Oxygen is required to restore flor strain viability and lipid biosynthesis under fermentative conditions

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Abstract

To further elucidate the biosynthesis of lipids in flor strains under fermentative conditions, the transcription levels of the lipid biosynthetic genes ACS1, ACS2, ACC1, OLE1, ERG1, ERG11, ARE1 and ARE2, as well as the lipid composition and cell viability of a flor strain were compared with that of a non-flor strain during hypoxic and aerobic fermentations in the absence of lipid nutrients. While no significant differences in transcription levels or lipid compositions were observed between the two strains when oxygen was not limiting, significant differences were seen during hypoxic fermentation. In this last condition, the flor strain, in spite of higher levels of transcription of hypoxic genes, lost the abilities to desaturate fatty acids and complete ergosterol biosynthesis, and showed a dramatic loss of viability. In contrast, the non-flor strain, which showed lower transcription levels, was able to reach a balanced lipid composition and maintained a higher cell viability. One possible explanation is that the flor strain requires a higher amount of oxygen than the non-flor strain in order to carry out the oxygen-dependent steps of lipid biosynthesis under fermentative conditions.

Introduction

The structural and functional properties of the plasma membrane and the activities of proteins and enzymes involved in transport and stress perception are considerably influenced by the lipid composition of the cell (Chapman, 1975; Quinn & Chapman, 1980). The yeast Saccharomyces cerevisiae derives cell lipids via the assimilation of external nutritional supplies (Thurston et al., 1981; Herraiz et al., 1990) and/or the activation of lipid biosynthesis that requires acetyl-CoA (Parks, 1978; Schweizer et al., 1984). When mitochondrial activity is absent, acetyl-CoA is produced in the cytoplasm by the enzyme acetyl-CoA synthase (ACS), which uses acetic acid as a substrate. The activity of ACS of S. cerevisiae varies according to the growth conditions. Two genes, ACS1 and ACS2, are known to encode the ACS isoenzymes, which are differently regulated: ACS1 is repressed by glucose and anaerobiosis, while ACS2 is not. Thus, ACS2 is essential for lipid biosynthesis in the absence of oxygen and in the presence of excess glucose (van der Berg et al., 1996; de Jong-Gubbels et al., 1997). The two gene products are inhibited by long-chain fatty acyl esters (van der Berg et al., 1996), and they differ with respect to their kinetic properties and acetate affinities.

Acetyl-CoA is carboxylated to malonyl-CoA by acetyl-CoA carboxylase (ACC; encoded by ACC1) (Hasslacher et al., 1993), with the elongation of fatty acid chains, which takes place on the fatty acid synthase (FAS) complex in the cytoplasm, started by ACC (Fig. 1). The final product is palmitoyl-CoA, which is released by the FAS complex, and that can be elongated to stearoyl-CoA. Palmitic (C16:0) and stearic (C18:0) acids are transformed into palmitoleic (C16:1) and oleic (C18:1) acids by the oxygen-dependent Δ⁹-desaturase, which is encoded by the OLE1 gene (Stukey et al., 1989; Kandasamy et al., 2004).

Acetyl-CoA is also needed for sterol synthesis, because it is the precursor of hydroxymethylglutaryl-CoA (HMG-CoA), the substrate of the regulatory enzyme of the pathway,
HMG-CoA reductase (encoded by the HMG1/2 genes). This leads to the formation of mevalonic acid, from which squalene can be produced (Parks, 1978) (Fig. 1). Cyclization of squalene to lanosterol is catalysed by the O2-dependent squalene epoxydase, which is encoded by ERG1 (Lees et al., 1995). Furthermore, the demethylation of lanosterol to ergosterol, which is catalysed by Erg1p, requires oxygen.

The maintenance of a low level of free sterols is a critical factor for cell growth, and the synthesis of sterol esters depends on the availability and requirement of sterols within the cell. The genes that encode the enzymes devoted to the accumulation of sterol esters are ARE1 and ARE2. Are1p is mainly involved in the esterification of sterol intermediates that accumulate in hypoxia. Are2p is the key enzyme in the production of sterol esters under aerobicosis. Moreover, while Are2p shows preferential activity towards ergosterol, the Are1p activities have similar efficiencies for ergosterol and its precursors. The accumulation of sterol esters can be considered as a survival mechanism that is involved in the detoxification of excess sterols, sterol precursors and/or fatty acids, which can lead to membrane perturbations (Mullner & Daum, 2004).

