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Saccharomyces cerevisiae strains with different degrees of ethanol tolerance exhibit different adaptive responses to produced ethanol

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Two *Saccharomyces cerevisiae* strains with different degrees of ethanol tolerance adapted differently to produced ethanol. Adaptation in the less ethanol-tolerant strain was high and resulted in a reduced formation of ethanol-induced respiratory deficient mutants and an increased ergosterol content of the cells. Adaptation in the more ethanol-tolerant strain was less pronounced. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 75–78.

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Introduction

Although the high ethanol tolerance of the yeast *Saccharomyces cerevisiae* has received widespread attention throughout the last five decades, the mechanisms underlying it are still far from understood. It is generally accepted that some *S. cerevisiae* strains are more ethanol-tolerant than others [15], and that every individual *S. cerevisiae* strain will exhibit an adaptive response to ethanol; ie cells of a given strain grown in the presence of ethanol are more ethanol-tolerant than the same cells grown in the absence of ethanol [11,12]. As yet, however, the mechanisms underlying this adaptive response are unclear, and the literature seems not to comprise any comparative studies on the ethanol-adaptive responses of strains with different degrees of ethanol tolerances.

It is well known that respiratory deficient (RD) mutants may occur spontaneously in *S. cerevisiae* [7]. Furthermore, it is well established that ethanol is a powerful inducer of RD mutants in *S. cerevisiae* [8–10,14]. So far, very little knowledge has been gained on the mechanisms leading to ethanol-induced respiratory deficiency. Taking into consideration, however, that ethanol is known to fluidize *S. cerevisiae* membranes [2], and that ethanol-induced RD mutations in *S. cerevisiae* cells are suggested to be caused by damage to the mitochondrial membrane rather than by DNA damage [8], it may be suggested that the membrane fluidizing effect of ethanol is responsible for the formation of ethanol-induced RD mutants in *S. cerevisiae*.

Membrane lipids, including ergosterol, are considered to play an essential role in the adaptive response to ethanol of *S. cerevisiae*, and a vast number of literature references exist on this issue (for recent reviews, see [13,16]). Ergosterol is a well known component of *S. cerevisiae* mitochondrial membranes [17,18] and a well known modulator of membrane fluidity in *S. cerevisiae*; ie a high ergosterol/phospholipid ratio in *S. cerevisiae* is typically associated with a low fluidity of the membranes [2,17]. Thus, it is likely that *S. cerevisiae* is able to adapt to ethanol by increasing its ergosterol content, resulting in a reduced frequency of ethanol-induced RD mutants. The existence of such an adaptive ability in *S. cerevisiae* seems, however, not to be reported.

In this study we show that *S. cerevisiae* strains with different degrees of ethanol tolerance adapt differently to produced ethanol, and that these adaptations are correlated to changes in the frequency of ethanol-induced RD mutants and in the lipid content of the cells.

Materials and methods

Microorganisms

The experiments were carried out with a wild-type strain, *S. cerevisiae* 1200, which is a highly ethanol-producing yeast [5], and a commercial strain of lager yeast, *S. cerevisiae* AJL 2155 (The Collection of Pure Cultures of Brewing Yeasts, Alfred Jørgensen Laboratory Ltd, Copenhagen, Denmark). Aerobic, early stationary phase cultures of these two yeast strains were previously shown to exhibit significantly different ethanol tolerances, as measured by viability; ie *S. cerevisiae* 1200 being more ethanol-tolerant than *S. cerevisiae* AJL 2155 [6]. The yeast strains were maintained at 4°C on YPD agar (containing per liter: 20 g glucose, 20 g peptone, 10 g yeast extract, and 20 g agar).

Media and fermentations

The chemostat fermentations were carried out using defined media (pH 5.0, C-source: glucose; N-source: $(NH_4)_2SO_4$) with ergosterol and oleic acid and with glucose as the growth limiting nutrient (DMD) [3].

Four chemostat experiments (Table 1) were carried out in a 2-L fermentor using the Braun Biostat MD fermenting system (B Braun Biotech Int GmbH, Melsungen, Germany).

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Table 1 Fermentation conditions and ethanol formation

Experiment[a]	Strain	Glucose in feed (% (w/v))	Ethanol in growth medium[b] (% (w/v))
1	AJL2155	2	0.6
2	1200	2	0.6
3	AJL2155	12	3.6
4	1200	12	4.2

[a]In each experiment the cells were grown anaerobically in a chemostat at 30°C, pH 5.0, and a dilution rate of 0.05 h^{-1} using a defined medium with glucose as the limiting growth factor, and cells were harvested at steady state. In each experiment, two steady states were reached.
[b]Values for each experiment are means of two steady states. Maximum variations in the data were ±5%.

