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Regulation of the Expression of the *Kluyveromyces lactis PDC1* Gene: Carbon Source-Responsive Elements and Autoregulation

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The yeast *Kluyveromyces lactis* has a single structural gene coding for pyruvate decarboxylase (*KlPDC1*). In order to study the regulation of the expression of *KlPDC1*, we have sequenced (EMBL Accession No. Y15435) its promoter and have fused the promoter to the reporter gene *lacZ* from *E. coli*. Transcription analysis in a *Klpdc1*Δ strain showed that *KlPDC1* expression is subject to autoregulation. The *PDC1* gene from *Saccharomyces cerevisiae* was able to complement the Rag$^-$ phenotype of the *Klpdc1*Δ mutant strain and it could also repress transcription of the *KlPDC1–lacZ* fusion on glucose. A deletion analysis of the promoter region was performed to study carbon source-dependent regulation and revealed that at least two *cis*-acting regions are necessary for full induction of gene expression on glucose. Other *cis*-elements mediate repression on ethanol. Copyright © 1999 John Wiley & Sons, Ltd.

KEY WORDS — *Kluyveromyces lactis*; pyruvate decarboxylase; autoregulation; promoter analysis

INTRODUCTION

The non-conventional yeast *Kluyveromyces lactis* is receiving increasing attention, not only for its use as a host for the production of heterologous proteins (Rocha *et al*., 1996), but also, because of its metabolic diversity from *Saccharomyces cerevisiae*. *K. lactis* is a respiratory yeast (Gancedo and Serrano, 1989), while in *S. cerevisiae* fermentation occurs even under aerobic conditions (De Deken, 1966). In spite of these differences in the regulation of fermentative and respiratory metabolism, the two yeasts are closely related and, in general, both the sequence and the function of structural genes are highly conserved (Stark and Milner, 1989; Goffrini *et al*., 1990; Lewis and Pelham, 1990; Bergkamp-Steffens *et al*., 1992; Jacoby *et al*., 1993; Prior *et al*., 1993).

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The physiological differences described above underline the importance of studying the metabolism of pyruvate at the branch point between respiration and fermentation in *K. lactis*, where these pathways seem to be regulated differently from *S. cerevisiae*.

The first step of the fermentative pathway, i.e. the conversion of pyruvate to acetaldehyde, is catalysed by pyruvate decarboxylase (PDC). In *S. cerevisiae*, the PDC system is rather complex and consists of three structural genes, *PDC1*, *PDC5* and *PDC6* (Schmitt *et al*., 1983; Kellermann *et al*., 1986; Schaaff *et al*., 1989; Seeboth *et al*., 1990; Hohmann, 1991). *PDC1* is responsible for most of the wild-type PDC activity, while *PDC5* only becomes active in *pdc1*Δ strains (Hohmann and Cederberg, 1990). The Pdc1 protein not only exerts a negative regulatory effect on the expression of *PDC5*, but also autoregulates the expression of its own gene (Liesen *et al*., 1996). The *PDC6* gene does not contribute to PDC activity and becomes active only when fused to an active promoter (Hohmann, 1991). PDC activity in *S. cerevisiae* is dependent on the regulatory factor Pdc2p (Hohmann, 1993; Raghuram *et al*., 1994) and on

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Table 1. *K. lactis* strains.

Strain	Genotype	Reference
PM6-7A	MAT**a**, *KlPDC1*, *adeT-600*, *uraA1-1*	Wésolowski-Louvel *et al.*, 1992
PMI	MAT**a**, *Klpdc1::URA3*, *adeT-600*, *uraA1-1*	Bianchi *et al.*, 1996
PMI/C1	MAT**a**, *Klpdc1::ura3*, *adeT-600*, *uraA1-1*	This work
MW341-5	MATα, *KlPDC1*, *lac4-8*, *leu2*, *lysA1-1*, *uraA1-1*	Wésolowski-Louvel M.
MW341-5/Δ	MATα, *Klpdc1::URA3*, *lac4-8*, *leu2*, *lysA1-1*, *uraA1-1*	This work
MM1-12D	MAT**a**, *Klpdc1::ura3*, *adeT-600*, *lac4-8*, *leu2*, *uraA1-1*	This work

the common transcriptional regulators of glycolytic gene expression, Gcr1p and Rap1p (Butler *et al.*, 1990; Chambers *et al.*, 1995; Liesen *et al.*, 1996). The binding sites for these factors are often found in close proximity (Chambers *et al.*, 1995). It has been suggested that only GCR1 sites located close to RAP1 sites are functionally important *in vivo* (Bitter *et al.*, 1991; Huie *et al.*, 1992). However, the RAP1–GCR1 box of *PDC1* has been identified as an element conferring general promoter activation, but not carbon source-dependent regulation (Butler *et al.*, 1990; Liesen *et al.*, 1996).

