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Insight into the *Kluyveromyces lactis* Pdr1p regulon

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Abstract

The overexpression of efflux pumps is an important mechanism leading to the development of multidrug resistance phenomenon. The transcription factor *KIPdr1p*, belonging to the Zn_2Cys_6 family, is a central regulator of efflux pump expression in *Kluyveromyces lactis*. To better understand how *KIPDR1*-mediated drug resistance is achieved in *K. lactis*, we used DNA microarrays to identify genes whose expression was affected by deletion or overexpression of the *KIPDR1* gene. Eighty-nine targets of the *KIPDR1* were identified. From those the transcription of 16 genes was induced in the transformant overexpressing *KIPDR1** and simultaneously repressed in the *Klpr1Δ* deletion mutant. Almost all of these genes contain putative binding motifs for the AP-1 like transcription factors in their promoters. Furthermore, we studied the possible interplay between *KIPdr1p* and *KIYap1p* transcription factors. Our results show that *KIYap1p* does not significantly contribute to the regulation of the *KIPDR1* gene expression in the presence of azoles. However, *KIPDR1* expression markedly increased in the presence of hydrogen peroxide and hinged upon the presence of *KIYap1p*. Our results show that although both *KIPdr1p* and *KIYap1p* transcription factors are involved in the control of *K. lactis* multidrug resistance further studies will be needed to determine their interplay.

Key words: *Kluyveromyces lactis*, multidrug resistance, DNA microarray, *KIYAP1*, *KIPDR1*

Introduction

Living organisms are constantly challenged by ever-changing variables in their environment, including fluctuating nutrient levels, osmotic imbalance, or exposure to toxic molecules. However, eukaryotic cells have distinct pathways that promote cell survival when confronted with environmental stresses. These pathways often lead to transcriptional activation of a set of genes whose products protect cells against the deleterious effects of the encountered stress. This transcriptional control results from the balanced action of diverse transcription factors which act alone or as interacting partners and may regulate and be regulated by others through complex feedback loops. Among these complex transcriptional responses all living organisms have evolved multidrug resistance (MDR) pathways, which confer resistance to a broad spectrum of unrelated chemicals. MDR is essentially based on the overexpression of membrane transporters able to export chemically different compounds from cells (Borst and Elferink 2002; Li and Nikaido 2004; Prasad and Kapoor 2005). MDR is a major concern for human health, as it leads to antibiotic resistance in pathogens and enables cancer cells to survive chemotherapy. The most detailed picture of the MDR network is available for the yeast *Saccharomyces cerevisiae* (reviewed in Moye-Rowley 2003a; 2003b; Fardeau *et al.* 2007; Paul and Moye-Rowley 2014). The network comprises 10 transcription factors regulating about 70 different target genes. In this network, the Pdr1p transcription factor has the largest set of potential targets (about 50). Pdr1p and its functional homologue Pdr3p, were identified in the early 1990s, as regulators of the basal level of drug resistance in *S. cerevisiae* (Balzi *et al.* 1987; Delaveau *et al.* 1994). Gain- or loss-of-function alleles of *PDR1* and *PDR3* confer resistance or sensitivity to many unrelated drugs, through constitutive modifications of the expression of ATP binding cassette transporters, major facilitator superfamily members or enzymes modifying the lipid composition of the plasma membrane

(reviewed in Kolaczowska and Goffeau 1999). Pdr1p and Pdr3p recognize the same DNA consensus motif (PDRE for pleiotropic drug response element) that has been found upstream of the majority of Pdr1p/Pdr3p-regulated genes. A feature unique to the *PDR3* gene was the presence of two PDREs in its promoter (Delahodde *et al.* 1995). This autoregulatory input was found to be essential for normal function of *PDR3* and is a key component for the regulated expression of this gene (Delahodde *et al.* 1995; Zhang and Moye-Rowley 2001). The Pdr1p function was found to be stimulated by Hsp70p, Ssz1p and Zuo1p by direct binding to this transcription factor (Hallstrom *et al.* 1998; Ducett *et al.* 2013). An interesting link between Pdr1p and the protein folding/degradation came from the finding that the gene encoding the key proteasomal transcriptional regulator Rpn4p is a transcriptional target of Pdr1p (Devaux *et al.* 2001; Owsianik *et al.* 2002). Thakur *et al.* (2008) proposed that Pdr1p/Pdr3p can act as xenobiotic receptor proteins. Their data support the notion that direct binding of drug leads to remodeling of the protein to a more potent transcriptional activator.

Another transcriptional regulator found to influence MDR in yeast, was the basic region-leucine zipper (bZip)-containing factor Yap1p that regulates most of the known antioxidant genes and plays a major role in the adaptive response to oxidative stress (Rodriguez-Pousada *et al.* 2010). Most of the drug resistance effects caused by this transcription factor occur when the protein is overproduced. Drugs that act through a mechanisms that invokes an oxidative stress response typically are sensitive to the presence of Yap1p expressed at normal gene dosage (Paul and Moye-Rowley 2014).

Kluyveromyces lactis is a yeast species phylogenetically close to *S. cerevisiae*. However, these two yeast species display some important differences. *K. lactis* is a yeast with predominantly respiratory metabolism, whereas *S. cerevisiae* primarily uses fermentation under aerobic conditions. Glucose is mainly consumed via glycolysis in *S. cerevisiae*, while *K. lactis* utilizes mainly the pentose phosphate pathway. The NAD(P)H- redox and thiol-

