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Membrane Fluidity Adjustments in Ethanol-Stressed *Oenococcus oeni* Cells

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The effect of ethanol on the cytoplasmic membrane of *Oenococcus oeni* cells and the role of membrane changes in the acquired tolerance to ethanol were investigated. Membrane tolerance to ethanol was defined as the resistance to ethanol-induced leakage of preloaded carboxyfluorescein (cF) from cells. To probe the fluidity of the cytoplasmic membrane, intact cells were labeled with doxyl-stearic acids and analyzed by electron spin resonance spectroscopy. Although the effect of ethanol was noticeable across the width of the membrane, we focused on fluidity changes at the lipid-water interface. Fluidity increased with increasing concentrations of ethanol. Cells responded to growth in the presence of 8% (vol/vol) ethanol by decreasing fluidity. Upon exposure to a range of ethanol concentrations, these adapted cells had reduced fluidity and cF leakage compared with cells grown in the absence of ethanol. Analysis of the membrane composition revealed an increase in the degree of fatty acid unsaturation and a decrease in the total amount of lipids in the cells grown in the presence of 8% (vol/vol) ethanol. Preexposure for 2 h to 12% (vol/vol) ethanol also reduced membrane fluidity and cF leakage. This short-term adaptation was not prevented in the presence of chloramphenicol, suggesting that de novo protein synthesis was not involved. We found a strong correlation between fluidity and cF leakage for all treatments and alcohol concentrations tested. We propose that the protective effect of growth in the presence of ethanol is, to a large extent, based on modification of the physicochemical state of the membrane, i.e., cells adjust their membrane permeability by decreasing fluidity at the lipid-water interface.

Malolactic bacteria are lactic acid bacteria that are able to carry out malolactic fermentation (MLF). The control of their activity is an important aspect of the technology of commercial wine production. MLF consists of the decarboxylation of L-malic acid to L-lactic acid, which decreases total acidity and improves the stability and quality of wine. *Oenococcus oeni* is recognized as the principal microorganism responsible for MLF under stress conditions, such as those prevailing in wine. However, inoculation of *O. oeni* starter cultures directly into wine leads to significant cell mortality and, consequently, failure of MLF. The reactivation and adaptation of starter cultures to wine conditions prior to inoculation into wine considerably enhance survival of the bacteria (for a review of MLF see reference 28). Ethanol tolerance appears to be a crucial parameter for the activity of *O. oeni* cells in wine.

The role of ethanol as an agent affecting the physicochemical state and biological functions of various cell membranes has been extensively studied. Ethanol toxicity is now generally attributed to the interaction of ethanol with membranes at the aqueous interface, resulting in a perturbed membrane structure and function (47). In an extensive review of the biological effects of ethanol, Jones (24) argues that membrane-located effects of ethanol are the result of the dielectric disruption of the aqueous phase, of competition with water for membrane polar sites, and of selective location within the polar region of membrane surfaces or proteins. However, the exact range of biochemical and physiological processes affected by ethanol is, for the most part, undefined.

The importance of the membrane lipid composition with respect to ethanol tolerance has been extensively studied with *Saccharomyces cerevisiae* (43; for a review see reference 34) and bacteria (12, 21). Ethanol tolerance has been strongly correlated with adaptive changes in plasma membrane composition, with many studies of yeast suggesting a role for acyl-chain unsaturation (31, 42, 43).

Ethanol tolerance has been associated with high plasma membrane fluidity in both yeast (1, 38) and bacteria (3, 10). The fluidization response can be interpreted on the basis of the hypothesis of "homeoviscous adaptation" (39) as a counteraction to the physicochemical effect of ethanol on membranes (21). This model, while being widely reported, is apparently not universally applicable to all organisms. Exceptions have been reported for *Bacillus subtilis* (35) and *Escherichia coli* (12) cells, in which plasma membranes isolated from cells grown in the presence of ethanol were more rigid than those from the control cells.

While membrane lipid composition has been considered important for cellular stress tolerance, other factors have also received extensive consideration. One widely studied aspect of the ethanol-stress response is the induction of heat shock proteins (hsp's) (22, 29). However, the relative contribution of hsp's to ethanol tolerance has been questioned (11, 40). Although hsp's and membrane composition are both likely to be

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of importance in ethanol tolerance, the relative contribution of each and the mechanisms of action remain unresolved.

