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Differential control of isocitrate lyase gene transcription by non-fermentable carbon sources in the milk yeast *Kluyveromyces lactis*

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Abstract **The *KlICL1* gene, encoding isocitrate lyase in *Kluyveromyces lactis*, is essential for ethanol utilization. Deletion analyses identified two functional promoter elements, CSRE-A and CSRE-B. Transcription is activated on ethanol, but not on glucose, glycerol or lactate. Expression depends on the KlCat8p transcription factor and KlSip4p binds to the promoter elements. Glycerol diminishes *KlICL1* expression and a single carbon source responsive element (CSRE) sequence is both necessary and sufficient to mediate this regulation. The glycerol effect is less pronounced in *Saccharomyces cerevisiae* than in *K. lactis*. Mutants lacking *KlGUT2* (which encodes the glycerol 3-phosphate dehydrogenase) still show reduced expression in glycerol, whereas mutants deficient in glycerol kinase (*Klgut1*) do not. We conclude that a metabolite of glycerol is required for this regulation.**
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1. Introduction

The yeasts *Kluyveromyces lactis* and *Saccharomyces cerevisiae* share a similar life-cycle and are closely related from an evolutionary point of view, but *S. cerevisiae* is believed to have undergone a whole genome duplication that *K. lactis* did not [1,2]. Both yeasts can grow on fermentable carbon sources as expected from their natural environments (i.e. sugar-rich fruit juices and milk with lactose). *S. cerevisiae* is specialized in alcoholic fermentation whereas *K. lactis* is Crabtree-negative and relies on a more respiratory metabolism. Accordingly, major differences are observed in the regulatory circuits which govern their central metabolism (for reviews see [3–5]). *S. cerevisiae* and *K. lactis* are also able to grow on alternative, non-fermentable, carbon sources such as ethanol or glycerol. Sugar phosphates are then supplied through gluconeogenesis and the energy demand is met by respiration. If ethanol is to be used as a sole carbon source, the glyoxylate cycle becomes essential as an anaplerotic pathway to replenish C4-metabolites to the tricarboxylic acid cycle (for reviews see [6,7]). Gluconeogenesis and the glyoxylate cycle are coordinately regulated in *S. cerevisiae.* Thus, transcription of the genes encoding the respective enzymes proceeds in the presence of ethanol and is repressed by glucose (for reviews see [7–10]).

Glucose repression in *S. cerevisiae* is mediated by the Snf1-complex, named after the catalytic Snf1p (=Cat1p) subunit, a member of a family of serine/threonine kinases, which is highly conserved in all eukaryotes [11]. Snf1p is closely associated with the regulatory protein Snf4p (=Cat3p [12]), which stimulates its kinase activity by binding the auto-inhibitory domain [13]. A third component of the complex is either one of three subunits encoded by *SIP1*, *SIP2* or *GAL83* [14,15]. If activated, the Snf1-complex phosphorylates Cat8p, which binds DNA at the so-called carbon source responsive element (CSRE) promoter sequences (for carbon source responsive element [16,17]). CSREs are found in the promoters of genes encoding gluconeogenic and glyoxylate cycle enzymes and account for the above mentioned coordinate regulation (see [7] and references therein). Consequently, *cat8* mutants fail to grow on non-fermentable carbon sources such as glycerol or ethanol. *CAT8* gene expression is negatively regulated by the general Mig1p repressor in *S. cerevisiae* [16]. Besides Cat8p, the zinc cluster protein Sip4p has also been shown to bind to CSRE sequences, but a *sip4* deletion does not display a marked growth defect on non-fermentable carbon sources [18].

