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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.**

Keywords: Ethanol metabolism; Isocitrate lyase; Carbon source responsive element; *CAT8*; *SIP4*; Yeast

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

Table 2
Oligonucleotides used

Designation	Sequence
OLI-26	5′acgaagcttGCAGCAGAAGCCTTAACGGAG3′
OLI-13	5′acagtcgacACGATGGGAGAATTGAATC3′
OLI-54	5′acgagtcgacTCCGCCATTAAACCGAAT3′
OLI-55	5′acgagtcgacTTAAACCGAATGTGATAG3′
OLI-32	5′acgctcgagTTCTTCCCCTCCCTACAT3′
OLI-56	5′acgagtcgacTTTCGCATGAATGGGGAAA3′
OLI-38	5′acgagtcgacGGTTCCTCAGCCAAATAG′ 3′
OLI-39	5′acgagtcGACGGCTTGAAACTGGTC3′
OLI-41	5′acgagtcgacGCACATACATGGTACCGT3′
OLI-8	5′ggagctcgagGAATTAGAGCCATGATTGG3′
OLI-33	5′acgagtcgacGTTCAATTATACCTTAAG3′
OLI-60	5′ccgtagatctCCGAATGTGATAGTTTTTCC3′
OLI-61	5′ccgtagatctATGATTCAATTCTCCCATC3′
OLI-62	5′ccgtagatctGAAAGTTGACTCAGTTGACTC3′
OLI-64	5′ccgtctcgaGGAAAGTTGACTCAGTTGACTC
OLI-65	5′ccgtctcgaGCGAAAATGTCCGTGAATTC
OLI-66	5′ccgtagatctTTCTTCCCCTCCCTACATCC
OLI-50	5′acgagatcTCGCATGAATGGGGtcgacaca3′
OLI-51	5′acggtcgaCCCCATTCATGCGAgatctaca3′
OLI-52	5′acgagatcTCGGTTTAATGGCGtcgacaca3′
OLI-53	5′acggtcgacGCCATTAAACCGagatctaca3′
OLI-80 D Xba	5′gacgagatcTCGCATGAATGGGGtctagacgag3′
OLI-81 D-Xba	5′ctcgtctagaCCCCATTCATGCGAgatctcgtc3′
OLI-76 ACS2	5′acgaagcttTGCAATTTATCCGACGACAT3′
OLI-77 ACS2	5′agaggatccGAGCTGAACGGTGCAACA3′
OLI-06.139	CGTAGCATCAGGTGGAATAGAGCAAGATAACAGCAGACTTTTATTGCAGCAGAT*C*TTCGTACGCTGCAGGTCGAC
OLI-06.140	CATTCATCCATCCTTTAGATTTCTCAATTGCAGTCTCCCACATCTTCCAACCT*C*CGCATAGGCCACTAGTGGATCTG
OLI-06.141	GCCAGTAAGGTTTTGCTGGGATCAGCTGTGTCTGCTGCCGCAGTGTTTGGCGCTG*C*TTCGTACGCTGCAGGTCGAC
OLI-06.142	GTTGAATTTCATCTTGTCTCTTTTTGGAATCCCAGCCCAATTCATCTCCCC*C*CGCATAGGCCACTAGTGGATCTG
OLI-06.102-Ov	CGATGACTTACAAACCATCGAA
OLI-06.103-Ov	ACAATTCGGTACCCAACAATTC
OLI-06.104-Ov	TCCCAGGTATTGCTGAAAGAAT
OLI-06.105-Ov	TCGTCGTATTCTTGCTTTGAGA
CSRE-KlICL1AL	CGGGTATGATTAACAGCGACA
CSRE-KlICL1AR	ATGGATGTAGGGAGGGGAAG
CSRE-KlICL1BL	CTTCCCCTCCCTACATCCAT
CSRE-KlICL1BR	CGTGTTTTGTTCGTCCATTG
KlKHT3-6-CW	GTATGCGTCAGTCGGATCGT
KlKHT3-9-CW	ATACCTGTATGGTCCATTGC

Capital letters represent the sequences present in the respective genes. Small letters show nucleotides added to the original sequences. Bases underlined indicate the restriction sites used for cloning. Italics correspond to sequences of the pUG6 vector.

50 °C for 30 min and a denaturation step at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing-extension at 60 °C for 1 min.

2.6. Chromatin immunoprecipitation analyses (ChIP)

For ChIP assays, cells were pre-grown in YNB glucose (2%) medium to OD_{600} of 1–1.5 and shifted to ethanol (3%) for 5 h for derepression before cross-linking with formaldehyde. Immunoprecipitation of sheared chromatin was performed with α-HA-antibodies (Santa Cruz) for 1 h at 4 °C. After reversal of cross-links and DNA extraction, input and ChIP were analysed using 25 cycles of standard PCR conditions.