redox reactions are a key differential point between both yeast species (Lamas-Maceiras *et al.* 2007). Unlike *S. cerevisiae*, *K. lactis* did not undergo a whole genome duplication (WGD) and has lower redundancy of genes involved in metabolism and signalling compared to *S. cerevisiae* (Bussereau *et al.* 2006; González-Siso *et al.* 2009; Rodicio and Heinisch 2013). *K. lactis* has emerged as one of the most important yeast species not only for basic research but also for industrial biotechnology. Because *K. lactis* rapidly achieves high culture densities and produces high yields of recombinant proteins, it has been used to produce proteins on an industrial scale in the food and feed industry for the past two decades (van Ooyen *et al.* 2006, Spohner *et al.* 2016). The ability to tolerate multiple stresses is a highly desirable phenotype in organisms used as cell factories (Teixera *et al.* 2011). It is, thus, crucial to understand the molecular basis underlying this phenomenon to be able to circumvent it or to explore it to design more robust industrial strains. In *K. lactis* only a handful of proteins involved in multidrug resistance have been characterized so far (Chen 2001; Takacova *et al.* 2004; Gbelska *et al.* 2006; Balkova *et al.* 2009; Goffa *et al.* 2014). Recently, we have characterized the *KIPdr1p*, the only homolog of *ScPdr1p/ScPdr3p* transcriptional regulators in *K. lactis* and have made *in silico* predictions based on the occurrence of transcription factor recognition sequences in the *KIPdr1p* target gene promoters (Balkova *et al.* 2009; Hervay *et al.* 2011). To better understand how *KIPDR1* mediated drug resistance is achieved in *K. lactis*, in this study we explored the transcriptional response of *K. lactis* to identify genes whose expression was affected by deletion or presence of the hyperactive *KIPdr1*_p* containing the L273P gain-of-function mutation (Balazfyova *et al.* 2013). We looked also for the possible interplay between *KIYap1p*, transcription factor playing a central role in oxidative stress defence, and *KIPdr1p*, transcription factor involved in *K. lactis* multidrug resistance development.

Materials and methods

Strains and culture conditions

The following yeast haploid strains were used: *K. lactis* PM6-7A (*MATa uraA1 ade2 Rag⁺ pKD1⁺*) (Chen *et al.* 1992), as well as their isogenic mutant strains *K. lactis* PM6-7A/*pdr1Δ* (*MATa, uraA1, ade2, pdr1::kanMX, Rag⁺, pKD1⁺*) deleted in *KIPDR1* ORF (Balkova *et al.* 2009) and *K. lactis* MW179-1D (*MATα lac4-8 ura A1-1 leu2 met A1-1 Ade⁻ trp1 Rag⁺*), *K. lactis* MW179-1D/*yap1Δ* (*MATα lac4-8 ura A1-1 met A1-1 Ade⁻ trp1 yap1::LEU2 Rag⁺*) (Billard *et al.* 1997). Cells were grown on glucose-rich (YPD) medium (2% glucose, 1% yeast extract, 2% bactopectone), or minimal (YNB) medium containing 0.67% yeast nitrogen base without amino acids and appropriate nutritional requirements. The media were solidified with 2% bactoagar. The *Escherichia coli* XL1-Blue strain was used as a host for plasmid constructions and propagation. The bacterial strains were grown at 37 °C in Luria-Bertani (LB) medium (1% tryptone, 1% NaCl, 0.5% yeast extract, pH 7.0) supplemented with 100 µg ml⁻¹ ampicillin for selection of transformants.

Oligonucleotides used in this work were purchased from Microsynth (Balgach/Switzerland) and are listed in Table 1.

*Construction of the *K. lactis yap1Δpdr1Δ* mutant strain*

A linear DNA fragment (3073 bp) containing the disruption cassette *Klpdr1::kanMX* (Balkova *et al.* 2009) was used to transform the *K. lactis* strain MW179-1D/*yap1Δ*. After transformation, cells were selected in YPD supplemented with 200 µg ml⁻¹ geneticin. Two rounds of re-plating on YPD containing geneticin were done to eliminate the false positives. The correct replacement in the *K. lactis* genome was verified by PCR using pairs of specific primers (Tab. 1). Genomic DNAs isolated from the parental MW179-1D/*yap1Δ* and the

deletion mutant candidates were amplified with two pairs of primers. Internal primers, P2 and P5, were designed for annealing inside the *kanMX* and external primers P1 and P3, were designed in the sequence of the *K. lactis* genome, flanking to *KIPDR1*.

Genetic manipulations, transformations and DNA preparations

Standard protocols for generating recombinant DNAs, the restriction enzyme analyses, gel electrophoresis and hybridization were used (Sambrook *et al.* 1989). Plasmid DNA from *E. coli* was prepared by the alkaline-lysis method. *K. lactis* strains were transformed by electroporation (Sánchez *et al.* 1993) using Bio-Rad gene pulser at 1.0 kV, 25 μ F and 400 Ω in 0.2 cm cuvettes.

Drug susceptibility testing

Yeast cultures were grown overnight in YNB containing glucose, diluted to a density of 1.0×10^7 cells ml^{-1} and 10-fold dilutions were performed. Cell suspensions in 5 μ l aliquots were spotted onto YPD plates containing drugs. The growth was scored after 3 days of incubation at 28 °C.

β -glucuronidase reporter assay

The *K. lactis* transformants expressing the *P_{KIPDR1}-gusA* or *P_{KIPDR5}-gusA* fusion constructs were grown in selective YNB medium and exposed to 0.025 $\mu\text{g ml}^{-1}$ of ketoconazole for 60 min. Enzyme activity was measured in permeabilized cells as described previously (Imrichova *et al.* 2005).

Quantitative real-time PCR

Yeast cells were grown in minimal medium containing glucose to the mid-logarithmic phase. Total RNA was isolated by the hot acidic phenol extraction method (Ausubel *et al.* 1989).

RNA concentrations were determined spectrophotometrically. First strand cDNA was synthesized from 1 µg of total RNA in a 20 µl reaction volume using 200 U of the Revert AidTM H Minus M-MuLV Reverse Transcriptase (MBI Fermentas, Vilnius, Lithuania). Quantitative real-time PCRs were performed in triplicate using the 7900 HT Fast Real-Time PCR System (Applied Biosystems, Foster City). Independent PCRs were performed using the same cDNA for both the gene of interest and the *KIAC1* gene, using the ABsoluteTM QPCR SYBR Green ROX Mix (Thermo Scientific, ABgene, Germany). The PCR conditions consisted of polymerase activation at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 30 s and annealing/extension at 60 °C for 1 min. A dissociation curve was generated at the end of each PCR cycle to verify that a single product was amplified using software provided with the 7900 Sequence Detection System. The change in fluorescence of SYBR Green dye in every cycle was monitored by the system software, and the threshold cycle (C_T) above the background for each reaction was calculated. The C_T value of *KIAC1* RNA was subtracted from that of the gene of interest to obtain the ΔC_T value. The ΔC_T value of an arbitrary calibrator (e.g. untreated sample) was subtracted from the ΔC_T value of each sample to obtain a $\Delta\Delta C_T$ value. The gene expression level relative to the calibrator was expressed as $2^{-\Delta\Delta C_T}$.

