Functional roles of the fatty acid desaturases encoded by KLOLE1, FAD2 and FAD3 in the yeast Kluyveromyces lactis

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Functional properties of cell membranes depend on their composition, particularly on the relative amount of saturated, unsaturated and polyunsaturated fatty acids present in the phospholipids. The aim of this study was to investigate the effect of cell membrane composition on cell fitness, adaptation and stress response in Kluyveromyces lactis. To this purpose, we have deleted the genes FAD2 and FAD3 encoding Δ12 and Δ3 desaturases in Kluyveromyces lactis, thus generating mutant strains with altered fatty acid composition of membranes. These strains were viable and able to grow in stressing conditions like hypoxia and low temperature. Deletion of the Δ9 desaturase-encoding gene KLOLE1 resulted in lethality, suggesting that this enzyme has an essential role in this yeast. Transcription of the desaturase genes KLOLE1, FAD2 and FAD3 and cellular localization of the corresponding enzymes, have been studied under hypoxia and cold stress. Our findings indicate that expression of these desaturase genes and membrane composition were modulated by hypoxia and temperature stress, although the changes induced by these and other assayed conditions did not dramatically affect the general cellular fitness.

INTRODUCTION

Fatty acids (FAs) are essential components of functional cytoplasmic and organelle membranes, but they are also involved in carbon source storage and protein modification. Proteins and lipids are the major components of cell mass and the carbon flux through the FAs biosynthetic pathway is relevant in cell proliferation. Acetyl-CoA and NADPH are the substrates consumed in FAs biosynthesis: acetyl-CoA is carboxylated by acetyl-CoA carboxylase to generate malonyl-CoA. Malonyl-CoA and NADPH are used by the FA synthase to elongate the carbon backbone and reduce the carbonyl groups, respectively, to generate long chain FAs. Interestingly, these enzymes are considered major targets in cancer therapies (Beckers et al., 2007; Flavin et al., 2010). Membrane FAs vary in length and in number of double bonds (desaturation) depending on the species and also on the environmental conditions. A proper FAs membrane composition is critical for membrane function and consequently for cell viability. Unsaturated FAs (UFAs) and polyunsaturated FAs (PUFAs) determine functional membrane properties like fluidity, but are also precursors of molecules with important biological activities in mammals (Funk, 2001). Many PUFAs, which cannot be synthesized

Abbreviations: DOT, dissolved oxygen tension; ER, endoplasmic reticulum; FAs, fatty acids; FAMEs, fatty acid methyl esters; LFH, long flanking homology; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids; SFH, short flanking homology; UFAs, unsaturated fatty acids; UI, unsaturation index.

Six supplementary figures are available with the online Supplementary Material.
by humans but have to be assimilated from the diet, are called essential FAs. They have been recognized to be beneficial for many chronic diseases, such as cardiovascular diseases, cancer and mental illnesses (for an overview, see Riediger et al., 2009), however in general essential FAs are not abundant in natural foods.

The yeast *Saccharomyces cerevisiae* has always been considered a useful model organism for studies of higher eukaryotes. For example, repression of respiration induced by glucose, i.e. the Crabtree effect (Crabtree, 1929), has been compared to the aerobic glycolysis of cancer cells, i.e. the Warburg effect (Warburg, 1956), and many other common features between carbon metabolism regulation in this yeast and tumour cells have been identified (Diaz-Ruiz et al., 2011). Although studies on lipid metabolism have been conducted in *S. cerevisiae* (Natter & Kohlwein, 2013), this is not always the appropriate model yeast to study the properties of FAs conserved in evolution. *S. cerevisiae* is a very specialized and simple yeast in which biosynthesis of FAs is restricted to saturated and mono-unsaturated FAs (MUFAs), essentially accomplished by acetyl-CoA carboxylase and FA synthase enzymes, that synthesize palmitic and stearic acids, and a unique Δ9 desaturase enzyme (Ole1) that produces only the monounsaturated palmitoleic and oleic acids.