We have isolated and sequenced the unique *PDC* gene from the yeast *K. lactis* (Bianchi *et al.*, 1996). As opposed to *S. cerevisiae*, a *K. lactis* strain, lacking the *KlPDC1* (formerly *KlPDCA*) gene, was able to grow at the same rate as the wild-type on glucose media, highlighting the fact that pyruvate chanelling through fermentation and respiration in these yeasts is regulated in different ways (Pronk *et al.*, 1996). The transcription of *KlPDC1* is induced by glucose, repressed by ethanol (Bianchi *et al.*, 1996) and regulated by the *RAG3* gene product, which has a high homology to the Pdc2 protein from *S. cerevisiae* (Prior *et al.*, 1996). In *K. lactis* the PDC activity is also induced by oxygen shortage (Kiers *et al.*, 1998). In *S. cerevisiae*, ethanol repression of the *PDC1* gene, as well as autoregulation, have been shown to be mediated by a novel *cis*-acting element, composed of short repetitive sequences called *ERA* sequences (ethanol repression and autoregulation) and placed downstream of the RAP1–GPR1 box. The *ERA* sequences have been subsequently found upstream of several glycolytic genes of *S. cerevisiae*.

In this report, we give evidence that autoregulation and oxygen depletion are involved in transcriptional regulation of the *KlPDC1* gene and that the *S. cerevisiae PDC1* gene can substitute *KlPDC1* gene in function and, partially, in autoregulation. On the other hand, we also show that carbon source regulation relies on multiple *cis*-acting elements of the promoter region, which allow glucose induction and ethanol repression of *KlPDC1* transcription.

MATERIALS AND METHODS

Strains, media and general genetic methods

The *E. coli* strain used was DH5aF′ (φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) *U169 deo rec1 end1 sup44* λ *THI-1 gyrA96 relA1*). *K. lactis* strains are listed in Table 1. The two *KlPDC1* strains PM6-7A and MW341-5 have essentially the genetic background of the standard wild-type strain CBS2359 and behave identically as far as genes involved in the Rag$^-$ phenotype are concerned (Wésolowski-Louvel M., personal communication). The *ura3* strain PMI/C1 has been obtained after 5-fluoro-orotic acid selection of resistant clones (McCusker and Davis, 1991) from strain PMI, which is a *Klpdc1::URA3* integrative transformant of PM6-7A (Bianchi *et al.*, 1996). Strain MM1-12D has been obtained from a cross between strains PMI/C1 and MW341-5.

Genetic procedures for crossing and sporulation have previously been described (Wésolowski *et al.*, 1982). Rich medium was 1% yeast extract and 2% (w/v) peptone (YP). Synthetic minimal (SM) selective medium contained 0·7 g/l of yeast nitrogen base without amino acids (Difco), supplemented with the appropriate amino acids and nucleotides. Glucose and ethanol were added to a final concentration of 2%. Antimycin A was used at a final concentration of 5 μM. Solid medium contained 20 g/l Bacto-agar (Difco).

Plasmids and plasmid constructions

All plasmids constructed in this work were obtained using standard techniques (Sambrook

Table 2. PDC activity in *K. lactis* wild-type and *Klpdc1*Δ strains.