Microarrays and data analyses

All microarray experiments were performed using the 30K *Kluyveromyces lactis* NRRL Y-1140 microarray (MYcroarray, 5692 Plymouth Road, Ann Arbor, MI 48105, USA).

Exponentially growing (1×10^7 cells ml⁻¹) *K. lactis* PM6-7A cells (wild-type, PM6-7A/*pdrl*Δ and the wild-type transformed with multicopy plasmid carrying the gain-of-function allele of *KIPDR1** gene) (Balazfyova *et al.* 2013), were collected and total RNA was isolated using RNeasy midi kit (Qiagen GmbH, Germany). 1 µg of total RNA was linearly amplified and

labelled using Amino allyl MessageAmpII aRNA Amplification kit (Ambion, USA) with two different fluorescent dyes; AlexaFluor647 and AlexaFluor555 (Life Technologies, Germany). 4 µg of labelled RNA was hybridized (18 h at 45 °C) in 6x SSPE with addition of formamide (10%), Tween 20 (0,01%) and microarray-specific control oligos (1%, MYcroarray, USA). After washing, microarray images and two-color GPR output files were obtained using the microarray scanner InnoScan 900 and Mapix software version 7.3.1 (Inopsys, France). The two-color GPR files were processed using the R version 3.0.2 [R Core Team (2014). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL (<http://www.R-project.org>)] and functions available in the limma package (Smyth 2005). Briefly, the two-color GPR files were imported using the `read.maimages()` function, background-subtracted using the “minimum” method and within-array-normalized using the “loess” method. The between-array normalisation was achieved using the “Aquantile” method. For further analysis, only genes with $|\log_2FC| > 2$ were selected and confirmed using qPCR.

Microarray data accession number

Microarray data can be found at the Gene Expression Omnibus database (<https://www.ncbi.nlm.nih.gov/geo>) under the series number GSE76160.

Results

*Transcriptional response of *K. lactis* to alterations in the *KIPDR1* expression*

Information of the role of *KIPdr1p* in *K. lactis* multidrug resistance is still fragmentary and important insight may be obtained if one could identify the complete set of genes controlled by *KIPdr1p*. We decided to search for genes differentially expressed in the

presence or in the absence of *KIPdr1p* using DNA microarrays. To amplify the sensitivity of the screen we used the cDNA from the *K. lactis* wild-type cells, transformant containing the gain-of-function allele *KIPDRI** on multicopy plasmid that led to amino acid substitution (L273P) in the *KIPdr1p* (Balazfyova *et al.* 2013) and the isogenic deletion mutant *Klpdr1Δ*. We hoped that the identification of differentially expressed genes in these cell types will provide a first information on the *KIPdr1p* target genes. Compared with wild-type cells, the mRNA levels for 16 genes were significantly higher in the transformant overexpressing *KIPDRI** and simultaneously lower in the *Klpdr1Δ* deletion mutant (cutoff value 1.0; $p < 0.01$; fold change > 2). Table 2 summarizes this analysis. The differentially expressed genes could be grouped into three main categories: transport, post-translational modification and cytoskeleton. There were 57 genes at least two-fold upregulated in the *KIPDRI** overexpressing strain compared with wild-type cells (Table 3). As expected, the gene designated KLLA0F21692g encoding *KIPdr5p*, the main drug efflux pump belonging to ABC transporter superfamily was upregulated. Amongst the genes with the mRNA level at least two-fold higher in the *KIPDRI** overexpressing strain were orthologues of *S. cerevisiae* *ERG6* (KLLA0A01738g), encoding the delta(24)-sterol-C-methyltransferase converting zymosterol to fecosterol in the ergosterol biosynthesis, *FAS2* (KLLA0C15983g), encoding the α subunit of fatty acid synthase and the *PLB1* (KLLA0C05940g) encoding phospholipase B. Thus, the results obtained suggest that the lipid composition of the plasma membrane is one of the key determinants of multidrug resistance in *K. lactis* similarly as it is proposed for *S. cerevisiae* (Kolaczowska *et al.*, 2012; Kodedova and Sychrova 2015) and *Candida albicans* (Mukhopadhyay *et al.* 2002; Pasrija *et al.* 2005).

The additional upregulated genes in the *KIPDRI** overexpressing transformant are involved in functional categories of transcription, translation, intracellular trafficking, signal transduction and defense mechanisms. The 16 genes for which transcript levels were at least

two-fold lower in the *Klpdr1Δ* deletion mutant compared with the wild-type cells are summarized in Table 4. These genes are enriched in functions related to translation/protein fate, transport and defense mechanisms. Taking together, the microarray analyses have shown that processes related to translation are particularly responsive to the absence of *KIPdr1p*.

Besides the 16 genes for which the transcript level changed, positively or negatively, by more than two-fold in response to the presence/absence of the *KIPdr1p*, several genes showed smaller effects on transcript levels. Some of these might turn out to be significant targets when tested under different physiological or genetic conditions.

The MEME software suite version (Bailey *et al* 2009) was used in this study to search for shared common upstream sequences in the promoter regions of 16 genes that were up-regulated in the presence of *KIPDR1** gene and simultaneously downregulated in the absence of *KIPdr1p*. A search in the promoter regions (defined for this purpose as 800 bp immediately upstream from ATG translation start codon of the ORF), allowed us to identify 2 different motifs: C(A)C(G)AG(C)AAA(G)G(A), T(C)C(T)CT(A)T(C)T(A)GC(T)TTC(T). Although, they does not show any similarity to the four types of PDREs described in *S. cerevisiae* by DeRisi *et al.* (2000). Taking advantage of the evolutionary relatedness of *K. lactis* to *S. cerevisiae*, we searched the promoter region of each gene listed in Table 2 for a match to the consensus *S. cerevisiae* YRE, STRE, PDRE and the Stb5p binding site according to information gathered in YEASTRACT database (Monteiro *et al.* 2008).