In other yeasts, like *Kluyveromyces lactis*, the balance of glucose metabolism between respiration and fermentation does not depend on glucose concentration (Crabtree-negative yeasts) but on oxygen availability (Kiers et al., 1998). The composition of their FAs is enriched with the polyunsaturated linoleic and α-linolenic acids generated by the Δ12 (Fad2) and ω3 (Fad3) desaturases (Kainou et al., 2006; Micolonghi et al., 2012). Studies on this yeast indicate that glucose and oxygen signalling and responses are linked (Micolonghi et al., 2011). This regulation also involves biosynthesis of FAs through the activity of the hypoxic regulator KIMga2 (Micolonghi et al., 2012; Ottaviano et al., 2015). Similar to *K. lactis*, PUFAs are present also in other ascomycetes yeasts, in which desaturase enzyme and/or genes have been previously studied, such as *Pichia pastoris* (Yu et al., 2012), *Hansenula polymorpha* (Sangwallek et al., 2014) and *Saccharomyces kluveri* (Oura & Kajiwara, 2008), and in basidiomycetes as well (Rossi et al., 2009). The relative abundance of MUFAs and PUFAs in the phospholipids determines the properties of the membranes and their functioning. The FA composition of the membranes responds to different environmental stimuli and contributes to adaptation to changing growth and/or stress conditions. FAs with different physical-chemical properties, derived from carbon backbone structure, length and saturation, can be synthesized and used to assemble membranes with the appropriate characteristics: this mechanism is known as ‘homeoviscous adaptation’ (Sinensky, 1974). Typical conditions controlling the expression of the desaturase gene(s) and hence the FA composition in *S. cerevisiae* are temperature (Nakagawa et al., 2002), pH, presence of ethanol (Yazawa et al., 2011) and hypoxia (Vasconcelles et al., 2001). In yeasts with enriched composition of unsaturated FAs, the environmental conditions might have different effects on the synthesis and abundance of specific unsaturated FA molecules (Rossi et al., 2009).

With the aim to establish functional roles of unsaturated and polyunsaturated FAs in *K. lactis* membranes, we studied the transcription of *K. lactis* desaturase genes KIOLE1, FAD2 and FAD3 and the cellular localization of the corresponding enzymes when environmental aeration and temperature were varied. We also generated fad2Δ and fad3Δ deletion mutants in order to study fatty acid composition and metabolic properties of the resulting strains under different conditions.

### METHODS

#### Strains, media and growth conditions

The *K. lactis* wild-type strains used in this study were MWL9S1 (MATα, lysA1, trp1, leu2, metA1-1, ura3) and MWL9S1 Δ(Δ12, Δω3) [MWL9S1 Δfad2 Δfad3] (Wesolowski-Louvel, 2011; Micolonghi et al., 2011). Deletion mutants were obtained by the short flanking sequences-PCR method (SFH; Wesolowski-Louvel, 2011) or the long flanking sequences-2 steps PCR method (LFH; Wach, 1996). In both cases, the kanMX4 module from pFA6a was used (Wach et al., 1994). Double-deleted strains were obtained by crossing the deletion mutant strains in the MWL9S1 and CSK1/1 backgrounds followed by selection of tetrad showing 2:2 segregation of the G418 resistance phenotype. Insertion mutants with GFP fusions were selected by the SFH-PCR method using plasmid pYMD27 (EUROSCARF) as template (Janke et al., 2004). Strains are summarized in Table 1; oligonucleotides are listed in Table 2. Transformants were obtained by electroporation (Salani & Bianchi, 2006). Standard YP medium was composed of 10 g l⁻¹ yeast extract (Becton Dickinson) and 10 g l⁻¹ bactopeptone (Becton Dickinson). The pH of the medium was adjusted to 6.2 with 1 N NaOH and the medium was autoclaved at 121°C for 20 minutes before use.

#### Table 1. *K. lactis* strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MWL9S1</td>
<td>MATα, lysA1, trp1, leu2, metA1-1, ura3</td>
<td>Micolonghi et al. (2011)</td>
</tr>
<tr>
<td>LDA3</td>
<td>MATα, lysA1, trp1, leu2, metA1-1, ura3, fad3Δ::kanMX4</td>
<td>This work</td>
</tr>
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<td>This work</td>
</tr>
<tr>
<td>CSK1/1</td>
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<td>Wesołowski-Louvel (2011)</td>
</tr>
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</tr>
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<td>This work</td>
</tr>
<tr>
<td>LD3G</td>
<td>MATα, lysA1, trp1, leu2, metA1-1, ura3, FAD3-GFP</td>
<td>This work</td>
</tr>
<tr>
<td>LD2G</td>
<td>MATα, lysA1, trp1, leu2, metA1-1, ura3, FAD2-GFP</td>
<td>This work</td>
</tr>
</tbody>
</table>

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10 g l⁻¹ peptone (Becton Dickinson) supplemented with carbon sources as needed, typically 20 g l⁻¹ glucose (YPD medium). Synthetic medium contained 6.7 g l⁻¹ yeast nitrogen base without amino acids (Becton Dickinson) supplemented with auxotrophic requirements and carbon source as needed. Solid media contained 20 g l⁻¹ bacto-agar (Becton Dickinson). Genetecin (Sigma–Aldrich) was supplemented at a final concentration of 100 µg ml⁻¹. Comparative growth in normoxic (sparger air flow at 0.4 l min⁻¹, 1.2 bar) and hypoxic (without air flow) conditions and growth at low temperature (15°C) were performed in a Biotaf® (B. Braun–Sartorius Stedim) bioreactor composed of four 1-litre vessels. At the equilibrium point with atmosphere, oxygen concentration in YPD medium is 6.8 mg l⁻¹ and 8.2 mg l⁻¹ at 28°C and 15°C, respectively. These values were used to set the 100% relative concentration of dissolved oxygen (DOT) at the start point.

