A dual signalling pathway for the hypoxic expression of lipid genes, dependent on the glucose sensor Rag4, is revealed by the analysis of the KlMGA2 gene in Kluyveromyces lactis

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INTRODUCTION

Kluyveromyces lactis is a yeast that is generally recognized as safe and is used in both basic research and applied microbiology. It is one of the few yeasts capable of growing on lactose as the sole carbon source and for this reason it was studied as a model for lactose metabolism and exploited as a source of eukaryotic β-galactosidase. Another field of application of this yeast is the production of heterologous proteins (van Ooyen et al., 2006). Its genome has been sequenced and annotated (Dujon et al., 2004) and is available at http://cbi.labri.fr/Genolevures.

Glucose metabolism in K. lactis is not governed by the Crabtree effect as strongly as in the fermentative yeast Saccharomyces cerevisiae. This implies that the balance between respiration and fermentation in this yeast depends on oxygen availability and not on glucose concentration (Weirich et al., 1997; Kiers et al., 1998; González-Siso et al., 2000). The key point of the balance between respiration and fermentation is the branch point of pyruvate metabolism, with pyruvate being either oxidized by the respiratory enzyme pyruvate dehydrogenase (PDH) or decarboxylated by the fermentative enzyme pyruvate decarboxylase (PDC).

In S. cerevisiae, the expression of aerobic and anaerobic genes, and other haem-dependent transcription factors like Rox1 (Kastaniotis & Zitomer, 2000), is regulated by haem and the regulator Hap1 (Zitomer & Lowry, 1992; Hon et al., 2003). The genomic response to oxygen, in particular the transcriptome modulation in hap1 and rox1 mutants, has been described (Becerra et al., 2002; Kwast et al., 2002). Moreover, Hap1- and Rox1-independent oxygen response pathways have been described: transcription of the hypoxic gene OLE1 depends on cytochrome c oxidase (Kwast et al.,...
1999) and requires the transcription factor Mgα2 (Jiang et al., 2001). Another oxygen-sensing pathway includes the regulators of sterol biosynthesis Upc2 and Ecm22 (Vik & Rine, 2001) and is triggered by sterol depletion rather than haem depletion (Davies & Rine, 2006). An integrated predictive model of oxygen sensing (Kundaje et al., 2008) allowed the confirmation of previously described participants and also the proposition of new candidates in the aerobic/anaerobic scenario of yeast.

The data about oxygen-dependent regulation are still fragmentary in K. lactis. Few steps of haem biosynthesis have been investigated (González-Dominguez et al., 1997; Blanco et al., 2005). The expression of KIHEM1 is clearly promoted by hypoxia (Nuñez et al., 2008). The transcriptional regulator KfHap1 is also expressed in an oxygen-dependent fashion but its role in respiratory and/or fermentative carbon metabolism is still to be elucidated (Lamas-Maceiras et al., 2007; Bao et al., 2008). A large-scale transcriptional analysis of about 60 K. lactis genes that are putatively oxygen-dependent (Blanco et al., 2007) indicated that the hypoxic response of this yeast is very different from S. cerevisiae and limited to very few ORFs, namely KLLA0A09075g (OYE2), KLLA0C05566g (OLE1) and KLLA0F14058g (GSH1).

We have recently demonstrated that the glucose sensor Rag4 is involved in the hypoxic regulation of KIPDC1 (Micolonghi et al., 2011). In this work, we have studied the role of KIMGA2, the orthologue of the MGA2 gene from S. cerevisiae, in the hypoxic response of K. lactis. The deletion of KIMGA2 produced specific growth phenotypes and altered the expression of hypoxic genes correlated with lipid metabolism. However, a predominant function of the glucose sensor Rag4 was found in the hypoxic induction of tested genes. Overlapping functions of KIMGα2 and Mgα2 were demonstrated by mutual exchange of the genes in the corresponding mutant strains.

**METHODS**

**Plasmids.** The plasmids used in this work are listed in Table 1. Vector KpKIMGA was obtained by amplifying KIMGA2 from the GDK genome with primers III132 and III133 (Table S1, available with the online version of this paper) and cloning the BamHI-digested PCR product into the BamHI site downstream of the ADH promoter of the replicative shuttle vector Kp426ADHSD11 (Uccellietti et al., 2005). The KIMGA2 amplification product was also cloned in the pYES vector under the GAL1 promoter (pYES[PLAG]KIMGA2), KpScMGA was obtained by cloning the pYES[PLAG]MGA2HA sequence, excised from pYES[PLAG]MGA2HA vector (Bhattacharya et al., 2009) by BamHI digestion, into the BamHI site of Kp426ADHSD11.