Flor yeasts are S. cerevisiae wine strains able to shift from fermentative to oxidative metabolism depending on the nutritional and environmental conditions. In particular, at the end of the alcoholic fermentation, when the sugar is depleted, they are able to form a biofilm (flor) on the free surface of the wine and to oxidize low amounts of ethanol to acetaldehyde. In addition, they create a reducing environment in the wine under flor and are, for these reasons, used mainly for biological wine ageing in the presence of oxygen (Farris et al., 1993; Valero et al., 2002). They are also able to carry out alcoholic fermentation while at the bottom of the fermenting vessel. Thus, their utilization as starters for fermentation in Sherry-like wines has also been suggested in order to reduce the total time required for fermentation and ageing (Zara et al., 2008b). However, the use of flor strains as fermentation starters is impaired under oxygen-limiting conditions and in the absence of lipid nutrients. Under these conditions, flor strains are unable to attain a balanced lipid composition and they undergo a dramatic loss of viability (Mannazzu et al., 2008).

In this context, with the aim of further elucidating the biosynthesis of lipids in flor strains under fermentative conditions, the transcription levels of the lipid biosynthetic genes ACS1, ACS2, ACC1, OLE1, ERG1, ERG11, ARE1 and ARE2 as well as the lipid composition and cell viability of a flor strain were analysed and compared with that of a non-flor strain during hypoxic and aerobic fermentations.

### Materials and methods

#### Strains and culture conditions

The S. cerevisiae strains used were L2056, a commercial oenological strain (Lallemand, Montreal, Canada) and M25, a flor strain belonging to the Culture Collection of DiS-AABA (Dipartimento di Scienze Ambientali Agrarie e Biotecnologie Agroalimentari, University of Sassari, Sardinia, Italy), which is used for industrial must fermentation and wine ageing. Bench-scale fermentations were carried out in triplicate in SJ medium (2 g L⁻¹ YNB w/o amino acids, 7 g L⁻¹ ammonium sulphate, 120 g L⁻¹ glucose, 120 g L⁻¹ fructose, 30 mg L⁻¹ leucin, 20 mg L⁻¹ histidine and 20 mg L⁻¹ uracil). Briefly, the yeast strains were precultured aerobically in YEPD (20 g L⁻¹ glucose, 10 g L⁻¹ yeast extract, 20 g L⁻¹ peptone; liquid : air ratio, 1 : 10), and then 5 x 10⁵ cells mL⁻¹ were inoculated into 100-mL flasks containing 75 mL SJ medium (liquid : air ratio, 7.5 : 10). These were then incubated at 20 °C statically (hypoxic synthetic juice, HSJ) or in a shaker (150 r.p.m.) (oxygenated synthetic juice, OxSJ). The flasks, incubated statically, were equipped with glass capillary stoppers, and Oxoid strips saturated with resazurin (Anaerobic Indicator BR0055B, Oxoid Ltd, Basingstoke, UK) were fixed to the glass capillary stoppers in the headspace of the flask. The colour change from pink to white indicates a lack of oxygen in the headspace of each flask. Yeast growth was determined by the total and viable counts. On the basis of the analyses of the
growth curves, sampling times corresponding to the mid-exponential phase (MEP), early stationary phase (ESP), late stationary phase (LSP) and death phase (DP) were individuated under both cultural conditions. A flaks was inoculated for each sampling time, and cells were analysed for the determination of residual respiratory activity, lipid composition and the transcription levels of genes involved in lipid biosynthesis.