The degree to which the effects of ethanol on membrane composition and fluidity share common features has not previously been explored with *O. oeni*. In addition, no direct measurements of the effect of growth in the presence of ethanol on the mobility of membrane components have been established. In this report we investigated the contribution of membrane fluidity changes to ethanol-stress tolerance and the relationship of those changes to the changes in fatty acid (FA) composition of *O. oeni* membranes. Ethanol-stress tolerance was examined by monitoring the leakage of preloaded carboxyfluorescein (cF) from the cells. The effect of ethanol challenge on the organization and dynamics of the plasma membrane in intact cells of *O. oeni* was assessed by the in vivo spin label technique. Three nitroxide spin labels were used to obtain motional anisotropies of the nitroxide moiety at different depths of the plasma membrane. The molecular-order parameter *S* derived from electron spin resonance (ESR) spectra provided a measure of membrane structural order. This parameter was studied in relation to the concentration of ethanol in nonadapted cells and in ethanol-adapted cells grown in the presence of 8% ethanol or after a short exposure to 12% ethanol. This study was undertaken to test the hypothesis that ethanol may be toxic to *O. oeni* because of its effects on the plasma membrane and that adaptation can partially or completely reverse these membrane effects via changes in membrane composition and/or organization. Adaptation was assessed as the reduction in ethanol-induced cF leakage. The relationship between membrane order and acyl-chain composition in the tolerance of *O. oeni* cells is discussed.

MATERIALS AND METHODS

Strain, medium, and growth conditions. *O. oeni* GM (Microlife Technics, Sarasota, Fla.) was cultured at 30°C in FT80 medium (8) at pH 4.5 without the addition of Tween 80 but containing 10 g of DL-malic acid per liter. Glucose and fructose were autoclaved separately and added to the medium just before inoculation at final concentrations of 2 and 8 g/liter, respectively. Stock cultures (kept frozen at −80°C) were grown until early stationary phase (48 h), diluted 100-fold in fresh medium, incubated for 24 h, and then used to obtain 1% inoculated cultures.

Cell adaptation. For adaptation to ethanol during growth, *O. oeni* cells were cultured at 30°C in 500 ml of FT80 medium, pH 4.5, with 8% (vol/vol) ethanol and recovered after 48 h (late exponential phase). For short-term adaptation, *O. oeni* was cultured in 500 ml of the same medium at 30°C. Exponential-phase cells (24 h) were harvested by centrifugation, suspended in the same medium containing 12% (vol/vol) ethanol, and incubated for 2 h at 30°C. The same procedure was repeated in the presence of chloramphenicol (CAP; 80 μg/ml) in order to inhibit de novo protein synthesis as observed by Jobin et al. for *O. oeni* (23).

ESR spectroscopy. The membrane fluidity of intact *O. oeni* cells was studied by the ESR spin probe technique. Spin-labeled stearic acids were used to probe membrane fluidity. In this molecule the nitroxide doxyl group (stable radical) is attached in a rigid, stereospecific manner to stearic acid so that the motion of the nitroxide group directly reflects the motion of the labeled part of stearic acid. The ESR spectral shape of spin-labeled stearic acids depends on the motion and angular orientation of the nitroxide group with respect to the membrane lipid-water interface (30). Depending on the position of the doxyl group along the carbon chain (at the 5th, 12th, or 16th C atom), it is possible to probe the motional freedom in membranes at the lipid-water interface, in the middle of the monolayer, and in the core of the bilayer, respectively.

5-Doxyl-stearic acid (5-DS) is commonly used to probe the membrane lipid-water interface. A typical ESR spectrum of 5-DS-labeled *O. oeni* cells is presented in Fig. 1. The anisotropic character of the spectral shape results from the restricted angular freedom of the radical group of 5-DS in the plasma membrane. The spectral parameters $A_{\parallel}$ and $A_{\perp}$ indicate the outer and inner hyperfine splittings in experimental spectra (as shown in Fig. 1). The membrane-order parameter *S* relates to membrane fluidity and can be calculated as the ratio between the observed hyperfine anisotropy ($A_{\parallel} - A_{\perp}$) to the maximum theoretically obtainable value of 25 G, which corresponds to the completely rigid orientation of 5-DS (27). Thus, the order parameter can be calculated as follows: $S = (A_{\parallel} - A_{\perp})/25$.