3. Results

3.1. The KlICL1 promoter contains two functional CSREs elements

Regulatory elements in the *KlICL1* promoter were first delimited by testing a set of sequential deletions from the 5′-end of the promoter for the reporter β-galactosidase activity under different growth conditions (Fig. 1A). Constructs carrying either 831 nt or 811 nt upstream from the ATG translation start codon confer high level expression on ethanol, which is repressed by glucose. A further deletion of 7 nt (−811 to −804) causes a 40% reduction of the initial activity on ethanol and expression is slowly reduced by approximately 60% when sequences up to nt −732 are removed. All further deletions resulted in only basal level activities. Thus, all activating sequences are located between nt −811 and −690. This was confirmed by a construct lacking specifically this region (pXW-61/62), which again displayed only basal level activities (Fig. 1B). Closer inspection of the respective DNA fragment revealed the presence of two similar sequences, which resemble the consensus for CSRE elements of *S. cerevisiae* (Fig. 1). Therefore, the two elements were designated CSRE-A and CSRE-B.

Further investigations showed that deletion of CSRE-A causes an approximately 45% reduction in specific β-galactosidase activities. An even stronger effect (70% reduction) was observed in the deletion of CSRE-B. A construct lacking both elements lacked expression (Fig. 1B). This suggests that the two CSRE elements are both necessary and sufficient for activating transcription under derepressing conditions.

Each of the elements was then tested individually. For this purpose, a construct (pXW-39) which lacks all regulatory elements but retains the putative TATA-box and the transcription start points of the *KlICL1* promoter (located at −226

