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Mechanisms underlying the toxicity of lactone aroma compounds towards the producing yeast cells

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ABSTRACT

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Aims: To study the fundamental mechanisms of toxicity of the fruity aroma compound γ-decalactone, that lead to alterations in cell viability during its biotechnological production by yeast cells; *Yarrowia lipolytica* that is able to produce high amounts of this metabolite was used here as a model.

Methods and Results: Lactone concentrations above 150 mg l^{-1} inhibited cell growth, depolarized the living cells and increased membrane fluidity. Infrared spectroscopic measurements revealed that the introduction of the lactone into model phospholipid bilayers, decreased the phase transition temperature. Moreover, the H^+-ATPase activity in membrane preparations was strongly affected by the presence of the lactone. On the other hand, only a slight decrease in the intracellular pH occurred.

Conclusions: We propose that the toxic effects of γ-decalactone on yeast may be initially linked to a strong interaction of the compound with cell membrane lipids and components.

Significance and Impact of the Study: These findings may enable the elaboration of strategies to improve yeast cell viability during the process of lactones bioproduction.

Keywords: Infrared spectroscopy, lactone, membrane fluidity, toxicity, *Yarrowia lipolytica*.

INTRODUCTION

A series of natural fruity aroma compounds possess a lactone structure (Maga 1976). Several of these valuable lactones can be obtained by biotechnological ways using microorganisms, mainly yeast strains (Endrizzi *et al.* 1996). γ-Decalactone, for example, is a peach-like odorous compound that has been produced industrially through the biotransformation of long-chain hydroxy-fatty acids by some yeast species (Aguedo *et al.* 2000). During such a process, lactone concentration in the medium reaches several hundreds of milligrams per litre, and a maximum concentration as high as 11 g l^{-1} has been reported (Page and Eilerman 1989). These high concentrations may limit the development of industrial applications as the metabolites become toxic towards the producing yeast (Feron *et al.* 1997). The decline in cell viability has been clearly associated with the increase in lactone concentration in the culture supernatant (Feron *et al.* 1997; Dufossé *et al.* 1999). Moreover, the antimicrobial properties of some lactones have been reported: for example, 2-deceno-δ-lactone, at a concentration of 100 mg l^{-1} inhibits the growth of bacteria (Nago *et al.* 1993). Also, 6-pentyl-α-pyrone is toxic towards a producing strain of *Trichoderma viride* (Bonnarme *et al.* 1997) and γ-decalactone was reported to inhibit the growth of the producing yeast *Sporidiobolus salmonicolor* (Feron *et al.* 1996). Generally, microorganisms and animal cells (Adams *et al.* 1998) are able to stand and, in many cases, to metabolize lactones up to a strain-dependent concentration threshold beyond which this compound becomes toxic (Feron *et al.* 1996). Some strategies have been developed to reduce lactone toxicity within the fermentation medium (Dufossé *et al.*

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1997, 1999), however, little attention has been given to the mechanisms underlying the observed toxicity. Nevertheless, a better knowledge of the fundamental action of these compounds should be useful to elaborate adequate techniques to improve the performance of processes of lactone production through biotransformation by yeast.

Yarrowia lipolytica is a non-pathogenic food spoilage dimorphic yeast (Barth and Gaillardin 1996), that is able to excrete large amounts of γ-decalactone when growing in the presence of methyl ricinoleate (MR) (Aguedo *et al.* 2000), making this species a good model to study lactone toxicity. In the present study, the mechanisms underlying the toxicity of γ-decalactone in yeast were specified by evaluating the effects of that compound on vital functions and membranes of the yeast *Y. lipolytica*. As fundamental mechanisms were to be elucidated, the experiments were conducted without the presence of the lipid substrate (MR) to avoid additional interactions between this oily phase and the lactone as well as the yeast cells.

MATERIALS AND METHODS

Strain and culture conditions

Yarrowia lipolytica W29 (ATCC20460; CLIB89) was cultured at 27°C, 140 rev min^{-1} in 500-ml baffled Erlenmeyer flasks containing 200 ml of liquid medium containing per litre: 15 g glucose, 2·5 g NH_4Cl, 0·1 g yeast extract and salts as previously described (Pagot *et al.* 1998). Yeasts were inoculated at $6{\cdot}5 \times 10^6$ cells ml^{-1} and grown for 19 h until cells were in late log growth phase.

Fluorescence measurements

All fluorescence measurements were done with a spectrofluorometer F-4500 (Hitachi Instrument co., Tokyo, Japan) equipped with a stirred and thermostated (27°C) cuvette holder, connected to an acquisition and processing system (Hitachi).

Intracellular pH determination with pyranine

The Intracellular pH (pHi) was determined by using the pH-sensitive probe pyranine, introduced into the yeast cells by electroporation as previously described (Aguedo *et al.* 2001).