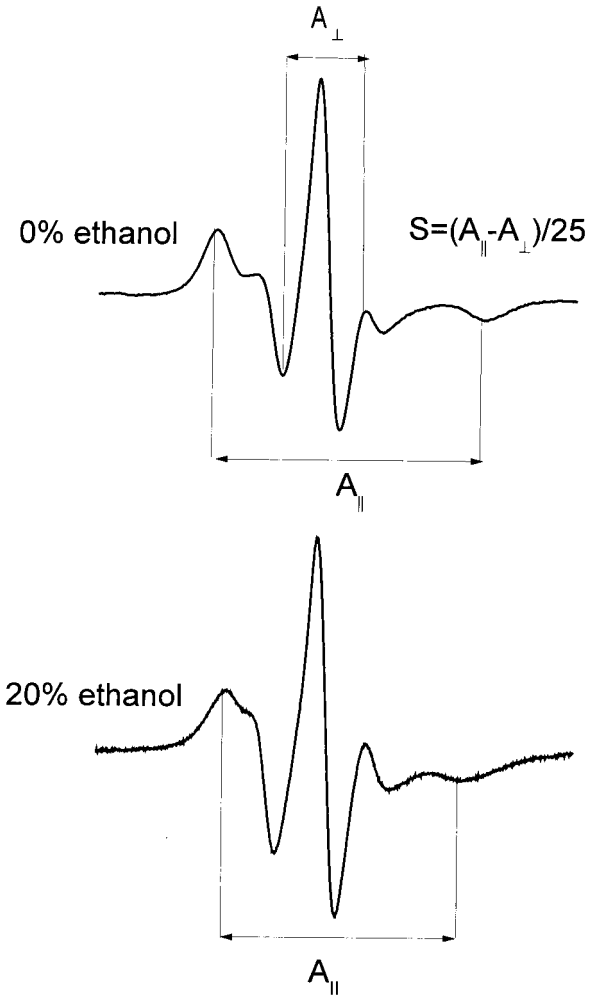

FIG. 1. EPR spectra of 5-DS in *O. oeni* cells in the absence or presence of 20% (vol/vol) ethanol. $A_{\parallel}$ and $A_{\perp}$ represent the outer and inner hyperfine splittings, respectively. The order parameter *S* is calculated as indicated in the figure.

From this formula it is clear that $S = 1$ for completely rigid order and $S = 0$ for completely isotropic motion. This means that in the case of completely rigid orientation of 5-DS $A_{\parallel}$ is maximal and $A_{\perp}$ is minimal, and in the case of completely isotropic motion the outer and inner splittings are equal. With membrane fluidization $A_{\parallel}$ decreases and $A_{\perp}$ increases, so that the order parameter decreases.

ESR spectra were recorded at room temperature with an X-band ESR spectrometer (Bruker, Rheinstetten, Germany; model 300E). Microwave power was 5 mW, the modulation amplitude was 3 G, and the scan range was 100 G. All spin probes were from Sigma, St. Louis, Mo., and were stored as a 0.1 M stock solution in ethanol at −20°C.

Cell labeling. For labeling, 1 mM spin probe solution was freshly prepared from the stock solution by dilution in water. Cells (250 ml) were recovered and washed three times with 5 mM EDTA plus 0.15 M KCl in P2 buffer (30 mM KP_i, 0.34 M CH_3COONH_4; pH 7.0) to chelate manganese ions. The cells were resuspended in the same buffer, and 25 μl of the cell suspension was introduced in a 2-mm (inner-diameter) capillary tube, centrifuged, resuspended in 20 μl of 1 mM spin probe solution, and incubated for 2 min. The cells were subsequently centrifuged, and the supernatant was removed completely. To avoid reduction of

the nitroxide spin probe, the cells were washed with ferricyanide (120 mM), and after centrifugation and removal of the supernatant, the pellet was ready for ESR spectrum recording.

Stress conditions. After the ESR measurements of the control cells (nonstressed), the pellet in the capillary was resuspended into 20 μl of 100 mM ferricyanide. From this, a 25-μl aliquot was mixed with 25 μl of a solution with twice the desired concentration of ethanol in 100 mM ferricyanide. This cell suspension was then divided into two capillaries: one was centrifuged immediately, and the other was centrifuged after 15 min. ESR spectra were recorded in both pellets. ESR measurements were repeated in the same cells after the ethanol was washed out by resuspending the pellets in 20 μl of 100 mM ferricyanide for 10 min.

Loading cells with cF. Cells were harvested at the end of the exponential growth phase (optical density at 600 nm [OD_{600}], approximately 0.4) by centrifugation, washed twice with 50 mM potassium phosphate buffer (pH 7.0), and concentrated in the same buffer to an OD_{600} of 20. Cells were deenergized with 2-deoxyglucose (at a final 2 mM concentration) by incubation at room temperature for 30 min to avoid cF extrusion from the cells by energy-dependent pumps (5, 6) and consequently to ensure that cF leakage was a consequence of ethanol-induced membrane damage. The cells were washed and resuspended in 50 mM KP_i buffer (pH 7.0) to an OD_{600} of 20. A stock solution of 2.3 mg of 5(6)-carboxyfluorescein diacetate (cFDA) (Molecular Probes, Eugene, Oreg.) per ml was prepared in acetone and stored at −20°C in the dark. cFDA was added to the cell suspension to a final 50 μM concentration and kept at 30°C for 15 or 60 min in the case of cells pregrown in the presence of 8% (vol/vol) ethanol. Immediately after labeling, the cells were spun down, washed once, and resuspended in 50 mM KP_i (pH 7.0) to an OD_{600} of 2.0 for fluorimetric analyses. Inside cells the uncharged, esterified, prefluorochrome cFDA is converted by cytoplasmic esterases into fluorescent cF that is negatively charged at physiological pH and, consequently, will accumulate inside cells with an intact cytoplasmic membrane (R. P. Haugland, *Handbook of Fluorescent Probes and Research Chemicals*, 1996, Molecular Probes).

