol as a compatible solute within the cells in the exponential growth phase (Onishi, 1963). A characteristic of salt-tolerant yeasts might be the ability to change their membrane functions in order to keep compatible solutes within the cells in response to osmotic stress. Passive permeability properties are likely to be related to the content and composition of lipids, which also affects the fluidity of the plasma membrane. Beside the change in lipid composition, the protein content of membranes of cells of *Z. rouxii* grown in 15% NaCl was higher than that of control cells. An active glycerol transport mechanism regulated by water activity has been found in the yeast *Debaryomyces hansenii* (Adler *et al.*, 1985) and in *Z. rouxii* itself (Zyl *et al.*, 1990), while yeasts such as *S. cerevisiae* and *Candida utilis* take up glycerol by passive diffusion (Gancedo *et al.*, 1968; Cooper, 1982). The active transport mechanism might be controlled to accumulate glycerol within cells under osmotic stress. The action of plasma membrane ATPase has been reported to affect several stress tolerances of *S. cerevisiae* and *Schizosaccharomyces pombe* (Panaretou & Piper, 1990).

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