Respiratory activity

Cell samples were collected on days 1, 2, 3, 4 and 7, harvested by centrifugation at 4 °C and suspended in a volume of cold water twice the pellet volume. Oxygen uptake was measured at 30 °C using a Clark-type oxygen electrode in a 1-mL stirred chamber containing 1 mL of air-saturated respiration buffer (0.1 M-phthalate-KOH, pH 5.0) (Oxygraph System, Hansatech Instruments, UK). Antimycin A (AA), an inhibitor of the respiratory chain, was added in the reaction vessel at the concentration of 2 μM. In order to avoid de novo synthesis of respiratory structures induced by the contact with oxygen, specific inhibitors of cytoplasmic and mitochondrial protein synthesis were added. Erythromycin, which inhibits mitochondrial protein synthesis, was added at a concentration of 2 mg mL⁻¹; cycloheximide, an inhibitor of cytoplasmic protein synthesis, was added at a concentration of 50 μg mL⁻¹. Respiratory rates were expressed as nmol of O₂ consumed min⁻¹ mg⁻¹ of dry weight. Dry mass was determined by weighing 1 mL of cell suspension oven-dried overnight at 90 °C. The respiratory activity values were evaluated in two independent experiments.

Cell lipid extraction

Cells were collected by centrifugation (5 min at 625 g), washed in sterile water and freeze dried. Lipid extraction was performed according to Taylor & Parks (1978), modified as described by Belviso et al. (2004). Briefly, 1.3 mL of 2.4 g L⁻¹ pentadecanoic acid in chloroform was added as the internal standard. The lipid extract was dried in a rotary evaporator (Rotovapor, Laborota 4000, Heidolos Instruments, Schwabach, Germany) and dissolved in 5 mL chloroform for storage at − 25 °C (for no longer than 2 weeks).

Determination of cellular fatty acid content

Cell samples were collected as described for lipid extraction. The methyl esters of the fatty acids were obtained according to Christie (1982), and then analysed by GC (DANI GC 1000 DPC, equipped with an FID detector, DANI Instruments, Milan, Italy) on a DB-5 capillary column (30 m length, 0.25 mm i.d., 0.25 μm film thickness) (J&W Scientific Inc., Folson, CA). The operating conditions were as follows: temperature from 80 to 120 °C at 4 °C min⁻¹, from 120 to 220 °C at 5 °C min⁻¹, from 220 to 280 °C at 7 °C min⁻¹ and 280 °C for 10 min; injector temperature 290 °C; detector temperature 290 °C; carrier gas nitrogen; carrier gas flow 1.0 mL min⁻¹; and injection volume 2 μL. The results were expressed as mg g⁻¹ dry weight of the cells.

Determination of cellular sterols

Cell samples were collected as described for lipid extraction. Sterols were analysed by HPLC, as already described (Xu et al., 1988). One millilitre of each lipid extract was dried in a rotary evaporator (Rotovapor) and then dissolved in 1 mL toluene. The lipids were saponified with 4 mL 10% KOH in 90% ethanol at room temperature overnight. Then, after the addition of 10 mL water, the unsaponified fraction was extracted three times by gently shaking for 3 min with 15 mL diethyl ether. The organic phase was dried in a rotary evaporator (Rotovapor), and then dissolved in isopropanol. Ergosterol, lanosterol and squalene were separated by HPLC (Jasco PU-980, equipped with a Jasco UV-2075 Plus detector, Jasco, Tokyo, Japan) on a Supelco LC-18 column (15 cm × 4.6 mm; particle size, 5 μm, Supelco, Sigma-Aldrich, St. Louis, MO). The operating conditions were as follows: methanol: water mobile phase (96:4; v/v); flow rate, 1 mL min⁻¹; injection volume, 20 μL; and detection at λ 205 nm. The results were expressed as mg g⁻¹ dry weight of cells.

Gene transcription analysis

For each strain, 10⁶ cells were collected and subjected to RNA extraction and reverse transcription using the following kits: RNAqueous® – 4 PCR (Ambion, TX) and SuperScript™ First-Strand Synthesis System for real-time PCR (Invitrogen, Breda, the Netherlands). The cDNA was quantified using an iCycler iQ real-time PCR detection system (Bio-Rad Laboratories, Milan, Italy). The reaction mix contained fluorescein 10 nmol L⁻¹, forward primer 0.2 μmol L⁻¹, reverse primer 0.2 μmol L⁻¹ and 50 ng cDNA template in Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, Cat No.11733-038). The primer pairs used are listed in Table 1. The reaction conditions were as follows: one cycle at 95 °C for 3 min, 50 cycles at 95 °C for 20 s, 60 °C for 30 s and 72 °C for 30 s. The final step was one cycle at 72 °C for 3 min. Transcription data from different samples were normalized using the ACT1 gene transcripts, as already described (Zara et al., 2008a). The results are means ± SDs of five repeats from two independent experiments.