**Strains.** The Escherichia coli strains used for cloning were DH5αF’ and K12-ER2925. The yeast strains used are listed in Table 1. The GDK strain was obtained by integrating the circular plasmid KpPLF (Micolonghi et al., 2011) at the KIPDC1 promoter site in the HNK20 strain. Integration was verified by PCR using primer pairs 110 and 19, and 110 and 112. Strain GDK/Klmga2Δ was obtained by disruption of the ORF KLLA0E17953g with the KanMX4 cassette. The deletion cassette was constructed according to the method described by Wach et al. (1994). The flanking regions of the ORF were amplified with Phusion DNA polymerase (Finzymes) using the oligonucleotide pairs I21 and I46, and I22 and I47. The amplified flanking sequences were used as primers for a second amplification step, using the NotI fragment of pFA6a–KanMX4 as a template. After transformation of GDK, the homologous integration of the cassettes into the genome was verified by PCR using the primer pairs I11 and H14, and I11 and I13.

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Media. Yeast cells were cultivated at 28 °C. Yeast-rich medium (YP) was 1 % yeast extract and 1 % peptone (Becton Dickinson). Yeast-selective synthetic media contained 0.7 % yeast nitrogen base without amino acids (Becton Dickinson), appropriately supplemented. Glucose was added at a final concentration of 2 % in rich (YPD) and selective media (SD), or 5 % in GAA medium [YP medium with 5 μM antimycin A (Sigma-Aldrich)]. Galactose was added at a final concentration of 2 %. The unsaturated fatty acids (UFAs) – oleic and palmitoleic acids (Sigma-Aldrich) – were used at a final concentration of 2 %. The unsaturated fatty acids (UFAs) – oleic and palmitoleic acids (Sigma-Aldrich) – were used at a final concentration of 10 μg ml⁻¹, from a DMSO stock solution. Geneticin (G418; Sigma) was added at a final concentration of 100 μg ml⁻¹. Bacterial cells were cultivated in Luria–Bertani medium: 1 % tryptone (Becton Dickinson), 0.5 % yeast extract (Becton Dickinson), 1 % NaCl. Ampicillin was added at a final concentration of 100 μg ml⁻¹. Solid media contained 2 % bacto-agar (Becton Dickinson). Yeast cells were grown normoxically in Erlenmeyer flasks at 28 °C on an orbital shaker at 175 r.p.m. (typically 100 ml medium in 500 ml flask) or in a bioreactor (B-Braun Biostat®Q) at 28 °C with stirring at 150 r.p.m. and air flow of 0.5 vol min⁻¹ at 1.2 bar. Hypoxia was routinely obtained by incubation in tightly capped tubes completely filled up with normoxic cultures from flasks, as previously described by Micolonghi et al. (2011). The eventual accumulation of metabolites with repressive activity, like ethanol (Bianchi et al., 1996) or carbon dioxide/HCO₃⁻ (M. M. Bianchi, unpublished data), did not affect the induction of transcription of the hypoxic reporter gene KLPDC1. The hypoxic condition in the bioreactor was achieved by closing the inlet air valve: oxygen concentration in the medium dropped to zero within 10 min. The hypoxic induction profile of KLPDC1 transcription was identical in cells from tubes or from the bioreactor for the investigated incubation times (Fig. S1).

RNA extraction and analysis. Yeast total RNA was extracted from cultures grown in YPD medium to OD₆₀₀ 1 using the hot phenol protocol (Köhler & Domdey, 1991) and quantified by measuring the absorption at 260 nm. Northern blot assays were performed by electrophoresis in agarose/formaldehyde gels and transferred to membranes following a standard procedure as described by Bianchi et al. (1996). Probes were obtained by PCR using GDK genomic DNA as a template and the primers reported in Table S1. [α-³²P]ATP-labelling of the probes was performed using the Random Primed Labelling Kit (Roche), following the instructions provided by the manufacturer.

Oxygen consumption and ethanol production assays. The oxygen consumption rate was measured at 28 °C by using a polarographic electrode in air-saturated 1 mM sodium phosphate (pH 7) plus 20 mM glucose solution where mid-exponentially growing cells (OD₆₀₀ between 2 and 3) were added at different final densities (0.2–1.0 OD units ml⁻¹). Cells, grown in YPD medium, were washed and suspended in 1 mM phosphate buffer before addition to the air-saturated solution. Ethanol production was determined enzymically (Ethanol kit; Nzytech Genes and Enzymes) as follows. GDK and mutant strains were cultivated in the bioreactor in YPD medium under normoxic conditions (air flow at 0.5 vol min⁻¹, 1.2 bar) until they reached OD₆₀₀ 1. Then hypoxia was induced by closing the air flux. Withdrawn samples were immediately centrifuged and clear supernatants were frozen at −20 °C for subsequent analysis, which was performed following the manufacturer’s instructions.