RNA analysis. Transcription analysis was performed by Northern blotting. Total RNAs were prepared by the hot phenol procedure as described by Bianchi et al. (1996). Probes were obtained by PCR using wild-type genomic DNA as a template and the primers reported in Table 2. (α⁻³²P)ATP labelling of the probes was performed using the Random Primed Labelling kit (Roche Diagnostics), following the instructions provided by the manufacturer. Quantification of signals was performed with Phoretics 1D plus (Nonlinear Dynamics) using rRNAs as loading references.

Fatty acids analysis. Cells were grown in bioreactors to OD₆₀₀=1–2 and then subjected to hypoxic or temperature shifts as described above. Lyophilized cells of K. lactis were homogenized in liquid nitrogen and contained 6.7 g l⁻¹ yeast nitrogen base without amino acids (Becton Dickinson) supplemented with auxotrophic requirements and carbon source as needed. Solid media contained 20 g l⁻¹ bacto-agar (Becton Dickinson). Genetecin (Sigma–Aldrich) was supplemented at a final concentration of 100 µg ml⁻¹. Comparative growth in normoxic (sparger air flow at 0.4 l min⁻¹, 1.2 bar) and hypoxic (without air flow) conditions and growth at low temperature (15°C) were performed in a Biotaf® (B. Braun–Sartorius Stedim) bioreactor composed of four 1-litre vessels. At the equilibrium point with atmosphere, oxygen concentration in YPD medium is 6.8 mg l⁻¹ and 8.2 mg l⁻¹ at 28°C and 15°C, respectively. These values were used to set the 100% relative concentration of dissolved oxygen (DOT) at the start point.

RNA analysis. Transcription analysis was performed by Northern blotting. Total RNAs were prepared by the hot phenol procedure as described by Körner & Domdey (1991) from cultures grown to OD₆₀₀=1–2 and then subjected to hypoxic or temperature shifts. Cells were collected by centrifugation, washed with water and stored frozen at −70°C until extraction. Northern blotting was performed by electrophoresis of RNAs in agarose/formaldehyde gels followed by transfer to membranes and hybridization with probes following a standard procedure as described by Bianchi et al. (1996). Probes were obtained by PCR using wild-type genomic DNA as a template and the primers reported in Table 2. (α⁻³²P)ATP labelling of the probes was performed using the Random Primed Labelling kit (Roche Diagnostics), following the instructions provided by the manufacturer. Quantification of signals was performed with Phoretics 1D plus (Nonlinear Dynamics) using rRNAs as loading references.

Fatty acids analysis. Cells were grown in bioreactors to OD₆₀₀=1–2 and then subjected to hypoxic or temperature shifts as described above. Lyophilized cells of K. lactis were homogenized in liquid nitrogen and were extracted with a chloroform/methanol (2 : 1, v/v) mixture, containing 100 µg butylated hydroxytoluene (BHT) as antioxidant. Nonadecanoic acid (C19:0) was added as internal standard before extraction. After filtration, the collected supernatants were dried by nitrogen at room temperature, and the residue was dissolved by boron trifluoride (BF₃, 10% in methanol; Merck). The mixture was kept at 95°C for 15 min in order to trans-esterify lipids, and then extracted by hexane. The resulting fatty acid methyl esters (FAMEs) were analysed with a gas chromatograph (GC-HP5890 series II) equipped with flame ionization detector (FID) using a capillary column SPB PUFA 30 m × 0.25 mm i.d. with a 0.2 µm film thickness (Supelco) (Reverberi et al., 2012). Fatty acids were identified by comparison of retention times to authentic standards. Alternatively, the extracts from flask-cultured cells were filtered onto celite and anhydrous Na₂SO₄, solvents were then removed. Reaction with sodium methanoate in methanol was used to generate FAMEs from the glycerides, using glycerol triundecanoate as the internal standard (Raimondi et al., 2014). FAMEs were analysed in a quadrupole GC-MS system (HP5890 HP5972; Agilent Technologies) equipped with CP–Select CB column (Varian). Peak areas in the total ion chromatograms were used to determine their relative amounts. The unsaturation index (UI) was calculated as follows (Heipieper et al., 2000): [(%C₁₆ : 1+%C₁₈ : 1)+(%C₁₈ : 2×2)+(%C₁₈ : 3×3)]/100.

Respiration. Respiration studies were performed using a Clark oxygen electrode (Hansatech Instruments) as described in De Luca et al. (2009). Cells (1×10⁶) from late-log cultures (1–4×10⁶ cells ml⁻¹) were collected, washed with 1 ml sterile water, suspended in 1 ml sodium phosphate buffer (10 mM pH 7.4 containing 4 g l⁻¹ glucose) and loaded in the reaction vessel of the previously calibrated oxygen electrode chamber.