This analysis detected a site closely related to *S. cerevisiae* PDRE only in the KLLA0F10857g promoter (Table 5), suggesting that it may be the direct target of *KIPdr1p*. In most genes, however, putative YRE and Stb5p binding sites were identified.

Role of Kiyap1p in K. lactis QDR1, ENA5, VCX1 and ARF2 orthologs gene regulation

Since the promoters of the four genes above contain putative YRE motifs (Table 5), we also analysed the contribution of Kiyap1p to their expression. The transcript level of four selected genes were determined using qPCR in the parental wild-type, the *Klyap1Δ* and the *Klpdr1Δyap1Δ* mutant strains. As shown in Fig. 1 the strains grown in the drug-free culture exhibited only a slightly altered expression of the *K. lactis QDR1* and *ENA5* orthologs. After growth for 30 min in the presence of subinhibitory concentration of hydrogen peroxide, the transcript levels of *K. lactis QDR1* and *ENA5* orthologs increased significantly in the wild-type strain pointing to the induction of transcription by Kiyap1p. In the oxidative stress inducing conditions, the putative role of Klpdr1p in *K. lactis ENA5* ortholog expression could also be suggested. On the other hand, the mRNA abundances of the *K. lactis ARF2* and *VCX1* orthologs were reduced in the Kiyap1p deletion mutant but did not respond to Kiyap1p activation by hydrogen peroxide (Fig. 1.). Both genes shared a common expression pattern in the presence or in the absence of hydrogen peroxide in all three strains analysed (wild-type, *Klyap1Δ* and *Klpdr1Δyap1Δ* mutant).

Kiyap1p influences the K. lactis susceptibility to antifungal azoles

Based on the presence of one PDRE-like (Balkova *et al.* 2009) and two YREs (for Yap1-Responsive Element) in the *KIPDR1* gene promoter (Fig. 2A), we proposed that Kiyap1p, a bZIP(basic-leucine zipper) transcription factor involved in cellular response to oxidative stress, might also contribute to drug resistance phenomenon in *K. lactis*. We were particularly interested to see whether the elimination of the *KIYAPI* gene would influence the phenotype of the *Klpdr1Δ* deletion mutant strain. In order to test this possibility, a double deletion mutant *Klpdr1Δyap1Δ* was constructed. Both single *Klpdr1Δ*, *Klyap1Δ* and the double deletion *Klpdr1Δyap1Δ* mutant strains were subsequently tested for their ability to

grow in the presence of antifungal azoles. As the Fig. 2B demonstrates, deletion of the *KIYAP1* gene in the *Klpdr1Δ* mutant background moderately increased the susceptibility of the mutant to all antifungal azoles tested. As *KIYap1p* has been implicated in the response to oxidative and metal stress (Billard *et al.* 1997, Imrichová *et al.* 2005) in *K. lactis*, the increased susceptibility of *Klyap1Δ* mutant cells compared with the isogenic *K. lactis* wild-type in the presence of H₂O₂ was expected. As the Fig. 2B shows, the double deletion mutant was able to grow in the presence of H₂O₂ comparably with the wild-type or the *Klpdr1Δ* mutant strain. At present, we cannot exclude the possibility that the *Klpdr1Δyap1Δ* double deletion leads to activation of some unknown compensatory pathway(s) protecting cells from oxidative stress elicited by H₂O₂.

Role of KIYap1p in KIPDR1 gene regulation

Since the *KIPDR1* gene promoter contains putative YRE-like motifs (Fig. 2A), we also analysed the contribution of *KIYap1p* to its expression (Fig. 3A). *KIYap1p* does not seem to contribute to the regulation of the *KIPDR1* gene expression neither in the absence nor in the presence of the ketoconazole as the deletion of *KIYAP1* gene did not significantly affect the amount of the *KIPDR1* mRNA. However, in the presence of hydrogen peroxide (1mM) activating *KIYap1p*, significantly increased expression of *KIPDR1* was observed in the wild-type strain and attenuated in the *Klyap1Δ* mutant (Fig. 3A). These results demonstrate that the *KIPDR1* expression responds to oxidative stress and requires the presence of *KIYap1p*.

To further investigate the possible role of YRE motif in the *KIPDR1* promoter, a β -glucuronidase assay was set up. The *KIPDR1* promoter region was fused to the *gusA* gene (*P_{KIPDR1}-gusA*) and the construct was introduced into the wild-type cells, the *Klyap1Δ* deletion mutant and the *Klpdr1Δyap1Δ* double deletion mutant. As the Fig 3B shows, all strains showed about the same β -glucuronidase activity, indicating that the deletion of *KIYAP1* has

no significant effect on the basal expression of *KIPDR1*, neither in the absence, nor in the presence of antifungal azole. These results corroborate those obtained when *KIPDR1* transcript levels were monitored in the same *K. lactis* strains used.

Discussion

Changes in the *K. lactis* transcriptome profile were examined in the presence of gain-of-function allele *KIPDR1** and in the absence of *KIPDR1* gene. This analysis led to an appreciation of the global reorganization of gene expression as a result of the presence of gain-of-function *KIPDR1** allele. The increased amount of the *KIPDR1* gene or in the *K. lactis* cells stimulates mainly the processes associated with transport, intracellular signalling, replication, ranscription and protein modification. Our results also show, that genes for proteins involved in translation or protein fate, and several chaperones also change their expression upon the *KIPDR1* gene deletion or overexpression. This suggest that specific alterations in protein turnover may occur and those changes may be required to aid protein folding or removing aggregated or misfolded proteins. It has been proposed that the response to chaperones and proteins from translational aparatus is important for switching cellular activity from biosynthetic toward protective functions (Godon *et al.* 1998). Global regulation of protein synthesis is a well-known cellular response to several different types of stresses. The inhibition of protein synthesis seems to be an evolutionary conserved response that inhibits global translation while promoting continued translation of defense genes that protect cells against and/or repair the resulting damage (Harding *et al.* 2003; Shenton *et al.* 2006). Our data indicate that transcriptional control of *KIPdr1p* target genes is the component of such adaptive response. However, the role of *KIPdr1p* in the regulation of genes involved in protein metabolism may be indirect.