Fluorescence measurements using diS-C_3(3)

Yeast cells from 100 ml of culture broth were harvested by centrifugation (6000*g*, 5 min), washed three times and resuspended in 3 ml of physiological saline (9 g l^{-1} NaCl). A volume of that cell suspension was added to a 4-ml cuvette (final O.D.$_{600}$ of 0·25) containing 0·1 mol l^{-1} Tris-HCl buffer (pH 7) prepared with deionized water. 3,3-Dipropylthiacarbocyanine (diS-C_3(3)), (Molecular probes Eugene, OR, USA) was added from an ethanol stock solution to obtain in the cuvette a final concentration of 10^{-7} mol l^{-1}. Fluorescence scans were then monitored at different time intervals and the maximum emission wavelength (λ_{max}) was noted for each scan. The excitation wavelength was 515 nm (10 nm slit width), and emission was scanned between 550 and 600 nm (10 nm slit width).

Fluorescence polarization measurements with DPH

Membrane fluidity was assessed by measuring fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) (Sigma, St Quentin Fallavier, France). The excitation wavelength was set at 360 nm (slit width of 10 nm) and the emission at 450 nm (slit width of 20 nm). The measured fluorescence intensities were corrected for background fluorescence and light scattering from the unlabelled samples. Fluorescence anisotropy (r) was calculated as follows:

$$r = \frac{I_{vv} - G \cdot I_{VH}}{I_{vv} + 2G \cdot I_{VH}} \quad \text{and} \quad G = \frac{I_{HV}}{I_{HH}}$$

I_{VV} and I_{VH} are the fluorescence intensities determined at vertical and horizontal orientations of the emission polarizer when the excitation polarizer is set in the vertical position. Similarly for I_{HV} and I_{HH} with the horizontal excitation polarizer. G is a correction factor for background fluorescence and light scattering. Yeast cells suspension was diluted into physiological saline to obtain an O.D.$_{600}$ of 1·4 in a 2-ml quartz cuvette. γ-Decalactone (Fig. 1) from a 235-mmol l^{-1} stock solution in ethanol, or ethanol alone for the reference, was added to the cell suspension. After 5 min, 4·5 μl of a

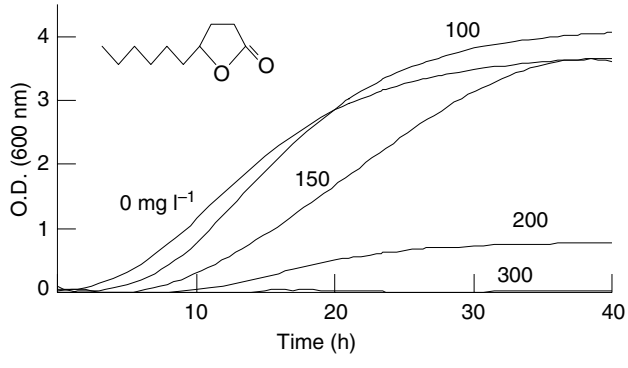

Fig. 1 Growth of *Yarrowia lipolytica* in a medium containing different concentrations (indicated on the curves) of γ-decalactone (structure presented on the graph). The curves were fitted using the software Slide®

1·8-mmol l^{-1} DPH solution in tetrahydrofuran were added in the cuvette. After a 20-min probe insertion period (this was found to be necessary to obtain stabilized *r* values), a minimum of four *r* values were determined and a mean value was calculated.

ATR-FTIR studies

Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) experiments were conducted on a Vector 22 FTIR spectrometer (Bruker, Karlsruhe, Germany). Data processing was done with the Opus software (Bruker) with a wavenumber accuracy of 0·1 cm^1. The model phospholipid, i.e. dimyristoyl-L-α-phosphatidylcholine (DMPC) (Sigma), was deposited (150 μl from a 20-g l^{-1} chloroformic solution) on the IR-transparent ZnSe window to form a solid film. The window was covered with a water-tight cell, enabling the introduction of a solution in order to hydrate the lipidic film. Temperatures were measured precisely with a thermocouple inserted in the cell. Hydration of DMPC was performed at 30°C with 2 ml of a deionized water solution containing the lactone. The temperature was first decreased to 14°C and then increased from 14 to 30°C (0·8°C min^{-1}). During this heating step, ten scans were taken (4 cm^{-1} resolution) every 0·5°C and an average spectrum was produced.

Activity of the plasma membrane H^+-ATPase

Yeast plasma membranes used for the enzyme activity determination were prepared as described by Goffeau and Dufour (1988). For protein determination, a portion (20 μl) of plasma membrane preparation was solubilized by boiling for 5 min after addition of 980 μl of a 0·5-mmol l^{-1} NaOH solution. The protein content was then assayed using the Lowry method with BSA as a standard. The assays for H^+-ATPase activity were carried out by incubation of the plasma membrane (25–50 μg of proteins) at 27°C in a final volume containing 6 mmol l^{-1} ATP, 12 mmol l^{-1} $MgCl_2$, 50 mmol l^{-1} MES/NaOH at pH 6·5 and 10 mmol l^{-1} NaN_3. To determine the activity in the presence of γ-decalactone, this compound was added from a stock solution in ethanol, to the reaction medium. For each tested concentration, a reference containing the same quantity of ethanol alone was determined.