Fluorescence microscopy. Exponentially growing cells on YPD medium were observed with a Zeiss Axio Imager Z1 fluorescence microscope with an AxioVision 4.8 digital image processing system, and objective lens ×63 oil. The fluorescence was observed using filter sets for DAPI (365 nm excitation and 445/450 nm emission), DASPMI (550/
25 nm excitation and 605/670 nm emission) and GFP (470/40 nm excitation and 525/50 nm emission).

**Ethanol.** Ethanol present in the culture supernatants was measured by enzymatic reactions using the Ethanol kit (NZYTech) as indicated by the supplier.

### RESULTS AND DISCUSSION

#### Deletion of FAD2 and FAD3 genes

Genes encoding FAs desaturases in *K. lactis* have been identified previously: the Δ9-desaturase (Ole1) is encoded by KLOLE1 (KLLA0C05566g; Micolorghi et al., 2012). The Δ12 and ω3 desaturases are respectively encoded by FAD2 and FAD3 genes (KLLA0F07095g and KLLA0B00473g). Characterization of the *K. lactis* Fad2 and Fad3 desaturases-encoding genes was previously performed only in the *S. cerevisiae* heterologous system (Kainou et al., 2006) and since then no further studies were carried out in *K. lactis*. In order to investigate the functional role of FAs desaturases in *K. lactis*, we have deleted FAD2 by replacement with the *kanMX4* marker gene using the long flanking homology (LFH) procedure (Wach, 1996) in strains MWL9S1 and CSK1/1, and deleted FAD3 using the short flanking homology (SFH) procedure (Węsolsowski-Louvel, 2011) in strain MWL9S1. The latter strain is defective in the non-homologous end-joining recombination by deletion of the KINEJ1 gene (Węsolsowski-Louvel, 2011). The obtained mutant strains were named LDA2, LDK2 and LDA3, respectively, and were viable under the selection conditions (standard YPD medium plus genetecin). Conversely, several attempts failed to generate deletion mutants of KLOLE1 by both the SFH and LFH procedures in both CSK1/1 and MWL9S1 strains, even in presence of oleic acid. In previous papers (Micolonghi et al., 2012; Ottaviano et al., 2015), we showed that UFAs could suppress the slow-growth phenotype deriving from KIMGA2 deletion (in GDK genetic context) indicating an effective uptake of oleic and palmitoleic acids in this strain. Thus we tested uptake of UFAs in the wild-type strains CSK1/1 and MWL9S1 and fad2Δ and fad3Δ deletion mutants, by assaying growth in the presence of Tween 80 (a source of oleate; Ottaviano et al., 2015) and cerulenin, an inhibitor of fatty acid synthase. Results, reported in Fig. 1 (a), demonstrate that these strains could uptake oleic acid from the medium and could grow bypassing the biosynthetic block caused by cerulenin. Identical results were obtained using free oleic acid (0.5 mM) instead of Tween 80 (not shown). Having demonstrated effective uptake of UFAs by these strains, the failure to obtain KLOLE1 deletion mutants suggested that this gene was essential under standard growth conditions and that the Δ9-desaturase might have other function(s) in addition to

![Fig. 1. FA uptake and FA composition of strains.](image)

**Fig. 1.** FA uptake and FA composition of strains. (a) Growth of wild-type strains MWL9S1 and CSK1/1 and mutant strains LDA2, LDA3 and LDK2 (serial dilutions) spotted on YP plates (YP) and on YP plates supplemented with: 0.2 % Tween 80 (YP+T80), 0.2 % Tween 80 and 10 µg ml⁻¹ cerulenin (YP+C+T80), or 10 µg ml⁻¹ cerulenin (YP+C). Plates were incubated at 28 °C for 4 days. (b) Saturated (C16 : 0 and C18 : 0) and unsaturated (C16 : 1, C18 : 1, C18 : 2 and C18 : 3) FA content (µg per mg of dry biomass) of the wt strain MWL9S1 (blue bars), the fad3Δ strain LDA3 (red bars) and the fad2Δ strain LDA2 (green bars). Error bars show standard deviations of three experiments (±SD). Biological repetitions gave similar results. (c) Total FA content and UI of the wild-type and deletion mutant strains.
FA desaturases in the yeast *K. lactis*

Among them, the PUFAs 18:2 and 18:3, generated by the *fad2* (oleic (18:1), linoleic (18:2) and the C18 ones being the most represented (about 75%). In unsaturated forms, accounted for >99% in all the strains, treatment under normoxic conditions (Fig. 1b). FAs with medium under normoxic conditions (Fig. 1b). FAs with the FA composition of wild-type and deleted strains was on standard medium.