Measurement of cF efflux. cF-loaded cells were washed twice and resuspended in 50 mM KP_i buffer (pH 7.0) to a final OD_{600} of 2.0. At time zero cell suspensions were placed in a water bath at 30°C and incubated without and with ethanol (8, 12, and 16% [vol/vol]). Samples (200 μl) were withdrawn at intervals and immediately centrifuged to remove the cells. To measure the cF labeling capacity, labeled cells were lysed by incubation at 70°C for 15 min and the debris was removed by centrifugation. The fluorescence of the supernatant was measured with a Perkin-Elmer LS 50B luminescence spectrometer (excitation wavelength at 490 ± 5 nm and emission wavelength at 515 ± 5 nm). From the fluorescence of the supernatants and the total labeling capacity, the intracellular concentrations of cF at the sampling time points were calculated.

FA analysis. Total lipids were extracted with chloroform-methanol-water from 30 to 40 mg (dry weight) of cells according to the method of Bligh and Dyer (4) and methyl esterified by a 15-min incubation at 95°C in boron trifluoride-methanol (32). The FA methyl esters were extracted with hexane, separated on a CP-Sil-88 fused silica capillary column (Chrompack; 50 m by 0.25 mm by 0.20 μm [film thickness]), and analyzed by gas chromatography-mass spectrometry (GC-MS) (Hewlett-Packard 5970B-series gas chromatograph-mass spectrometer). Electron impact spectra were obtained at 70 eV of electron energy. The following operating conditions were used: injection temperature of 250°C and oven temperature of 50°C initially, rising to 275°C at 6°C/min, with maintenance at this temperature for 10 min. The FAs were identified with the aid of FA methyl ester standards (Sigma), and the identity was confirmed using the NIST Mass Spectral Library. Replicate determinations indicated that the relative error (standard deviation of the mean × 100%) of the values was less than 8%. The average results of three independent experiments are presented. In semiquantitative analysis, the percentage of each FA was calculated by the ratio peak area/sum of total identified peak areas × 100. In quantitative analysis, peak areas were related to that of the internal standard (C_{22}) and then converted to micrograms by using the area of the nearest standard peak for the calculation.

RESULTS

Ethanol-induced cF leakage. The ethanol-induced leakage of the fluorescent probe cF from *O. oeni* cells into the supernatant was studied to determine the cellular resistance to the disruptive effect of ethanol. The increased cF leakage rate values at high ethanol concentrations indicated that cell membranes were sensitive to ethanol in all the conditions shown (Fig. 2). Exposure of nonadapted control cells to 16% ethanol resulted in a rapid loss of cF, suggesting an immediate disorganization of the plasma membrane. However, cells grown in the presence of 8% (vol/vol) ethanol were able to retain cF more efficiently, for all the concentrations of ethanol tested, and even in the absence of ethanol. Leakage rates in these adapted cells were less than 50% of those in nonadapted control cells. *O. oeni* cells preincubated with 12% (vol/vol) ethanol for 2 h were less leaky for cF during the challenge with increasing ethanol concentrations than were the nonadapted cells. The presence of CAP during this 2-h incubation in 12% (vol/vol) ethanol did not significantly change the cF-efflux kinetics.

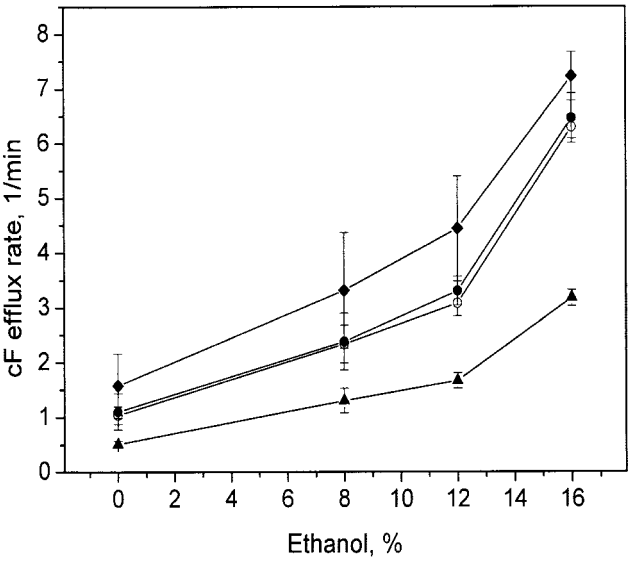

FIG. 2. Effect of ethanol on the rate of cF efflux from deenergized *O. oeni*. The cells were loaded with cF by incubation at 30°C in 50 μM cFDA. The efflux of cF was measured by spectrofluorimetry at 30°C in 50 mM potassium buffer (pH 7.0) in cells grown without ethanol (◆), preexposed to 12% (vol/vol) ethanol for 2 h in the absence (○) and in the presence (●) of CAP, and grown in the presence of 8% (vol/vol) ethanol (▲).