In all experiments, the effective fermentor volume was 1.5 L, the dilution rate was 0.05 h^{-1}, the stirring rate was 200 rpm, the temperature was 30°C, and the pH was monitored with an installed Ingold pH electrode (Mettler-Toledo GmbH, Steinbach, Germany) and maintained at 5.0 by the addition of 2 M NaOH. The fermentor was supplied with sterile feed using a Watson Marlow 101 U pump (Watson Marlow, Wilmington, MA, USA). The fermentor was flushed continuously with nitrogen (99.998% purity). *S. cerevisiae* AJL 2155 was grown in experiments 1 and 3, and *S. cerevisiae* 1200 was grown in experiments 2 and 4 (Table 1). The glucose concentration in the medium reservoir was 2% (w/v) in experiments 1 and 2 and 12% (w/v) in experiments 3 and 4 (Table 1). The steady state concentration of produced ethanol in the growth medium was 0.6% (w/v) in experiments 1 and 2 and approximately 4% (w/v) in experiments 3 and 4 (Table 1). The steady state glucose concentration in the growth medium in all four experiments was below 0.05% (w/v) (data not shown). Steady state, by definition, was reached when the cell dry weight (CDW), the glucose concentration, and the ethanol concentration in the growth medium were constant in three consecutive samples withdrawn over a period of 9 h, after at least five residence times with the given conditions in the experiment. In each experiment, two steady states were reached, and from each steady state yeast cells were harvested and treated as described below.

Ethanol shock treatment

The yeast cells were washed twice with sterile water. The cell pellet was resuspended in 10 ml defined medium without glucose (DM) with 18% (v/v) ethanol. In addition, an unstressed control; ie cells suspended in DM without ethanol, was carried out for each cell harvest. Both the ethanol stressed cells and the unstressed control were incubated at 30°C for 2 h in a rotary shaker (100 rpm).

Determination of cell survival

After the 2-h incubation period, the cells were diluted appropriately and plated on YPD agar in triplicate. The plates were incubated for 72 h at 30°C before counting. The survival of cells was taken as the percentage of survivors after exposure to ethanol compared with the unstressed control.

Determination of frequency of RD mutants

RD mutants were identified by overlaying the yeast colonies on the above mentioned plates with a 2,3,5-triphenyltetrazoliumchloride (TTC) staining medium (containing per liter: 0.5 g TTC, 5 g glucose, and 6.25 g agar). After incubation at 30°C for 1 h, RD mutants were identified as white colonies that were unable to reduce the tetrazolium salt from colourless to red. The frequency of RD mutants was taken as the percentage of white colonies compared with the total number of cells on a plate. Thus, the detection limit of this analysis was 0.25%. The respiratory deficiency was confirmed by an inability of the white colonies to grow on YPG agar (containing per liter: 30 ml glycerol, 20 g peptone, 10 g yeast extract, and 20 g agar), and at the same time, an ability to grow on YPD agar [7].

Lipid analyses

The extraction of lipids from the yeast cells was performed as described previously [6]. The separation of phospholipids from neutral lipids and the ergosterol and phospholipid content analyses were performed according to [3].

Miscellaneous analytical procedures

Glucose and ethanol were determined as described in [4], and CDW was determined as described in [6].

Results

Ethanol tolerance

In all four experiments, harvesting at steady state and subsequent exposure of both yeast strains to 18% (v/v) ethanol for 2 h resulted in a loss of viability (Table 2). At a low concentration of produced ethanol in the growth medium, however, the *S. cerevisiae* 1200 cells were more ethanol-tolerant, as measured by viability, than the *S. cerevisiae* AJL 2155 cells (Table 2). The ethanol tolerance of the *S. cerevisiae* AJL 2155 cells increased dramatically, when these cells were grown to steady state in the presence of an increased concentration of produced ethanol, whereas in the *S. cerevisiae* 1200 cells, it only increased slightly (Table 2).

Respiratory deficient mutants

In all four experiments, no spontaneous RD mutants were found in the unstressed controls (data not shown). In all four experiments, however, exposure of the cells to 18%

Table 2 Percentage survival and frequency of respiratory deficient mutants among the survivors after exposure to 18% (v/v) ethanol for 2 h at 30°C of *S. cerevisiae* AJL 2155 and 1200 cells from anaerobic chemostat fermentations

Experiment	Strain	Survival[a] (%)	RD mutants[a] (%)
1	AJL 2155	<0.1	15.4
2	1200	9.2	2.2
3	AJL 2155	20.6	0.7
4	1200	36.3	0.3

[a]Values for each experiment are means of two steady states. Maximum variations in the data were ±10%.

(v/v) ethanol for 2 h resulted in the formation of RD mutants among the survivors (Table 2). One explanation for this formation of RD mutants during ethanol exposure could be that the resulting RD mutants reflected the frequencies of spontaneous RD mutants in the experiments, and that these frequencies were so low that they were not possible to detect in the unstressed controls due to the detection limit of the analysis (0.25%); ie the apparent increase in RD mutants could simply be due to a decline in parental strains. This explanation, however, is not consistent with the facts that parental strains are more ethanol-tolerant, as measured by viability, than their RD mutants [1,10], and that ethanol is a strong inducer of RD mutants in *S. cerevisiae* [8–10,14]. Thus, our results suggest that the formation of RD mutants among the surviving yeast cells in each experiment was induced by the ethanol exposure.