Fatty acid composition analysis. Fatty acid composition of membranes was performed by gas chromatography. The GDK and deletion mutant strains were cultivated in normoxia to OD₆₀₀ 1 and then shifted to hypoxia. After incubation, cells were collected by centrifugation, washed with sodium phosphate buffer (50 mM, pH 7) and lipids were extracted with chloroform/methanol/water; fatty acid methyl esters (FAMEs) were prepared by 15 min incubation at 95 °C in boron trifluoride/methanol and extracted with hexane. FAMEs were then analysed as described by Heipieper et al. (2000). The fluidity of the membrane lipids, expressed as the fatty acid unsaturation index (UI), was calculated as follows: [(% C16 : 1 + C18 : 1) + (% C18 : 2 x 2) + (% C18 : 3 x 3)]/100.

β-Galactosidase assays. Total protein extracts were obtained by breaking cells with glass beads (Sigma-Aldrich) in breaking buffer (100 mM Tris/HCl, pH 8; 1 mM DTT; 20 %, w/v glycerol). β-Galactosidase activity was measured as described by Miller (1972).

Western blotting. Cultures were grown to OD₆₀₀ 1 and were harvested and then suspended at 50 OD units ml⁻¹ in sterile water. An equal volume of 0.2 M NaOH was added and cells were incubated at room temperature for 5 min, then centrifuged for 2 min at 10,000 g. After elimination of the supernatant, the pellets were suspended in Laemmli buffer (60 mM Tris/HCl, pH 6.8; 50 %, w/v glycerol; 2 % SDS; 5 % β-mercaptoethanol; 2 % bromophenol blue). The samples were boiled for 5 min, kept on ice for 2 min and centrifuged at 10,000 g for 1 min, then loaded on a 10 % acrylamide gel for SDS-PAGE. After electrophoretic separation, the proteins were transferred to a PVDF transfer membrane (Amersham Hybond-P). Western blotting was performed with mouse anti-FLAG primary antibodies (Sigma-Aldrich) and horseradish peroxidase-conjugated anti-mouse antibodies (Santa Cruz Biotechnology). Detection was performed with ECL Western blotting detection reagents (Amersham).

RESULTS

Identification and hypoxic expression of KIMGA2

We explored the K. lactis genome (http://genolevures.org) for the presence of MGA2 homologues and identified the coding sequence KLLA0E17953g that was named KIMGA2. BlastP alignment showed conservation of the two proteins, with 39 % identity and 54 % similarity (Fig. S2). Smart analysis of KIMGA2 revealed the presence of putative functional domains which were identical and arranged similarly to Mga2: the IPT (Ig, plexin, transcription factor) DNA-binding domain, the ankyrin-like, protein–protein interaction domain and the transmembrane (TM) domain. The amino acidic sequence LPKYxxxxxxxxxKxxKxK, involved in the proteolytic maturation of Mga2 (Shcherbik et al., 2004), was also found in KIMga2. A coiled-coil domain was present only in Mga2, close to the TM domain. In addition to MGA2, the S. cerevisiae genome contains the ohnologous gene SPT23 derived from the whole genome duplication event (http://wolfe.gen.tcd.ie/ygob).

In S. cerevisiae, Mga2 is responsive to oxygen levels and is involved in the expression of hypoxic genes. Therefore we investigated whether KIMGA2 transcription was modulated by oxygen availability. RNA was extracted both from cells grown aerobically in standard Erlenmeyer flask conditions, and after shifting from normoxic to hypoxic conditions in tubes, as described previously (Micolonghi et al., 2011). KIMGA2 transcription was assayed by Northern blotting, results of which are reported in Fig. 1(a). The expression level of KIMGA2 was significantly increased during the
hypoxic shift (20 min), and this level was maintained until approximately 150 min.

**Metabolic defects of the mutant strain GDK/\(Klmg2\Delta\)**

In order to investigate the possible roles of \(Klmg2\) in the hypoxic response, we deleted \(KIMGA2\) by disruption with the selection marker \(KanMX4\) (Wach et al., 1994). The GDK/\(Klmg2\Delta\) strain showed reduced growth on YPD plates with respect to the wild-type (Fig. 2a). Transformation with the cognate gene (vector KpKIMGA; Table 1) fully restored wild-type growth. We tested GDK/\(Klmg2\Delta\) growth in the bioreactor (Fig. 2b) under fully aerated conditions in YPD medium. After a lag phase, the aerobic growth of the mutant strain started with a specific growth rate \((\mu)\) of \(0.13\pm0.01\ \text{h}^{-1}\), compared with \(0.42\pm0.07\ \text{h}^{-1}\) of the wild-type strain (mean \(\pm\ SD\) of three independent experiments). After induction of hypoxia to exponentially growing cells (Fig. 2c), the growth rate of both strains slowed, although the difference between the wild-type \((\mu=0.054\pm0.011\ \text{h}^{-1})\) and the mutant strains \((\mu=0.034\pm0.003\ \text{h}^{-1})\) was reduced. These results suggested that deletion of \(KIMGA2\) might impair both respiratory and fermentative metabolism.

