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Characterization of the transcription factor encoding gene, *KlADR1*: metabolic role in *Kluyveromyces lactis* and expression in *Saccharomyces cerevisiae*

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In *Saccharomyces cerevisiae*, Adr1 is a zinc-finger transcription factor involved in the transcriptional activation of *ADH2*. Deletion of *KlADR1*, its putative ortholog in *Kluyveromyces lactis*, led to reduced growth in glycerol, oleate and yeast extract-peptone medium suggesting, as in *S. cerevisiae*, its requirement for glycerol, fatty acid and nitrogen utilization. Moreover, growth comparison on yeast extract and peptone plates showed in *K. lactis* a KlAdr1-dependent growth trait not present in *S. cerevisiae,* indicating different metabolic roles of the two factors in their environmental niches. *KlADR1* is required for growth under respiratory and fermentative conditions like *KlADH*, alcohol dehydrogenase genes necessary for metabolic adaptation during the growth transition. Using in-gel native alcohol dehydrogenase assay, we showed that this factor affected the Adh pattern by altering the balance between these activities. Since the activity most affected by KlAdr1 is KlAdh3, a deletion analysis of the *KlADH3* promoter allowed the isolation of a DNA fragment through which KlAdr1 modulated its expression. The expression of the *KlADR1-GFP* gene allowed the intracellular localization of the factor in *K. lactis* and *S. cerevisiae,* suggesting in the two yeasts a common mechanism of KlAdr1 translocation under fermentative and respiratory conditions. Finally, the chimeric *Kl/ScADR1* gene encoding the zinc-finger domains of KlAdr1 fused to the transactivating domains of the *S. cerevisiae* factor activated in *Scadr1*Δ the transcription of *ADH2* in a ScAdr1-dependent fashion.

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INTRODUCTION

Four major alcohol dehydrogenase (*ADH*) genes are present in the yeast *Saccharomyces cerevisiae*. *ADH1* encodes the fermentative enzyme involved in the production of ethanol (Lutstorf & Megnet, 1968; Bennetzen & Hall, 1982). *ADH2* codes for a glucose-repressible enzyme involved in the oxidation of the ethanol produced during fermentation (Ciriacy, 1975, 1979; Denis *et al.*, 1981). *ADH3* codes for a mitochondrial enzyme, a component of the ethanol/acetaldehyde shuttle (Young & Paquin, 1985; Bakker *et al.*, 2000), while *ADH5* codes for a minor activity whose physiological role has not yet been defined (Dickinson *et al.*, 2003).

Genetic analysis of the *ADH2* locus led to the identification and isolation of *ADR1* (Ciriacy, 1979; Denis & Young, 1983), a gene coding for a carbon source-responsive zinc-finger transcription factor, the major regulatory protein involved in the transcriptional activation of *ADH2*. This factor is also required for the induction of genes that are regulated by glucose repression (Young *et al.*, 2003), for the biogenesis of peroxisomes and for ethanol, glycerol and fatty acid utilization (Simon *et al.*, 1991; Young *et al.*, 2003; Tachibana *et al.*, 2005).

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Abbreviations: ADH, alcohol dehydrogenase; β-gal, β-galactosidase; RE, repression ethanol; YE, yeast extract; YNB, yeast nitrogen base; YP, yeast extract-peptone.

S. cerevisiae is a Crabtree-positive yeast in which respiration is completely repressed by glucose even under aerobic conditions, growth being supported solely by fermentation. In contrast, in the Crabtree-negative *Kluyveromyces lactis*, both respiratory and fermentative pathways coexist during growth on glucose (De Deken, 1966), although respiration appears dispensable since antimycin A does not inhibit growth on glucose (Rag^+ phenotype) (Goffrini *et al.*, 1989; Wésolowski-Louvel *et al.*, 1992).

In *K. lactis*, four *ADH* genes have also been identified (*ADH1–ADH4*) (Saliola *et al.*, 1990, 1991; Shain *et al.*, 1992). *KlADH1* and *KlADH2* encode two enzymes preferentially expressed on fermentative carbon sources and *KlADH3* and *KlADH4* code for two mitochondrial enzymes, which are probably components of the ethanol/acetaldehyde shuttle (Saliola & Falcone, 1995; Bakker *et al.*, 2000; Overkamp *et al.*, 2002; Saliola *et al.*, 2008). The latter two genes are regulated at the transcriptional level by carbon source-dependent growth conditions and their expression is mutually exclusive (Saliola & Falcone, 1995). In fact, *KlADH4* is specifically induced by ethanol added to the medium or produced by fermentation. On the other hand, this compound specifically represses the expression of *KlADH3,* which, differently from *KlADH4*, is highly expressed on all respiratory carbon sources with the exception of ethanol (Saliola & Falcone, 1995). The corresponding activities have been identified by genetic analysis in native PAGE by an ADH-specific staining assay and characterized at the biochemical level (Lutstorf & Megnet, 1968; Mazzoni *et al.*, 1992; Saliola & Falcone, 1995; Bozzi *et al.*, 1997). Although a deletion analysis of the *KlADH4* and *KlADH3* promoters led to the identification of regulatory elements, no transcription factors directly involved in their activation have yet been found (Saliola *et al.*, 1999, 2007; Mazzoni *et al.*, 2000). The identification of a putative ortholog of *ScADR1* in the genome of *K. lactis* (KLLA0F13046g) (Dujon *et al.*, 2004; Bussereau *et al.*, 2006) led us to investigate its role in the regulation of *ADH* genes and in the metabolism of this yeast.

KlADR1 is required for growth in glycerol, oleate and yeast extract-peptone (YP) medium. Measurements of glycerol, ethanol and oxygen reoxidation rate showed that KlAdr1 regulates both respiratory and fermentative metabolisms displaying an Adh pattern with an altered balance between the four different Adh activities. Since the activity mostly affected by KlAdr1 was KlAdh3, a deletion analysis of the *KlADH3* promoter allowed the isolation of a DNA fragment through which KlAdr1 modulates the expression of *KlADH3* and, indirectly, that of *KlADH4*.

In addition, the *KlADR1-GFP* gene allowed the intracellular localization of the fusion protein under different growth conditions in both yeasts.

Finally, a chimeric *K. lactis/S. cerevisiae* Adr1 containing the zinc-finger domains of *K. lactis* activated in *Scadr1*Δ the transcription of *ADH2* under de-repressing conditions like the endogenous factor.

METHODS

Strains, media and culture conditions. The strains used in this work are reported in Table 1.

Cultures were grown under shaking conditions at 28 °C in YP [1 % Difco yeast extract (YE), 2 % Difco Bacto-peptone] or in minimal yeast nitrogen base (YNB) medium (6.7 g l^{-1} Difco YNB) supplemented by different carbon sources at concentrations specified below, while *S. cerevisiae* cultures were also supplemented by Bacto Casamino Acids (Difco) at a concentration of 2.5 mg ml^{-1}. Oleate medium contained 0.1 % oleic acid, 0.02 % Tween 40, 0.1 % YE and 0.67 % YNB. Ergosterol was added to YPD plated at 20 μg ml^{-1}. Antimycin A was supplemented at a concentration of 5 μM in YP plates containing 5 % glucose. Geneticin (G418) concentration in the medium was 100 μg ml^{-1} (*K. lactis*) or 200 μg ml^{-1} (*S. cerevisiae*). Minimal medium was supplemented with auxotrophic requirements at the final concentration of 10 μg ml^{-1}. *Escherichia coli* strain DH5α was used for the propagation of plasmid DNA. Cultures were grown at 37 °C on LB medium (0.5 % YE, 1 % Difco tryptone, 0.5 % NaCl) supplemented with 100 μg ml^{-1} ampicillin.