RESULTS

Yarrowia lipolytica growth in the presence of γ-decalactone

The growth of *Y. lipolytica* was monitored in a medium containing glucose as carbon source. Yeast cells growth in the presence of 100 mg l^{-1} γ-decalactone was not altered and the biomass concentration at the stationary phase was even slightly higher than in the absence of the lactone. With 150 mg l^{-1}, the growth rate was significantly reduced and the stationary phase was reached after 35 h. With 200 mg l^{-1} the growth was very weak. There was no growth for 300 mg l^{-1} (Fig. 1). These experiments enabled to establish the γ-decalactone toxicity threshold towards *Y. lipolytica*.

Effect of γ-decalactone on intracellular pH

We evaluated the effects of γ-decalactone on the pHi of *Y. lipolytica* with the fluorescent probe pyranine introduced into the cells by electroporation. Octanoic acid was used as a reference compound as its acidifying action on the pHi was reported previously in *Saccharomyces cerevisiae* (Viegas and Sá-Correia 1995) and then in *Y. lipolytica* (Aguedo *et al.* 2001). An acidic extracellular pH (pHe) slightly influenced pHi (0·5 pH units). The intracellular acidification in the presence of γ-decalactone was weak and it reached about 0·5 pH units with 300 mg l^{-1}, both in pH 6·5- and 3·9-media, indicating no pH-dependent effect. The action of octanoic acid on pHi was sharper: with 80 mg l^{-1}, a pHi decrease of 1·7 units was observed and pHi reached 5·4 (Fig. 2). The decrease in pHi induced by γ-decalactone is weak when compared to that obtained with octanoic acid, whose toxic action is known to be due to intracellular acidification in yeast.

Influence of γ-decalactone on membrane potential

The uptake of diS-C_3 (3) by living cells is Δψ-dependent and the interaction of the probe with intracellular

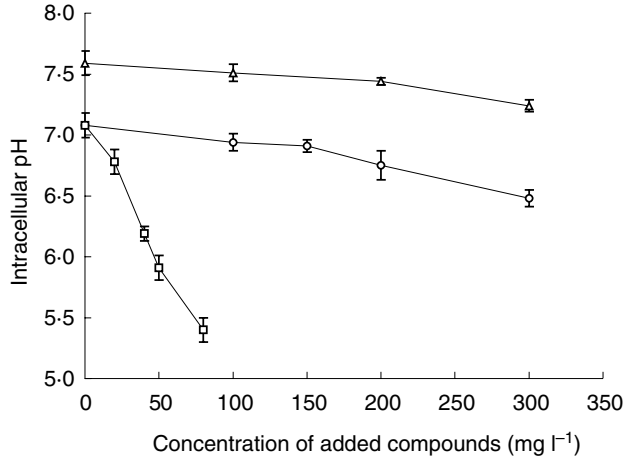

Fig. 2 Intracellular pH of *Yarrowia lipolytica* as a function of γ-decalactone concentration in pH 3·9 (○) and pH 6·5 (△) media, or octanoic acid concentration (□) in a pH 3·9 medium

components brings about a shift of the emission λ_{max} to longer wavelengths. This method is rapid and simple for following the evolution of $\Delta\psi$ in living cells. As described by Gášková *et al.* (1998, 2001), the emission λ_{max} variations of diS-C_3 (3) can be caused in two ways, a depolarization or hyperpolarization of the plasma membrane and also a permeabilization of the cells. To differentiate between these actions, 150 μmol l^{-1} KCl were added after stabilization of λ_{max} values: a drop in the emission λ_{max} to the values obtained with the reference, indicated that no membrane permeabilization occurred. Otherwise, if permeabilization occurred, the interaction of the probe with intracellular components still gave high λ_{max} values after the addition of KCl: an illustration of this is given by the values obtained with heat-deactivated cells, in which λ_{max} rapidly reached a value of 578 and it hardly changed after the addition of KCl (Fig. 3a). The reference medium contained glucose and 0·5% ethanol; the λ_{max} values for these conditions where around 575 and dropped to 572 upon the addition of KCl (Fig. 3a–c). With 50 (Fig. 3b) or 100 mg l^{-1} γ- decalactone (Fig. 3c), the λ_{max} values obtained were slightly higher than that of the reference, indicating hyperpolarization: KCl indeed, dropped λ_{max} to the same final values. With 200 (Fig. 3b) or 400 mg l^{-1} γ-decalactone (Fig. 3c), λ_{max} values were higher than that obtained with previous lactone concentrations, reaching 577·5 and approaching values of heat-deactivated cells. The addition of KCl brought about only a slight decrease, indicating this time, membrane permeabilization together with a dissipation of $\Delta\psi$ (Gášková *et al.* 1998).

This technique showed that the highest tested toxic lactone concentrations induced a dissipation of $\Delta\psi$.