The efficiency of respiratory metabolism was tested by growing the mutant and the wild-type strains on non-fermentable carbon sources (ethanol plus glycerol) in Erlenmeyer flask cultures, under standard aerobic bench conditions (Fig. S3a). The aerobic growth rate of the wild-type strain was almost identical on ethanol plus glycerol \((\mu=0.28\ \text{h}^{-1})\) and glucose \((\mu=0.30\ \text{h}^{-1})\). In the mutant strain, the aerobic growth rate on ethanol plus glycerol \((\mu=0.08\ \text{h}^{-1})\) was strongly reduced even with respect to the growth rate on YPD \((\mu=0.12\ \text{h}^{-1})\). The respiratory capacities of the two strains were compared by measuring the rate of oxygen consumption by exponentially growing cells suspended at various densities in air-saturated sodium phosphate buffer. The rate of oxygen consumption of the GDK/\(Klmg2\Delta\) strain was \(75\pm1.4\ \%\) of that of the wild-type (Fig. S3b), thus confirming the detrimental effect of \(KIMGA2\) deletion on respiration.

In \(K.\ lactis\), the impaired growth phenotype on rich glucose medium supplemented with the mitochondrial inhibitor antimycin A (GAA medium) is known as the Rag-
phenotype (Wesolowski-Louvel et al., 1992) and is usually ascribed to defective glycolysis and/or fermentation. The GDK/Klmga2Δ strain exhibited a Rag—phenotype, and this was fully recovered by the KpKlMGA vector (Fig. 2a). In order to assess if the fermentative metabolism was affected in the deletion mutant, we measured ethanol production in bioreactor cultures after induction of the hypoxic shift (Fig. S4a, b). The total amount of ethanol produced by the two strains was identical. Surprisingly, the rate of production seemed to be higher for the mutant strain, especially if its slower growth rate was taken into account. Furthermore, the phenotype of the mutant strain, Rag−, was not suppressed by overproduction of the fermentative enzyme KlPDcd1 (Fig. S4c) in clones transformed with the multicopy plasmid KEpPDC (Destruelle et al., 1999).

**Phenotype suppression of the deleted strain GDK/Klmga2Δ**

The Rag− phenotype, in association with the slow growth phenotype on YPD medium and reduced respiration, suggested that KLMGA2 might not be necessarily involved in glycolysis and/or fermentative metabolism but could be involved in other mechanisms affecting the general cell fitness. In S. cerevisiae, Mga2 is a regulatory protein involved in the synthesis of UFAs. We tested whether the presence of palmitoleic (16:1cis) and oleic (18:1Δ9cis) acids could suppress the GDK/Klmga2Δ growth phenotype. Results (Fig. 3a) indicate that the addition of UFAs was beneficial to the mutant strain, suggesting a functional correlation mediated by Klmga2 between UFA biosynthesis and defective growth. We determined the fatty acid compositions of wild-type and mutant cells. Percentages of saturated C16:0 and C18:0 fatty acids (Fig. 3b) were similar. A parameter to express membrane fluidity is UI, calculated from the fatty acid composition. The UI is a value for the average number of double bonds per fatty acid (Heipieper et al., 2000; Cialfi et al., 2011): the lower the UI, the lower the fluidity of membranes. Thus, the relative composition of the three C18 unsaturated fatty acid species, which varied significantly between the two strains, yielded a lower UI for the mutant (1.04 ± 0.07) with respect to the wild-type (1.29 ± 0.01). The addition of ergosterol to YPD or GAA plates or the presence of hydrogen peroxide in YPD plates (Kelley & Ideker, 2009) did not result in a growth phenotype variation in the Klmga2Δ strain (data not shown).