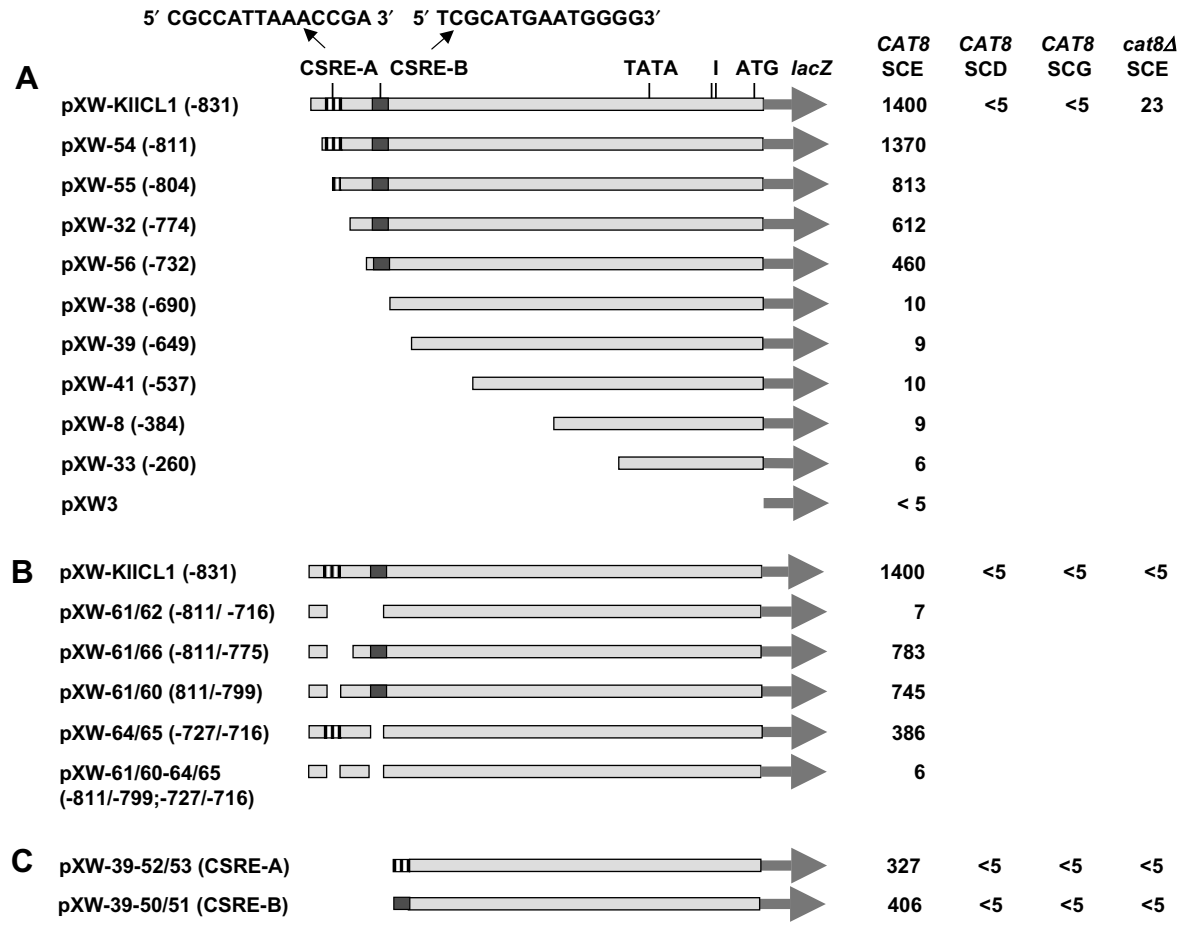

Fig. 1. *KlICL1* promoter analysis. The *KlICL1* promoter and part of the coding sequence was fused to the bacterial *lacZ* gene in the vector pXW3 and truncated constructs were obtained by PCR (see Section 2). Positions are numbered relative to the ATG translational start codon. Plasmids were transformed into the strains MW190-9B (*KlCAT8 lac4-8*) or Kbc3 (*Klcat8 lac4-8*) both lacking β-glactosidase. For preparation of crude extracts, cells were grown on SC lacking uracil, with 4% glucose. The culture was either collected at an OD_{600} of approximately 2 (SCDura; repressing conditions) or was used after centrifugation to inoculate cultures containing the indicated media to an OD_{600} of 0.5 (*KlCAT8*) or 1.5 (*Klcat8* mutant). The cells were incubated for a period of 6 h to allow for induction of enzyme synthesis. Repeated β-galactosidase determinations of different transformants did not differ by more than 30%.

and −113/−95 as described previously [25]) was employed. CSRE-A and CSRE-B were each reconstituted from pairs of 12 nt oligonucleotides and inserted into the reporter construct. Neither of these elements conferred a high level reporter gene expression in *K. lactis*. Therefore, CSRE-A and CSRE-B were again reconstituted, but from 14 nt oligonucleotides. These elements were fully functional and conferred high β-galactosidase activities on ethanol (Fig. 1C) and acetate (111 and 223 mU/mg, for CSRE-A and CSRE-B, respectively). Low basal activities were observed on glucose, glycerol (Fig. 1C) and lactate (<5 mU/mg). It should be noted that the kinetics of β-galactosidase activity in time-course experiments with constructs which carried either CSRE-A or CSRE-B did not reveal any significant differences. Thus, these data did not provide any evidence for a recognition of the CSREs by different factors. For both constructs activities started to increase 3 h after a shift from glucose to ethanol medium, and the strains carrying the CSRE-B construct always showed the higher activity.

For further investigation, we tested a *Klcat8* deletion, which lacks the ability to derepress *KlICL1* transcription on non-fermentable carbon sources [19,25]. Neither one of the pXW-39 derivatives employed, nor the construct carrying the entire *KlICL1* promoter, displayed significant β-galactosidase activities when ethanol was used as carbon source (Fig. 1). This strongly indicates that regulation through CSRE sequences depends on the presence of the KlCat8p protein. However, attempts to demonstrate KlCat8p binding have failed so far.

In *S. cerevisiae*, Cat8p is required for the transcription of *SIP4*, which encodes a second regulatory protein that has been shown to bind to CSRE sequences. We therefore tested whether KlSip4p might bind to the identified elements within the *KlICL1* promoter. A strain with an HA-tagged version of the *KlSIP4* gene was employed for ChIP analysis. Cells were pre-grown in glucose and shifted to ethanol medium for five hours. Chromatin precipitated with anti-HA antibodies was subjected to PCR with specific oligonucleotides to detect *KlICL1*-CSRE sequences. An unrelated sequences (from the *KlKHT3* gene) and a strain with an untagged *KlSIP4* gene, were used as controls. As demonstrated in Fig. 2, the CSRE-A and CSRE-B elements of the *KlICL1* promoter were enriched in the precipitate over the input DNA. This indicates that KlSip4p is able to bind to both CSREs. The weaker

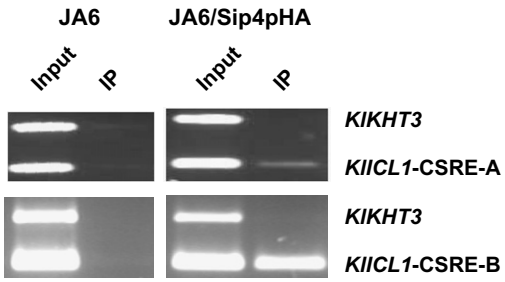

Fig. 2. ChIP data for binding of KlSip4p-HA to CSRE sequences. Cells were shifted for 5 h to ethanol medium prior to the isolation of cross-linked chromatin. PCR reactions were carried out with oligonucleotides listed in Table 2 specific for CSRE-A (CSRE-KlICL1AL and CSRE-KlICL1AR) and CSRE-B (CSRE-KlICL1BL and CSRE-KlICL1BR), and the *KlKHT3* promoter (KlKHT3-6-CW and KlKHT3-9-CW) as a negative control. Reactions were also performed on input DNA to confirm the function of the PCR reaction. Analyses were exerted as described in [39].

signal for CSRE-A correlates with the weak influence of deletion of this element in the wild-type promoter context. This suggests that CSRE-B has a higher affinity for KlSip4p than CSRE-A.