In-gel ADH staining activity and β-galactosidase assay. Preparation of cellular extracts, clear native PAGE, electrophoresis conditions and ADH staining assay were carried out as previously described (Saliola & Falcone, 1995).

The construction of the *lacZ/KlADH3* fusion vectors used in this study for the deletion analysis of the *KlADH3* promoter (pCG1, pCG17, pCG13, pCG11, pCG7, pCG12, pCG29, pCG30, pCG31 and pCG32, respectively) was as previously described (Saliola *et al.*, 2007). The vector pCG33 was obtained from pCG32 following *Sac*I digestion and religation. This plasmid contains the first 529 bp of the promoter fused to the *KlADH3* gene.

Yeast transformants, used for β-galactosidase (β-gal) analysis, were selected for leucine prototrophy. Colonies were collected after 3 days of growth, inoculated into 2 ml of YNB containing 0.2 % glucose and

Table 1. Yeast strains used in this study

Strains	Genotype	Reference
MW179-1D	*MATα metA1 ade-T600 leu2 trpA1 ura3 lac4*	Saliola *et al.* (2008)
MW179-1D/Kladr1Δ	*Kladr1* :: *Kan*MX4	This study
MW179-1D/Kladh3Δ	*Kladh3* :: *URA3*	This study
MW179-1D/Kladr1Δ Kladh3Δ	*Kladh3* :: *URA3*	This study
MW179-1D/Klgut2Δ	*Klgut2* :: *Kan*MX4	Saliola *et al.* (2008)
BY4741	*MAT*a *his3Δ0 leu2Δ0 met15Δ0 ura3Δ0*	Euroscarf coll
BY4741/adr1Δ	*adr1* :: *Kan*MX4	Euroscarf coll

grown for a further 2 days. Finally, 200 µl of these cultures was inoculated in 10 ml of YNB containing 2 % ethanol or 2 % glycerol plus 0.1 % glucose and grown for a further 60 h ready for β-gal determination. Parental and deleted strain cultures grown on ethanol showed a higher biomass yield (roughly 3×10^8 cells ml^{-1}) than those grown in 2 % glycerol plus 0.1 % glucose (roughly 2×10^8 cells ml^{-1}). Four independent clones were inoculated for each construct and each β-gal value represents the media from at least three independent determinations. β-Gal values with a standard deviation >20 % were excluded from the determinations. β-Gal activity (nmol min mg^{-1} protein) was measured in crude extract prepared by breakage of yeast cells with glass beads according to Kaiser *et al.* (1994).

Assay methods. Glucose, glycerol and ethanol concentrations in culture supernatants were measured using commercial kits from R-Biopharm according to the manufacturer's instructions.

Amplification of *KlADR1* and construction of centromeric and multicopy plasmids. The *KlADR1* gene was amplified as *Xba*I fragments from the *K. lactis* genome with the following primers: forward: 5′-GGGTCTAGacctaccagcaagtgacgcttc-3′ and reverse: 5′-GGGTCtagagatataatcgccacaatttccac-3′ (in capital letters are sequences introduced to produce a *Xba*I site). The primer sequences were located at –1216 bp upstream of the ATG and +561 bp downstream of the stop codon. The amplified *KlADR1* was cloned as a 5.9 kbp *Xba*I fragment into the *Xba*I site of the pTZ19 vector (Pharmacia) to harbour pTZ19/KlADR1. The 5.9 kbp *KlADR1 Xba*I fragment was also cloned in the *Xba*I site of the *K. lactis* centromeric KCplac13 to harbour KCp-KlADR1, in the *Spe*I site of the multi-copy pKL plasmid (Saliola *et al.*, 2008) to harbour pKlADR1 and in the *Xba*I site of the *S. cerevisiae* centromeric pRS416 plasmid (pRS-KlADR1).

Construction of plasmids with the *KlADR1* gene fused in frame with the GFP gene. pTZ19/KlADR1 was digested with *Hpa*I (site located at the 3′ end terminal part of the gene 66 nucleotides before the stop codon) and ligated with the 0.8 kbp *Eco*ICRI-digested GFP gene fragment. Analysis of *E. coli* clones transformed with the ligated fragments allowed the isolation of pTZ18/KlADR1-GFP. This plasmid contains the *KlADR1* gene fused in frame with the GFP at the *Hpa*I site. This gene codes for a chimeric protein in which KlAdr1 is devoid of the last 22 amino acids as compared to the WT protein. pTZ18/KlADR1-GFP was digested with *Xba*I, and the purified KlADR1-GFP 6.8 kbp fragment was cloned in the *Xba*I and *Spe*I sites of the centromeric (KCplac13) and multi-copy (pKL) *K. lactis* plasmids, and into *S. cerevisiae* pRS416 to harbour KCp-KlADR1-GFP, pKlADR1-GFP and pRS-KlADR1-GFP, respectively.

Construction of the centromeric KCplac13 vector containing the chimeric *KlGUT2-GFP* gene has already been described (Saliola *et al.*, 2008).

Construction of the *KlADR1* deletion cassette. The deletion cassette was constructed by digesting pTZ19/KlADR1with *Stu*I–*Bgl*II and cloning the purified KanMX4 *Eco*RV–*Bgl*II-digested fragment obtained from the pFA6a vector at these sites. The final plasmid isolated (pTZ19/Kladr1Δ) contained a deletion of 95 % of *KlADR1*. The cassette was linearized by *Xba*I digestion and transformed into strain MW179-1D for gene replacement. Transformants were selected on plates containing G418. Resistant clones were first analysed by Southern blot: DNA prepared from putative deleted strains was transferred to filters and hybridized concomitantly with ^{32}P DNA probes containing the deleted portions of the *KlADR1* gene and the KanMX4 gene. The correct integration of the cassette into the *KlADR1* locus was finally confirmed by PCR using the primers previously used for its amplification and the KanMX4 K1 : 5′-ggatgtatgggctaaatgtacg-3′ and K2 : 5′-gtttcatttgatgctcgatgag-3′ primers. One of these deletants, named MW179-1D/Kladr1Δ, was further characterized.

Construction of strains with the *KlADR1* and *KlADH3* deletion. The strains MW179-1D/Kladh3Δ and MW179-1D/Kladr1Δ Kladh3Δ have been constructed transforming the parental strain with the linearized Kladh3 :: URA3 DNA deletion cassette (Mazzoni *et al.*, 1992). Putative deleted clones selected for uracil prototrophy were analysed for KlAdh3 activity on native gel. Strains unable to express this activity were further verified by PCR for *KlADH3* deletion with the primers (forward) 5′-ggtgaaaacgtcaagggctgg-3′, (reverse) 5′-agatggcaacccagtcaccag-3′ and *URA3* (forward) 5′-gcagtactctgcgggtgttatac-3′.

Construction of centromeric and multi-copy *ScADR1* plasmids. A pRS314 plasmid containing the *ScADR1* gene with its own promoter and terminator (Young *et al.*, 1998) was digested with *Spe*I–*Sal*I, and the 6.2 kbp purified fragment was cloned in the *K. lactis* KCplac13 centromeric and pKL multicopy vectors (Saliola *et al.*, 2008), digested with the same enzymes, to harbour KCp-ScADR1 and pKL-ScADR1, respectively.