**Expression of hypoxic genes in GDK/Klmga2Δ strain**

The KIPDC1 gene, encoding the unique K. lactis pyruvate decarboxylase enzyme, is tightly regulated at the transcriptional level and is induced by hypoxia (Bianchi et al., 1996; Destruelle et al., 1999; Camattari et al., 2007): its mutation generates Rag− strains. In order to find out whether Klmga2 was involved in KIPDC1 transcriptional regulation, hypoxic KIPDC1 expression was assayed in the mutant strain (Fig. 4a). Induction of KIPDC1 transcription was maximal 20–90 min after the hypoxic shift. Transcriptional induction was still effective in the GDK/Klmga2Δ strain although the increase of PDC1 transcripts was slightly reduced. GDK and the mutant strain harboured the reporter gene lacZ fused downstream of the KIPDC1 promoter (Destruelle et al., 1999). Transcription results were confirmed by measuring the β-galactosidase activity (Fig. 4b), which was only twofold higher in the mutant under hypoxia, in comparison with a fourfold induction in the GDK strain.

In S. cerevisiae, the fatty acid desaturase gene OLE1 is induced by hypoxia (Kwast et al., 1999) and is a consolidated target gene of Mga2 (Kwast et al., 1999; Jiang et al., 2001; Vasconcelles et al., 2001). We identified ORF KLLA0C05566g as the orthologue gene of OLE1 in K. lactis and named it KIOLE1. Northern blot analysis of KIOLE1 transcription is reported in Fig. 4(c). KIOLE1 expression

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**Fig. 3.** Growth of the GDK/Klmga2Δ strain on UFAs. (a) The mutant and wild-type strains were streaked onto YPD and GAA plates with and without supplemented UFAs. (b) Fatty acid compositions of the wild-type (black bars) and mutant (white bars) strain. Values of relative (total=100) amounts of palmitic (C16:0), palmitoleic (C16:1cis), stearic (C18:0), oleic (C18:1Δ9cis), linoleic (C18:2Δ9,12cis,cis) and linolenic (C18:3Δ9,12,15cis,cis,cis) acids are reported. Results are the mean ± SD of two independent lipid extractions.
Fig. 4. Hypoxic transcription of KIPDC1 and KIOLE1. (a) Northern blot analysis of KIPDC1 in the wild-type strain (GDK) and in the GDK/Klmga2Δ mutant strain (Klmga2Δ). The KIPDC1 probe is described by Bianchi et al. (1996). (b) β-Galactosidase activities from crude extracts of normoxic cultures (N) and after 90 min of hypoxic shift (H) of the wild-type (white bars) and mutant (grey bars) strains. Results are mean ± SD of three independent cell cultures and protein extractions. (c) Effect of the hypoxic shift on KIOLE1 transcription in wild-type (GDK) and GDK/Klmga2Δ mutant (Klmga2Δ) strains. (d) Effect of the rag4 mutation. mRNA samples were taken from strains grown on YP medium: wild-type (PM6-7A) and PM6-7A/VV32 mutant (rag4). In Northern blot figures, samples are as follows: N, normoxic; 20 and 90, hypoxic incubations. rRNA is shown as a reference.

was induced by hypoxia in both the wild-type and the GDK/Klmga2Δ strains. Quantitative analysis of signals from repeated experiments (data not shown) demonstrated that the level of hypoxic expression of KIOLE1, similar to KIPDC1, was reduced but not abolished in the mutant strain. OLE1 is repressed by UFAs in S. cerevisiae (Choi et al., 1996). We tested the effect of UFAs on the hypoxic induction of KIOLE1. Results (Fig. S5a) showed that UFAs only slightly reduced KIOLE1 transcription in normoxic cells. Interestingly, when normoxic cells were shifted to hypoxia in the presence of UFAs, KIOLE1 induction could not be prevented, but when the hypoxic incubation followed the UFA treatment, KIOLE1 induction did not occur. UFA repression of KIOLE1 was effective when carried out after the hypoxic incubation, although transcription could not be reduced to the normoxic level within 90 min. Unlike in S. cerevisiae, transcription of KIOLE1 (Fig. S5b) was not induced by cobalt chloride (Vasconcelles et al., 2001) or by incubation at low temperature (Nakagawa et al., 2002).

The list of Mga2 target genes from the YEASTRACT database (http://www.yeastract.com; Teixeira et al., 2006; Monteiro et al., 2008; Abdulrehman et al., 2011) was used to extract potential targets of KIMGA2. We identified ORFs KLLA0B02717g, KLLA0F15224g, KLLA0C03542g, KLLA0F15202g and KLLA0C12694g as probable orthologue genes of FAS1 (fatty acid synthetase, β subunit), ERG1 (squalene epoxidase), ELO1 (UFA elongase), ATFI (alcohol acyltransferase) and OAR1 (mitochondrial 3-oxoacyl reductase), respectively. The K. lactis genes were named as usual. Their expression was analysed by Northern blotting. The genes KIFAS1 and KIERG1 were induced by hypoxia and their expression and/or induction was clearly KIMGA2-dependent (Fig. 5a, b), KIATFI1 was also induced by hypoxia and was dependent on KIMGA2 (data not shown). Conversely, KIELO1 and KIOAR1 transcription was not induced by hypoxia and was not affected by the absence of KIMGA2 (data not shown).