Influence of γ-decalactone on membrane fluidity

Fluorescence anisotropy (r) gives an inverse indication of membrane fluidity: decreasing values of r indicate an increased fluidity (Shechter 1997). Increased membrane fluidity can be obtained by increasing the medium temperature (Tracey and Watson 1997): we evaluated r at temperatures from 27 to 40°C. A linear relation between r and the measurement temperature was obtained, r was around 0·155 for 27°C and 0·120 for 40°C (Fig. 4a).

γ-Decalactone had a strong effect on fluorescence anisotropy: at 50 mg l^{-1}, the r value dropped to 0·133 and then declined almost linearly with concentration. With 200 and 250 mg l^{-1} it was equal to the r value obtained previously at 40°C and with 300 mg l^{-1}, r was even lower (Fig. 4b). So, the lactone increased membrane fluidity in a concentration-dependent manner and even non-toxic concentrations were sufficient to modify fluorescence anisotropy.

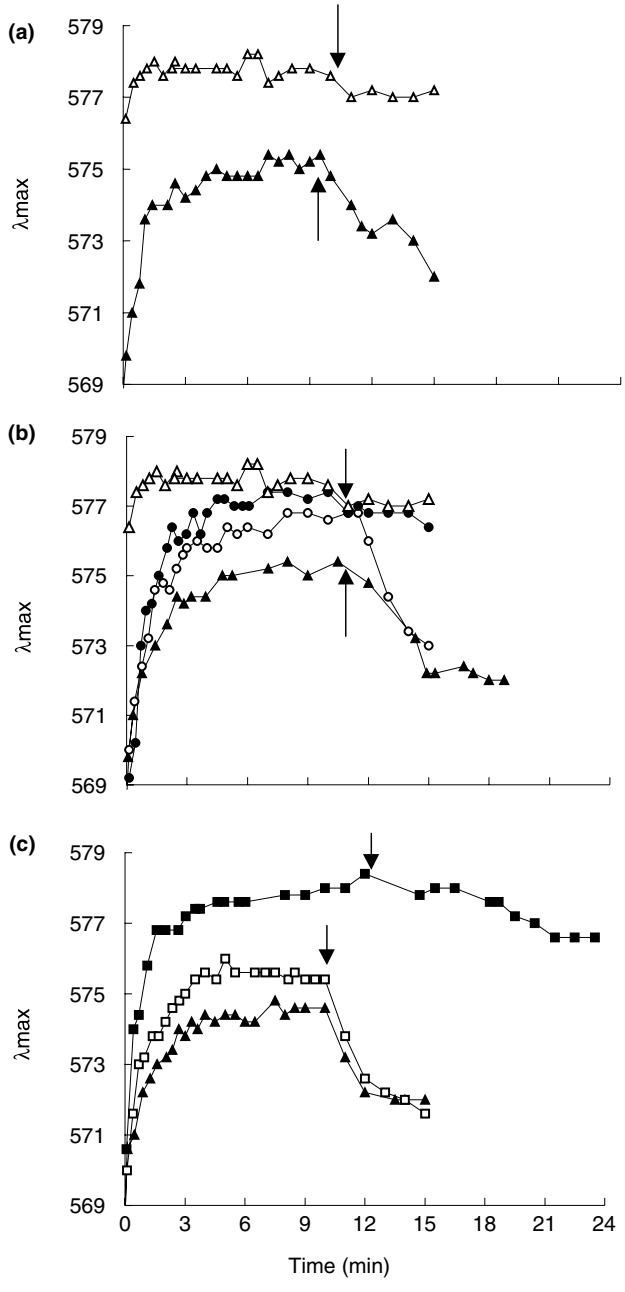

Fig. 3 Changes in maximum emission wavelength of diS-C_3 (3). Reference cells in presence of glucose and ethanol 0·5% (▲). Signals obtained with heat-deactivated cells (△) (a), 50 mg l^{-1} (○) or 200 mg l^{-1}γ-decalactone (●) (b), 100 mg l^{-1} (□) or 400 mg l^{-1}γ-decalactone (■) (c). The arrows indicate the addition of 150 μmol l^{-1} KCl in the cuvette

Interaction of γ-decalactone with a model phospholipid bilayer

The *in vitro* action of γ-decalactone was investigated by Fourier transform infrared spectroscopy measurements, by