Our data suggest that under the tested conditions, the *KIPdr1p* transcription factor might render cells resistant to chemical stress in several ways. In fact, we show that some of the genes overexpressed in the transformant containing *KIPDR1** encode proteins that reduce intracellular accumulation of toxic compounds. Modulation of several proteins involved in lipid metabolism and transport is also an essential part of the cellular response to xenobiotics.

Of note, *PLB1*, the homolog of the *S. cerevisiae* gene that encodes phospholipase B, was upregulated in azole-resistant *C. glabrata* isolate (Caudle *et al.* 2011) and also found to be overexpressed in our *KIPDR1** containing transformant. Although *C. glabrata PLB1* contains a PDRE in its promoter, we were not able to find any consensus sequence reminding the PDRE in *K. lactis*.

Moreover, increased expression of the *FAS2*, the ortholog of *S. cerevisiae* gene encoding one of the subunits of fatty acid CoA synthase, in *K. lactis* overexpressing the *KIPDR1** allele was found. Recently, it was shown that the product of *KIMG2*, the hypoxic regulatory gene, is involved in lipid biosynthesis especially in the regulation of transcript levels of several biosynthetic genes (*KIOLE1*, *KIFAS1*, *KIERG1*, *KLATF1*) under hypoxic shift (Micolonghi *et al.* 2012; Ottaviano *et al.* 2015), suggesting that the *KIMga2p* modulates membrane functioning or membrane-associated functions, both cytoplasmic and mitochondrial.

Our analysis led to the identification of several genes that appear to be directly or indirectly regulated by *KIPdr1p*. Promoter sequence analysis of *K. lactis* genes upregulated in *KIPDR1** containing transformant and downregulated in *Klpr1Δ* mutant revealed that this regulatory effect is not always dependent upon the presence of a PDRE-like binding consensus. Inspection of the target gene promoters for the common sequences revealed that in most of them the proposed binding site for the Stb5p (ZnCys₆ containing transcription factor) can be found. Studies in *S. cerevisiae* have shown that Stb5p forms a heterodimer with Pdr1p

and functions as transcriptional activator of multidrug resistance genes (Akache and Turcotte 2002). On the other hand, in *C. glabrata* CgStb5p functions as negative regulator of multidrug resistance (Noble *et al.* 2013). The relevance of *KlStb5p* in *K. lactis* multidrug resistance has to be experimentally verified.

Most of the genes upregulated in the *KIPDR1** bearing strain contain putative YRE consensus sequences in their promoters. Our data suggest that *K. lactis* orthologs of *QDR1* (multidrug transporter of the major facilitator superfamily) and *ENA5* (P-type ATPase) regulation primarily involves *KlYap1p*, and *KlARF2* and *KlVCX1* regulation might involve some other regulatory proteins. Like other pre-WGD yeast species, *K. lactis* has fewer Yap paralogs (5) than *S. cerevisiae* (7) (Bussereau *et al.* 2006). Besides *KlYap1p* other proteins are likely to contribute.

KlYap1p and *ScYap1p* have identical bZIP DNA binding domains and *KlYap1p* is predicted to recognize the canonical YRE motif TGACAAA according to Veide Vilg *et al.* (2014). Gene regulatory specificity may also be achieved by transcription factor homo- or heterodimerization. The AP-1 factors in metazoans have a high capacity for heterodimerization, whereas the *S. cerevisiae* AP-1 factors appear to preferentially form homodimers (Reinke *et al.* 2013). Study of specific stress response pathways in *K. lactis* have shown that besides *KlYap1p*, the main oxidative stress response factor and *KlYap8p*, regulating the transcriptional response of cells to As(III) and *t*-BOOH, other proteins are likely to contribute (Veide Vilg *et al.* 2014). Cells lacking *KlRox1p* exhibit diminished *KlYCF1* expression and are As(V) sensitive (Torres *et al.* 2012), whereas deletion of *KlHAP1* results in increased resistance of *K. lactis* cells to H₂O₂ and cadmium (Lamas-Maceiras *et al.* 2007). The mechanisms by which these proteins mediate arsenic- and peroxide-dependent

transcriptional responses and whether the transcription factors could dimerize in *K. lactis* remains to be elucidated (Veide Vilg *et al.* 2014).

Extensive analysis of the suite of Pdr1p-regulated genes were accomplished in *S. cerevisiae* (DeRisi *et al.* 2000; Devaux *et al.* 2001; Lucau-Danila *et al.* 2005, Fardeau *et al.* 2007) and *C. glabrata* (Vermitsky *et al.* 2006; Ferrari *et al.* 2009; Tsai *et al.* 2010). An important overlap between Pdr1p-induced genes encoding ABC transporters and proteins involved in lipid metabolism in *S. cerevisiae* and in *C. glabrata* was found (Paul *et al.* 2014). As two YRE-like sequences for Kiyap1p binding were identified in the *KIPDR1* promoter, we were interested if the Kiyap1p may exert an direct or indirect action upon the *KIPDR1* gene regulation. The deletion of the *KIYAP1* gene does not significantly influence the transcript level of *KIPDR1* nor the fusion construct P_{*KIPDR1*}-gusA activity. Upon Kiyap1p activation by hydrogen peroxide, the *KIPDR1* expression was markedly increased and was apparently dependent on Kiyap1p.

We propose a functional connection of the Kiyap1p activity with multidrug resistance network in *K. lactis*. Kiyap1p could act either in concert with KIPdr1p on a common gene or participates in the sequential activation of an unknown target gene. It became clear that other transcription factors whose function is not primarily linked to multidrug resistance, are also involved in the transcription control of drug efflux pumps. In *S. cerevisiae*, Yap1p confers diazaborine resistance via the functional *PDR3* gene (Wendler *et al.* 1997; Teixeira *et al.* 2010). Yap1p has been found to be required also for the stress-regulated expression of some genes through the STRE element and a cross-talk could exist between the apparently separate pathways involving a STRE-binding protein (either the Msn2p/Msn4p complex or another regulatory protein) (Dumond *et al.* 2000). Our experimental data suggest a connection of multidrug resistance with the cellular stress-response system. This could be accomplished by

direct or indirect modulation of expression of some MDR genes by *K/Yap1p* (and/or some other transcription factor under stress conditions). Elucidation of numerous interactions between MDR, YAP and other transcription factors will require further analyses. The elucidation if *K/Yap1p* acts either in concert with the *K/Pdr1p* on a common gene or participates in the sequential activation of unknown target gene(s) remains to be elucidated.