Expression of hypoxic genes in a rag4 mutant strain

We have previously demonstrated (Micolonghi et al., 2011) that hypoxic induction of KIPDC1 transcription was dependent on the glucose sensor Rag4. We assayed the hypoxic expression of KIMGA2 (Fig. 1b) and of the structural hypoxic genes KIOLE1 and KIERG1 (Figs 4d, 5c, respectively) in the parental strain PM6-7A and the rag4 mutant strain VV32 (Betina et al., 2001). Although the induction of transcription was slightly reduced in the PM6-7A strain with respect to the GDK strain, results clearly indicate that hypoxic induction of KIMGA2 and KIOLE1 was dependent on the RAG4 gene. In these experiments, RNA was prepared from cultures grown on YP medium, without glucose to minimize the interference of glucose regulation. Interestingly, the hypoxic transcription of KIERG1 (Fig. 5c) was completely absent in both PM6-7A and VV32 strains. However, when RNA was prepared from cultures grown on glucose medium, the usual hypoxic induction of KIERG1 transcription was observed but only in the wild-type strain (Fig. 5d), suggesting the involvement of a combined mechanism of regulation by the carbon source and oxygen levels. The latter finding was also demonstrated by Northern blot analysis of GDK RNA from YP cultures (data not shown) where hypoxic induction of transcription was no longer observed for KIERG1.
Reciprocal suppression by Mga2 and KlMga2 in the deletion mutant strains

In \textit{S. cerevisiae}, the single deletion of \textit{MGA2} or \textit{SPT23} produces a viable mutant strain while the double-deletion strain is auxotrophic for UFAs (Zhang \textit{et al.}, 1999; Chellappa \textit{et al.}, 2001). The main physiological defect of the double-deletion mutant is inappropriate membrane composition due to impaired UFA biosynthesis. In fact, UFA auxotrophy of the double-deletion strain can be suppressed by \textit{OLE1} overexpression (Zhang \textit{et al.}, 1999). In the mutant strain \textit{GDK/Klmga2}, \textit{KIOLE1} transcription was still operating and was inducible by hypoxia. As a consequence, altered UFA biosynthesis might not be the unique cause of the reduced growth rate of this strain. We tested whether \textit{MGA2} from \textit{S. cerevisiae} could suppress \textit{GDK/Klmga2} phenotypes. We transformed the \textit{GDK/Klmga2} strain with vector \textit{KpScMGA}, harbouring the \textit{ScMGA2} gene under the control of the constitutive \textit{ADH1} promoter. The transformed clones (Fig. 2a) fully recovered growth on YPD and GAA plates. Fig. 5(b) shows the effect of the heterologous expression of \textit{MGA2} on the hypoxic induction of \textit{KIERG1} transcription: the gene from \textit{S. cerevisiae} could apparently functionally substitute for \textit{KlMGA2} in transcription regulation, although hypoxic induction of \textit{KIERG1} in the transformed clones was not as high as in the wild-type strain. Western blot analysis of crude extracts from the \textit{K. lactis}-transformed clones (Fig. 6a) showed a maturation pattern of Mga2 similar to that reported in \textit{S. cerevisiae} (Shcherbik \& Haines, 2007), involving ubiquitination and proteasomal proteolysis.

In \textit{S. cerevisiae}, hypoxic induction of \textit{OLE1} transcription depends upon Mga2. We tested whether \textit{KlMga2} could substitute for Mga2 in this regulation. To this purpose, we cloned \textit{KlMGA2} in the regulated expression vector \textit{pYES} (Table 1) and transformed the \textit{BY4741/mga2\Delta} strain. The expression of \textit{OLE1} in normoxic and hypoxic conditions is

\textbf{Fig. 5.} Hypoxic transcription of \textit{KIFAS1} and \textit{KIERG1} in the wild-type (GDK) and mutant (\textit{Klmga2\Delta}) strains. (a, b) Transcription of \textit{KIFAS1} (a) and \textit{KIERG1} (b). The additional panel on the right (b) shows \textit{KIERG1} transcription analysis in the deletion-mutant strain transformed with vector \textit{KpScMGA}. To reduce clonal variability and plasmid-loss effects, RNA was extracted from a pool of independent transformants grown on selective medium, supplemented with 0.2\% Casamino acids. (c, d) Effect of the \textit{rag4} mutation on \textit{KIERG1}. mRNA samples were extracted from YP and YPD cultures of the wild-type strain (PM6-7A) and from the mutant strain PM6-7A/VV32 (\textit{rag4}). N, normoxic; 20, 60, 90, hypoxic incubations.