Construction of the chimeric *K. lactis/S. cerevisiae ADR1* plasmid. Construction of the *K. lactis/S. cerevisiae ADR1* gene was performed in two steps. First, the 5′ end of *KlADR1* (containing 980 bp of the promoter and the sequence coding for the first 184 amino acids, comprising the zinc-finger domains of the protein) was amplified by PCR using the following primers: forward 5′-tgtcacgtgcctccaccctct-3′ and reverse-5′ GGGTCTAGAgatataatcgccacaatttccac-3′. The amplified fragment was digested with *Pml*I–*Xba*I and cloned in the *Eco*ICR–*Xba*I-digested sites of pRS416 to harbour pRS-5′KlADR1. In the second step, the 4.1 kbp fragment of the *ScADR1* gene, containing the DNA sequence that codes for the portion of the protein comprised from amino acid 164 to amino acid 1323 plus 600 bp of the terminator, was amplified by PCR with the following primers: forward 5′-gggactagtaccaagaaagtgtcgagaact-3′ and reverse 5′-gggtctagagatataatcgccacaatttccac-3′. The PCR product was digested with *Spe*I–*Xho*I, purified and ligated with the pRS-5′KlADR1 plasmid digested with *Xba*I–*Xho*I. The final plasmid was called pRS-Kl/ScADR1 and contained the *K. lactis/S. cerevisiae* chimeric gene.

DASPMI and GFP fluorescence microscopy. DASPMI (2-[4dimethylaminostryl]-1-methylpyridinium iodide) mitochondria-specific staining of live cells was carried out according to Yaffe (1995).

Cells transformed with GFP plasmids were grown under selective conditions to the exponential phase and then shifted for 6 h in YP containing carbon sources at concentrations specified in the text. To assess the fluorescent signal, cells were analysed with an Axio Observer inverted microscope (Zeiss) then scanned in a series of 0.3 µm sequential sections with AxioVision software (Zeiss) that allows 2D reconstruction.

General methods. DNA manipulation, plasmid engineering and other techniques were performed according to standard procedures. Yeast transformation was performed by electroporation with a Biorad Gene Pulser apparatus. Putative KlAdr1-binding sites were retrieved using the FIMO tool of the MEME suite (Bailey *et al.*, 2009).

Respiratory rates were measured at 30 °C using a Clark-type electrode according to the method of Ferrero *et al.* (1981).

RESULTS

Carbon source utilization in *Kladr1Δ*

The *K. lactis ADR1* gene was amplified by PCR using primers designed on the basis of the reported Génolevures genome sequence (Dujon *et al.*, 2004; Bussereau *et al.*, 2006). The role of this gene was studied by deleting *KlADR1* with the KanMX4 gene and one of the deleted strains, named MW179-1D/Kladr1Δ, was further characterized.

Growth tests on *Kladr1*Δ were performed in rich YP medium and minimal medium (YNB) in the presence of respiratory and fermentative carbon sources. The growth of the parental and *Kladr1*Δ strains showed it to be similar on YNB plates containing glucose, lactate or ethanol, whereas in glycerol the mutant displayed a highly reduced growth (Fig. 1a). Unexpectedly, *Kladr1*Δ showed reduced growth on YP medium that was not ameliorated by the addition of glycerol (YPG) (Fig. 1a), suggesting the requirement of KlAdr1 for the utilization of glycerol and for the mobilization of nutrient sources contained in YP medium.

The two strains showed a comparable growth on YPD, while the addition of antimycin A, an inhibitor of the respiratory transport chain, reduced the growth of the mutant (Fig. 1a). The addition of ergosterol, a structural component required for membrane fluidity and permeability (Parks *et al.*, 1995), to plates containing YPD-antimycin A recovered the growth of *Kladr1*Δ to nearly parental levels (Fig. 1a). Finally, *Kladr1*Δ was unable to grow on plates containing oleate as a sole carbon source (Fig. 1a) like its *S. cerevisiae* counterpart (Simon *et al.*, 1991).

The growth of the *Kladr1*Δ in YP medium (Fig. 1a) was compared to that of *S. cerevisiae adr1*Δ and was extended to plates containing only YE or peptone. *Kladr1*Δ showed reduced growth on YP and severely reduced growth on YE and peptone, revealing that these phenotypes are specific to *Kladr1*Δ (Fig. 1b). In fact, the WT, the *Kladr1*Δ complemented with KCp-KlADR1 and also the *Klgut2*Δ strain, unable to grow under glycerol conditions like *Kladr1*Δ (Fig. 1b) (Saliola *et al.*, 2008), showed comparable growth ability on these media (Fig. 1b). In contrast, *Scadr1*Δ and its parental strain were unable to grow on these nutrient sources, suggesting a different role of these two factors in the mobilization of complex resources like peptone and YE (Fig. 1b).

The decreased fermentative capability of *Kladr1*Δ, deduced from sensitivity to antimycin A (Goffrini *et al.*, 1989), were associated with reduced respiratory capability. In fact, *Kladr1*Δ showed a reduction of 50 % O_2 respiration rate in the presence of glucose and ethanol, as compared to its parental strain (glucose: 45±6 vs 22±3; ethanol: 51±6 vs 27 ±3 µl O_2 per hour per milligram dry weight), while in lactate, the oxygen consumption was reduced to 30 % (41±3 vs 14±1).