Data analysis

Unless otherwise stated, all experiments were carried out in triplicate from independent precultures. Statistical analyses of the data were performed using ANOVA, followed by the Tukey-Kramer HSD test (all-pair comparison) using the JMP version 3.1.5 software (SAS Institute Inc., Cary, NC).
Chemicals
Where not otherwise stated, all of the chemicals were purchased from Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany). Organic solvents and pentadecanoic acid were from Fluka Chemie GmbH (Buchs, Switzerland).

Results

Growth kinetics, cell viability and oxygen consumption rates
The growth kinetics of the two strains were followed by the total and viable counts during the adaptation to progressive oxygen depletion (HSJ) and under aerobic conditions (OxSJ). Four growth phases were observed under both culture conditions: MEP, ESP, LSP and DP (Fig. 2). Oxygen availability had a positive effect on cell growth. In OxSJ, the two strains reached cell numbers that were about the same as those produced in HSJ. Moreover, as observed by Valero et al. (2002), oxygen availability led to significant increases (P < 0.05) in viable plate counts and in the numbers of generations. In particular, M25 and L2056 produced 7.2 and 7.1 generations in OxSJ, while they reached 5.1 and 4.9 generations in HSJ, respectively.

The resazurin test indicated that the two strains differed in the time required for the consumption of the oxygen in the headspace of the flask during growth in HSJ. In particular, the M25 strain showed oxygen depletion immediately after entry into the ESP (days 4–5 of fermentation), while strain L2056 underwent oxygen depletion at the LSP (days 6–7 of fermentation). Cells of the two strains, sampled during growth in HSJ, also showed differences in the potential respiratory capability, expressed as the oxygen consumption rate (Fig. 3). In particular, in L2056 the oxygen consumption rate remained unchanged until the third day of fermentation, while in M25 it decreased significantly after entry into the ESP.

Table 1. Primer pairs utilized for RT-PCR reactions

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>ACT1</td>
<td>5’-CGTCCGTTGAGATGTT-3’</td>
<td>5’-TACAGGTCGCCGTT-3’</td>
</tr>
<tr>
<td>ACS1</td>
<td>5’-GCTGACCCGTCGTT-3’</td>
<td>5’-GCTGACCCGTCGTT-3’</td>
</tr>
<tr>
<td>ACS2</td>
<td>5’-CTGACCCGTCGTT-3’</td>
<td>5’-CTGACCCGTCGTT-3’</td>
</tr>
<tr>
<td>ACC1</td>
<td>5’-GAAGGGAAAGTTGTT-3’</td>
<td>5’-GAAGGGAAAGTTGTT-3’</td>
</tr>
<tr>
<td>ARE1</td>
<td>5’-GCTGACCCGTCGTT-3’</td>
<td>5’-GCTGACCCGTCGTT-3’</td>
</tr>
<tr>
<td>ARE2</td>
<td>5’-GCTGACCCGTCGTT-3’</td>
<td>5’-GCTGACCCGTCGTT-3’</td>
</tr>
<tr>
<td>OLE1</td>
<td>5’-ACCCAGCGGTATCCGTT-3’</td>
<td>5’-ACCCAGCGGTATCCGTT-3’</td>
</tr>
<tr>
<td>ERG1</td>
<td>5’-ATCCAGCGGTATCCGTT-3’</td>
<td>5’-ATCCAGCGGTATCCGTT-3’</td>
</tr>
<tr>
<td>ERG2</td>
<td>5’-CGGCACTGCTTGTT-3’</td>
<td>5’-CGGCACTGCTTGTT-3’</td>
</tr>
</tbody>
</table>

Fig. 2. Total and viable counts of the M25 flor strain and the L2056 commercial oenological strain under progressive oxygen depletion (HSJ) and aeration (OxSJ). Closed circles and diamonds indicate sampling times corresponding to MEP, ESP and LSP under both conditions of fermentation. Data are means ± SD of three independent experiments.