\textbf{Fig. 6.} Heterologous expression of \textit{MGA2} and \textit{KlMGA2}. (a) Western blot analysis of two \textit{KpScMGA}-transformed clones (1 and 2) of strain \textit{GDK/Klmga2\Delta}. M, molecular mass marker with sizes indicated on the left; nt, non-transformed \textit{GDK/Klmga2\Delta}; V, \textit{GDK/Klmga2\Delta} transformed with an empty vector. Signals at 120 and 100 kDa might correspond to the entire and matured proteins, respectively. The higher molecular mass signal represents the polyubiquitinated form. (b) Northern blot analysis of \textit{OLE1} in the wild-type strain \textit{BY4742} and in the \textit{mga2}-deleted strain (\textit{BY4741/mga2\Delta}) transformed with \textit{pYES\textsuperscript{[FLAG\textit{KlMGA2}]}} (+\textit{KIMGA2}) or with the empty vector (V). A pool of transformants was grown for 6 h on glucose (glu) or galactose (gal) medium and then subjected to normoxic (N) and hypoxic (H) incubations.
reported in Fig. 6(b). Induction of OLE1 could be detected in hypoxic conditions only when KLMGA2 was previously expressed on galactose medium, indicating that KIMga2 could functionally replace Mga2 in hypoxic transcription regulation. The level of OLE1 induction by KIMga2 was similar to that in the wild-type strain.

**DISCUSSION**

Environmental oxygen is a fundamental component for living organisms, especially aerobic organisms that use molecular oxygen for energy and essential metabolic reactions. These organisms, from bacteria to mammals, developed systems for oxygen sensing and developed responses to hypoxic conditions. In some cases, the oxygen-sensing mechanism is believed to function via seizing by cytoplasmic reactions using molecular oxygen as a substrate, like haem biosynthesis or protein hydroxylation.

We have recently shown that transcription of the pyruvate decarboxylase gene (KIPDC1) in response to hypoxia is mediated by the glucose sensor Rag4 in *K. lactis* (Micolonghi et al., 2011). By extending the analysis to other hypoxic genes like KLMGA2, we show here that Rag4 is actually a master hypoxic sensor heading a dual regulatory cascade that governs the expression of two sets of hypoxic structural genes. The first set includes KIPDC1 and KIOLE1: the latter, similar to KIPDC1, is induced by hypoxia independently of KIMga2. Both genes require Rag4 for induction. The second set includes KIERG1, KIFAS1 and KIATF1: the regulatory pathway leading to their hypoxic induction is mediated by KLMGA2, which is, in turn, induced by hypoxia in a Rag4-dependent fashion. These findings indicate a close correlation between the oxygen-dependent regulation of glucose metabolism and lipid biosynthesis. Actually, the catabolism of glucose through glycolysis and PDC produces C2 compounds which, in the form of cytoplasmic acetyl-CoA, are the substrates of fatty acid and ergosterol biosynthesis. Rag4 is the unique glucose sensor, and because of its nature and function, it is a good candidate for environmental oxygen sensing. In *S. cerevisiae*, where two glucose sensors are present for high and low glucose concentrations (Rgt2 and Snf3, respectively), carbon metabolism is predominantly regulated and oriented towards fermentation by glucose while oxygen seems to have only a marginal role: for example, the branch-point PDC genes are not induced by hypoxia (Kwast et al., 2002). In contrast, our findings indicate that the balance between respiration and fermentation in *K. lactis* seems to be determined equally by glucose and oxygen through the single sensor Rag4. Signal transduction downstream of Rag4 has been extensively studied (Rolland et al., 2006; Neil et al., 2007; Hnatova et al., 2008), especially as far as the expression of the glucose transporter Rag1 is concerned. The involvement of the glucose-signalling cascade in the hypoxic response and the hypoxic regulation of *RAG1* are currently under investigation, as is the role of glucose in hypoxic induction and in fatty acid biosynthesis, especially when they are mediated by KIMga2.