3.2. Glycerol regulates *KlICL1* expression through CSRE promoter sequences

The data presented above suggest that glycerol causes a reduction of *KlICL1* expression, even in the presence of ethanol. Therefore, specific isocitrate lyase activities were determined after overnight growth on glucose and transfer of the cells to synthetic medium with either ethanol or ethanol plus glycerol as carbon sources. As shown for the wild-type strain KB6-2C (Fig. 3A), an approximately 75% drop in activity was detected with glycerol plus ethanol, as compared to the ethanol control. Similar results were obtained in other genetic backgrounds, i.e. the effect is not strain specific, although considerable variations were observed for specific isocitrate lyase activities between different strains (data not shown). This was also observed when cultures were grown on rich media or when cells were pre-grown under inducing conditions (i.e. with ethanol as carbon source). The effect of glycerol is mediated by the promoter of *KlICL1*, as demonstrated by measurements of β-galactosidase activities in the *lacZ* fusion (pXW-KlICL1). As observed for isocitrate lyase, a reduction in β-galactosidase synthesis was caused by the presence of glycerol (Fig. 3A). These data were confirmed by the results of real-time RT-PCR experiments. As evident from Table 3, the relative levels of *KlICL1*-mRNA were 7–10-fold higher (after 6 h and 8h following the shift from glucose, respectively) in cells grown on ethanol alone as compared to those where glycerol was added. Addition of glucose to the ethanol medium had an even stronger effect, with a 250-fold decrease in mRNA abundance after 8 h. Consistent with published data, growth on either glucose or glycerol alone also led to a basal level expression [19,23,25].

We also followed the KlIcl1p activity after addition of glycerol to ethanol grown cells to investigate the dynamics of the down-regulation of *KlICL1* expression. Activities remained largely unaffected in the wild-type for the first 2 h (Fig. 3B). In this time interval, one would hardly detect changes in the level of gene expression, but rather the effect of proteolytic events. After this period, KlIcl1p activity significantly decreased, likely due to dilution during growth. This was also true for β-galactosidase activities in the respective reporter strain (Fig. 3B). Thus, glycerol most likely acts on *KlICL1* transcription rather than through inactivation of KlIcl1p, consistent with the previously reported glucose-effect [25].

To investigate whether the observed effect is due to a regulatory system exclusively operating in *K. lactis*, we tested the behaviour of the *S. cerevisiae* isocitrate lyase on synthetic medium. Although activities are lower in the cultures containing glycerol, they always reached more than 70% of those of the ethanol cultures (on rich media the glycerol effect was more pronounced, data not shown). This also holds true for the *K. lactis* enzyme tested in a *S. cerevisiae icl1* deletion (Fig. 3C). Vice versa, isocitrate lyase activities are lower on glycerol in a *K. lactis* strain which expresses *ScICL1* (Fig. 3C). This strongly indicates that glycerol acts distinctively on *K. lactis* metabolism.

To characterize the promoter elements mediating the glycerol regulation, a *lacZ* fusion of the *KlACS2* promoter (governing expression of a gene which encodes an acetyl-CoA synthase isozyme) was obtained. It confers β-galactosidase production on non-fermentable carbon sources which remains unaltered on glycerol (Fig. 3D) and is independent of Cat8p (i.e. activity levels decrease by a maximum of 10% in a *Klcat8* deletion strain; not shown). *KlICL1*-CSRE-B was introduced into this construct and again tested under different conditions. As shown in Fig. 3D, specific β-galactosidase activities in the hybrid construct carrying the CSRE-B element are 4–5-fold higher in ethanol but not in the presence of glycerol. The activity of both CSRE-A and CSRE-B was also affected by glycerol when tested individually with the pXW-39 reporter. These results clearly demonstrate that glycerol can regulate either CSRE-element.