K. lactis mainly grows under aerobic conditions (De Deken, 1966) as opposed to *S. cerevisiae,* where respiration

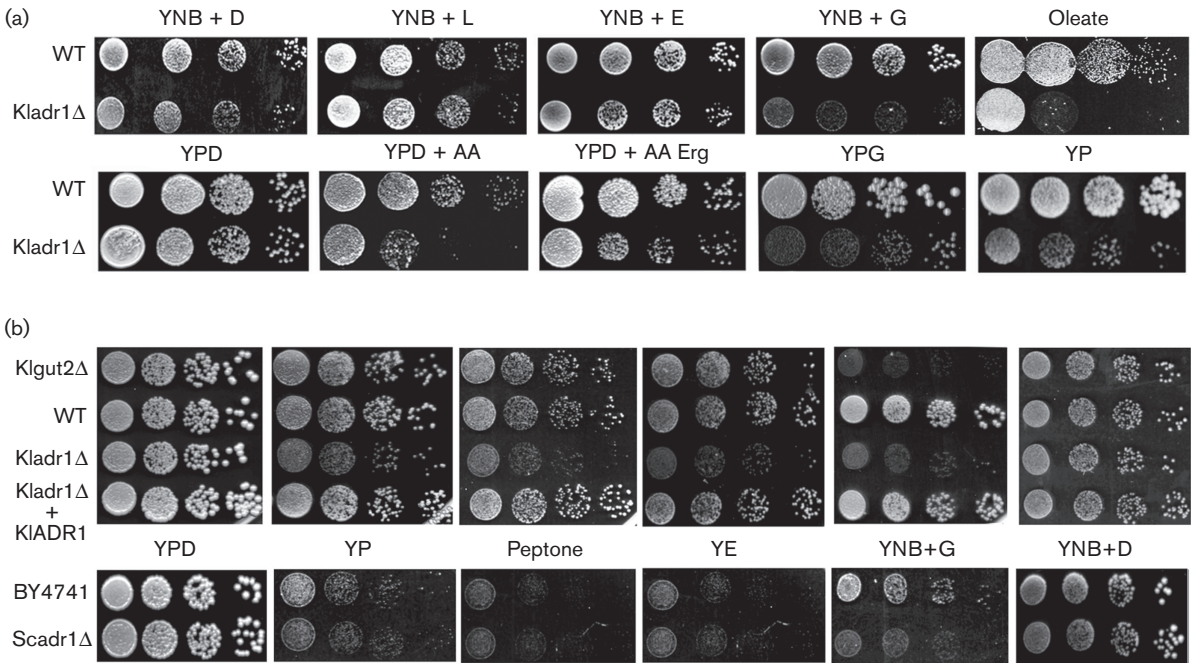

Fig. 1. (a) Growth testing of *K. lactis* MW179-1D (WT) and *Kladr1*Δ. (b) Growth test comparison of *K. lactis* and *S. cerevisiae* parental and *adr1*Δ strains. *KlGut2*Δ and *KlAdr1*Δ harbouring the centromeric KCp-KlADR1 were used as controls for growth on YNB plates supplemented by glycerol and peptone, and YE and YP plates. Cells were grown on plates containing YNB or YP medium supplemented by glucose (YNB+D, YPD), ethanol (YNB+E), glycerol (YNB+G, YPG) and lactate (YNB +L) at a concentration of 2 %; in oleate medium; or on plates containing only peptone, YE or YP medium (YP). YPD plates were also supplemented by antimycin A (YPD+AA) or antimycin A plus ergosterol (YPD+AA Erg). *K. lactis* cultures were pre-grown in YNB containing 1 % glucose, and *S. cerevisiae* cultures were pre-grown in YNB containing 1 % glucose supplemented with casamino acids. Cultures were adjusted to the same density, and 5 µl of serial 10-fold dilutions was spotted onto the medium indicate. The initial concentration was 1×10^7 cells ml^{-1}.

is completely repressed by glucose. However, when the concentration of oxygen in the culture was reduced to a critical point, as compared to the cellular biomass and glucose concentration, cells began to ferment, accumulating ethanol in the medium (Lemaire & Wésolowski-Louvel, 2004). Accordingly, determination of the main parameters describing the growth behaviour of *Kladr1*Δ can provide insight into the respiratory-fermentative capabilities of the mutant. These parameters, reported in the curves of Fig. 2, display (at time intervals) cell growth, glucose consumption and the production of ethanol and glycerol. The growth curve of the mutant showed a profile not very different from that of WT (Fig. 2). Glucose consumption was also very similar in the two strains, at least over the first 18 h, and was followed by a slightly delayed consumption of glucose and higher accumulation of ethanol (2.4 vs 1.9 g l^{-1}) in *Kladr1*Δ (Fig. 2). Glycerol is one of the end products of fermentation; its determination in the supernatant of the two strains showed a significantly lower amount, and absence of its reoxidation, in *Kladr1*Δ as compared to WT (Fig. 2).

In-gel native PAGE analysis of Adh pattern in *Kladr1Δ*

The four *ADH* genes identified in *K. lactis* (*KlADH1*–*KlADH4*) are regulated at the transcriptional level by carbon source-dependent growth conditions (Saliola & Falcone, 1995). The corresponding activities have been identified, in native gel from *adh* mutant extracts, by ADH staining assay (Mazzoni *et al.*, 1992; Saliola & Falcone, 1995) that allows dissection of the *S. cerevisiae* ADH system (Lutstorf & Megnet, 1968; Ciriacy, 1975, 1979).

ScAdr1 is the transcription factor responsible for the activation of the *S. cerevisiae ADH2* gene (Ciriacy 1979; Denis & Young, 1983). To test whether KlAdr1 is involved in the activation of *KlADH* genes, we analysed the *K. lactis* Adh pattern. Cellular extracts from respiratory and fermentative-grown cultures of the two strains were fractioned on native PAGE and stained for ADH activity. The cultures grown in 7 % and 1 % glucose-containing medium represent conditions completely fermentative or as mixed respiratory-fermentative (Saliola & Falcone, 1995). These differences were confirmed by the Adh patterns (Fig. 3a, lanes 1 and 2). Comparison of the two Adh patterns (Fig. 3a) showed reduced levels of both cytosolic (Adh1 and Adh2) and mitochondrial activity (Adh3 and Adh4) in *Kladr1*Δ (lanes 5–8 vs lanes 1–4), indicating that KlAdr1 can regulate the expression of these genes.

To better assess its role in the expression of *KlADH* genes, both *KlADR1* and *ScADR1* were over-expressed from centromeric and multi-copy plasmids and their expression was compared, on cultures grown in different carbon sources, by Adh pattern. The strain harbouring the centromeric KCp-KlADR1 displayed in glucose a reduced amount of KlAdh4 (Fig. 3b, lanes 1 and 2 vs Fig. 3a, lanes 1 and 2), an activity that increases during fermentation or growth in ethanol (Fig. 3a and b, lane 4) (Saliola & Falcone, 1995). The strain transformed with KCp-ScADR1 showed an Adh pattern almost identical to that of the untransformed strain (Fig. 3b lanes 5–8).

In contrast, the strains over-expressing either factor displayed highly increased levels of KlAdh3 (Fig. 3c), even in ethanol (lanes 4 and 8), a compound that specifically represses the expression of this gene (Saliola & Falcone,

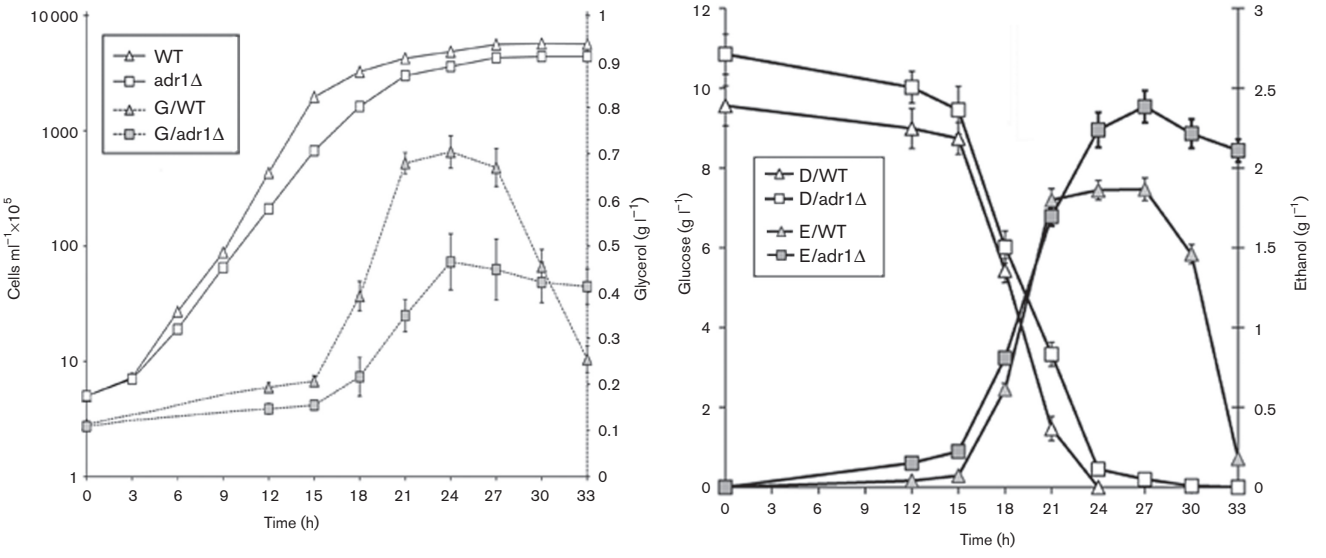

Fig. 2. Growth curves and metabolite determination of WT and *Kladr1*Δ. Cultures were grown in YP medium containing 1 % glucose. Glycerol (G), glucose (D) and ethanol (E) were determined at time intervals in culture supernatants. adr1Δ=*Kladr1*Δ. Each value is the average of three independent determinations.