The regulatory gene KLMGA2 has been identified in *silico* using the *S. cerevisiae* MGA2 sequence as a reference. Genes homologous to MGA2 are present in all of the ascomycete genomes provided by the Génolevures database (http://genolevures.org). The proteins encoded by these genes are highly conserved in sequence. Except for a coiled-coil domain absent in KLMga2, all the known functional domains present in Mga2 are conserved in KLMga2, suggesting an evolutionary maintenance of the mechanism of action. Mga2 is a dormant membrane protein in the endoplasmic reticulum which is activated by ubiquitination and proteasomal maturation at a specific sequence that is also present in KLMga2. Whether or not the hypoxic shift activates proteolytic maturation of KIMga2 is currently under investigation; however, the heterologous expression and proteolysis of ScMga2 in *K. lactis* supports the hypothesis that KLMga2 might also be subjected to the same maturation mechanism. In *S. cerevisiae*, this process is triggered by hypoxia, a reduction in UFA content and low temperature (Hoppe et al., 2000; Jiang et al., 2001; Nakagawa et al., 2003). We have shown here that transcription of KLMGA2 is steadily induced by hypoxia. This effect might be an alternative mechanism of regulation of KLMga2 activity in *K. lactis* or an additional step to post-translational maturation. Our findings indicate that KLMga2 and Mga2 are actually functionally identical and exchangeable proteins. Consequently, their physiological roles might be very similar although not necessarily identical, for example in lipid biosynthesis as discussed below.

Similar to OLE1 of *S. cerevisiae* (Jiang et al., 2002), KIOLE1 transcription is induced by hypoxia and repressed by UFAs. Oxygen shortage promptly induces KIOLE1 transcription overcoming UFA repression, while a preliminary incubation with UFAs blocks hypoxic induction. Interestingly, repression by UFAs of hypoxia-induced KIOLE1 transcription occurs slowly. This might be the consequence of the stabilization of KIOLE1 mRNA as demonstrated in *S. cerevisiae*, where activation of OLE1 transcription and mRNA stabilization appear to be separate functions of Mga2 (Kandasamy et al., 2004). Since we have demonstrated that hypoxic induction of KIOLE1 transcription is independent of KLMga2, a possible role for this protein in KIOLE1 mRNA decay might be envisaged. The fermentative gene KIPDC1 is strongly and rapidly induced by hypoxia, but similar to KIOLE1, only the basal level and intensity of induction of KIPDC1 transcription is affected by KLMga2.

The deletion of KLMGA2 generates important phenotypes: a slow growth rate on both fermentative and respiratory carbon sources and sensitivity to antimycin A (Rag- phenotype). Interestingly, the latter is not to be ascribed to defective glycolysis or fermentation, because ethanol biosynthesis of the mutant strain under hypoxia is unaffected, but rather to the involvement of KLMga2 in a UFA biosynthetic step(s)
requiring a mitochondrial function(s), as suggested by the suppression of the Rag− phenotype by addition of UFAs. The correlation between Klmga2 and mitochondrial functions is also suggested by the reduced respiration capacity of the mutant strain, possibly affecting the growth rate on respiratory carbon sources and/or on glucose in normoxic conditions.

*K. lactis* membranes contain linoleic (C18:2Δ9,12cis,cis) and linolenic (C18:3Δ9,12,15cis,cis,cis) acid, both of which are absent in *S. cerevisiae*, synthesized by the desaturases *KlpfAD2* (A12) and *KlpfAD3* (A15) (Kainou et al., 2006). Since oleic acid accumulates and ω6 (linoleic acid) and ω3 (linolenic acid) fatty acids are reduced in the Klmga2Δ mutant strain, the Klmga2 protein might modulate the activity of the Δ12 and Δ15 desaturases and might reasonably be involved in the regulation of membrane fluidity through the synthesis of polyunsaturated lipids. In *S. cerevisiae*, this effect is obtained by regulating only Ole1 desaturase. In comparison with the wild-type strain, the Klmga2Δ strain has a reduced UI, a measure of membrane fluidity, which might affect membrane functions of organelles – including mitochondria – and cellular fitness. Therefore, the phenotypic suppression by UFAs (palmitoleic and oleic acid) on the growth rate of the mutant strain might be due to the increased cellular content of ω9 mono-unsaturated fatty acids, which compensates for the reduced UI in the mutant strain. However, the relative composition of unsaturated/polyunsaturated fatty acids did not change with hypoxia in both the wild-type and mutant strains (data not shown). The lack of suppression by ergosterol on the growth rate of the Klmga2Δ strain could depend on the absence of genes involved in the uptake of sterols in *K. lactis* (Snoek & Steensma, 2006).

Finally, our results indicate that Klmga2 is a possible regulator that functions in the hypoxic response, membrane structure and functions, and respiration. However, Rag4 played a significant role in mediating both glucose and oxygen signalling and controlling the expression of a larger set of genes involved in carbon metabolism and lipid biosynthesis. The regulation of respiration and fermentation and the fatty acid composition of membranes suggest that *K. lactis* might be a good higher eukaryote model for studying the hypoxic response, as an alternative to the extremely specialized and fermentative yeast *S. cerevisiae*.

**ACKNOWLEDGEMENTS**

This work was supported by MIUR (2006051483), Istituto Pasteur Fondazione Cenci-Bolognetti and Sapienza Università di Roma (C26A1075W).

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Edited by: D. Mattanovich