We also investigated whether lactate has similar effects as glycerol on *KlICL1* expression. The results obtained strongly depended on the genetic background, since some of the wild-type strains are not able to grow on this carbon source. This is the case for KB6-2C, where lactate has no significant effect on isocitrate lyase synthesis (with activities varying between 82 and 75 mU/mg protein after 8 h of shift to ethanol/lactate medium). However, in the *lacZ* mutant strain (MW190-9B) we found that lactate interferes with isocitrate lyase expression to a similar extent as glycerol (i.e. a reduction of KlIcl1p activity from 178 to 25 mU/mg protein after 8 h).

3.3. Glycerol metabolism is required to exert regulatory effects

To investigate how glycerol interferes with *KlICL1* expression, different deletions which affect carbohydrate metabolism were employed. Both a *rag2* and a *tim1* deletion (lacking phosphoglucose isomerase and triosephosphate isomerase, respectively) still showed diminished expression on glycerol. Thus, specific isocitrate lyase activities where reduced by at least 60% after the addition of glycerol as compared to those of the ethanol grown cultures (i.e. for the 8 h samples from 246 to 66 mU/mg protein for *rag2* and from 41 to 12 mU/mg protein for *tim1*, on ethanol or glycerol plus ethanol, respectively).

To more directly target glycerol metabolism, *KlGUT1* and *KlGUT2* (which encode the glycerol kinase and the mitochondrial glycerol 3-phosphate dehydrogenase, respectively) were also deleted individually from the haploid *K. lactis* genome. As expected, neither strain retained the ability to grow on