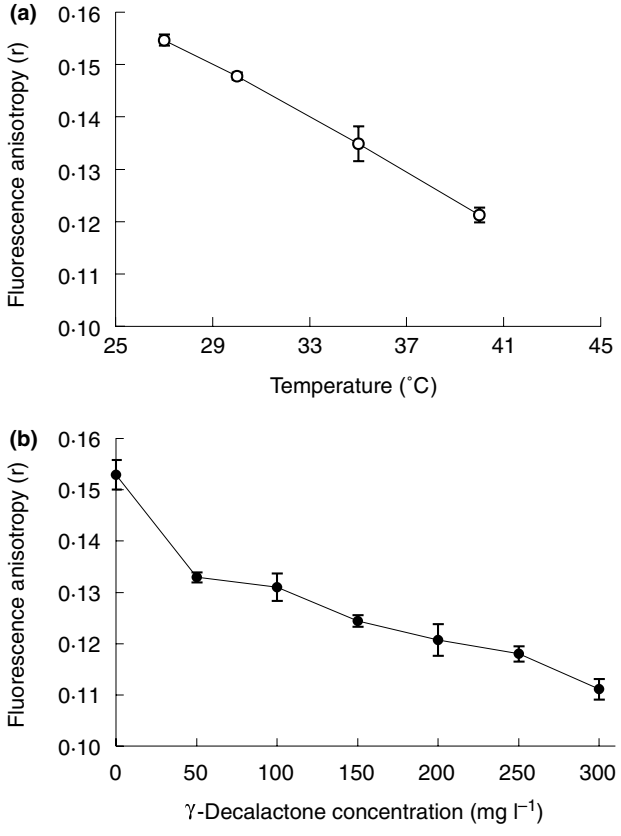

Fig. 4 Fluorescence anisotropy of yeast cells evaluated: at temperatures higher than 27°C (a) or at 27°C in the presence of γ-decalactone (b)

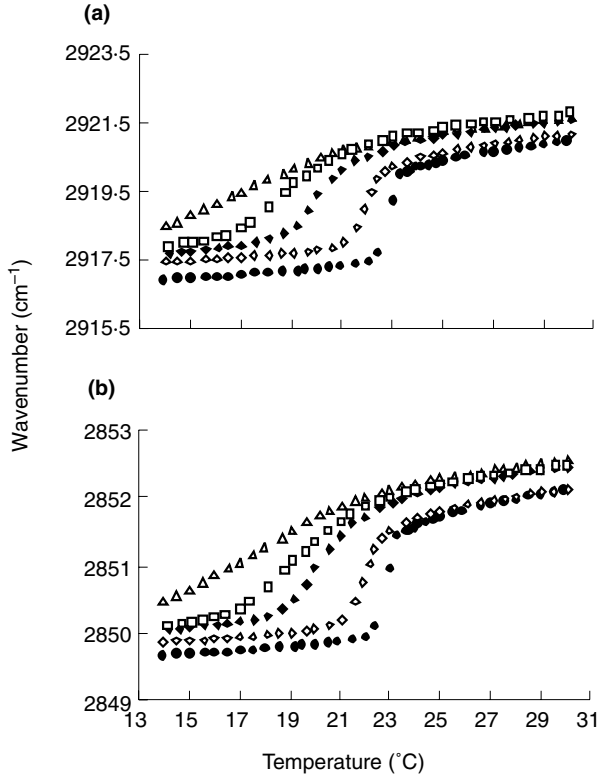

Fig. 5 Temperature dependence of CH_2 symmetric (a) and asymmetric (b) stretching vibration frequency of DMPC bilayers hydrated with water containing 0·5% ethanol (reference) (●), γ-decalactone at 50 (◇), 100 (◆), 175 (□) and 300 mg l^{-1} (△)

determining the effect of its incorporation in model phospholipid bilayers, composed of DMPC. The CH_2 stretching signals of the fatty acyl chains of phospholipids give information about the physical state of a lipid bilayer. This C–H bond gives a typical symmetric stretching (ν_s CH_2) band at 2851 (±3) cm^{-1} and one asymmetric stretching (ν_a CH_2) band at 2920 (±4) cm^{-1} (Akyüz and Davies 1998). Monitoring the shift of these wavenumber values for increasing temperatures, is a mean to detect a gel-to-liquid-crystalline phase transition (Lewis and McElhaney 1998). Such a phase transition is characteristic of aqueous phospholipid bilayers. For DMPC (hydrated with a solution containing 0·5% ethanol), the transition temperature was detected at 22·8°C (Fig. 5). When γ-decalactone was introduced (with the hydration solution), signal values of both stretching bands became higher, the transition was broadened and strongly shifted to lower temperatures in a lactone concentration-dependent manner (Fig. 5a and 5b). The diffusion of γ-decalactone into model membranes led to an important decrease in the transition temperature.

Activity of the plasma membrane H^+-ATPase

The effect of γ-decalactone on the activity of the membrane enzyme H^+-ATPase was determined on yeast membrane preparations. When γ-decalactone was present, the specific activity of H^+-ATPase was strongly affected: it dropped to 50% for concentrations between 100 and 150 mg l^{-1}, and to 20% for 300 mg l^{-1} (Fig 6). Thus, γ-decalactone can strongly lower the activity of H^+-ATPase, a vital membrane enzyme.

DISCUSSION

Yeast growth was slowed down in the presence of 150 mg l^{-1} of γ-decalactone, indicating that the cells can adapt themselves to such a concentration. Growth inhibition was observed at concentrations higher than 200 mg l^{-1}. With 100 mg l^{-1}, the high biomass concentration reached within the stationary growth phase may be due to a metabolism of the lactone (Aguedo *et al.* 2000).