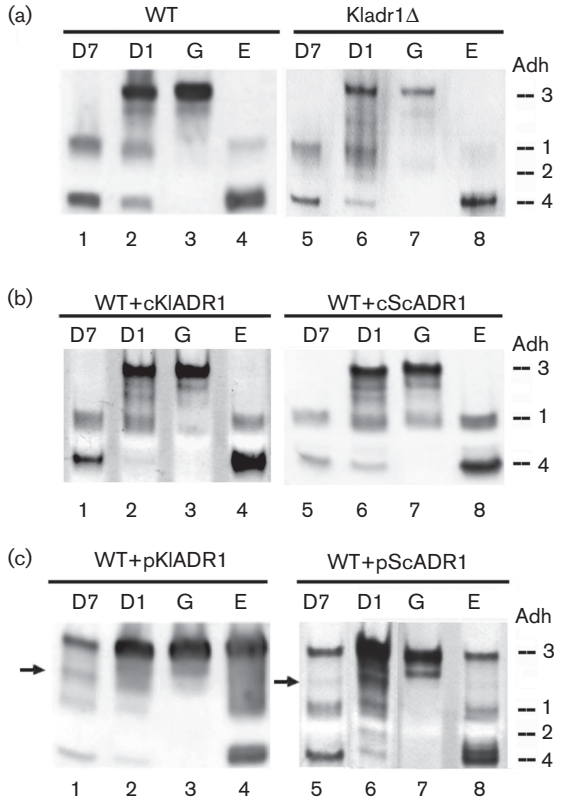

Fig. 3. In-gel native Adh pattern from extracts of *K. lactis* cultures grown in fermentative/respiratory carbon sources. Adh-stained activities from extracts of (a) WT and *Kladr1*Δ. (b) WT transformed with centromeric KCp-KlADR1 (WT+cKlADR1) or KCp-ScADR1 (WT+cScADR1) plasmids. (c) WT transformed with the multi-copy pKlADR1 (WT+pKlADR1) or pKL-ScADR1 (WT +pScADR1) plasmids. Cellular extracts were prepared from cultures grown on YP containing 7 % (D7) or 1 % (D1) glucose, 2 % glycerol (G) or 2 % ethanol (E). The migrating position of each activity is also reported. The arrows indicate the hetero-tetramers formed by KlAdh3 and KlAdh4 activity.

1995; Saliola *et al.*, 2007). Since the regulation of *KlADH3* and *KlADH4* genes is mutually exclusive (Saliola & Falcone, 1995), the increased Adr1-dependent expression of *KlADH3* alters the balance between the two genes leading to the formation of hetero-tetramers between the activity of KlAdh3 and KlAdh4 (see arrows in Fig. 3c; and the next section) (Saliola *et al.*, 2007). Indeed, strains over-expressing *ADR1* displayed very high levels of KlAdh3, but lower levels of KlAdh4. However, the over-expression of *ScADR1* was unable to complement the growth-deficient glycerol phenotype of *Kladr1*Δ (data not shown).

Deletion analysis of the *KlADH3* promoter in *Kladr1Δ*

An extensive deletion analysis of the *KlADH3* promoter fused to the *lacZ* gene reporter allowed the functional identification of positive and negative elements. Suggesting very complex regulation of this gene (Saliola *et al.*, 2007). The positive (+) and the short *cis*-acting repression ethanol (RE) element previously identified are reported in Fig. 4(a).

To test whether KlAdr1 is involved in the control of *KlADH3*, deleted fragments of its promoter fused to *lacZ* were transformed in parental and *Kladr1*Δ strains (Fig. 4a). β-Gal values were determined under ethanol-repressive and glycerol plus 0.1 % glucose-inducing conditions (see Fig. 3a, lanes 4 and 3, respectively) to bypass the inability of *Kladr1*Δ inability to grow in glycerol. pCG1, pCG17, pCG13, pCG11 and pCG12 containing promoter fragments of increased deleted length showed comparable levels of activity in both parental and mutant strains (Fig. 4a). In contrast, the *Kladr1*Δ strain transformed with pCG7 (first 529 bp of the promoter) showed comparable levels of β-gal activity under glycerol and ethanol conditions (323 and 371 nmol min mg^{-1} protein, respectively). These values are very different to those measured in the parental strain (445 and 83, respectively) (Fig. 4a), suggesting the presence on the $DNA_{529\text{-}297}$ fragment of an element conferring constitutive β-gal activity. In this process, KlAdr1 seems to modulate the positive (+) factor element conferring a role of repressor in ethanol and one of activator in glycerol. However, the β-gal values shown by the two pCG11-containing strains suggested that the KlAdr1-dependent role noted for pCG7 is completely abolished by the presence of another element(s) located in the $DNA_{660\text{-}529}$ fragment (Fig. 4a). Analysis of pCG8 (Δ660–529) confirmed the role of the two elements (Fig. 4a). In fact, the repressive role of KlAdr1 ($Adr1_{RE}$) and the presence of an antagonistic dominant activator partner on the $DNA_{660\text{-}529}$ fragment are in agreement with β-gal measured in the parental strain and its *Kladr1*Δ strain (79 and 18 vs 616 and 150) (Fig. 4a). The low β-gal value measured under the two conditions in the parental strain with pCG8 (79 and 18), as compared to *Kladr1*Δ (616 and 150), is probably due to the repressive role of $Adr1_{RE}$ in the absence of the dominant $DNA_{660\text{-}529}$-positive element. Conversely, the different β-gal values measured under ethanol conditions (31 vs 59) in the two pCG1-containing strains can be interpreted in the same way.

To confirm the interfering role of KlAdr1 in the regulation of *KlADH3* and *KlADH4* (see Fig. 3c), we transformed *Kladr1*Δ, *Kladh3*Δ and *Kladh3*Δ with promoter constructs containing different combinations of positive and negative elements fused to *KlADH3*, used as a gene reporter (Fig. 4b–d) (Saliola *et al.*, 2007). The extracts from the ethanol- and glycerol-grown cultures of the two transformed strains were fractioned in native gel and their Adh staining activity compared (Fig. 4b).

The patterns were similar in the two strains transformed with pCG29, pCG30, pCG31 and pCG32 (Fig. 4b) (Saliola *et al.*, 2007), whereas the activity of pCG33 extracts (see scheme of Fig. 4c) showed interesting results. The expression of this plasmid, together with pCG32 used as a control, confirmed the role of KlAdr1 in this gene (Fig. 4d). In fact, the double