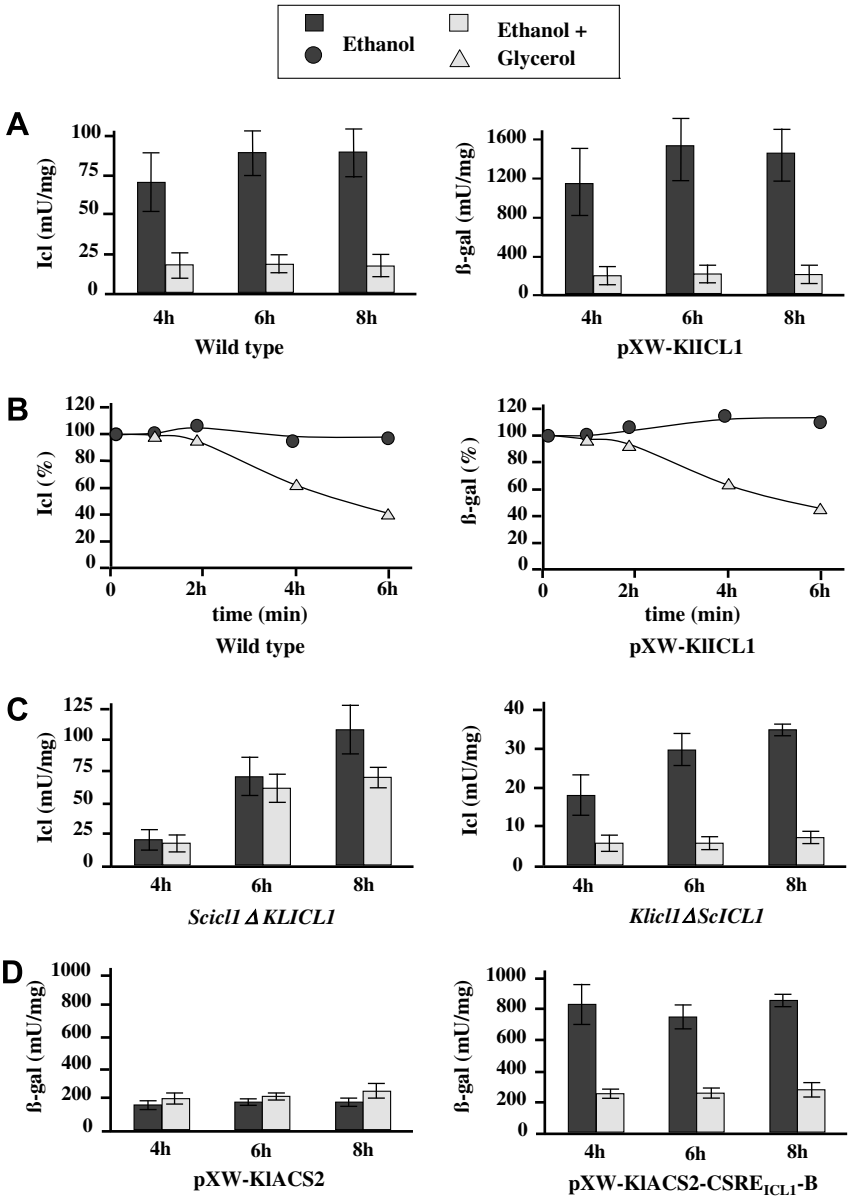

Fig. 3. Glycerol regulation of isocitrate lyase synthesis. A, C, and D represent assays to assess the effect of glycerol. Cells were grown overnight on SCD or SCDura (transformants) and then transferred to SCE to an OD_{600} of 0.5. The culture was divided and 3% glycerol was added to one aliquot. Samples were obtained at different times for enzymatic determinations. (A) Wild-type (KB6-2C); pXW-KlICL1 (MW190-9B (*Klura3 lac4-8*) transformed with pXW-KlICL1). (C) *Scicl1ΔKlICL1* (FMY401 (*Scicl1::LEU2*) carrying plasmid KlICL1YIp integrated at the *URA3* locus) and *Klicl1ΔScICL1* (Klicl1Δ13 transformed with ScICL1pCXJ). (D) pXW-KlACS2 and pXW-KlACS2-CSRE$_{ICL1}$-B (MW190-9B (*Klura3 lac4-8*) transformed with the respective plasmids). (B) Glycerol inactivation assays. Strains were pre-grown on SCD or SCDura (transformants). For derepression cells were transferred to YEPE medium and incubated overnight. Inactivation was started by addition of glycerol to a final concentration of 3% at time zero to one half of the culture. The remaining culture was further incubated on ethanol. Samples were taken at the times indicated. Wild-type (MW190-9B *ura3 lac4-8*); pXW-KlICL1 (MW190-9B carrying pXW-KlICL1).

glycerol as a sole carbon source (Fig. 4A). Glycerol causes a reduction in isocitrate lyase synthesis in the strain lacking the dehydrogenase (Fig. 4D), whereas expression was not affected in the kinase-less strain (Fig. 4C). Thus, glycerol must be at least initially phosphorylated to negatively regulate *KlICL1* expression.

Finally, we also tested whether glucose has any effect on *KlICL1* transcription in the *Klgut1* deletion mutant. Cells grown on medium containing glucose as a sole carbon source displayed only basal level isocitrate lyase activities (<2 mU/mg protein) in wild-type and the *Klgut1* deletion. Growth on ethanol plus glucose showed intermediate activities in wild-type and the *Klgut1* mutant (Fig. 4E and F). Thus, in this strain background, glucose leads to a 3-fold decrease in *KlICL1* expression in either strain. Therefore, the deregulation observed for the *Klgut1* deletion is specific for glycerol. However, it seems that KlGut1p has a negative regulatory influence on expression, since activities in the deletion strain are significantly higher than those of the corresponding wild-type. The molecular reason for this effect escapes our knowledge.

Table 3
Relative *KlICL1*-mRNA levels determined by real-time RT-PCR

Carbon source	Relative mRNA levels (%)	
	6 h	8 h
3% ethanol	100.00	100.00
4% glucose	0.40	0.78
3% ethanol + 4% glucose	n.d.	0.39
3% glycerol	0.42	0.44
3% ethanol + 3% glycerol	14.50	9.56

Relative expression levels were determined in comparison to the *KlACT1* gene expression as described in Section 2. n.d., not determined.

4. Discussion

Previous reports indicated that *KlICL1* transcription is subject to carbon catabolite repression similar to its counterpart in *S. cerevisiae* [25]. The promoter deletion analyses presented here identified two putative CSRE sequences which we propose to confer the transcriptional regulation. Several lines of evidence lead to this conclusion: (i) CSRE-A and CSRE-B show similarities to the consensus CSRE sequence from *S. cerevisiae* (Table 4). However, it should be noted that a functional CSRE in *K. lactis* requires 14 bp, whereas in *S. cerevisiae* 12 bp have been shown to suffice. Moreover, a G at position N_{10}, which is found in CSRE-B, abolishes activity in *S. cerevisiae* [32]. (ii) Each of the elements is sufficient to confer transcription on ethanol to promoters lacking activating sequences. Both the original deletion analyses and the insertion of each element into the basal promoter show a slightly stronger effect for CSRE-B. Yet, for the intact *KlICL1* promoter, the effects seem to be largely additive. (iii) The elements depend on a functional KlCat8p, since the respective deletion mutant lacks reporter gene expression on all carbon sources. This is reminiscent of *S. cerevisiae*, where Cat8p has been shown to act through CSRE sequences. (iv) Whereas direct binding of KlCat8p to the *KlICL1* regulatory elements (CSRE-A and CSRE-B) could not be detected, ChIP showed