Octanoic acid was chosen here as a reference for its known action on yeast pHi (Viegas and Sá-Correia 1995; Aguedo

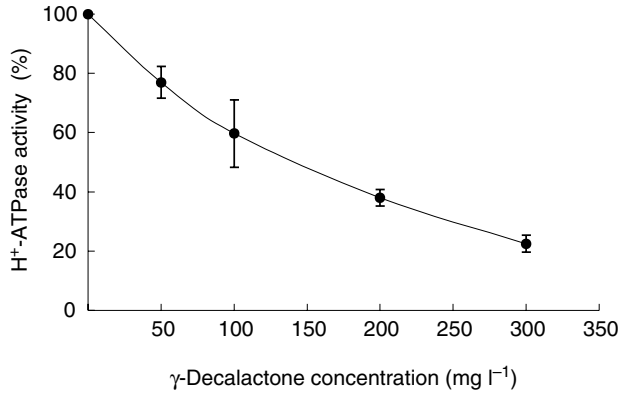

Fig. 6 Activity of H^+-ATPase in the presence of γ-decalactone

et al. 2001). Its drastic effect, like that of many lipophilic acids, is in itself responsible for its antimicrobial activity (Pampulha and Loureiro-Dias 1989). The weak acidification in pHi observed in the presence of γ-decalactone may not by itself explain the growth inhibition observed at concentrations higher than 200 mg l^{-1}. In the case of the cells exposed to the lactone, the slight decrease in pHi may be the consequence of an increased influx of protons across the plasma membrane. Consistent with this hypothesis are the results indicating that the pHi values were lower, both in the absence or in the presence of lactone, when the medium pH was lower (pH 3·9 compared with 6·5) (Fig. 2).

The slight hyperpolarization observed with lactone concentrations of 50 or 100 mg l^{-1} γ-decalactone (Fig. 3b and c) may be the consequence of a degradation of part of the molecules reaching the cell interior, once hydrolysed they can bring about an increase in dissociated carboxylic groups concentration and so an excess in negative charges. The λ_{max} values observed with 200 or 400 mg l^{-1} γ-decalactone (Fig. 3b and c), comparable to that induced by the addition of KCl, indicated a membrane permeabilization together with a dissipation of $\Delta\psi$ (Gášková *et al.* 1998).

The fluorescence anisotropy measurements demonstrated the *in vivo* action of the lactone on yeast membranes. Some structurally different odorant compounds (ionone, citral and amyl acetate) were previously shown to increase the fluidity of liposomes, leading to a disruption of $\Delta\psi$ (Enomoto *et al.* 1991). Decanoic acid was also shown to increase membrane fluidity in living cells of *S. cerevisiae* (Alexandre *et al.* 1996). γ-Decalactone action appears to be exerted at the membrane lipids level, making its fluidizing action comparable to that of β-pinene (Uribe *et al.* 1985) or of cyclic hydrocarbons (Sikkema *et al.* 1994). γ-Decalactone concentrations of 50 and 100 mg l^{-1} decreased fluorescence anisotropy of DPH, nevertheless such concentrations did not inhibit yeast growth. Therefore, yeast cells are able to withstand variations in membrane fluidity and deleterious effects may be related to the exceeding of a threshold, which could be here around a *r* value of 0·12.

γ-Decalactone introduction into DMPC multibilayers strongly modified the phase transition profile and increased the disorder within the lipid chains. Independently of temperature (in the tested range), the introduction of γ-decalactone concentrations higher than 50 mg l^{-1} gave ν_a CH_2 and ν_s CH_2 at higher frequencies, indicating a global increased disorder. These results are correlated with the above-mentioned fluorescence anisotropy measurements performed with living yeast cells, i.e. lactone increased membrane fluidity. Actually, the effects of lactone on fluorescence anisotropy values and on global vibration frequencies of the phospholipid acyl chains were in both cases comparable to the effects of heating, expressing a strong disordering action. The lipid-to-lactone molar ratios we tested, varied from 7·25 : 1 to 0·85 : 1, which is in the same concentration range as those where β-ionone and menthone have been reported to modify DMPC phase transition by interacting with the lipid acyl chains (Bouchard *et al.* 1996).

The *in vitro* inhibition of H^+-ATPase activity was almost linearly correlated to γ-decalactone concentration. H^+-ATPase is the main element controlling proton fluxes in yeast (Haworth *et al.* 1991). Decanoic acid inhibits H^+-ATPase activity *in vitro,* whereas cells grown in the presence of decanoic acid have an activated H^+-ATPase activity, which shows an adaptation of the cells to the acid (Alexandre *et al.* 1996; Piper *et al.* 2001). In our study, when non-adapted cells were put in direct contact with the lactone, a decrease in acidification of the extracellular medium was observed (not shown): this may indicate that the strong inhibition observed *in vitro* with 300 mg l^{-1}, may also occur *in vivo* and may lead to an insurmountable inhibition of the H^+-ATPase activity. However, these results demonstrated that the lactone can strongly alterate membrane proteins activity. For the mechanism of the inhibition of H^+-ATPase activity, two explanations have been proposed: an induction of changes in membrane structure which leads to a modification in bindings between native lipids and the membrane protein (Shechter 1997) or a competition of the lipophilic compound with inorganic phosphate at the catalytic site level of the enzyme (De Meis 1989). However, our results cannot distinguish between a direct or an indirect effect of the lactone on H^+-ATPase.