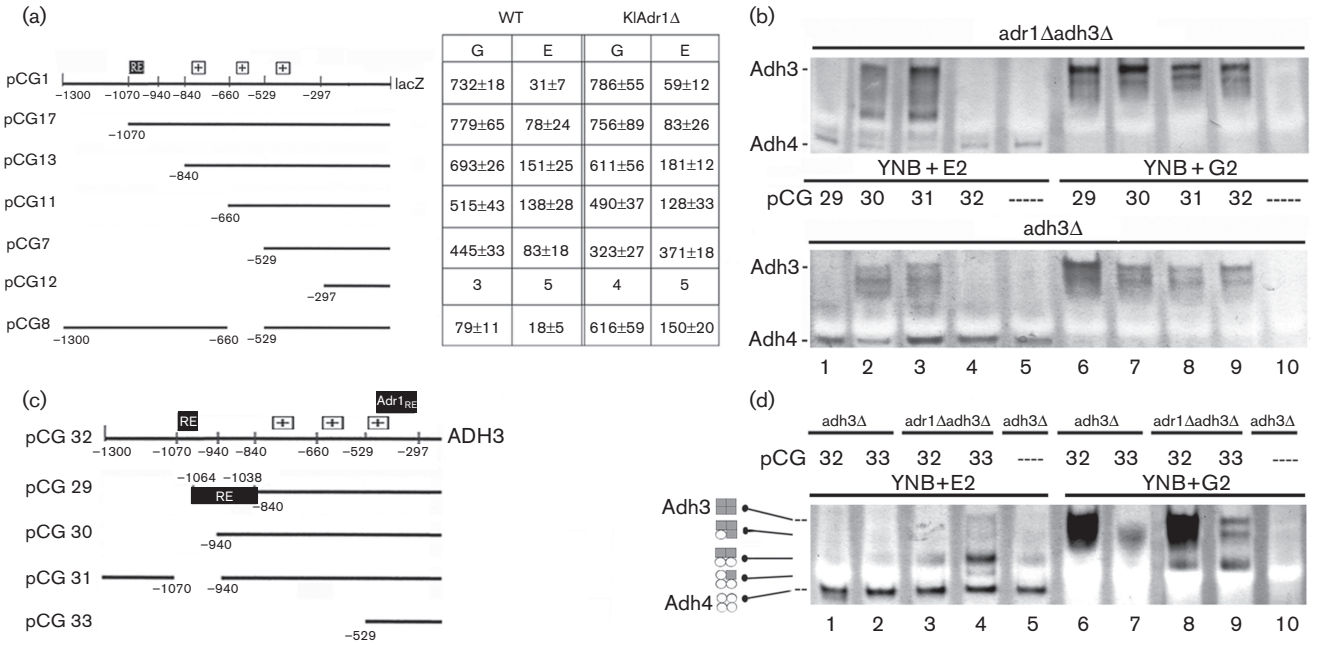

Fig. 4. Deletion analysis of the *KlADH3* promoter fused to the *lacZ* and *KlADH3* gene reporters. (a) β-Gal activity (nmol min mg^{-1} protein) measured in WT and *Kladr1*Δ cells transformed with various promoter fragment constructs fused to *lacZ*. (b) Adh staining activity of extracts of *Kladr1*Δ, *Kladh3*Δ (*adr1*Δ*adh3*Δ) and *Kladh3*Δ (*adh3*Δ) cells transformed with various promoter fragment constructs fused to *KlADH3*. (c) Centromeric plasmids carrying *KlADH3* under different promoter forms used in this analysis. (d) Adh staining activity of extracts of *Kladr1*Δ, *Kladh3*Δ and *Kladh3*Δ cells transformed with pCG32 and pCG33. A scheme reports the homo-/hetero-tetrameric composition of each Adh band in the figure. Cells were grown for 60 h in YNB medium under repressing (E=2 % ethanol) and de-repressing (2 % glycerol and 0.1 % glucose) conditions. Each β-gal value is the average of at least three independent clones analysed. The Adh pattern was analysed in three independent clones for each construct. The position on the promoter of positive (+) and negative (RE and $Adr1_{RE}$) elements is also reported.

mutant harbouring pCG33 showed the presence of KlAdh3 in both substrates (Fig. 4d, lanes 4 and 9), as compared to *Kladh3*Δ (lanes 2 and 7). However, the highly reduced levels of KlAdh3 in ethanol (Fig. 4d, lanes 4), as compared to glycerol (Fig. 4d, lane 9) and to the β-gal values in Fig. 4(a) (371 vs 323 nmol min mg^{-1} protein), were interpreted according to the scheme reported on the left of Fig. 4(d). The absence of KlAdr1 leads to the contemporary expression of the two genes, as shown by the assembly of both KlAdh3 and KlAdh4 homo- and hetero-tetramers (Fig. 4d, lanes 4, 8 and 9 and Fig. 4b, lanes 2 and 3) in a manner similar to the over-expression of *KlADR1* in WT (see Fig. 3c).

Cellular localization of KlAdr1

In *S. cerevisiae*, expression of the *ScADR1-GFP* fusion gene allowed the intracellular localization of ScAdr1 under repressed and de-repressed conditions (Sloan *et al.*, 1999; Huh *et al.*, 2003; Moreno-Cermeno *et al.*, 2013).

In order to visualize the localization of KlAdr1 in *K. lactis*, the centromeric KCp-KlADR1-GFP plasmid was inserted into the parental and *Kladr1*Δ strains. Growth of the transformed *Kladr1*Δ strain on glycerol-containing plates confirmed the functionality of the *KlADR1-GFP* gene (data not shown). To visualize the fusion protein, WT-transformed cells were grown to the mid-exponential phase in YNB containing 1 % glucose and shifted for 6 h on fermentative (glucose 7 %) under respiratory conditions (glycerol 2 % and ethanol 2 %). As shown in Fig. 5(a), cells grown in glucose 7 % displayed a diffuse fluorescence suggesting a mainly cytoplasmic localization of KlAdr1-GFP. In contrast, cells shifted in glycerol or ethanol showed a weak, localized fluorescent signal in glycerol and a stronger one in ethanol, indicating that a substantial amount of the chimeric protein was localized within the nucleus (Fig. 5a). These results suggested a common mechanism of gene activation in the two yeasts under fermentative/repressive and respiratory/derepressive conditions.

Cellular localization of KlGut2 in *Kladr1Δ*

Since *KlGUT2* is required for growth in glycerol (Saliola *et al.*, 2008) and, in *S. cerevisiae*, this gene is under the control of Adr1 (Tachibana *et al.*, 2005), we analysed the localization of KlGut2 in *Kladr1*Δ by means of its fusion with GFP.

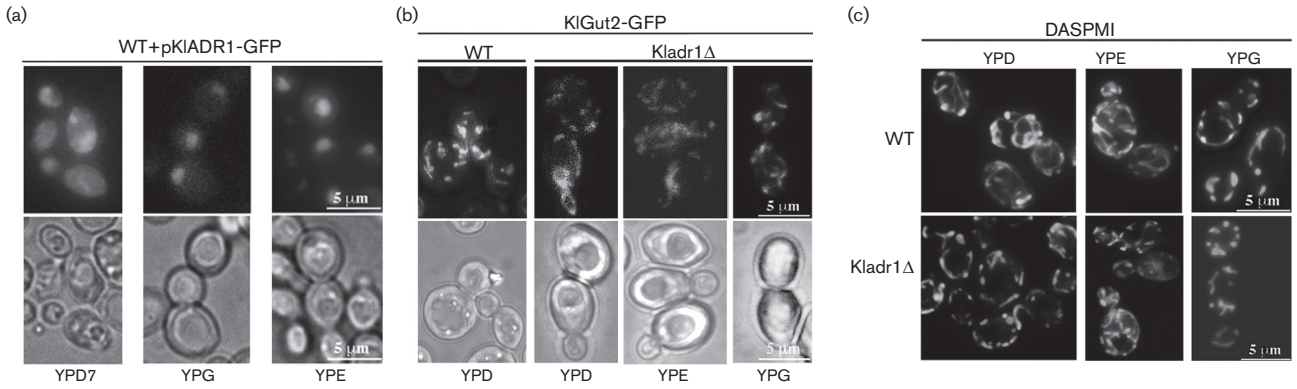

Fig. 5. Fluorescence analysis of WT and *Kladr1*Δ cells transformed with the centromeric KCp-KlADR1-GFP or KCp-KlGUT2-GFP plasmids or treated with DASPMI. Micrographs show (a) the intracellular localization of KlAdr1-GFP in the WT under fermentative and respiratory conditions; (b) the intracellular localization of KlGut2-GFP in the WT and in the *Kladr1*Δ; and (c) the mitochondrial morphology of WT and *Kladr1*Δ cells stained with DASPMI. Cells were grown to late exponential phase in YNB supplemented by 1 % glucose. Cells were harvested, then shifted for 6 h on YP containing 7 % (YPD7) or 1 % glucose (YPD), 2 % glycerol (YPG) or 2 % ethanol (YPE) with/without DASPMI.