The FA composition of wild-type and deleted strains was determined after growth in a bioreactor on standard YPD medium under normoxic conditions (Fig. 1b). FAs with chain lengths of 16 and 18 carbons, in their saturated and unsaturated forms, accounted for >99% in all the strains, the C18 ones being the most represented (about 75%). In the wild-type, the unsaturated FAs were palmitoleic (16:1), oleic (18:1), linoleic (18:2) and α-linolenic (18:3) acids. Among them, the PUFAs 18:2 and 18:3, generated by the consecutive activity of Δ12 and ω3 desaturases, respectively, constituted 39% and 15.4% of total FAs, and were mainly responsible for a UI of 1.52 (Fig. 1c). In the *fad3* strain LDA3, the deletion of *FAD3* led to lack of α-linolenic acid (18:3). While all the other FAs remained unchanged (*P* > 0.05), linoleic (18:2) acid was highly accumulated (62.7%; *P* < 0.05), evidently due to the lack of ω-desaturase activity. As a result of linoleic acid accumulation, the UI of the *fad3* strain LDA3 remained similar to that of the wild-type (1.48 vs 1.52; *P* > 0.05). *FAD2* deletion in LDA2 mutant strain resulted in the block of the desaturation pathway, not proceeding beyond oleic acid (18:1); thus linoleic (18:2) and α-linolenic (18:3) acids were both absent. Oleic acid was the most abundant FA in this mutant, which showed a higher concentration of C18:1 than in the other strains (71.6%; *P* < 0.05). Also palmitoleic acid (16:1) occurred at a higher concentration in the *fad2* strain LDA2 (15.2%; *P* < 0.05), while the saturated FAs showed no significant difference. Despite the increase of oleic and palmitoleic acids, the lack of PUFAs in the *fad2* strain LDA2 resulted in a significant decrease of UI (0.87; *P* < 0.05). No significant difference in the total amount of FAs was observed among the wild-type and the two *fad3* or *fad2* mutant strains (Fig. 1c; *P* > 0.05).

### Growth of the *fad2* and *fad3* strains

Exponentially growing cells of the wild-type strain MWL9S1 and of the deleted strains LDA2 (*fad2*) and LDA3 (*fad3*) were inoculated in bioreactor vessels containing YPD medium and grown in normoxia or hypoxia at 28°C or 15°C (Fig. S1, available in the online Supplementary Material). Under normoxic condition, aeration was sufficient to prevent oxygen limitation conditions at both temperatures. Growth profiles showed that all the strains grew less abundantly in hypoxia than in normoxia at both 15°C and 28°C, because of the low energy gain under limited aeration. Regardless of the aeration, growth rates and biomass yields at low temperature were reduced, in accordance with the mesophilic adaptation of *K. lactis*. Specific growth rates of the three strains at the exponential phase of the four growth conditions were also measured for a more detailed analysis (Fig. 2a).

At 28°C, under both normoxic and hypoxic conditions, the wild-type and the mutants presented similar biomass yields.

### Respiration of the *fad2* and *fad3* strains

FA desaturases in the yeast *K. lactis*

![Image](http://mic.microbiologyresearch.org)
and similar specific growth rates, indicating that the deletion of the genes involved in the biosynthesis of PUFAs did not affect significantly biomass production at this temperature. Interestingly, when cultured at 28 °C in normoxia, the LDA2 (fad2Δ) and LDA3 (fad3Δ) mutants presented lower oxygen consumption than the wild-type. In fact, the relative dissolved oxygen tensions (DOT) decreased to 20–30 % for approximately 12 h during the growth phase of the mutants, while values of 5–15 % were observed for 15 h in the wild-type (Fig. S2a). This finding may be related to the fact that desaturases are oxidative enzymes that consume molecular oxygen as electron acceptor (Los & Murata, 1998). Lower oxygen consumption might thus derive from reduced desaturase activity, especially in the case of the strain LDA2 (fad2Δ) in which the UI is particularly low.

At 15 °C, the three strains presented similar biomass production (Fig. S1), but the two mutants exhibited a decrease (20–25 %) of the specific growth rates at the exponential phase (Fig. 2a), compared to the wild-type, indicating that the defective production of PUFAs in the mutants may have altered their ability to cope with lower temperature. However, the specific growth rate was always higher in the LDA2 (fad2Δ) than in the LDA3 (fad3Δ) strain, also indicating that the limitation to MUFAs was less detrimental than linoleic acid accumulation for growth performance at maximal rate at 15 °C and 28 °C. Furthermore, the finding that the specific growth rate of the LDA2 (fad2Δ) strain at 28 °C was slightly higher than that of the wild-type, suggested that at this temperature the increase of MUFAs might be sufficient for optimal growth. Coherently with different growth rates, biomass production and oxygen solubility, DOT profiles were higher (>70 %) at 15 °C than at 28 °C (Fig. S2b). In order to investigate the consistency of growth differences at low temperature, we assayed growth on YPD plates at 8 °C (Fig. 2b). The three strains grew poorly but especially the fad2Δ strain LDA2 showed an extremely reduced growth rate, thus exacerbating the effect of biosynthetic limitation of this strain at very low temperature.