Strain	Vector	PDC-activity (mU/mg) 2% Glucose	PDC-activity (mU/mg) 2% Ethanol	Rag phenotype
PM6-7A (wt)		215 (± 84)	54 (± 19)	+
PMI/C1 (*Klpdc1*Δ)		<1	<1	−
PMI/C1 (*Klpdc1*Δ)	KCpPDC (*KlPDC1*)	237 (± 69)	22 (± 9)	+
PMI/C1 (*Klpdc1*Δ)	KEpPDC (*KlPDC1*)	3516 (± 400)	226 (± 25)	+
MW341-5 (wt)		212 (± 58)	51 (± 1)	+
MW341-5/Δ (*Klpdc1*Δ)		<1	<1	−
MW341-5/Δ (*Klpdc1*Δ)	pK3C1 (*ScPDC1*)	890 (± 168)	950 (± 141)	+
MW341-5/Δ (*Klpdc1*Δ)	pK3C1-8 (*Scpdc1-8*)	<1	<1	−

The *Klpdc1*Δ strains were transformed either with the gene coding for pyruvate decarboxylase from *K. lactis* (*KlPDC1*) on a centromeric and a multicopy vector, or with the gene from *S. cerevisiae* (*ScPDC1* and *Scpdc1-8*) on a centromeric vector. Cells from YP medium precultures were grown overnight in selective medium containing 2% glucose or 2% ethanol as carbon source to appoximately $OD_{600}=1$. The transformants with empty vectors (KCp491 and KCplac13) showed no additional PDC activity to the untransformed strains (not shown). Values are averages of three or more independent determinations. Standard errors are reported in parentheses.

et al., 1989). A 4·3 kb chromosomal *Hin*dIII fragment containing the *KlPDC1* gene (Bianchi *et al.*, 1996), was cloned into the unique *Hin*dIII sites of the *K. lactis* multicopy and centromeric vectors KEp6 (Chen *et al.*, 1988) and KCp491 (Prior *et al.*, 1993), respectively. The resulting vectors, harbouring the *URA3* gene from *S. cerevisiae* as a selectable marker, were named KEpPDC and KCpPDC. The 1·2 kb 5′ upstream region from *KlPDC1* was sequenced from the 4·3 kb chromosomal *Hin*dIII fragment. This promoter region was then amplified by PCR with oligonucleotide primers bearing *Bam*HI and *Xba*I restriction sites at their 5′ ends. After purification and endonuclease digestion, the promoter fragment has been cloned into the plasmid pFDY180, a pBR322 derivative containing the 3·2 kb *lacZ* gene from *E. coli*. The amplified promoter region has been controlled by sequencing. A 4·4 kb *Bam*HI–*Nhe*I fragment containing the *lacZ* gene under the control of the 1·2 kb promoter fragment was then ligated into the centromeric vector KCp491. This plasmid, named pMD12 (Figure 1), was used to construct all further pMD-derivatives, either by using the naturally occurring restriction sites or by amplifying different promoter fragments with oligonucleotide primers carrying the appropriate restriction sites at their 5′ ends to facilitate cloning steps. All amplification steps were performed with the proof-reading VENT-Polymerase (NEB).

In order to transform *K. lactis* with the *PDC1* gene from *S. cerevisiae* and with its *pdc1-8* mutant allele (kindly given by Dr Stefan Hohmann, University of Göteborg), two 5 kb fragments, harbouring the whole open reading frames of *PDC1* and *pdc1-8* alleles together with 1 kb from their 5′ upstream region, were ligated into the *Pst*I site from the centromeric LEU2 vector KCplac13 (kindly given by Dr Wésolowski-Louvel, University of Lyon). The resulting plasmids were named pK3C1 and pK3C1-8, respectively.

Yeast transformation procedures

K. lactis was transformed using the electroporation procedure as described previously in Bianchi *et al.* (1996).

Northern analysis

400 ml of YP medium in a 1000 ml Erlenmeyer flask were inoculated with wild-type PM6-7A or MW341-5 cells and incubated overnight with shaking (175 rpm) at 28°C to 4×10^7 cells/ml ($OD_{600}=1·2$). The grown culture was divided into six 50 ml aliquots and transferred to 250 ml flasks; glucose, galactose, fructose, glycerol and ethanol were added to a final concentration of 2%. Carbon source was omitted from one of the six YP aliquots

Table 3. β-galactosidase activity in *K. lactis Klpdc1*Δ double transformants repressed by heterologous Pdc1p from *S. cerevisiae*.