These regulatory relationships are less well studied in *K. lactis*. In contrast to *S. cerevisiae*, gluconeogenesis and the glyoxylate pathway seem to be regulated independently. Thus, *Klcat8* mutants lack the ability to utilize ethanol but still grow on glycerol as a sole carbon source. KlCat8p is not required for synthesis of the gluconeogenic enzymes fructose 1,6-bisphosphatase and phosphoenolpyruvate carboxykinase. Yet, it is essential for the expression of *KlICL1* and *KlMLS1*, which encode the glyoxylate cycle enzymes isocitrate lyase and malate synthase, respectively [19]. Transcription of *KlACS1* and *KlACS2* (which encode isozymes of acetyl coenzyme A synthases), *KlJEN1* and *KlJEN2* (which encode carboxylic acid permeases) and *KlQCR8* (which encodes the 11 kDa subunit VIII of the mitochondrial bc1-complex) are also regulated by KlCat8p [20–22]. Analogous to *S. cerevisiae*, KlCat8p

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Abbreviations: CSRE, carbon source responsive element; nt, nucleotide; PCR, polymerase chain reaction

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phosphorylation depends on the *KlSNF1* (=*FOG2*) product [23]. The *GAL83*/*SIP1*/*SIP2* gene family of *S. cerevisiae* is represented by the sole *FOG1* gene in *K. lactis* [24].

In previous works, we isolated and characterized the *KlICL1* gene, which is essential for ethanol utilization and whose expression is repressed by glucose and strongly increased on ethanol [25]. Derepression requires a functional KlCat8p transcription factor [19,25]. In contrast to the enzyme from *S. cerevisiae*, KlIcl1p is not subject to catabolite inactivation [25].

In this paper, we describe the identification of two CSRE elements in the *KlICL1* promoter and show that these are bound by the KlCat8p dependent transcription factor KlSip4p. We also demonstrate that the activity of these CSREs is not only down-regulated by glucose but also by glycerol.

2. Materials and methods

2.1. Strains, media and enzymatic analysis

The *K. lactis* strains used in this work are listed in Table 1. The *S. cerevisiae* strain FMY401 (*MATα icl1::LEU2 ura3(fs) leu2–3,112 his3–11,15*) was also employed. In order to obtain a *Klcat8* mutant strain that lacks β-galactosidase activity, strains MW190-9B (*lac4*) and IG8 (*Klcat8*) were crossed on plates containing 5% malt extract and 3% agar and diploids were selected by complementation of auxotrophic markers. After sporulation, the segregant Kbc3 (Table 1), which does not grow on ethanol medium and fails to produce β-galactosidase, was chosen for further studies. That this strain is indeed mutated in *KlCAT8* was confirmed by polymerase chain reaction (PCR). For genetic manipulations in *Escherichia coli*, strain DH5α from InVitrogene (Karlsruhe/Germany) was used.

Media, preparation of crude extracts and enzymatic determinations have been described, previously [25].

2.2. Nucleic acid preparations, hybridization and sequencing

Yeast cells were transformed by the method of Klebe et al. [26] modified as described in [27]. Other DNA manipulations were performed by the standard methods described in [28].

Custom sequencing was done by SCT (Oviedo/Spain) or Secugen (Madrid/Spain).

2.3. Plasmids

2.3.1. KlICL1lacZ fusions. A SalI/HindIII fragment containing 831 nucleotides (nt) from the 5′ non-coding region and 26 bp of the *KlICL1* coding sequence (Gene Bank accession number AY124768) was obtained by PCR with the oligonucleotides OLI-13 and OLI-26 (Table 2) and KlICL1KEp [25] as template, and cloned in frame to *lacZ* into pXW3 [29] to yield plasmid pXWKlICL1 (Fig. 1) or into pUK1921 [30] to give pUK-KlICL1. Unidirectional deletions were obtained by PCR using pXW-KlICL1 as template and oligonucleotides listed in Table 2 together with the universal primer. The amplified products were cloned into pXW3, and the resulting plasmids were named according to the oligonucleotide number.

Starting with pUK-KlICL1, internal promoter deletions were introduced by inverse PCR. By using suitable inside–out oligonucleotides (see Table 2) all plasmid sequences but the ones to be deleted were amplified. From the pUK-derivatives the *KlICL1* promoter deletions were cloned into pXW3 as SalI/HindIII fragments. The plasmids were named according to the corresponding oligonucleotide pairs.

Construct pXW-39 (see Fig. 1) was used to study the function of putative CSRE elements. All the inserts were obtained by annealing two complementary oligonucleotides (Table 2) and cloned into pXW-39 digested with BamHI/SalI to give plasmids pXW-39-#, with # corresponding to the numbers of the complementary oligonucleotides.