At a low concentration of produced ethanol in the growth medium, the frequency of ethanol-induced RD mutants was lower in the *S. cerevisiae* 1200 cells as compared to the *S. cerevisiae* AJL 2155 cells (Table 2). The frequency of ethanol-induced RD mutants in the *S. cerevisiae* AJL 2155 cells decreased markedly with the raised concentration of produced ethanol, whereas in the *S. cerevisiae* 1200 cells, it only decreased slightly (Table 2).

Lipid content

At a low concentration of produced ethanol in the growth medium, no differences in the lipid contents between the two yeast strains could be observed (Table 3). When the ethanol concentration in the growth medium was increased, the ergosterol/phospholipid ratio increased in the *S. cerevisiae* AJL 2155 cells, due primarily to a decrease in the phospholipid content, whereas it did not change in the *S. cerevisiae* 1200 cells (Table 3).

Discussion

In this study the effect of produced ethanol on the ethanol tolerance, the frequency of ethanol-induced RD mutants, and the lipid content in *S. cerevisiae* has been investigated using steady state cells from anaerobic, glucose-limited chemostat cultures. The cells have been grown at a constant specific growth rate of 0.05 h^{-1}, thereby avoiding growth rate-dependent changes of the ethanol tolerance, the frequency of ethanol-induced RD mutants, and the lipid content.

We have previously shown that the two yeast strains used in this study exhibit different ethanol tolerances, as determined by viability; ie *S. cerevisiae* 1200 being more ethanol-tolerant than *S. cerevisiae* AJL 2155 [6]. In our previous study, however, we used aerobic, early stationary phase cultures of the two yeast strains for the ethanol tolerance experiments [6]. In our present work, using a different experimental set-up for the growth experiments but exactly the same method for the ethanol tolerance experiments, a different picture emerges. When grown in the presence of a low concentration of produced ethanol, the *S. cerevisiae* 1200 cells are more ethanol-tolerant than the *S. cerevisiae* AJL 2155 cells (Table 2). When grown in the presence of a higher concentration of produced ethanol, however, the less ethanol-tolerant strain of *S. cerevisiae* exhibits a very high adaptive response to produced ethanol, whereas the adaptive response in the more ethanol-tolerant strain is less pronounced (Table 2). In fact, in the presence of an increased concentration of produced ethanol, the two yeast strains almost exhibit similar degrees of ethanol tolerance, due to the higher ethanol adaptive response in the less ethanol-tolerant strain. Thus, our results clearly demonstrate that the adaptive response in *S. cerevisiae* to produced ethanol is strain-dependent.

Furthermore, the results in Table 2 show that the more ethanol-tolerant strain is more resistant to the formation of ethanol-induced RD mutants than the less ethanol-tolerant strain, when grown in the presence of a low concentration of produced ethanol. These results agree with previously reported findings [6,9,10]. Concurrently, the results in Table 2 seem to be the first of their kind, suggesting that a less ethanol-tolerant strain adapts to produced ethanol, resulting in a reduced formation of ethanol-induced RD mutants, whereas the change in the more ethanol-tolerant strain is less pronounced. In addition, the two yeast strains exhibit differences in their way of changing lipid content in response to the increased ethanol concentration; ie where the less ethanol-tolerant strain increases its ergosterol/phospholipid ratio, no changes occur in the more ethanol-tolerant strain (Table 3). These results indicate that the ergosterol/phospholipid ratio is involved in the ethanol-adaptive response of the less ethanol-tolerant strain. An increase in ergosterol content in response to increased ethanol concentrations in *S. cerevisiae* has previously been reported [3,13], and our findings suggest that the increased ergosterol/phospholipid ratio causes a decreased membrane fluidity [2,17], thereby resulting in a better counteraction against the membrane fluidizing effect of ethanol [2], and thus a reduced frequency of ethanol-induced RD mutants.

In conclusion, the results of the present work demonstrate that the adaptive response to produced ethanol is high in a less ethanol-tolerant *S. cerevisiae* strain, whereas it is less pronounced in a more ethanol-tolerant strain. Furthermore, they suggest that the less ethanol-tolerant strain adapts to produced ethanol in a way which causes a reduced formation of ethanol-induced RD mutants. Finally, our results indicate that the ergosterol content of the cells may be involved in this adaptive response to produced ethanol.

Table 3 Phospholipid and ergosterol content in *S. cerevisiae* AJL 2155 and 1200 cells from anaerobic chemostat fermentations

Experiment	Strain	Ratio of[a]		
		PL:CDW (mg g^{-1})[b]	E:CDW (mg g^{-1})	E:PL (mg mg^{-1})
1	AJL 2155	29.5	1.9	0.06
2	1200	33.2	1.9	0.06
3	AJL 2155	20.5	2.4	0.12
4	1200	34.0	1.8	0.05

[a]Values for each experiment are means of two steady states. Maximum variations in the data were ±10%.
[b]In the calculations a mean molecular weight of 760 g mol^{-1} was used for the phospholipids. PL, phospholipid; E, ergosterol; CDW, cell dry weight.

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