Fig. 3. Oxygen consumption rates in the M25 (squares) and the L2056 (diamonds) strains sampled during hypoxic fermentations. Cell samples were collected at days 1, 2, 3, 4 and 7 and both total (closed symbols) and AA-insensitive (open symbols) oxygen consumption rates were analysed. A and B, M25 total and AA-insensitive oxygen consumption rates, respectively. C and D, L2056 total and AA-insensitive oxygen consumption rates, respectively. Data are means ± SD of two independent experiments. Where not visible, the SDs lie within the symbols.
of fermentation (ESP) in contrast to M25, which showed a strong reduction of oxygen consumption rate after day 2. In the two strains, reductions in oxygen consumption rates were concomitant with reductions in the percentages of viable/total cells to about 60%. Interestingly, the two strains retained the ability to carry out respiratory oxygen consumption in the presence of very high sugar concentrations. In contrast, oxygen consumption was undetectable in the laboratory strain BY4743 under the same culture conditions (data not shown). This indicates that both wine strains are less sensitive to glucose repression than laboratory strains, which do not show any detectable respiratory activity due to glucose repression of mitochondrial enzyme biosynthesis (Slonimski, 1953).

M25 also showed a peak in the nonrespiratory (AA insensitive) oxygen consumption rate (Ferrero et al., 1981) on day 2, while this remained unchanged in L2056. Biofilm formation was prevented under oxygen depletion.

**Transcription of genes involved in lipid biosynthesis during progressive oxygen depletion**

As expected, due to an excess of glucose (Kratzer & Schueller, 1995), during growth in HSJ, ACS1 transcription was repressed in the two strains (data not shown). The L2056 and M25 strains showed similar trends for ACS2 and ACC1 transcription, with a peak in the transcription levels of the two genes at ESP (Fig. 4). Both strains induced significant transcription of OLE1 ($P < 0.05$), in agreement with the hypoxic regulation of this gene, which depends on a low-oxygen responsive element located in the promoter (Nakagawa et al., 2001; Jiang et al., 2002; Rosenfeld & Beauvoit, 2003) (Table 2). M25, which was the first to undergo oxygen depletion, showed the highest levels of OLE1 transcription. Potentially due to the observed lack of oxygen, M25 also significantly increased ERG1 transcription at ESP (Fig. 5). The two strains also showed an increase in ERG11 transcription levels at ESP, which was followed by a decrease at LSP in M25. ERG11 induction in M25 and L2056 was in agreement with the hypoxic regulation of this gene. Although they differed in relative abundance, the transcripts of the ERG1 and ERG11 genes appeared to be coregulated in M25 but not in L2056 (Fig. 5). ARE1 and ARE2 showed a peak at ESP for the M25 strain, while L2056 did not show marked variations in the transcription levels of these two genes (Fig. 5).

**Transcription of genes involved in lipid biosynthesis during aerobic fermentation**

While ACS1 transcription is severely repressed by lack of oxygen and high glucose concentrations (Kratzer & Schueller, 1995), that of ACS2 is not. Accordingly, the transcription of ACS1 was repressed in OxSJ (data not shown), while that of ACS2 was induced at MEP in the two strains and showed significant decreases in the subsequent growth phases. For M25, ACC1 and ACS2 showed a similar trend in transcription levels, thus suggesting an active production of malonyl-CoA at MEP in this strain (Fig. 4). L2056 did not show any variation in the transcription levels of ACC1. OLE1, which encodes the $\Delta^9$-desaturase involved in the biosynthesis of palmitoleic (C16:1) and oleic (C18:1) acids in *S. cerevisiae* (Stukey et al., 1989), showed limited variations in the transcription level in the two strains (Table 2). Similarly, ERG1, ERG11, ARE1 and ARE2 were transcribed at basal levels and did not show significant variations under aerobic conditions (data not shown).