In the parental strain, the expression of the *KlGUT2-GFP* gene showed mitochondrial localization of the chimeric protein under glucose conditions (Fig. 5b) (Saliola *et al.*, 2010), while fluorescence distribution was reduced in intensity and slightly more diffuse in *Kladr1*Δ. On the contrary, under glycerol and ethanol conditions, the mutant displayed a reduced fluorescence signal, altered in definition and slightly dispersed throughout the cell (Fig. 5b).

In *S. cerevisiae*, Adr1 coordinates the pathways that generate acetyl-CoA and NADH from non-fermentable substrates (Young *et al.*, 2003), playing a role in the mitochondrial network dynamic (Cherry & Denis, 1989). Similarly, *Kladr1*Δ showed highly reduced oxygen reoxidation rates, the inability to utilize respiratory substrates like glycerol, and also that the intracellular distribution of KlGut2 is slightly more diffuse. Therefore, cells were analysed by the DASPMI vital dye assay to test the effect of deletion of *KlADR1* on the mitochondrial network. Under glycerol conditions, this assay visualized a mitochondrial network frame with a punctuated shape slightly more accentuated in the mutant, as compared to the WT (Fig. 5c).

Expression of *KlADR1* in *S. cerevisiae*

The over-expression of *ScADR1* in *K. lactis* influenced the Adh pattern like the endogenous gene (Fig. 3c, lanes 5–8 vs lanes 1–4). Thus, a complementation test was performed to see whether KlAdr1 is capable of activating transcription of the *S. cerevisiae ADH2* gene. To this end, pRS-KlADR1-GFP was transformed into the *Scadr1*Δ strain and the extracts were tested on native PAGE for Adh2 activity. Indeed, as can be seen from Fig. 6(a), the extracts of four *KlADR1-GFP*-transformed clones showed the presence of Adh2 only under derepressing conditions (lanes 3–6 vs lanes 9–12), as compared to the parental (lanes 1 and 2) and *Scadr1*Δ (lanes 7 and 8) strains. However, this activity (Fig. 6a, lanes 3–6) was not displayed at levels comparable to those of WT (lane 2). The presence of the KlAdr1-GFP protein in the transformants was also analysed under fluorescent microscopy. The weak fluorescent signal visualized under repressing and de-repressing conditions indicated that KlAdr1 is expressed in *S. cerevisiae* (Fig. 6b). The protein would appear to be distributed in the cytoplasm under repressing conditions, while in glucose 0.2 %, fluorescence distribution suggested its nuclear localization (Fig. 6b).

To discover why the *S. cerevisiae* strain expressing *KlADR1-GFP* displayed reduced levels of KlAdh2, we focused on the zinc-finger DNA-binding domains of this protein being more highly conserved between the two factors (Fig. 6c) (Parua *et al.*, 2010). The scheme of Fig. 6c also shows the identity between the domains of the two proteins, the phosphorylated serine of Adr1 (*S) (Denis *et al.*, 1992) and the putative one of KlAdr1 (§S). The location of this serine ($^{\S}S_{312}$) suggested the presence of an extra domain of roughly 50 amino acids in *K. lactis*, as compared with that of ScAdr1 ($^{*}S_{230}$) (Fig. 6c, – aa insertion).

A chimeric *ADR1* gene was constructed to test whether the ability of KlAdr1 to activate ScAdh2 depends on its DNA-binding domain. The chimeric gene (*Kl/ScADR1*) contains the *K. lactis* promoter and the sequence coding for the zinc-finger domains in frame with the *ScADR1* sequence encoding the transactivating domains (Bemis & Denis, 1988) (scheme of Fig. 6c). This chimera, when transformed into the *Scadr1*Δ strain under derepressed conditions, allowed the appearance of the native ScAdh2 activity like the endogenous factor (Fig. 6d, lanes 7 and 8 vs lanes 1 and 2). These results indicated that the zinc-finger domains of KlAdr1 can recognize UAS1 or other UAS1-like elements of its promoter activating the transcription of *ScADH2* in a ScAdr1-dependent manner (Shuster *et al.*, 1986).

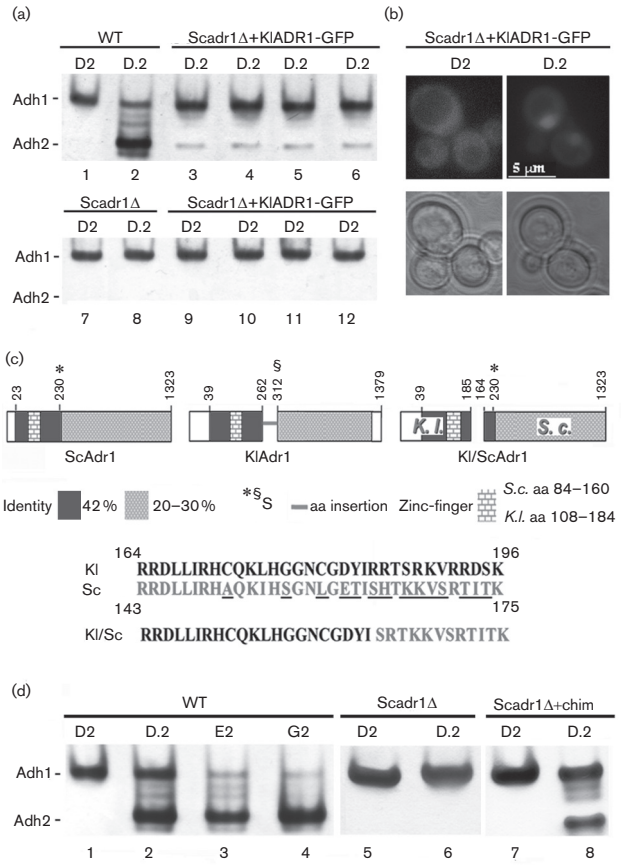

Fig. 6. Expression analysis of *KlADR1-GFP* in *S. cerevisiae* by in-gel native Adh pattern and GFP localization. (a) In-gel native Adh pattern from extracts of BY4741 (WT), *Scadr1*Δ (Scadr1Δ) and *Scadr1*Δ transformed with KCp-KlADR1-GFP. (b) Micrographs showing the intracellular localization of KlAdr1 in *Scadr1*Δ cells transformed with KCp-KlADR1-GFP. (c) A scheme showing the identities between *K. lactis* and *S. cerevisiae* Adr1 domains and the construction strategy for the chimeric fusion Kl/ScAdr1 factor. (d) Expression analysis of the *Kl/ScADR1* chimeric gene in the *Scadr1*Δ by in-gel native Adh pattern from extracts of BY4741 (WT), *Scadr1*Δ (Scadr1Δ) and *Scadr1*Δ transformed with pRS-Kl/ScADR1 (Scadr1Δ+chim). Cellular extracts were prepared from YNB-casamino acid-supplemented cultures containing 2% (D2) or 0.2% (D.2) glucose, ethanol 2% (E2) or glycerol 2% (G2). Cells transformed with KCp-KlADR1-GFP were pre-grown to the early exponential phase in 2% glucose, shifted in repressing (D2) and de-repressing (D.2) glucose medium and grown for a further 6 h.