Ethanol stress and adaptation at the membrane lipid-water interface. Because the interaction of ethanol with the membrane is thought to occur at the membrane lipid-water interface (2, 14, 20), we studied the effects of ethanol by using the lipid-water interface membrane probe 5-DS. Figure 3a shows the effect of addition of ethanol to intact *O. oeni* cells on the order parameter *S*, as calculated from ESR spectra of 5-DS (Fig. 1). The *S* value in nonadapted cells was 0.71 and decreased to 0.58 directly upon the addition of 20% (vol/vol) ethanol. Such behavior is indicative of significant, instantaneous disordering of the membrane lipid-water interface. The data for membrane order closely parallel those assessing ethanol effects on cF leakage. Interestingly, the value of 12% (vol/vol) ethanol appears to be the point for the onset of drastic increases in both membrane fluidity and permeability. The shape of the spectra did not change when spectra were rerecorded within 15 min of the initial recording (data not shown), indicating that ethanol causes an immediate disordering effect. Spectra of 5-DS were recorded also from cells grown in the presence of 8% (vol/vol) ethanol. The value of *S* obtained from these adapted cells was approximately 0.03 U higher than that

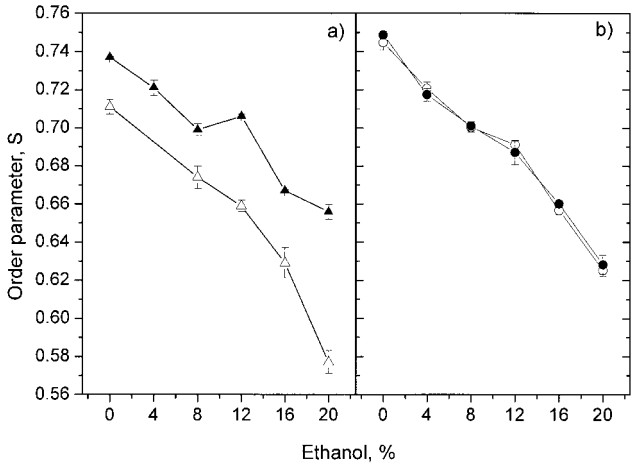

FIG. 3. Effect of ethanol on the molecular-order parameter *S* calculated from ESR spectra of 5-DS-labeled intact *O. oeni* cells. (a) Nonadapted cells (△) or cells grown in the presence of 8% (vol/vol) ethanol (▲). (b) Cells preexposed to 12% (vol/vol) ethanol for 2 h in the presence (○) or in the absence (●) of CAP.

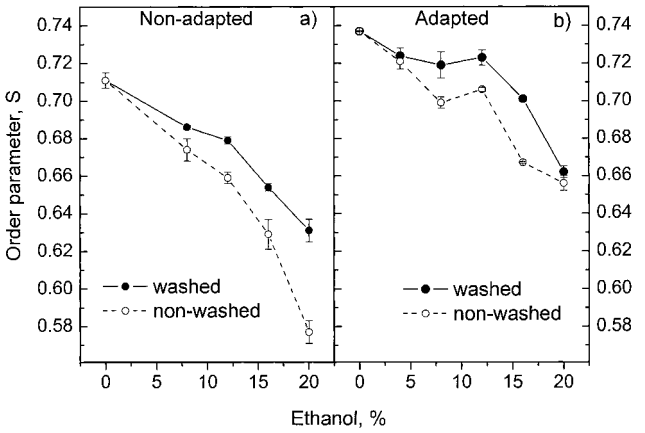

FIG. 4. Effect of ethanol on the molecular-order parameter *S* calculated from ESR spectra of 5-DS-labeled intact *O. oeni* cells. The measurements were made with nonadapted cells (a) or cells grown in the presence of 8% (vol/vol) ethanol (b) and with washed or nonwashed (control) cells.

from nonadapted cells, indicating a higher order at the membrane lipid-water interface (Fig. 3a). Stressing these ethanol-adapted cells with increasing concentrations of ethanol showed that the *S* values were always significantly higher than those in the nonadapted cells. The difference in *S* was particularly evident at the highest concentration of ethanol tested (20%). These results indicate that cells grown in the presence of ethanol are more resistant to ethanol-induced disordering.

In *O. oeni* cells, incubation with 12% (vol/vol) ethanol induces enhancement of the synthesis of a specific stress protein, Lo18, and a significant amount of this protein has been found to be associated with the membrane (22). Based on this, it seemed reasonable to assume that membrane resistance to ethanol disordering is associated with de novo protein synthesis, a possibility that we tested by a short adaptation of cells (without growth) to ethanol in both the presence and the absence of CAP. Values of *S* calculated from 5-DS spectra in cells preexposed to 12% (vol/vol) ethanol for 2 h were higher than those in control cells (Fig. 3b), i.e., the lipid-water interface region of the membranes in cells preexposed to ethanol was more rigid than that in the nonadapted cells. The ethanol concentration-dependent decrease of *S* in these ethanol-exposed cells followed the same trend as that in the control cells, although the spin label was always less mobile. When cells were preexposed to 12% (vol/vol) ethanol in the presence of the protein synthesis inhibitor CAP, the results obtained were similar to those in the absence of CAP at all concentrations of ethanol tested. These results suggest that de novo protein synthesis does not play a significant role in membrane adaptation to ethanol-induced disorder under nongrowing conditions.