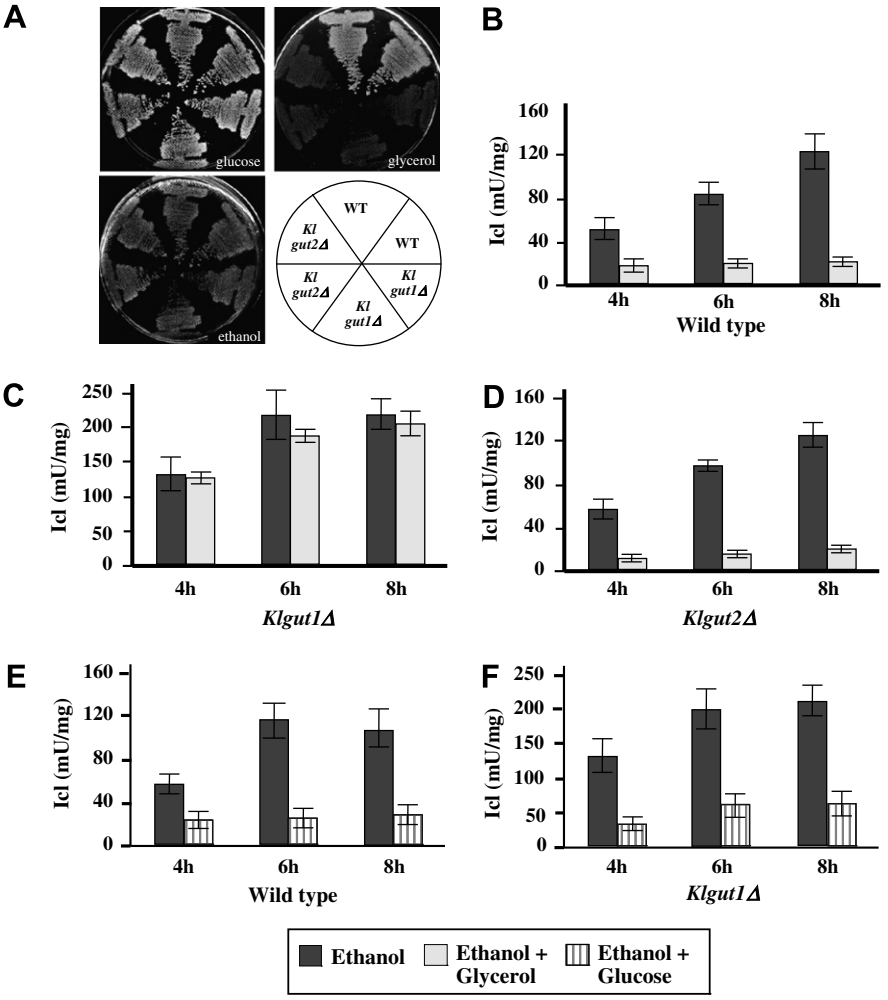

Fig. 4. Glycerol effect in ***Klgut1*Δ** and *Klgut2*Δ mutant strains. (A) Growth behaviour of the null mutants as compared to the wild-type strain. Strain MW270-7B (WT) and the isogenic deletion mutants *Klgut1::KanMX* (*Klgut1*Δ) and *Klgut2::KanMX* (*Klgut2*Δ) were grown on YEPD and then replica-plated onto SCD (glucose), SCE (ethanol) and SCG (glycerol) and incubated for 48 h at 28 °C. (B, C, D) Regulation by glycerol. Crude extracts for enzymatic determination were prepared from cells grown as described in the legend of Fig. 3A, (C, D) Wild-type (MW270-7B); *Klgut1*Δ (*Klgut1::KanMX*) and *Klgut2*Δ (*Klgut2::KanMX*). (E, F) Regulation by glucose. Cells were grown overnight on SCD and then inoculated in SCE with an OD_{600} of 0.5. The cultures were each divided into two and 4% glucose was added to one set. Samples were obtained at the indicated times for enzymatic determinations. Wild-type (MW270-7B) and *Klgut1*Δ *(Klgut1::kanMX)*.

Table 4
Alignment of known and putative CSRE sequences from *K. lactis*

Gene (promotor)[a]	Sequence (5′N_0 to N_{13}3′)[b]
KlICL1$_{CSRE-A(-811/-824)}$	5′C GCC ATT AAA CCG A3′
KlICL1$_{CSRE-B(-729/-742)}$	5′C CCC ATT CAT GCG A3′
KlMLS1$_{(-726/-713)}$	5′T CCC ATT TGT GCG A3′
KlACS2$_{(-1020/-1007)}$[c]	5′A CCC CTC TAT CGG T3′
KlSIP4$_{(-467/-454)}$	5′T CCC TTT CAG CCG G3′
KlSIP4$_{(-609/-596)}$	5′A GCC TTT CAT CCG G3′
KlSFC1$_{(-1052-1039)}$	5′T GCC ATT GAC CCG G3′
LAC4$_{(-1123/-1110)}$ [3]	5′C CCC TTT CAT CCG A3′
***Kl*-consensus**[c]	**5′H SCC WTT NRN SCG R3′**
Sc-consensus [32]	**5′N YCC NYT NRK CCG N3**

[a]Numbers refer to the translation start codon (ATG), with the A counting as +1.
[b]Ambiguity code: H = C, A or T; Y = C or T; S = G or C; R = A or G; W = A or T; K = G or T.
[c]The putative *KlACS2* element has been omitted for generating the consensus.