The capability of the deleted strains to use different carbon sources was tested on YP plates supplemented with ethanol, glycerol or lactate (Fig. S3). Again, no significant differences were found among the wild-type, the single- and the double-deleted strains. The addition of 1 M NaCl did not show any effect of the deletion, compared to the wild-type strain. The deleted strains grew like the wild-type even in synthetic SD medium (not shown). Results suggest that the deletion of FAD2 and FAD3 desaturase genes and, consequently, the altered membrane composition in unsaturated and polyunsaturated FAs had little effect on growth in a wide range of conditions except at low temperature.

**Respiratory and fermentative metabolism in the deleted strains**

We have previously reported (Ottaviano et al., 2015) that *KIMGA2* deletion affected biosynthesis and respiration of FAs. In order to establish the occurrence of such correlation in the desaturase mutants, we measured respiration in the wild-type and in the *fad2Δ* and *fad3Δ* deletion mutant strains. Oxygen consumption rates of the three strains were measured using the Clark electrode with late-log growing cells from YP cultures containing different amounts of glucose. Results, reported in Fig. 3, indicate that wild-type cells consume oxygen at the same rate independently of the absence or the concentration of glucose. Conversely, the deleted strains showed oxygen consumption rates similar to the wild-type only in 20 g l⁻¹ glucose while they expressed lower rates of respiration in high glucose concentration (50 g l⁻¹) or in the absence of glucose. In order to verify whether these differences were correlated with the mitochondrial function, cells were stained with Dimethylaminostyrylmethylpyridiniumiodine (DASPMI) to visualize active mitochondrial membranes by fluorescence microscopy. Results (Fig. 4a) indicate that

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**Fig. 4.** Fluorescence microscopy analysis. (a) Functional mitochondrial membranes of wild-type (MWL9S1) and mutant (LDA3 and LDA2) cells grown on YP medium without glucose or supplemented with 20 g l⁻¹ or 50 g l⁻¹ glucose and stained with DASMI. (b) Cellular localization of desaturases KIOle1, Fad2 and Fad3 fused to GFP in strains LDOG, LD2G and LD3G, respectively (GFP column). Figure also shows cells under visible light (VIS column) and nuclei stained with DAPI (DAPI column). Bar, 0.5 μm.

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L. D. Angelis and others
Expression of KIOLE1, FAD2 and FAD3 genes

Transcription of genes involved in FAs biosynthesis is regulated by environmental stimuli such as hypoxia, temperature (Micolonghi et al., 2012; Ottaviano et al., 2015) and ethanol (Fig. S5). We investigated how hypoxia and low temperature could affect KIOLE1, FAD2 and FAD3 transcription in the deleted strains. Transcription was assayed by Northern blot analysis at 1 h and 4 h after shifting to hypoxia and to 15 °C in bioreactor (Fig. S5). Results after quantification are summarized in Table 3. Hypoxic induction of KIOLE1 and FAD2 transcription, present in the wild-type strain, was maintained also in the deleted strains but KIOLE1 induction was transient in the LDA2 (fad2Δ) strain. FAD3 showed repression of transcription by hypoxia in both wild-type and LDA2 strains. Effects of temperature were less pronounced: after a moderate increase of transcription at 1 h of treatment, the transcription level was reduced to that found in the wild-type at 28 °C or less, as was the case of KIOLE1 transcription in LDA3 (fad3Δ) and LDA2 (fad2Δ) strains. In the latter strain, the level of transcription of FAD3 was significantly higher than in the wild-type in normoxic conditions at 28 °C, indicating a moderate repressing activity of linoleic acid on this gene. This result suggests a regulatory role of linoleic acid on linolenic acid biosynthesis.

Table 3. Effect of hypoxia and low temperature on transcription of desaturase genes

<table>
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<tr>
<th>Gene</th>
<th>Condition</th>
<th>MWL9S1 (wt)</th>
<th>LDA3 (fad3Δ)</th>
<th>LDA2 (fad2Δ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OLE1</td>
<td>Normoxia</td>
<td>1</td>
<td>1.0 (±0.35)</td>
<td>1.7 (±0.79)</td>
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<td></td>
<td>Hypoxia 1 h</td>
<td>2.3 (±0.55)</td>
<td>2.7 (±1.22)</td>
<td>4.0 (±1.53)</td>
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<td></td>
<td>Hypoxia 4 h</td>
<td>4.8 (±2.32)</td>
<td>4.2 (±2.62)</td>
<td>0.8 (±0.47)</td>
</tr>
<tr>
<td>FAD2</td>
<td>Normoxia</td>
<td>1</td>
<td>1.0 (±0.28)</td>
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<td>0.8 (±0.21)</td>
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<tr>
<td></td>
<td>Hypoxia 4 h</td>
<td>2.3 (±0.29)</td>
<td>2.7 (±0.79)</td>
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</tr>
<tr>
<td>FAD3</td>
<td>Normoxia</td>
<td>1</td>
<td>1.5 (±0.62)</td>
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</tr>
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<td>Hypoxia 1 h</td>
<td>0.4 (±0.1)</td>
<td>0.4 (±0.16)</td>
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<td>Hypoxia 4 h</td>
<td>0.7 (±0.07)</td>
<td>0.5 (±0.24)</td>
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<tr>
<td>OLE1</td>
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<td>1.6 (±0.82)</td>
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<tr>
<td></td>
<td>15 °C 1 h</td>
<td>1.2 (±0.33)</td>
<td>1.3 (±0.12)</td>
<td>2.0 (±0.93)</td>
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<td></td>
<td>15 °C 4 h</td>
<td>0.7 (±0.43)</td>
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<td>1.4 (±0.43)</td>
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<td></td>
<td>15 °C 1 h</td>
<td>1.6 (±0.66)</td>
<td>2.1 (±0.56)</td>
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<td>1.8 (±0.94)</td>
<td>3.2 (±1.12)</td>
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<td>15 °C 4 h</td>
<td>0.5 (±0.17)</td>
<td>1.6 (±0.47)</td>
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mitochondrial membranes were functional in the three strains and mitochondrial morphologies were substantially unchanged with the exception of a fragmented morphology in the LDA2 (fad2Δ) strain in absence of glucose. This fragmentation was not due to a structural defect, since the same cells had a normal mitochondrial morphology in different concentrations of glucose: fragmentation could rather derive from the need of increased mitochondrial surface for full respiration when the absence of PUFAs could not provide optimal membrane composition.