The data enabled us to establish the mechanisms underlying the toxicity from a molecular to a physiological level: γ-decalactone may form hydrophobic interactions with the acyl chains of membrane phospholipids leading to a progressive increase in degree of disorder and so in global fluidity within cell membranes. With increasing lactone concentrations, the membrane fluidity reaches a 'critical' threshold at which the cell permeability is modified, as

shown by the drop in $\Delta\psi$ and, to a lesser extent, in pHi: the lactone becomes toxic. At the same time, vital membrane enzyme activities as H^+-ATPase, can be modified, leading to the alteration in cell functions.

According to the evidenced mechanisms, yeast cells resistance to high lactone concentrations may be theoretically improved by decreasing membrane fluidity. Such a strategy is possible through different ways: (i) by the addition of membrane 'rigidifying' agents in the medium such as sterols (Shechter 1997), (ii) by pre-culturing the cells in the presence of saturated fatty acids (i.e. palmitic or stearic acids) (Mizoguchi and Hara 1996) or (iii) at a temperature higher than that of the process (Beney and Gervais 2001). Nevertheless, the trapping of the lactone *in situ* by hydrophobic sorbents, as for example paraffin oils, remains also a good system to maintain yeast cell viability (Dufossé *et al.* 1999).

A point that should be emphasized, is that non-toxic concentrations of the lactone (below 200 mg l^{-1}) modified cell membrane fluidity *in vivo* (in correlation with the *in vitro* experiments). This fact suggests a potential use of that compound to prevent membrane damage that can be induced by some environmental stresses which lead to sharp decreases in membrane fluidity (Beney and Gervais 2001).

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REFERENCES

Adams, T.B., Greer, D.B., Doull, J., Munro, I.C., Newberne, P., Portoghese, P.S., Smith, R.L., Wagner, B.M., Weil, C.S., Woods, I.A. and Ford, R.A. (1998) The FEMA GRAS assessment of lactones used as flavour ingredients. *Food and Chemical Toxicology* **36**, 249–278.

Aguedo, M., Waché, Y. and Belin, J.-M. (2000) Biotransformation of ricinoleic acid into γ-decalactone by yeast cells: recent progress and current questions. *Recent Research Developments in Biotechnology and Bioengineering* **3**, 167–179.

Aguedo, M., Waché, Y. and Belin, J.-M. (2001) Intracellular pH-dependent efflux of the fluorescent probe pyranine in the yeast *Yarrowia lipolytica*. *FEMS Microbiology Letters* **200**, 185–189.

Akyüz, S. and Davies, J.E.D. (1998) Temperature dependent FTIR spectroscopic study of the interaction of α-tocopherol and α-tocopheryl acetate with phospholipid bilayers. *Journal of Molecular Structure* **415**, 65–70.

Alexandre, H., Mathieu, B. and Charpentier, C. (1996) Alteration in membrane fluidity and lipid composition, and modulation of H^+-ATPase activity in *Saccharomyces cerevisiae* caused by decanoic acid. *Microbiology* **142**, 469–475.

Barth, G. and Gaillardin, C. (1996) The dimorphic fungus *Yarrowia lipolytica*. In *Non-conventional Yeasts in Biotechnology* ed. Wolf, K. pp. 314–388. New York: Springer-Verlag.

Beney, L. and Gervais, P. (2001) Influence of the fluidity of the membrane on the response of microorganisms to environmental stresses. *Applied Microbiology and Biotechnology* **57**, 34–42.

Bonnarme, P., Djian, A., Latrasse, A., Feron, G., Giniès, C., Durand, A. and Le Quéré, J.-L. (1997) Production of 6-pentyl-α-pyrone by *Trichoderma* sp. from vegetable oils. *Journal of Biotechnology* **56**, 143–150.

Bouchard, M., Boudreau, N. and Auger, M. (1996) Membrane fluidity response to odorants as seen by ^{2}H-NMR and infrared spectroscopy. *Biochimica et Biophysica Acta* **1282**, 233–239.

De Meis, L. (1989) Role of water in the energy of hydrolysis of phosphate compounds – energy transduction in biological membranes. *Biochimica et Biophysica Acta* **973**, 333–349.

Dufossé, L., Souchon, I., Feron, G., Latrasse, A. and Spinnler, H.E. (1997) Strategies to overcome toxicity during flavour production by micro-organisms: the case of γ-decalactone from *Sporidiobolus salmonicolor*. *Rivista Italiana EPPOS*, (Spec Num) 284–298.

Dufossé, L., Souchon, I., Feron, G., Latrasse, A. and Spinnler, H.E. (1999) *In situ* detoxification of the fermentation medium during γ-decalactone production with the yeast *Sporidiobolus salmonicolor*. *Biotechnology Progress* **15**, 135–139.