Strain	Vectors	β-galactosidase activity	
		2% Glucose	2% Ethanol
MW341–5 (wt)	pMD12	159 (± 3)	35 (± 4)
MM1-12D (*Klpdc1*Δ)	pMD12	1113 (± 78)	1696 (± 117)
MM1-12D (*Klpdc1*Δ)	pMD12+pK3C1 (*ScPDC1*)	442 (± 46)	577 (± 61)
MM1-12D (*Klpdc1*Δ)	pMD12+pK3C1-8 (*Scpdc1–8*)	453 (± 92)	658 (± 127)
MM1-12D (*Klpdc1*Δ)	pMD12+KCplac13	1050 (± 155)	1701 (± 206)

Transformed cells, precultured in YP medium without any added carbon source, were inoculated in SM selective medium with glucose or ethanol and grown overnight to $OD_{600}=1$. Each value represents an average of at least three independent measurements. Standard errors are reported in parentheses. In the double transformants containing vectors pK3C1, pK3C1-8 and KCplac13, the PDC activities were identical to those reported for the single plasmid transformants reported in Table 2.

of the culture. The flasks were then incubated at 28°C with shaking (175 rpm) for a further 5 h growth. A 50 ml tube was filled up with a further 50 ml aliquot of YP culture, firmly capped, sealed and held at 28°C without shaking for 5 h. This sample was set up to obtain a condition of oxygen shortage. RNAs were then extracted and Northern analysis performed as described in Bianchi *et al.* (1996).

Enzyme assays

PDC activity was measured as described by Ullrich (1970) with minor modifications (Bianchi *et al.*, 1996). For the β-galactosidase assays, yeast cells were inoculated from a preculture, grown in YP medium without any carbon source added, in 50 ml of SM selective medium containing 2% glucose or 2% ethanol and incubated at 28°C overnight in 250 ml Erlenmeyer flasks with vigorous shaking (175 rpm) to 1 OD_{600}. Glass bead extraction of proteins and β-galactosidase assays were carried out according to Miller (1972) with modifications as described in Rose and Botstein (1983). Protein determination was performed according to Bradford (1976). The plasmids used for the assays contained a centromeric sequence from *K. lactis* and should therefore be stably present in one to two copies per cell, minimizing artifacts due to multicopy effects (Chen, 1996). Transformed cells were grown in selective medium for monitoring β-galactosidase activity.

RESULTS AND DISCUSSION

Complementation analysis of the Klpdc1Δ *strain*

In a previous work, we described the isolation of the pyruvate decarboxylase gene from *K. lactis* (*KlPDC1*) and its carbon source-dependent expression, i.e. induction on glucose and repression on ethanol. We also showed that *K. lactis* strains bearing a deletion in the *KlPDC1* gene do not grow on glucose media plus antimycin. This inability to grow by fermentation when respiration is blocked has been defined as a Rag^- phenotype (Goffrini *et al.*, 1990). The ability to grow on glucose in the presence of antimycin A and the carbon-source dependent PDC activity could be restored in a *Klpdc1*Δ strain by transformation with a 4·3 kb chromosomal *Hin*dIII fragment, which harbours the *KlPDC1* ORF and 1·2 kb of the 5′ upstream region, either on a centromeric or on a multicopy vector (KCpPDC and KEpPDC, respectively). These results, reported in Table 2, show that in the *Klpdc1*Δ centromeric transformants the PDC activities were very similar to those of the wild-type, demonstrating that 1·2 kb of the promoter region contained all the elements necessary for glucose- and ethanol-dependent gene regulation. When we transformed the same strain with the corresponding multicopy plasmid, the enzymatic activity was several times higher on both glucose and ethanol.

Table 2 also shows that, as expected from the high homology between the *KlPDC1* gene product

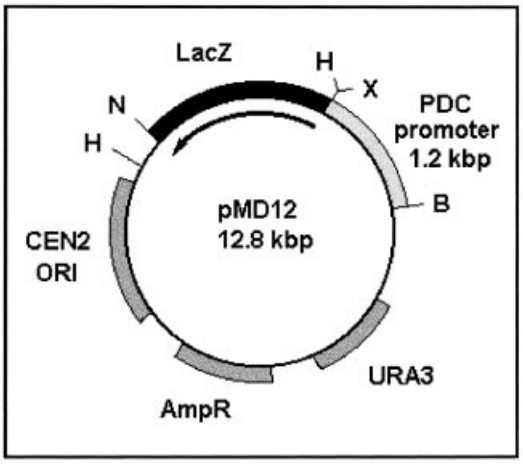


Figure 1. Structure of the Plasmid pMD12. The centromeric shuttle plasmid contains the CEN2/ORI region from *K. lactis*, the *URA3* marker from *S. cerevisiae* and the *LacZ* gene from *E. coli* under the control of the *KlPDC1* promoter. Relevant restriction sites: B=*Bam*HI, H=*Hin*dIII, N=*Nhe*I, X=*Xba*I.