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Figure captions

Figure 1. Relative gene expression levels in *Klyap1Δ* and *Klpdr1Δyap1Δ* mutant cells as measured by quantitative real time PCR. Fold-change is defined as the average ratio of gene expression in respective mutant cells compared to their parental cells. The gene transcript levels in parental strains were set as 1. Gene expressions were normalized by the *KIAC1* expression levels in hydrogen peroxide untreated and hydrogen peroxide treated cells respectively. Error bars indicate the SDs.

Figure 2. Localization of YRE and PDRE consensus elements in the *KIPDR1* promoter (A). Growth of *K. lactis* strains on solid YPD media supplemented with antifungal azoles and hydrogen peroxide (B). Spotting assays were performed with a 10-fold dilution of overnight cultures on YPD medium containing the drugs as indicated. The plates were incubated for 2 days at 28 °C. MIC miconazole, KET ketoconazole, ITR itraconazole.

Figure 3. Transcriptional regulation of the *KIPDR1* gene (A) measured in the wild-type, *Klyap1Δ* and *Klpdr1Δyap1Δ* strains grown in the presence of ketoconazole (0.025 μg ml⁻¹) for 1 h or hydrogen peroxide (1mM) for 30 min. Gene expression was normalized by the *KIAC1* expression level.. (B) β-glucuronidase activity of P_{*KIPDR1*}-gusA promoter construct without induction (no drug) and after induction by ketoconazole (0.025 μg ml⁻¹, 1h) in *K. lactis*. The obtained values are the average of at least three independent experiments, with error bars representing standard deviation

Figure 1. Relative gene expression levels in *Klyap1Δ* and *Klpdr1Δyap1Δ* mutant cells as measured by quantitative real time PCR. Fold-change is defined as the average ratio of gene expression in respective mutant cells compared to their parental cells. The gene transcript levels in parental strains were set as 1. Gene expressions were normalized by the *KIACT1* expression levels in hydrogen peroxide untreated and hydrogen peroxide treated cells respectively. Error bars indicate the SDs.

167x164mm (300 x 300 DPI)

Figure 2. (A) Localization of YRE and PDRE consensus elements in the *KIPDR1* promoter. (B) Growth of *K. lactis* strains on solid YPD media supplemented with antifungal azoles and hydrogen peroxide. Spotting assays were performed with a 10-fold dilution of overnight cultures on YPD medium containing the drugs as indicated. The plates were incubated for 2 days at 28 °C. MIC miconazole, KET ketoconazole, ITR itraconazole.

167x95mm (300 x 300 DPI)

Figure 3. Transcriptional regulation of the *KIPDR1* gene (A) measured in the wild-type, *Klyap1Δ* and *Klpdr1Δyap1Δ* strains grown in the presence of ketoconazole (0.025 $\mu\text{g ml}^{-1}$) for 1 h or hydrogen peroxide (1mM) for 30 min. Gene expression was normalized by the *KIACT1* expression level. (B) β -glucuronidase activity of P_{KIPDR1} -gusA promoter construct without induction (no drug) and after induction by ketoconazole (0.025 $\mu\text{g ml}^{-1}$, 1 h) in *K. lactis*. The obtained values are the average of at least three independent experiments, with error bars representing standard deviation.

225x81mm (300 x 300 DPI)

Table 1. List of oligonucleotides used in this study

Primers for qRT-PCR analysis	Nucleotide sequence (5'→3')
<i>KIACT1</i> – F	ACA CCT AAC CTA CCC AAA C
<i>KIACT</i> – R	TCA ATA ACT AAA GCA GCG AC
<i>KIPDR1</i> – F	ATT TCA ACC TGC CGT TTC
<i>KIPDR1</i> – R	CCG TTG CCA TTA CTA TCT CTA
<i>KIPDR5</i> – F	AGG AC GAG GGT GTG AAT
<i>KIPDR5</i> – R	CAT ATT CTT GAT CCA CGC AG
Primers for verification of <i>KIPDR1</i> gene deletion	
P1 <i>KIPDR1</i> – F	CCAAGAAGGAAAGTTAGCAGAGC
P2 KanMX – R	CTGTAACATCATTGGCAACGCTA
P3 <i>KIPDR1</i> – R	TATCACCGTACCATTGACCGT
P5 KanMX – F	AGACCGATAACCAGGATCTTGC

Table 2. Genes upregulated in *KIPDR1** compared to wild-type and down regulated in *Klpdr1Δ* compared to wild-type

Functional category	<i>K. lactis</i> systematic name	<i>S. cerevisiae</i> ortholog	Description*	Fold change in expression in	
				<i>KIPDR1</i> */ wt	<i>Klpdr1Δ</i> / wt
Transport					
Transmembrane transport	KLLA0F03278g	<i>QDR1</i>	MFS transporter,required for resistance to quinidine and azoles	2.4	-2.1
	KLLA0E19283g	<i>GUP1</i>	Plasma membrane protein, involved in glycerol transport	2.3	-2.1
Lipid transport and metabolism	KLLA0A12045g	<i>YGL262W</i>	Hypothetical protein	2.1	-2.0
Carbohydrate transport and metabolism	KLLA0F27423g	<i>PKPI</i>	Pyruvate dehydrogenase kinase	2.3	-2.0
Amino acid transport and metabolism	KLLA0F09317g	<i>CYS4</i>	Cystathionine beta-synthase	2.5	-2.1
Inorganic ion transport and metabolism	KLLA0F20658g	<i>ENA5</i>	Member of the Na+ efflux ATPase family	2.3	-2.0
	KLLA0F05632g	<i>VCX1</i>	Ca2+ transport, Ca2+/H+ and K+/H+ exchange protein	2.2	-2.1
Intracellular trafficking, secretion, vesicular transport	KLLA0B07975g	<i>GRX8</i>	GTPase activator activity	2.5	-2.2
	KLLA0C02299g	<i>SEC2</i>	Essential for post-Golgi vesicle transport and for autophagy	2.2	-2.1
	KLLA0C05126g	<i>GSP2</i>	GTP binding protein of the RAS superfamily	2.2	-2.1
	KLLA0F05225g	<i>ARF2</i>	GTP-binding protein of the ARF family	2.2	-2.2
Post-translational modification, protein turnover, chaperones	KLLA0C14322g	<i>SBA1</i>	Hsp90 associated co-chaperone	2.4	-2.0
	KLLA0F10857g	<i>YIL161W</i>	Hypothetical protein	2.4	-2.2
Cytoskeleton	KLLA0F23848g	<i>BZZ1</i>	Regulating actin polymerization	2.2	-2.1
	KLLA0C09460g	<i>RBL2</i>	Protein involved in microtubule morphogenesis	2.2	-2.1
Other	KLLA0B07403g	N/A	Hypothetical protein	2.3	-2.1