Endrizzi, A., Pagot, Y., Le Clainche, A., Nicaud, J.-M. and Belin, J.-M. (1996) Production of lactones and peroxisomal beta-oxidation in yeasts. *Critical Reviews in Biotechnology* **16**, 301–329.

Enomoto, S., Kashiwayanagi, M. and Kurihara, K. (1991) Liposomes having high sensitivity to odorants. *Biochimica et Biophysica Acta* **1062**, 7–12.

Feron, G., Dufossé, L., Mauvais, G., Bonnarme, P. and Spinnler, H.E. (1997) Fatty acid accumulation in the yeast *Sporidiobolus salmonicolor* during batch production of γ-decalactone. *FEMS Microbiology Letters* **149**, 17–24.

Feron, G., Dufossé, L., Pierard, E., Bonnarme, P., Le Quéré, J.L. and Spinnler, H.E. (1996) Production, identification, and toxicity of gamma-decalactone and 4-hydroxydecanoic acid from *Sporidiobolus* spp. *Applied and Environmental Microbiology* **62**, 2826–2831.

Gášková, D., Cadek, R., Chaloupka, R., Plasek, J. and Sigler, K. (2001) Factors underlying membrane potential-dependent and -independente fluorescence responses of potentiometric dyes in stressed cells: diS-C3(3). *Biochimica et Biophysica Acta* **1511**, 74–79.

Gášková, D., Denksteinova, B., Herman, P., Vecer, J., Plaseck, J. and Sigler, K. (1998) Changes in diS-C3(3) fluorescence in yeast report not only on membrane potential, but also on structural integrity of the plasma membrane. In *Fluorescence Microscopy and Fluorescent Probes* Vol. 2 ed. Slavík, pp. 147–152. New York: Plenum Press.

Goffeau, A. and Dufour, P. (1988) Plasma membrane ATPase from the yeast *Saccharomyces cerevisiae*. *Methods in Enzymology* **157**, 528–533.

Haworth, R.S., Lemire, B.D., Crandall, D., Cragoe Jr, E.D. and Fliegel, L. (1991) Characterization of proton fluxes across the cytoplasmic membrane of the yeast *Saccharomyces cerevisiae*. *Biochimica et Biophysica Acta* **1098**, 79–89.

Lewis, R.N.A.H. and McElhaney, R.N. (1998) The structure and organization of phospholipid bilayers as revealed by infrared spectroscopy. *Chemistry and Physics of Lipids* **96**, 9–21.

Maga, J.A. (1976) Lactones in foods. *CRC Critical Reviews in Food Science and Nutrition* **8**, 1–56.

Mizoguchi, H. and Hara, S. (1996) Effect of fatty acid saturation in membrane lipid bilayers on simple diffusion in the presence of ethanol at high concentrations. *Journal of Fermentation and Bioengineering* **81**, 406–411.

Nago, H., Matsumoto, M. and Nakai, S. (1993) 2-Deceno-δ-lactone-producing fungi, strains of *Fusarium solani,* isolated by using medium containing decano-δ-lactone as the sole carbon source. *Bioscience, Biotechnology, and Biochemistry* **57**, 2107–2110.

Page, G.V. and Eilerman, R.G. (1989) Process for the preparation of gamma and delta lactones. *International Patent* PCT WO 89/12104.

Pagot, Y., Le Clainche, A., Nicaud, J.-M., Waché, Y. and Belin, J.-M. (1998) Peroxisomal beta-oxidation activities and gamma-decalactone production by the yeast *Yarrowia lipolytica*. *Applied Microbiology and Biotechnology* **49**, 295–300.

Pampulha, M.E. and Loureiro-Dias, M.C. (1989) Combined effect of acetic acid, pH and ethanol on intracellular pH of fermenting yeast. *Applied Microbiology and Biotechnology* **31**, 547–550.

Piper, P., Ortiz Calderon, C., Hatzixanthis, K. and Mollapour, M. (2001) Weak acid adaptation: the stress response that confers yeasts with resistance to organic acid food preservatives. *Microbiology* **147**, 2635–2642.

Shechter, E. (1997) Fluidité membranaire. In *Biochimie et biophysique des membranes. Aspects structuraux et fonctionnels.* pp. 97–121. Paris: Masson.

Sikkema, J., de Bont, J.A.M. and Poolman, B. (1994) Interactions of cyclic hydrocarbons with biological membranes. *Journal of Biological Chemistry* **269**, 8022–8028.

Tracey, M.S. and Watson, K. (1997) Membrane fatty acid composition and membrane fluidity as parameters of stress tolerance in yeast. *Canadian Journal of Microbiology* **43**, 70–77.

Uribe, S., Ramirez, J. and Peña, A. (1985) Effects of β -pinene on yeast membrane functions. *Journal of Bacteriology* **161**, 1195–1200.

Viegas, C.A. and Sá-Correia, I. (1995) Toxicity of octanoic acid in *Saccharomyces cerevisiae* at temperatures between 8·5 and 30°C. *Enzyme and Microbial Technology* **17**, 826–831.