After stressing control and ethanol-adapted cells with increasing concentrations of ethanol, cells were resuspended in a solution of ferricyanide in order to wash out the ethanol. The order parameter was only partially recovered (Fig. 4), with the extent of recovery being more evident for the higher concentrations tested. In cells grown in the presence of ethanol, the extent of recovery was less pronounced than in nonadapted cells for all the ethanol concentrations tested, with no recovery for cells stressed with 20% (vol/vol) ethanol (Fig. 4b). A possible explanation for these observations is that preexposure to ethanol decreases the partitioning of ethanol into *O. oeni* membranes as was observed for a variety of other biological membranes (26, 36). Moreover, the observation that the partition coefficient of ethanol correlates inversely with the lipid order (33) is in line with the proposed explanation.

Ethanol stress at the membrane interior. To study the influence of ethanol deeper in the bilayer, we labeled *O. oeni* cells with 12-DS and 16-DS. These compounds probe membrane mobility at the level of the 12th and 16th carbon atoms of the acyl chains, respectively. Figure 5 shows a 12-DS spectrum (A, top) and a 16-DS spectrum (B, top) from cells in the absence of ethanol. The shapes of these spectra were different from that of 5-DS (Fig. 1) and are indicative of an increasing motional freedom towards the lipid hydrocarbon core (25). The presence of 20% ethanol causes narrowing of the lines of both the 12-DS and the 16-DS spectra (bottom spectra in Fig. 5A and B, respectively), which can be interpreted to mean that membrane fluidity has increased (18). Thus, ethanol has a

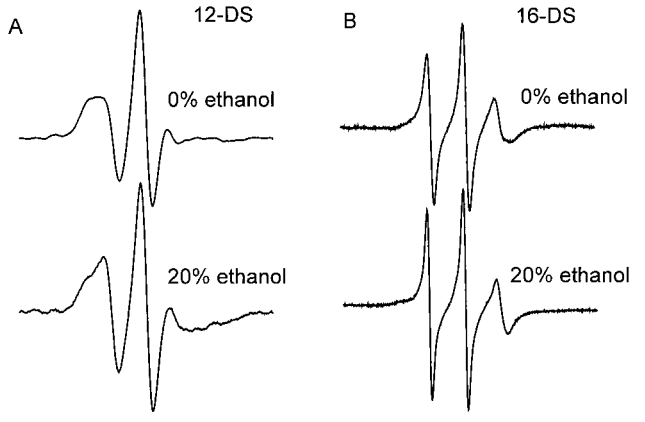

FIG. 5. EPR spectra of doxyl-stearate spin probes 12-DS (A) and 16-DS (B) in *O. oeni* cells in the absence or presence of 20% ethanol.

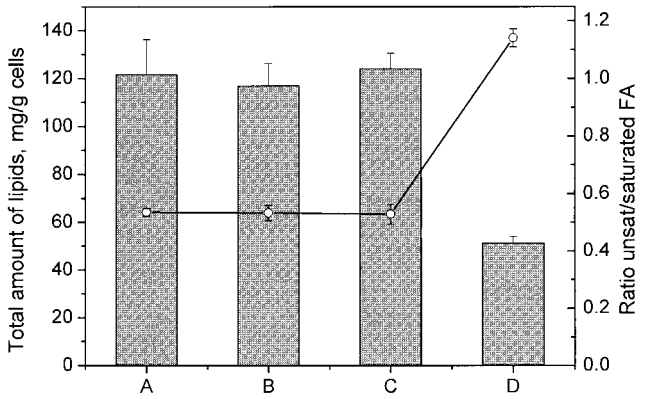

FIG. 6. Membrane lipid composition of *O. oeni* cells. The total lipid content (bars) and the unsaturation/saturation ratio (○) were measured in control cells (A), cells exposed to 12% ethanol for 2 h in the presence (B) or the absence (C) of CAP, and cells grown in the presence of 8% ethanol (D).

fluidizing effect on *O. oeni* membranes not only at the membrane lipid-water interface but also deeper in the bilayer. Adaptation to ethanol does not cause notable changes in the spectral shape of 12-DS and 16-DS (spectra not shown), thus suggesting that the effect of adaptation mainly relates to the membrane lipid-water interface.