* Description according to *K. lactis* annotation (http://www-archbac.u-psud.fr/genomes/r_klactis/r_klact_annotation.html)

Functional category	<i>K. lactis</i> systematic name	<i>S. cerevisiae</i> ortholog	Description*	Fold change in expression in <i>KIPDR1</i> * / wt
Transport				
Inorganic ion transport and metabolism	KLLA0E12133g	<i>VTC2</i>	Putative polyphosphate synthetase	4.0
Transmembrane transport	KLLA0F21692g	<i>PDR5</i>	ABC transporter involved in steroid/multidrug resistance	2.5
Lipid transport and metabolism	KLLA0C05940g	<i>PLB1</i>	Phospholipase B (lysophospholipase)	2.5
	KLLA0C15983g	<i>FAS2</i>	Fatty acid CoA synthetase subunit	2.5
	KLLA0A01738g	<i>ERG6</i>	S-adenosyl-methionine delta(24)-sterol C-methyltransferase	2.5
carbohydrate transport and metabolism	KLLA0E18348g	<i>YMR090W</i>	Predicted dehydrogenase	6.2
	KLLA0B09988g	<i>KRE2</i>	Alpha-1,2-mannosyltransferase	2.2
	KLLA0F23870g	<i>YNL115C</i>	Putative galactosyltransferase	2.0
Aminoacid transport and metabolism	KLLA0E09031g	<i>ARO2</i>	Chorismate synthase	2.5
	KLLA0E13838g	<i>AVT3</i>	Vacuolar transporter	2.1
	KLLA0F27995g	<i>N/A</i>	Putative arginase	2.0
Nucleotid transport and metabolism	KLLA0E22902g	<i>URA3</i>	Orotidine-5'-phosphate (OMP) decarboxylase	2.2
	KLLA0E05500g	<i>URA7</i>	Major CTP synthase isozyme	2.0
Intracellular trafficking, secretion, vesicular transport	KLLA0F17798g	<i>SED5</i>	Required for vesicular transport between the ER and the Golgi complex	2.6
	KLLA0E07249g	<i>ALR2</i>	Divalent ion transporter	2.5
	KLLA0F26895g	<i>MNR2</i>	Vacuolar membrane protein required for magnesium homeostasis	2.4
	KLLA0E23958g	<i>SEC16</i>	Required for ER transport vesicle budding	2.4
	KLLA0C10461g	<i>NEL1</i>	Activator of Sar1p GTPase activity	2.1
Energy production/conversion	KLLA0A09383g	<i>MTM1</i>	Mitochondrial carrier protein	2.5
	KLLA0E05159g	<i>SWC7</i>	Hypothetical protein	2.3
Nuclear structure	KLLA0F14344g	<i>NTR2</i>	Hypothetical protein	2.5
Replication, recombination,	KLLA0F13002g	<i>ORC3</i>	Subunit of the origin recognition complex (ORC)	2.5

repair	KLLA0C02607g	<i>ORC5</i>	Subunit of the origin recognition complex (ORC)	2.5
	KLLA0C16984g	<i>ORC4</i>	Subunit of the origin recognition complex (ORC)	2.1
RNA processing and modification	KLLA0D02728g	<i>SPP382</i>	Essential protein involved in spliceosome disassembly	2.1
Transcription	KLLA0B03047g	<i>SFP1</i>	Regulates transcription of ribosomal protein and response to nutrients and stress	2.4
	KLLA0D03234g	<i>RPB9</i>	RNA polymerase II subunit	2.1
Translation, ribosomal structure and biogenesis	KLLA0B00869g	<i>MRPL39</i>	Mitochondrial ribosomal protein of the large subunit	9.1
	KLLA0A05665g	<i>TRM2</i>	tRNA methyltransferase	2.5
	KLLA0A11814g	<i>SUI2</i>	Translation initiation factor	2.3
Post-translational modification, protein turnover, chaperones	KLLA0E00704g	<i>WSC4</i>	Endoplasmic reticulum (ER) membrane protein involved in the translocation of soluble secretory proteins	2.5
	KLLA0E07183g	<i>ATP11</i>	F1F0 ATPase complex assembly protein	2.5
	KLLA0B13882g	<i>CCT5</i>	T complex protein 1 epsilon subunit	2.5
	KLLA0E07898g	<i>TMA108</i>	Puromycin-sensitive aminopeptidase	2.2
Cytoskeleton	KLLA0A09141g	<i>SPC97</i>	Component of the gamma-tubulin complex	2.5
	KLLA0B13453g	<i>CAP1</i>	F-actin capping protein alpha subunit	2.5
Signal transduction mechanisms	KLLA0C07216g	<i>SKY1</i>	Involved in regulating proteins involved in mRNA metabolism and cation homeostasis	2.5
	KLLA0D05797g	<i>SIC1</i>	Cyclin-dependent kinase inhibitor (CKI)	2.4
	KLLA0C16269g	<i>LDB17</i>	Protein involved in the regulation of endocytosis	2.4
	KLLA0D10549g	<i>SST2</i>	Required to prevent receptor-independent signaling of the mating pathway	2.1
	KLLA0D15334g	<i>VOA1</i>	ER protein that functions in assembly of the V0 sector of V-ATPase	2.1
	KLLA0C05654g	<i>PKP2</i>	Dehydrogenase kinase	2.1
Cell cycle control, division and chromosome partitioning	KLLA0E21263g	<i>CDC40</i>	Cell division control protein	2.7
	KLLA0C00484g	<i>MRC1</i>	Hypothetical protein	2.6
	KLLA0F27005g	<i>MIH1</i>	M phase inducing protein tyrosine phosphatase	2.4
	KLLA0C15829g	<i>HSL7</i>	Protein kinase inhibitor	2.4
Secondary metabolites biosynthesis, transport and catabolism	KLLA0B14619g	<i>FMO1</i>	Flavin-containing monooxygenase	2.5
Defense mechanisms	KLLA0A03047g	<i>HAA1</i>	Transcriptional activator involved in adaptation to weak acid stress	2.8