![Fig. 4](https://academic.oup.com/femsyr/article-abstract/9/2/217/568634/221)

**Table 2.** $\text{OLE1}$ transcription levels in HSJ and OxSJ

<table>
<thead>
<tr>
<th>Growth phase</th>
<th>HSJ</th>
<th>L2056</th>
<th>M25</th>
<th>L2056</th>
</tr>
</thead>
<tbody>
<tr>
<td>M25 L2056</td>
<td>MEP 1.07 ± 0.06^a 0.54 ± 0.54^a 1.12 ± 0.00^a 0.56 ± 0.05^a</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>ESP 44.84 ± 6.41^b 10.85 ± 1.26^b 1.13 ± 0.22^a 1.36 ± 0.06^b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LSP 122.48 ± 9.57^c 17.92 ± 3.49^c 0.64 ± 0.06^b 1.71 ± 0.12^c</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

The transcription levels were normalized with respect to those of actin. Within the dataset for each strain, different letters indicate statistically different values ($P \leq 0.05$).
Lipid content

The comparison of cell lipid compositions under the two cultural conditions indicated that both strains take advantage of oxygen availability. In particular, under aerobic conditions, a significant increase in the total fatty acid (TFA) content was observed in L2056, which maintained a higher unsaturated fatty acid (UFA)/TFA ratio at ESP and LSP, with respect to oxygen depletion (HSJ). Similarly, in the presence of oxygen, M25 attained a UFA/TFA ratio that was comparable to that shown by L2056 in OxSJ (Fig. 6).

However, at MEP, irrespective of the availability of oxygen (as indicated by the resazurin test), M25 showed drastic differences in the UFA/TFA ratio under the two culture conditions, with respect to L2056. As supported by the role of oxygen in the maintenance of sterol biosynthesis, during the whole fermentation process, the two strains showed higher percentages of ergosterol in OxSJ, as compared with HSJ.

In HSJ, the flor strain was unable to attain a balanced lipid composition in terms of the UFA/TFA ratio (Mannazzu et al., 2008) in spite of the drastic increase in the OLE1 level of transcription. OLE1 induction by anoxia seems to

Discussion

Under oxygen deprivation, both strains needed to induce ACS2 at ESP to modulate their cellular lipid compositions in response to the progressive increase in ethanol levels (Chi & Arneborg, 1999), but they differed in ACC1 transcription. In particular, in M25, ACC1 transcription appeared to be insensitive to fatty acid repression; thus, this strain produced higher amounts of TFAs than L2056 during hypoxic fermentation. In contrast, in L2056, the transcription levels of this gene were regulated by fatty acid contents (Kamiryo et al., 1976).

In HSJ, the flor strain was unable to attain a balanced lipid composition in terms of the UFA/TFA ratio (Mannazzu et al., 2008) in spite of the drastic increase in the OLE1 level of transcription. OLE1 induction by anoxia seems to
depend on several factors, among which are the carbon source, genetic background, growth phase, the presence of UFAs and the length of time that cells are exposed to low oxygen levels (Martin et al., 2007). This gene is regulated at the levels of transcription and mRNA stability through distinct promoter elements (Martin et al., 2007) and two homologous endoplasmic reticulum proteins, Spt23p and Mga2p, which work as transcriptional coactivators (Hitchcock et al., 2001). The variations in membrane fluidity may be involved in the proteolytic maturation of Spt23p and Mga2p and therefore in the transcriptional activation of OLE1 (Nakagawa et al., 2003). In this context, differences in UFA/TFA and C16/TFA ratios could also be involved in the different regulation of OLE1 in M25 and L2056.

The discrepancy between the OLE1 transcription level and the UFA/TFA ratio could be due either to OLE1 posttranscriptional and/or posttranslational regulations or to the requirement of higher amounts of oxygen of the Ole1p of M25 compared with the one of L2056. Accordingly, the L2056 strain retained the ability to desaturate fatty acids despite the lower transcription level of this gene. This behaviour could be related to the observed differences in oxygen consumption as indicated by the resazurin test.

In M25, the increase in ERG1 transcription at ESP was accompanied by decreases in the squalene content (Mannazzu et al., 2008). Leber et al. (2001) reported that ERG1 transcription is regulated by ergosterol via a feedback mechanism. Accordingly, the ergosterol content shown by L2056 at MEP could be responsible for ERG1 repression. Differences were also seen in the levels of transcription of ERG11 in the two strains. In M25, the decrease of ERG11 transcription seen at LSP may be due to additional regulation mechanisms of this gene, as has been suggested by other studies (Jahnke & Klein, 1983; Daum et al., 1998; Leber et al., 2001; Valachovič et al., 2001; Higgins et al., 2003), and led to an increase in the lanosterol content and a consequent decrease in the ergosterol content (Veen et al., 2003; Mannazzu et al., 2008). In L2056, the progressive increase in the transcription level of ERG11 would be expected as a consequence of the oxygen depletion and the accumulation of ergosterol precursors.