Modification of fatty acyl composition of the phospholipid fraction by different treatments. Membrane lipid FA composition has been claimed to play a major role in determining membrane fluidity, especially in bacteria that typically lack sterols in their membranes (37). We determined the fatty acyl composition of the phospholipid fraction extracted from *O. oeni* cells grown under normal conditions (control), preexposed to 12% ethanol for 2 h in the presence or absence of CAP, or grown in the presence of 8% (vol/vol) ethanol. Palmitic acid ($C_{16:0}$) was the major component of the FA profile followed by myristic acid ($C_{14:0}$) (data not shown). The FA profile found was essentially similar to the results for *O. oeni* reported elsewhere (13, 16, 41), except that we could not detect lactobacillic acid. We found two minor peaks that could not be identified, which might represent lactobacillic acid; however, FA methyl ester standards (Sigma) and GC-MS analysis were not conclusive in this respect. Addition of 8% (vol/vol) ethanol to the growth medium increased the degree of FA unsaturation in these ethanol-adapted cells, while the total lipid content markedly decreased (Fig. 6). This was mainly due to a strong decrease in $C_{16:0}$ and an increase in the level of $C_{16:1}$ (data not shown). Moreover, it was observed that two additional unsaturated short-chain FAs were present in cells grown in the presence of ethanol (data not shown). The degree of unsaturation and the total amount of lipids were identical in control cells and in cells preincubated in 12% (vol/vol) ethanol for 2 h, irrespective of the presence of CAP (Fig. 6). Notably, these cells retained cF more efficiently (Fig. 2) and were more resistant to ethanol-induced membrane disordering (Fig. 3b).

DISCUSSION

There is virtually universal agreement in the literature that biological membranes are the primary target of ethanol injury, and it is widely assumed that membrane physical properties and lipid composition are the main factors involved in ethanol tolerance (for a review see reference 24). An important question arises as to the extent to which these aspects of membrane tolerance share common features. In the present paper, ethanol effects on the physical and chemical properties of the cytoplasmic membrane of *O. oeni* cells are described, also in relation to acquired tolerance to ethanol. We found similar trends in the ethanol dependencies of membrane permeability and fluidity. This might be taken as preliminary evidence that these two membrane properties are ruled by a common mechanism: i.e., ethanol-induced leakiness is the consequence of membrane physical disordering, and resistance to the permeabilizing effect of ethanol would result from an adaptive increase in membrane order. However, there was no clear correlation between adjustment of membrane physical properties and changes in the unsaturated/saturated ratio of membrane FAs.

From the ESR spectra of 5-DS it is clear that the fluidity of the cytoplasmic membrane in *O. oeni* cells instantaneously increases on addition of ethanol, in a concentration-dependent manner. 5-DS allows the order to be examined at the upper methylene segment of the lipid hydrocarbon chains, i.e., close to the lipid-water interface. From the disturbance of this relatively immobile membrane segment, it follows that ethanol partitions at least into the lipid-water interface. Ethanol also increased the freedom of motion of spin probes that were labeled along the FA near the hydrophobic core of the membrane, but only for high concentrations of ethanol (20% [vol/vol]). These results are in agreement with other studies showing that ethanol molecules reside mainly at the lipid-water interface near the lipid glycerol backbone of the hydrocarbon chains (14, 20).

The capacity for survival under what would normally be considered extremely adverse conditions, such as those prevailing in wine, requires specific cellular strategies that are of fundamental importance for microbial life in such extreme environments. For optimal biological performance, membranes should be maintained in a fluid, liquid-crystalline state (21). We found that ethanol-adapted *O. oeni* cells were able to respond to the fluidizing effect of ethanol by increasing the order at the membrane lipid-water interface and decreasing permeability. These results are consistent with the theory that bacterial cells possess adaptation mechanisms to compensate for the accumulation of toxic amphiphilic compounds in their membranes (47). Interestingly, the readdition of 8% (vol/vol) ethanol to cells grown in the presence of 8% (vol/vol) ethanol resulted in a membrane fluidity (at the position of the nitroxide label of 5-DS) that was similar to that in nonadapted cells in the absence of ethanol. This result implies that ethanol-induced adaptation in membrane fluidity is not only qualitatively but also quantitatively consistent with the homeoviscous theory validated for bacteria by Sinensky (38).