DISCUSSION

Role of KlAdr1 in the metabolism of *K. lactis*

In this study, we report the characterization of the *K. lactis ADR1* gene. In *S. cerevisiae*, Adr1 is a zinc-finger transcription factor that regulates the transcription of *ADH2* and other genes required for the utilization of respiratory carbon sources, peroxisomal biogenesis, fatty acid metabolism and other cellular functions (Denis & Young, 1983; Simon *et al.*, 1991; Young *et al.*, 2003; Tachibana *et al.*, 2005).

In *K. lactis*, KlAdr1 is required for growth in glycerol, oleate and YP medium (Fig. 1a). Moreover, the sensitivity of *Kladr1*Δ to antimycin A, its reduced oxygen reoxidation rate and the different accumulation of ethanol and glycerol in glucose-grown culture indicated a role for KlAdr1 under respiratory and fermentative conditions (Figs 1 and 2).

Yeast cells produce and accumulate glycerol from the intermediate of glycolysis dihydroxyacetone phosphate as the main compatible solute for redox balance and osmoregulation (Hohmann, 2002). It follows that the reduced glycolytic flux of *Kladr1*Δ is unable to support the accumulation of glycerol for the cytoplasmic reoxidation of excess NAD(P)H to maintain redox balance (Fig. 2) (Cialfi *et al.*, 2011). As a consequence, the metabolism of these cells becomes respiro-fermentative (González-Siso *et al.*, 2000) and the respiratory chain intervenes to neutralize excess redox (Overkamp *et al.*, 2002). Therefore, blocking of the respiratory chain by antimycin A inhibits the growth of this mutant, while the addition of ergosterol, a major structural component required for the control of membrane fluidity (Park *et al.*, 1995), can recover the growth deficiency (Fig. 1). These data and the growth-deficient oleate phenotypes (Fig. 1) indicated that the altered redox balance of *Kladr1*Δ is the limiting factor in the adaptation of plasma membrane fatty acid composition during the transition from respiratory to fermentative conditions (Heipieper *et al.*, 2000; Cialfi *et al.*, 2011).

Role of KlAdr1 in the ADH system of *K. lactis*

Under fermentative and respiratory conditions, Adhs are required for the maintenance of intracellular redox balance (Bakker *et al.*, 2000; Overkamp *et al.*, 2002; Saliola *et al.*, 2012). Since KlAdr1 intervenes in the regulation of these physiological conditions (Fig. 2), we showed, by in-gel native assays, that the deletion and over-expression of this factor altered the Adh pattern in all carbon sources. In particular, KlAdh3 was the activity most affected by its over-expression (Fig. 3). Moreover, extensive deletion analysis allowed the isolation of a *KlADH3* promoter fragment ($DNA_{529\text{-}297}$) in which KlAdr1 exerted its role in modulating the expression of this gene (Fig. 4).

In this process, the role of KlAdr1 is to tune *KlADH3*, repressing its expression in ethanol and inducing it in glycerol. We propose that the modulatory role of KlAdr1 in this gene is to avoid the contemporary expression of *KlADH3* and *KlADH4* and the assembly of hetero-tetramers of the two activities (Fig. 4d). In fact, the two Adhs displayed different biochemical properties (Bozzi *et al.*, 1997), in agreement with their specific activation during growth under mainly fermentative (*KlADH4*) and respiratory (*KlADH3*) conditions (Saliola & Falcone, 1995; Saliola *et al.*, 2006).

Using the FIMO tool of the MEME suite (Bailey *et al.*, 2009), we were able to identify on this DNA fragment a

putative $UAS_{509\text{-}490}$ element showing a significant identity with the UAS1 of *ScADH2* (Shuster *et al.*, 1986). Since *KlADH3* seems to be regulated by an intricate and concerted balance of many positive and negative factors (Fig. 4) (Saliola *et al.*, 2007), further research will be necessary to confirm the binding of KlAdr1 to this element, the roles and the identities of the other factors and their relation with KlAdr1.

Intracellular localization of KlAdr1 and KlGut2

Minor differences have been reported in the localization of Adr1 in *S. cerevisiae,* probably due to the diverse genetic context of the strains used (Sloan *et al.*, 1999; Huh *et al.*, 2003; Moreno-Cermeño *et al.*, 2013). In *K. lactis*, the diffuse fluorescence displayed under fermentative conditions suggested a mainly cytoplasmic localization of the KlAdr1-GFP. In contrast, in glycerol- and ethanol-grown cells, the fluorescent signal suggested the localization of the protein within the nucleus (Fig. 5a). We may emphasize that the nuclear localization of KlAdr1 is in agreement with the regulation of *KlADH3* in glycerol-inducing and ethanol-repressive media, as suggested by the deletion data (see Fig. 4a and d).

The localization of KlGut2-GFP was also performed, to provide insights into the role of KlAdr1 on *KlGUT2*, a gene that, in *S. cerevisiae*, is regulated by Adr1 (Tachibana *et al.*, 2005).

In *K. lactis,* KlGut2-GFP displayed mitochondrial localization (Saliola *et al.*, 2010) while fluorescence distribution was reduced in intensity in *Kladr1*Δ as compared to WT (Fig. 5b). However, under glycerol and ethanol conditions, the mutant displayed a stronger fluorescence signal and reduced intensity, definition and dispersal throughout the cell than in glucose (Fig. 5b). These results indicated that KlAdr1 is not directly involved in its regulation and, as for *KlADH3*, seems to modulate its expression; or it may be necessary to addressing KlGut2 into the mitochondrial compartment. In fact, as shown by DASPMI vital dye assay, *Kladr1*Δ cells displayed a mitochondrial network distribution only slightly punctuated as compared to WT. Although this result suggested a role for KlAdr1 in the maintenance of the mitochondrial network, like its *S. cerevisiae* counterpart (Cherry & Denis, 1989), the effect of its deletion seems more marked on the localization and distribution of KlGut2.

Expression of *KlADR1* in *S. cerevisiae*

By cross-complementation experiments, we were able to define the extent of KlAdr1- activating expression. *KlADR1* was shown to be expressed in *Scadr1*Δ and its product localized in the cytoplasm in glucose-repressing medium, and in the nucleus under de-repressing conditions (Fig. 6b). Moreover, KlAdr1 induced the expression of *ScADH2* under de-repressing conditions, although with reduced levels of ScAdh2 as compared to WT (Fig. 6a). This result suggested a limited functionality of KlAdr1 in the activation of *ScADH2* expression, while the introduction of the chimeric *Kl/ScADR1* gene in *Scadr1*Δ led to levels of ScAdh2 similar to those of the WT. It follows that the zinc-finger domains of KlAdr1 must bind to a *ScADH2* promoter element(s) regulating its expression in a ScAdr1-dependent fashion (Fig. 6d). We suggest that this domain of KlAdr1 binds to the same sequence of ScAdr1, while the transactivator domains have diverged between the two yeasts and cannot be fully recognized by the evolutionarily diverged *S. cerevisiae* apparatus (Bussereau *et al.*, 2006).

Finally, growth test comparison of the two *adr1*Δ yeast strains showed the inability of the *S. cerevisiae* parental and mutant strains to grow in the presence of rich nutrient sources without the addition of carbohydrate (YE, peptone or YP) (Fig. 1b). Since *K. lactis* has been isolated from mammalian fluids (milk) particularly rich in nutrients (Rodicio & Heinish, 2013), these data suggest that the adaptation to this habitat and/or the intracellular mobilization of nutrients as sources of carbon, nitrogen and co-factors could be a specific KlAdr1-dependent trait.

In conclusion, these factors have evolved and contribute to the metabolic adaptation of the two yeasts to their specific environmental niches.

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