	KLLA0E17963g	<i>IRA2</i>	GTPase-activating protein	2.4
	KLLA0E01430g	<i>AIM36</i>	Hypothetical protein	2.2
Other	KLLA0F22946g	<i>MCP2</i>	Predicted unusual protein kinase	2.5
	KLLA0C18546g	<i>TRM112</i>	Protein involved in methylation of tRNA, rRNA, and translation factors	2.5
	KLLA0D06149g	<i>PFA5</i>	Hypothetical protein	2.4
	KLLA0F11385g	<i>ABP140</i>	Predicted methyltransferase	2.4
	KLLA0F24002g	<i>NCS2</i>	Uncharacterized conserved protein	2.2
	KLLA0B02519g	<i>HYMI</i>	Hypothetical protein	2.2
	KLLA0D05445g	<i>FYV7</i>	Uncharacterized conserved protein	2.1

* Description according to *K. lactis* annotation (http://www-archbac.u-psud.fr/genomes/r_klactis/r_klact_annotation.html)

Table 4. Genes down regulated in *Klpdr1Δ* compared to wild-type

Functional category	<i>K. lactis</i> systematic name	<i>S. cerevisiae</i> ortholog	Decsription*	Fold change in expression in <i>Klpdr1Δ</i> / wt
Transport				
Inorganic ion transport and metabolism	KLLA0D09581g	<i>SMF2</i>	Divalent metal ion transporter involved in manganese homeostasis	-2.3
Carbohydrate transport and metabolism	KLLA0E25025g	<i>HXT2</i>	low-affinity hexose transporter	-2.4
Cell cycle control, division and chromosome partitioning	KLLA0C08800g	<i>BUD31</i>	RNA splicing	-2.4
	KLLA0E24508g	<i>SWD3</i>	Histone methyltransferase activity, essential subunit of the COMPASS (Set1C) complex, required in transcriptional silencing near telomeres	-2.3
Post-translational modification, protein turnover, chaperones	KLLA0E23463g	<i>UBP16</i>	Ubiquitin-specific protease	-2.1
Translation, ribosomal structure and biogenesis	KLLA0B00517g	<i>SLF1</i>	RNA binding protein that associates with polysomes, cellular copper ion homeostasis, regulation of translation	-2.5
	KLLA0C08371g	<i>RPL34B</i>	Ribosomal 60S subunit protein L34B	-2.3
	KLLA0E13717g	<i>NOP9</i>	Essential subunit 90S preribosome	-2.2
Signal transduction mechanisms	KLLA0E05808g	<i>YMR1</i>	Phosphatidylinositol 3-phosphate (PI3P) phosphatase; involved in various protein sorting pathways,	-2.7
Defense mechanisms	KLLA0D11660g	<i>CTA1</i>	Catalase A, peroxisomal	-2.2
Other	KLLA0C17006g	<i>PIB2</i>	Phosphatidylinositol 3-phosphate binding protein	-2.6
	KLLA0B05104g	N/A	Hypothetical protein	-2.3
	KLLA0C11671g	<i>YER156C</i>	Hypothetical protein	-2.3
	KLLA0D07876g	<i>YNL162W-A</i>	Hypothetical protein	-2.3
	KLLA0A09933g	<i>YMR295C</i>	Hypothetical protein	-2.1

KLLA0A01551g

FRA2

Hypothetical protein

-2.0

* Description according to *K. lactis* annotation (http://www-archbac.u-psud.fr/genomes/r_klactis/r_klact_annotation.html)

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Table 5. Putative regulatory sites in promoters of the *K/Pdr1p* regulated genes

<i>K. lactis</i> systematic name	<i>S. cerevisiae</i> ortholog	consensus recognition sequence					
		MEME		YEAstract
		YRE*	Stb5p binding site	STRE**	PDRE***
				TKACAAA TTACTCA TTACTAA	CGGNS	CCCCT	TCCGTGGA TCCACGGA TCCGGGTA
KLLA0C14322g	<i>SBA1</i>	+	+	+	+		
KLLA0F03278g	<i>YDR1</i>	+		+	+	+	
KLLA0F20658g	<i>ENA5</i>	+	+	+	+	+	
KLLA0A12045g	<i>YGL262W</i>	+	+	+	+	+	
KLLA0C02299g	<i>SEC2</i>	+	+		+		
KLLA0F23848g	<i>BZZ1</i>	+	+	+	+		
KLLA0B07403g	N/A	+	+		+		
KLLA0F09317g	<i>CYS4</i>	+	+	+	+		
KLLA0F27423g	<i>PKP1</i>	+	+	+			
KLLA0E19283g	<i>GUP1</i>	+	+	+	+	+	
KLLA0C05126g	<i>GSP2</i>	+	+	+	+		
KLLA0C09460g	<i>RBL2</i>	+	+	+	+		
KLLA0F05632g	<i>VCX1</i>	+	+	+	+	+	
KLLA0B07975g	<i>GRX8</i>	+	+		+		
KLLA0F10857g	<i>YIL161W</i>	+	+		+		+
KLLA0F05225g	<i>ARF2</i>	+	+	+	+		

*YRE Yap1 recognition element

**STRE stress response element

***PDRE pleiotropic drug resistance response element

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