We tested the fermentative growth of the fad2Δ and fad3Δ strains on plates containing high glucose concentration (50 g l−1) and the mitochondrial drug antimycin A (GAA medium) that blocks respiration. Slightly larger colonies could be observed for the LDA2 (fad2Δ) strain (Fig. S4a). Ethanol production of the wild-type and the LDA2 (fad2Δ) strains grown in the hypoxic bioreactor process on YP medium containing 50 g l−1 glucose was measured. Growth of the two strains was identical while a slightly higher accumulation of ethanol was detected at the end of the fermentation process with LDA2 (fad2Δ) (Fig. S4b, c). These results indicate that the deletion of either FAD2 and/or FAD3 genes might influence some aspects of the respiratory and fermentative metabolism even if the general cell fitness and growth were not particularly affected.

Effects of hypoxia and low temperature on FA composition

In order to cope with cold temperatures, microorganisms are known to utilize diverse changes in lipid composition that are to incorporate unsaturated, short-chain, branched, or cyclic FAs within membranes with the aim to maintain

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membrane fluidity and functionality (Chintalapati et al., 2004; Morgan-Kiss et al., 2006). We have determined the FA composition of the wild-type and the deleted strains after hypoxic or temperature shift in a bioreactor. Results are reported in Fig. 5(a, b). Calculations of the corresponding UIs are reported in Fig. 5(d). In the wild-type strain MWL9S1, we measured a hypoxic increase (Fig. 5a) in oleate (C18:1) and a decrease in α-linolenate (C18:3) that could be correlated with transcription of KIOLE1 and FAD3. On the contrary, linoleate decreased notwithstanding the hypoxic induction of FAD2 (Table 3). The FA composition of LDA3 (fad3Δ) was similar to the wild-type but without C18:3. The increased percentage of C16:0 in the wild-type and LDA3 strain after hypoxic shift might be ascribed to the hypoxic induction of the FAS1 gene (Miccoli et al., 2012). No significant variations of relative FA

Fig. 5. Fatty acid composition of the wild-type strain (MWL9S1) and the deleted strains LDA3 and LDA2 grown under hypoxia and 15°C. (a) The three strains were grown in a bioreactor (YPD medium, 28°C, normoxia; blue bars) up to 1–2 OD600 and then shifted to hypoxia for 1 h (red bars) and 4 h (green bars) before sampling. Content of each single FA is reported as % of the total FAs. (b) The three strains were grown in a bioreactor (YPD medium, 28°C, normoxia; blue bars) up to 1–2 OD600 and then shifted to 15°C for 1 h (red bars) and 4 h (green bars) before sampling. Values are reported as % of the total FAs content. Error bars in (a) and (b) are ±SD; three biological repetitions gave similar results. (c) FA determinations (% of the total FAs content) are reported for cells grown in YPD flasks for 48 h at 28°C or for 96 h at 15°C (blue bars). (d) UI of the three strains grown in a bioreactor and subjected to hypoxic and temperature shifts of 1 h and 4 h. (e) UI of the three strains grown in flasks at 28°C or 15°C for 48 h and 95 h, respectively.

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<td>MWL9S1</td>
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composition were observed in the LDA2 (fad2Δ) strain upon hypoxic shift. The hypoxic shift induced a reduction of the UFs of the wild-type and of the LDA3 (fad3Δ) strains after 4 h while no significant changes were observed in the LDA2 (fad2Δ) strain (Fig. 5d).