To obtain a *KlACS2-lacZ* fusion, a BamHI/HindIII fragment containing 809 nt from the 5′ non-coding region and 20 bp of the *KlACS2* coding sequence, was generated by PCR with the oligonucleotides OLI76 and OLI77 (Table 2) and genomic DNA from the wild-type strain KB6-2C as template and cloned in frame to *lacZ* into pXW3 [29] to yield pXW-KlACS2* (Fig. 3). *KlICL1*-CSRE-B, obtained by annealing the complementary oligonucleotides OLI80 and OLI81 (Table 2), was cloned into pXW-KlACS2 digested with BamHI (vector) and XbaI to give pXW-KlACS2-$CSRE_{ICL}$-B. Plasmid pXW-KlACS2 was prepared as a control by digesting pXW-KlACS2* with XbaI and BamHI, end-filling with the Klenow fragment of DNA polymerase I and religation.

To investigate the effect of glycerol on the *S. cerevisiae* enzyme in *K. lactis,* the deletion strain Klicl1Δ13, which carries the centromeric plasmid ScICL1pCXJ with the *ScICL1* gene, was used. For the *S. cerevisiae* studies, plasmid KlICL1YIp integrated at the *URA3* locus of the *ScICL1* deletion strain FMY401 was employed [25].

The respective promoter regions of all constructs described were verified by sequence analyses.

2.4. Construction of Klgut1 and Klgut2 deletions

The*Klgut1* deletion was obtained by transformation of the wild-type strain MW270-7B with a PCR product carrying the *KanMX* cassette flanked by *KlGUT1* sequences. Primers OLI-06.139 and OLI-06.140 were used with pUG6 [31] as a template. Transformants were selected on YEPD/sorbitol medium containing 110 mg/l G418 and replica-plated onto synthetic medium containing glycerol as a sole carbon source. Two colonies that did not grow on the latter medium had *KlGUT1* substituted by the *KanMX* cassette, verified by two different PCR approaches. A similar strategy was used for the construction of the *Klgut2* deletion strain, i.e. with the oligonucleotide pair OLI-06.141/06.142 as primers for the PCR reaction. Transformants selected on G418 that did not grow on synthetic medium with glycerol were shown to contain the correct deletion by PCR.

2.5. Quantification of expression levels by real-time RT-PCR

Total RNA was isolated with the FastRNA® Pro Red Kit from Bio 101® Systems and treated with deoxyribonuclease I (RNase-free). cDNA and PCR reactions were performed in an ABI PRISM® 7000 (Applied Biosystems) with the SuperScript™ III Platinum® SYBR® GREEN One-Step qRT-PCR kit from InVitrogene. All procedures were performed according the instructions of the manufacturers. The primers 06.102-Ov/06.103-Ov and 06.104-Ov/06.105-Ov (Table 2) were used to amplify internal sequences of *KlICL1* and *KlACT1* (control), respectively. PCR conditions were: an initial cDNA synthesis step at

Table 1
Kluyveromyces lactis strains employed in this work

Strain	Genotype	Source
KB6-2C	*MATa ura3-12 his3-35 ade⁻*	K.D. Breunig [36]
MW270-7B	*MATa leu2 uraA1-1 met1-1*	M. Wésolowski-Louvel [27]
MW190-9B	*MATa Klura3 lac4-8* Rag+	M. Wésolowski-Louvel [29]
Kbc3	*MATa ura3-12 lac4-8 Klcat8Δ*	This work
Klicl1Δ13	*MATa ura3-12 his3-35 ade⁻ Klicl1::ScHis3*	López [25]
PM6-7A-KltpiΔ	*MATα uraA1-1 adeT-600 tpi1::KanMx*	Compagno [37]
CBS2359/pgi1	*MATa rag2::loxP*	Steensma [38]
MW270-7B-Klgut1Δ	*MATa leu2 uraA1-1 met1-1*	This work
MW270-7B-Klgut2Δ	*MATa leu2 uraA1-1 met1-1*	This work
JA6/SIP4HA	*MATα trp1-11 ura3-12 adeA-600 lac4::KlSIP4-(HA)6::KlTRP1*	L.Schild unpublished data

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