The transcriptional profiles of ARE1 and ARE2 were similar to those of ERG1 and ERG11 in M25, thus suggesting that the induction of enzymes devoted to the disposal of metabolic intermediates of lipid biosynthesis may be required in response to the impairment of ergosterol biosynthesis caused by the lack of oxygen.

Thus, the two strains differed in terms of gene transcription levels in HSJ. However, the inability of M25 to reach a UFA/TFA ratio comparable to L2056 and its low ergosterol content were not due to low levels of transcription of the hypoxic genes OLE1, ERG1 and ERG11, but appeared to be a consequence of an inefficient utilization of oxygen in oxygen-dependent steps in lipid biosynthesis. Even though the two strains were potentially able to utilize oxygen for lipid biosynthesis (see AA-insensitive oxygen consumption rates) during hypoxic fermentation, this mechanism seemed to work in L2056 but not in M25. This could be due to the fact that flor strains have higher oxygen requirements than non-flor strains. Accordingly, during aerobic fermentations, the flor strain increased cell viability and biomass production and reached a cell lipid composition that was similar to that of L2056. This could be due, at least in part, to the transcription levels of ACS2 at MEP. Acs2p is essential for acetyl-CoA biosynthesis in the presence of high sugar concentrations, and it has a central role in the metabolic network. Thus, the transcription levels of ACS2 at MEP in OxSJ are in agreement with the increased biosynthetic requirements of the two strains in the presence of oxygen. The subsequent decreases in ACS2 transcription levels appear to be related to the reduction in biosynthetic activity at the end of the exponential phase. The levels of ACC1 transcription shown by the two strains were related to the fatty acid contents in the presence of oxygen (Valero et al., 2002; Mannazzu et al., 2008). In particular, according to Kamiyori et al. (1976), fatty acid accumulation at MEP appeared to regulate ACC1 transcription in the following phases.

OLE1, ERG1 and ERG11, which are subjected to hypoxic regulation (Turi & Loper, 1992; Defranoux et al., 1994; Kwast et al., 1998; Nakagawa et al., 2001; Jiang et al., 2002; Rosenfeld & Beauvoit, 2003), were repressed in both strains in OxSJ. In agreement with Kwast et al. (1998), who reported that oxygen availability ensures a high flux of metabolites through the oxygen-dependent steps of lipid biosynthesis, the two strains were able to efficiently desaturate fatty acids and facilitate the oxygen-dependent steps of sterol biosynthesis. Thus, the lack of induction of the transcription of hypoxic genes in OxSJ is symptomatic of the full functionality of the metabolic pathways involved in the biosynthesis of UFAs and sterols. Similarly, a basal level
of expression of ARE1 and ARE2 was sufficient to support sterol ester accumulation when oxygen was available. Accordingly, the two strains showed a higher accumulation of neutral lipids in OX5J, when lipid biosynthesis was more active. These results indicate similar behaviours of the flor and non-flor strains when oxygen is largely available.

In summary, our results are consistent with those obtained by Valero et al. (2002), who reported that when biofilm formation is impaired, flor yeasts reduce dramatically the UFA content. In particular, starting from MEP, in spite of the higher levels of transcription of the hypoxia genes, M25 was unable to redirect oxygen utilization towards fatty acid desaturation and sterol biosynthesis, similar to that observed in anaerobically grown cells by Rosenfeld et al. (2003).

In contrast, under aerobic fermentation, the two strains showed similar levels of transcription of the genes analysed and the flor strain attained a lipid composition that was comparable to that of the non-flor strain in terms of the UFA/TFA and C16/TFA ratios and the ergosterol content.

One possible explanation is that the flor strain requires a higher amount of oxygen than the non-flor strain in order to carry out the oxygen-dependent steps of lipid biosynthesis under fermentative conditions.

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