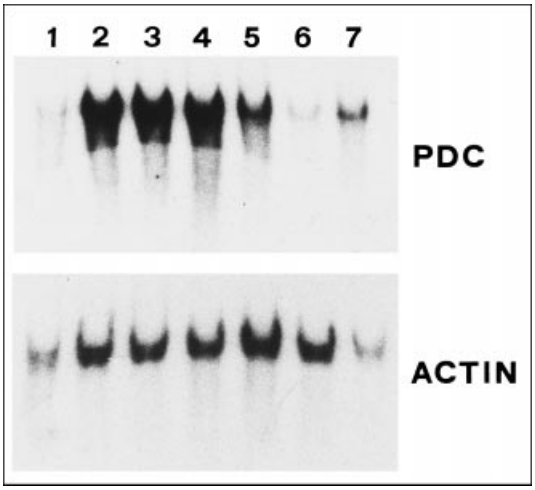


Figure 2. Northern analysis of *KlPDC1* transcripts in different growth conditions and carbon sources. RNAs were extracted from PM6-7A cells grown to OD_{600}>1·2 on basic YP medium. Lane 1, no added carbon source; lane 2, 2% glucose; lane 3, 2% galactose; lane 4, 2% fructose; lane 5, 2% glycerol; lane 6, 2% ethanol; lane 7, no added carbon source with oxygen limitation. RNAs were hybridized with a *KlPDC1* probe and a *K. lactis* actin probe for quantitation.

and its counterpart Pdc1p from *S. cerevisiae* (Bianchi *et al*., 1996), the *PDC1* gene of the latter yeast was able to complement the Rag$^-$ phenotype of a *Klpdc1*Δ strain of *K. lactis*. However, the *Klpdc1*Δ strain, harbouring the *PDC1* gene from *S. cerevisiae* on a centromeric vector, exhibited a 5–6-fold higher PDC activity than the *KlPDC1* transformants. This level of PDC activity was similar to that measured in *S. cerevisiae* on glucose (Hohmann, 1991) and, in contrast to the PDC activity in *S. cerevisiae*, was not repressed on ethanol. This indicates important differences between either the regulatory sequences and/or the transcription factors responsible for ethanol repression in these yeasts.

Autoregulation of KlPDC1 *expression*

In *S. cerevisiae*, the *PDC1* gene product has been shown to have a regulatory influence on the transcription of *PDC5* and on that of *PDC1* itself (Seeboth *et al*., 1990; Hohmann and Cederberg, 1990; Liesen *et al*., 1996). To investigate the presence or absence of this effect in *K. lactis*, we constructed the centromeric vector pMD12, which carried the 1·2 kb promoter region of *KlPDC1* fused to the reporter gene *lacZ* (see Figure 1). When introduced into the *KlPDC1* strain MW341-5, this vector yielded β-galactosidase activity, exhibiting the same pattern of regulation by glucose and ethanol as the PDC activity in wild-type strains (Table 3). On the other hand, pMD12 transformants of the *Klpdc1*Δ strain MM1-12D showed activities many-fold higher than the wild-type transformants on both carbon sources, thus revealing a strong repressive effect of the *KlPDC1* gene product on the transcription from its own gene promoter. The high β-galactosidase activity of MM1-12D transformants on ethanol also suggest that in *K. lactis* KlPdc1p is required for ethanol repression.