Besides long-term ethanol adaptation, achieved by cells growing in the presence of ethanol, cells need a means for rapid adjustment of ethanol-induced membrane disorder. Cells preexposed to 12% (vol/vol) ethanol (2 h) acquired membrane ethanol tolerance, although the plasma membranes from these cells were more disturbed by ethanol than were those from cells that were grown in the presence of 8% (vol/vol) ethanol. The tolerance

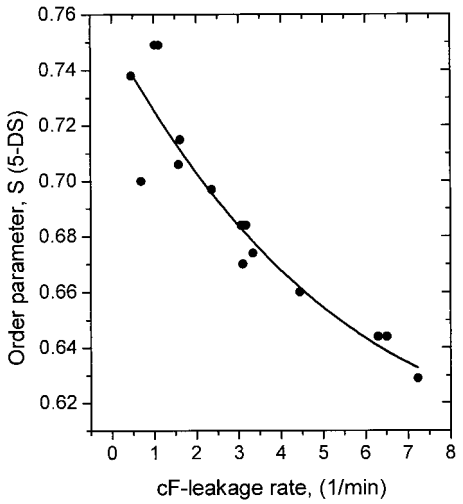

FIG. 7. Correlation between *S* values calculated from 5-DS spectra shown in Fig. 3 and cF leakage rate values from Fig. 2. The data relate to *O. oeni* cells grown without (control) or with 8% ethanol and cells preexposed for 2 h to 12% ethanol in the presence or absence of CAP. The cells were exposed to 0, 8, 12, and 16% ethanol during ESR spectrum recording and cF efflux measurements.

included adaptive changes in both order and permeability to negate the effect of ethanol. It has been suggested that ethanol-induced synthesis of stress proteins such as small hsp's is associated with the enhanced ethanol tolerance in bacteria (22, 29). It was recently shown that small hsp's interact with phospholipid bilayers and stabilize them (45). While such a mechanism may have provided tolerance to *O. oeni* cells that were grown in the presence of 8% (vol/vol) ethanol, it is unlikely that a similar mechanism operated during the 2-h preexposure to 12% (vol/vol) ethanol, since the adaptation was not prevented in the presence of the protein synthesis inhibitor CAP. This leaves the possibility that proteins or other compounds already existing in the cytoplasm are called upon to stabilize the cytoplasmic membrane, e.g., ethanol induces an increase in the affinity of cytoplasmic proteins for membranes by increasing their hydrophobicity. These results appear to imply that a mechanism(s) independent of de novo protein synthesis may be involved in the adaptive response of *O. oeni* cells to ethanol.

Figure 7 shows the correlation between *S* values, calculated from 5-DS spectra, and cF leakage rates in ethanol-treated cells grown in the presence or absence of 8% ethanol or preexposed to 12% (vol/vol) ethanol for 2 h. Although no causal relationship between permeability and lipid order was established, Fig. 7 shows a strong negative correlation ($r = -0.93$) between these parameters, which suggests that cF leakage rates are determined by the fluidity at the lipid-water interface. There are arguments that support a possible causal relationship. First, from membrane dynamics simulation it appeared that the order at the membrane region where the nitroxide moiety of 5-DS resides determines the ability of water and ions to diffuse across a membrane (44). Second, ethanol appears to reside at the lipid-water interface, which, together with the aforementioned permeability control at the upper methylene segment of the acyl chains, renders a direct link between ethanol-induced disorder and leakage plausible.

Adaptation of cells to ethanol would be most effective at the membrane lipid-water interface, where stress proteins are supposed to interact. Adaptive changes in the acyl-chain composition may adjust membrane fluidity in the core region, which might be less effective in the case of ethanol. This is probably the reason for the less consistent picture that emerges from the literature concerning ethanol-induced changes in acyl-chain composition (for a review, see reference 24). In the present work we found a significant increase in the degree of unsaturation of the FAs in cells grown in the presence of 8% (vol/vol) ethanol, which is supposed to lead to a decrease in the gel-to-liquid-crystalline transition temperature and to increased fluidity. However, as determined by ESR, the cytoplasmic membranes in *O. oeni* cells grown in the presence of ethanol were much more rigid than were those in control cells. The same phenomena were observed in *E. coli* cells, and liposomes made from the phospholipids of these cells displayed, as expected, increased fluidity (12). This observation could point to an important role for the protein content of membranes in regulating fluidity. Actually, we found that *O. oeni* cells grown in the presence of ethanol (8%) had a reduced lipid content, i.e., half of that of the control cells. The decreased mobility of the membranes observed in these ethanol-grown cells corroborates the idea that a selective decrease in the lipid content can increase the proportion of motionally hindered lipid molecules (25).

The membrane composition of cells preexposed to 12% (vol/vol) ethanol for 2 h was identical to that of control cells. However, these preexposed cells showed decreased membrane permeability and disordering under ethanol-stress conditions. Thus, these results suggest that ethanol adaptation at the membrane level does not hold for changing membrane lipid composition. Moreover, the increased level of unsaturation observed in cells grown in the presence of ethanol, more than a direct effect of ethanol adaptation, as has been suggested previously (15), is just a consequence of ethanol-induced inhibition of saturated FA synthesis (19, 21).

In this paper we conclude that *O. oeni* cells adjust their membrane permeability during ethanol adaptation by decreasing fluidity at the lipid-water interface. Thus, we hypothesize that the physical state of the membrane, rather than merely the membrane composition, may preclude an important role during ethanol adaptation by controlling other biological process, e.g., ATPase activity (9, 17) and transport systems (7, 46).

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