Temperature shift in the wild-type strain (Fig. 5b) produced an increase of C16:1 and a decrease of C18:1, suggesting a control of membrane composition partly on the length of the FAs. In fact, also an increase of the UI in the wild-type strain could be observed after shift at 15°C (Fig. 5d). The observed increase of α-linolenate might be the consequence of increased expression of FAD3 at 15°C. Temperature shift had little effect on FA composition in LDA2 and LDA3 strains: only a small increase of C16:1 was observed. Also UFs of the deleted strains showed no significant changes after 4 h at 15°C, indicating an altered response to temperature downshift in FA composition with respect to the wild-type.

In order to compare adaptation of membrane composition to low temperature of the wild-type and the desaturase-deficient mutants, strains were cultured at 28°C and 15°C in flasks of YPD for 48 h and 95 h, respectively. In accordance with previous observations, both the growth rate and final biomass yield were higher at 28°C than at 15°C. Growth profiles (OD) and metabolism (glucose consumption and ethanol production) did not differ substantially among the strains, especially at 28°C (Fig. S6). The growth temperature differently affected the FA profile of the wild-type and the mutants (Fig. 5c), likely as a result of altered biosynthetic condition of LDA2 and LDA3 strains. The wild-type produced a higher amount of α-linolenic acid (C18:3) at 15°C than at 28°C. This observation is in agreement with the fact that the degree of unsaturation, which increased after growth at 15°C (Fig. 5e), is a physiologic acclimation of the vast majority of mesophilic yeasts to low temperatures (Rossi et al., 2009). In strain LDA2, in which the biosynthesis of PUFA was precluded by the lack of α2 desaturases, the mono-unsaturated FAs C16:1 and C18:1 increased in the cultures grown at 15°C (Fig. 5c), similarly contributing to the large increase of the UI (Fig. 5e). Differently, the acclimation to 15°C of strain LDA3 did not cause any increase in linoleic acid. In this strain, the response to cold did not consist of any increase of the UI (Fig. 5e), but in the accumulation of shorter FAs such as palmitic and palmitoleic acids (Fig. 5c). The altered response of the LDA3 strain to cold adaptation suggests a role of linoleenic acid in this regulation.

Our results suggest that cold adaptation of membranes could be achieved in K. lactis either by increasing the fraction of MUFAs or PUFA (wild-type and LDA2 strains) or by increasing the proportion of short-chain FAs (strain LDA3), depending on the biosynthetic capability. Interestingly, stearate was undetectable in late-log cells and, in some conditions, C10 fatty acid was a significant fraction of the total FA composition.

Cellular localization of FA desaturases

In order to visualize the cellular localization of KIOle1, Fad2 and Fad3 enzymes, GFP fusions of KIOle1, FAD2 and FAD3 genes were integrated into the wild-type strain MWL951 (see Methods). The resulting strains (LDOG, LD2G and LD3G, respectively) were grown on YPD medium and the subcellular location of the three FA desaturases was determined by fluorescence microscopic analysis. The three enzymes were found in the endoplasmic reticulum (ER) (Fig. 4b) as was previously reported for S. cerevisiae Ole1 and for plant Fad2 and Fad3 enzymes (Dyera & Mullen, 2001). However, differences could be observed. In fact, Fad3 enzyme had a prevalent localization in the nuclear and cortical ER, while Ole1 and Fad2 were more uniformly localized in both nuclear/cortical and cytoplasmic ER (Du et al., 2004). As a control, cells were also stained with DAPI in order to visualize the nuclear DNA.

CONCLUSIONS

In previous reports (Micolonghi et al., 2012; Ottaviano et al., 2015), we showed that the deletion of the regulatory gene KIMGA2 affected FAs biosynthesis, especially under hypoxia and cold. The KIMGA2 deletion mutant exhibited a reduced growth rate that could be compensated by addition of UFAs. Results of the FAD2 and FAD3 deletion mutants presented here indicate that PUFA are dispensable in yeast and the only essential unsaturated FAs are oleate and/or palmitoleate. FAD2 deletion mutants have the same FA components as S. cerevisiae and are healthy and viable in a wide range of conditions and stresses in spite of a reduced UI. We showed here that adaptation of membrane composition to low temperature might be acquired either by increasing UI or by reducing FA length, or both. A combination of increased desaturation and chain shortening of FAs has been proposed as a mechanism for cold adaptation of membranes in Candida utilis (Suutari et al., 1997), resulting from the contribution of various effectors, including oxygen availability, growth rate and carbon source, in addition to temperature. Our results also pose the question of the nature of the selective pressure that ensures the maintenance of PUFA in K. lactis. The deletions of FAD2 and FAD3 genes have different phenotypic effects in other yeasts where they have been generated. For example, the deletion of FAD2 in Saccharomyces kluyveri (Watanabe et al., 2004) does not produce a growth phenotype while it causes a slow-growth phenotype in Pichia pastoris (Yu et al., 2012). In the latter yeast, the deletion of FAD3 does not generate a growth phenotype. Although these genes are dispensable in K. lactis, they are regulated in response to various stressing conditions like hypoxia, low temperature and ethanol, and might affect some cellular function such as mitochondrial morphology and respiration.

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