To examine whether the *PDC1* gene product from *S. cerevisiae* could restore normal transcriptional regulation of the construct pMD12 in a *Klpdc1*Δ strain, we transformed this strain with pK3C1, a centromeric vector containing the *PDC1* gene from *S. cerevisiae* (see Materials and Methods). The results, shown in Table 3, demonstrated that the Pdc1 protein from *S. cerevisiae* was able to exert a repressive function on the expression of the *lacZ* reporter gene. To demonstrate that it was the Pdc1 protein itself and not the absence of PDC activity that caused the repressive effect, we also tested the *pdc1-8* gene from *S. cerevisiae*, which carries a point mutation leading to an enzymatically inactive protein (Schaaff *et al*., 1989). Results also demonstrated that the inactive protein downregulated *KlPDC1* promoter-driven expression. The high values observed with the empty vector KCplac13 excluded the possibility

that the reduced β-galactosidase activity in transformants carrying two centromeric vectors was due to plasmid competition. Neither the Pdc1 nor the Pdc1-8 proteins were able to restore ethanol repression, thus suggesting the involvement in this regulatory circuit of ethanol-specific regulatory protein(s) unable to interact correctly with the heterologous pyruvate decarboxylase.

Transcription of KlPDC1 *in wild-type strains*

Oxygen shortage might become a critical condition when measuring the activity or the transcription of the fermentative gene *KlPDC1* in cells grown to high density in flasks. In fact, it has been recently demonstrated that PDC activity is induced in *K. lactis* cells grown in a chemostat at low oxygen supply (Kiers *et al.*, 1998). However, no data on *KlPDC1* transcription in cells grown under oxygen limitation have been reported to date.

In order to exclude any interference by oxygen on our subsequent analysis on carbon source regulation of *KlPDC1* transcription, we performed a Northern analysis as described in Materials and Methods. The results, shown in Figure 2 for strain PM6-7A, confirmed that *KlPDC1* transcription is induced by glucose and repressed by ethanol. Identical results were obtained with strain MW341-5. Glycerol and sugars, such as galactose and fructose, could also induce *KlPDC1* transcription, suggesting that the onset of one or more signals triggering the induction of this gene might depend on glycolytic steps downstream of sugar phosphorylation and/or steps involving C3 compounds, as reported by Boles and Zimmermann (1993) for the induction of *ScPDC1*.

Northern analysis also showed that *KlPDC1* transcription was induced by oxygen limitation in the absence of any added carbon source, but that this regulatory effect was absent in cells grown in shake flask up to $OD_{600}=1{\cdot}2$. This finding allowed us to perform the carbon source-dependent transcriptional analysis of the *KlPDC1* promoter, described below, while no attempt has been made up to now to identify elements involved in oxygen regulation.

Nucleotide sequence of the KlPDC1 *5′ upstream region*

The nucleotide primary structure of the 1·2 kb upstream region from the pyruvate decarboxylase structural gene *KlPDC1* was determined by sequencing (EMBL Nucleotide Sequence Database; Accession No. Y15435).

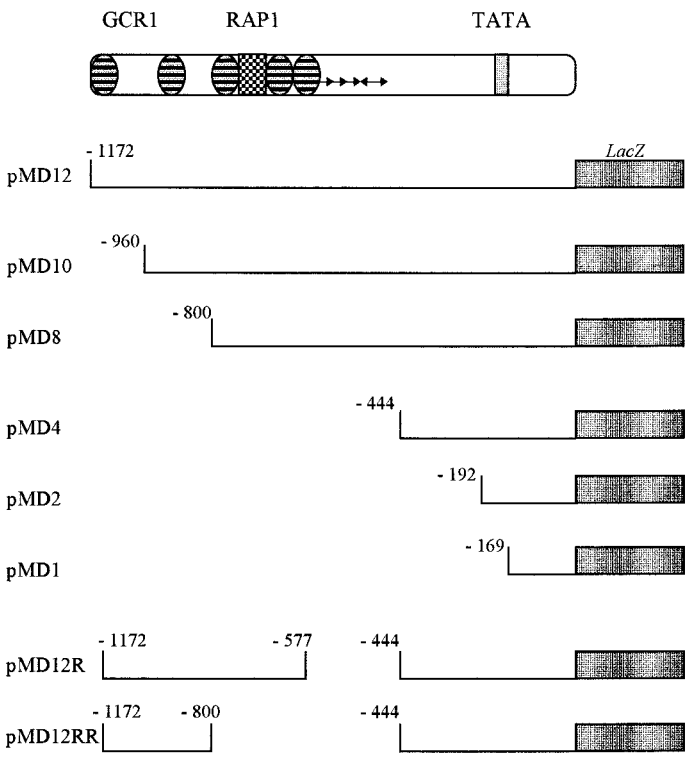
A computer analysis of the promoter sequence revealed several putative binding sites for common fungal transcription factors (Quandt *et al.*, 1995). Three binding sites for Gcr1p could be identified in the farther upstream region, respectively at positions -766, -865 and -1172 upstream from the ATG start codon. Two additional Gcr1p binding sites at positions -585 and -591 were found to form a UAS_{RPG} (Larson *et al.*, 1994), together with a putative Rap1p binding site located at -623. To date, little is known about the regulation of glycolytic gene expression in *K. lactis*. However, several transcription factors from *K. lactis* have been isolated by functional complementation of their *S. cerevisiae* counterparts (Jakobsen and Pelham, 1991; Gonçalves *et al.*, 1992; Na and Hampsey, 1993; Mulder *et al.*, 1994), demonstrating the close relationship between these yeasts. The *KlRAP1* homologue, on the contrary, was unable to complement a rap^{ts} mutant strain from *S. cerevisiae*, even if a binding of the Rap1p to the consensus sequence could be observed (Larson *et al.*, 1994; Ishii *et al.*, 1997).

Immediately downstream from the RAP1–GCR1-binding site, there are five repetitive sequences, T/GGAAATGG/T, showing a slight homology with the *ERA* sequences from *S. cerevisiae*. Two potential TATA boxes were found at the positions -177 and -108.

Identification of glucose and ethanol-dependent regulatory regions in the upstream sequence of KlPDC1

In order to study the importance of *cis*-acting sequences in *KlPDC1* expression, we generated a series of 5′ and internal promoter deletions, starting from the plasmid pMD12, and we introduced the different constructs in a wild-type strain. The effect of the deletions on expression was measured by assaying the levels of β-galactosidase activity in the transformants grown on glucose- or ethanol-containing media. The results are reported in Figure 3.

Deletions in the farther 5′ promoter region, removing one or two of the single Gcr1p putative binding sites (vectors pMD10 and pMD8), progressively reduced the activity on glucose. More extended deletions, which also removed the putative RAP1–GCR1 box and the downstream



	β–galactosidase activities				
	MW341-5 (*KlPDC1*)			MM1-12D (*Klpdc1Δ*)	
	2% Glc	2% EtOH	G/E	2% Glc	2% EtOH
pMD12	159 (±3)	35 (±4)	5.0	1113 (±78)	1696 (±117)
pMD10	83.6 (±2.6)	140 (±27)	0.6	167 (±19)	457 (±12)
pMD8	58 (±7)	143 (±19)	0.31	216 (±41)	n.d.
pMD4	36 (±1.5)	150 (±27)	0.24	40 (±4)	490 (±22)
pMD2	48 (±5.5)	388 (±21)	0.11	25 (±1)	335 (±8)
pMD1	7.8 (±0.8)	13.2 (±1.5)	0.6	n.d.	n.d.
pMD12R	172 (±26)	117 (±10)	1.6	638 (±58)	1159 (±56)
pMD12RR	47.2 (±9.4)	23.7 (±6.2)	2.0	585 (±5)	1242 (±82)

Figure 3. β-galactosidase activities of different pMD-transformants. The sequences indicated were cloned into the centromeric vector KCp491 and introduced either in the *KlPDC1* strain MW341-5 or in the *Klpdc1*Δ strain MM1-12D. β-galactosidase activities were determined after growth of the transformed cells to OD_{600}=1·0 on SM selective medium with the indicated carbon source (Glc=glucose, EtOH=ethanol). Each value represents an average of measurements from at least three different transformants. Putative controlling promoter elements of *KlPDC1* are schematically depicted in the upper part of the figure. The striped ovals indicate the positions of the putative GCR1 consensus sequences, while the squared box marks the positions of the RAP1 binding site. The dotted grey box indicates the TATA box. *ERA*-like sequences are marked by arrows (see text for details). G/E, ratio of activities measured in glucose grown cells to ethanol grown cells; 'n.d.', not determined.

region containing the *ERA*-like repetitive sequences (vectors pMD4 and pMD2), led to further reduced activity on glucose. The deletion of only the repetitive sequences in vector pMD12R had no effect on β-galactosidase activity of the transformants on glucose, suggesting that these sequences are not involved in glucose induction of *KlPDC1*. On the other hand, when the region containing the RAP1–GCR1 box was also absent (vector pMD12RR), a sharp decrease of the activity on glucose could be observed. These results suggest the existence of multiple *cis*-acting regulatory elements important for induction on glucose, which should be located between positions -577 and -1172. Site-directed mutagenesis of the putative Gcr1p and Rap1p binding sites present in this region will ultimately reveal whether these sequences play a role in *KlPDC1* expression.

As far as ethanol-grown transformants are concerned, the deletions of the regions between -1172 and -960 (vector pMD10) and between -444 and -192 (vector pMD2), resulted in substantially increased β-galactosidase activities, suggesting a role of these regions in ethanol repression of *KlPDC1*. The β-galactosidase activities obtained from vectors with internal deletions showed that the *ERA*-like sequences were not essential for ethanol repression, at least when the region containing the RAP1–GCR1 box was also absent (vector pMD12RR). However, the deletion of only the *ERA*-like sequences (vector pMD12R) led to derepressed expression of the reporter gene. These results indicate that the expression of *KlPDC1* on ethanol media might depend on complex interactions between positive and negative regulatory elements and/or factors.

The deletion of 23 bp between -192 and -169 (vector pMD1) reduced expression on both carbon sources to very low values. These values were identical to those measured with the promoter-less vector and the untransformed strain MW341-5 (data not shown), suggesting that the putative TATA box at position -177 might be the functional one.

On medium containing both glucose and ethanol, the β-galactosidase activity in pMD12 transformants of strain MW341-5 showed intermediate values (62 ± 11 β-galactosidase units). This finding is consistent with the analysis of *KlPDC1* transcription previously reported (Bianchi *et al.* 1996), where we showed that the presence of ethanol reduced the level of *KlPDC1* transcription on glucose. These data indicate that expression of pyruvate decarboxylase in *K. lactis* is modulated by two mutually non-exclusive effects of induction by glucose and repression by ethanol. It should be noted, however, that the construct bearing only the active TATA box (vector pMD2) still led to carbon source-dependent β-galactosidase activity. Again, intermediate values (200 ± 30 β-galactosidase units) of the reporter gene activity were observed on both glucose and ethanol media, suggesting that, in *K. lactis*, the basal level of transcription might also depend on the presence of carbon source-specific regulatory factors.

Promoter elements mediating autoregulation

As shown above, the *KlPDC1* expression is subjected to autoregulation. The transformation of the *lacZ* fusions into a *Klpdc1*Δ strain allowed us to indicate promoter regions involved in autoregulation.

Results reported in Figure 3 showed that all of the tested constructs, with the exception of vectors pMD4 and pMD2, led to higher activities in a *Klpdc1*Δ background than in the wild-type strain, indicating that autoregulatory factor(s) should require sequences upstream from position -444. However, two distinct regions seemed to mediate autoregulation with different efficiency. In fact, the deletion of the farther region, between -1172 and -800, had a stronger effect on the decrease of β-galactosidase activity (vectors pMD10 and pMD8) than the deletion of the region including the *ERA*-like sequences, between positions -577 and -444. A similar situation was observed when the transformants were grown on ethanol medium, although transcriptional activity on ethanol was in all cases higher than on glucose. This result supports the conclusion that the autoregulatory factors are the major repressing agents in *KlPDC1* expression. This differs from the situation in *S. cerevisiae*, where the β-galactosidase values, obtained on ethanol media in a *pdc1*Δ strain, never exceeded the values measured on glucose (Liesen *et al.*, 1996).

In conclusion, our results reveal some similarities between the two closely related yeasts *K. lactis* and *S. cerevisiae*, as far as autoregulation, carbon source-dependent expression and the promoter structure of *PDC1* genes are concerned. However, important differences have also been found, as one might expect for two yeasts in one of which fermentation is largely dispensable, as is the

case of *K. lactis*, or is the prevalent metabolic pathway, as is the case of *S. cerevisiae*. It is interesting to note that the putative binding sites for all of the transcription factors isolated from *K. lactis* so far are remarkably conserved, while the factors that ensure the different regulation of channelling of metabolites through either the fermentative or the respiratory pathway might rely on the diversity of specific regulatory agents.

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