KlSip4p-HA to be mainly associated with CSRE-B and, to a lower extent, with CSRE-.A. In line with this finding, *KlICL1* activation was shown to depend on KlSip4p in addition to KlCat8p (J.J. Krijger, L. Schild and K.D. Breunig, unpublished results). Interestingly, a putative CSRE sequence is also found in the promoter of *KlMLS1*, which encodes malate synthase, another key enzyme of the glyoxylate cycle (Table 4). This element is more similar to CSRE-B than to CSRE-A and the consensus from *S. cerevisiae* and it is likely to govern *KlMLS1* transcription in a similar manner. Two putative CSREs are also found in the *KlSIP4* promoter. Taken together, our data indicate that KlCat8p induces the expression of *KlSIP4*, which in turn activates *KlICL1* and *KlMLS1* transcription by binding to the respective CSRE sequences.

For *K. lactis*, transcription of seven genes has been shown to depend on KlCat8p, so far [19–22]. In contrast to *KlMLS1*, the promoters of all the other genes lack a consensus CSRE, which suggests that the influence of KlCat8p may be indirect. This hypothesis is supported by the fact that *KlACS1* and *KlJEN1* are expressed on lactate [20,21], a carbon source, which, as demonstrated here, does not activate the *KlICL1*-CSREs. The *KlACS2* promoter used in our *KlACS2-lacZ* fusion is expressed on ethanol and glycerol independently of KlCat8p. Thus, sequences which confer dependence on KlCat8p must be located upstream of position −732 nt contained in this construct. A sequence close to the consensus for *K. lactis* CSREs is located at position −1020 nt in the *KlACS2* promoter (Table 4). Homologs of most of the other *S. cerevisiae* genes, which are regulated by CSREs in their promoters, also lack such sequences in *K. lactis*.

In *K. lactis*, it has been shown that the degree of glucose repression is highly strain dependent [3]. This is also true for the strains used here, where in the KB6-2C background a complete loss of *KlICL1* expression is observed on glucose, while in MW270-7B, only a 3-fold decrease is detected. The heterogeneity of *K. lactis* strains most likely is also responsible for the observed variations in specific isocitrate lyase activities in wild-type strains, *rag2* and *tim1* mutants.

K. lactis seems to prefer the utilization of three carbon compounds over ethanol, as indicated by the down-regulatory effect of glycerol. This is a clear difference to *S. cerevisiae*, where such a distinction between non-fermentable carbon sources does not seem to occur. As shown here, and similar to the glucose effect, glycerol acts through transcriptional regulation of *KlICL1*, rather than through catabolite inactivation. Although we observed an effect of glycerol on ethanol-grown cells also in *S. cerevisiae*, which confirms previous observations [33], it is much more pronounced in *K. lactis*. This effect is species- rather than gene-specific. Thus, *KlICL1* expression is only marginally reduced by glycerol in *S. cerevisiae*, whereas *ScICL1* expression is strongly diminished by the addition of this compound in *K. lactis*. First hints as to how glycerol exerts this effect are provided by the mutant analyses presented here. The lack of this effect in a *Klgut1*-, but not a *Klgut2*-deletion strain, demonstrates that glycerol has to enter the cell and to get activated by phosphorylation prior to action. For lactate, a similar regulation requires the ability of the strain to grow on this carbon source. This is reminiscent of xylose in *S. cerevisiae*. There, too, metabolism of the five carbon compound is essential to exert catabolite repression [33]. Interestingly, in contrast to *S. cerevisiae*, a strain lacking the succinate dehydrogenase gene *KlSDH1* in *K. lactis* displays high isocitrate lyase and malate synthase activities on glucose and also on lactate [34]. Taken together, these data suggest that intracellular metabolites are responsible for keeping isocitrate lyase gene expression low on glucose, glycerol and lactate. Mutations in genes such as *KlGUT1* or *KlSDH1* would then cause a metabolic imbalance and result in a lack of down-regulation.

Since in the context of the *KlACS2-lacZ* reporter construct the glycerol control is mediated through the introduced *KlICL1*-CSRE-B, one can assume that signalling occurs through a pathway which connects with glucose repression at or downstream of the Snf1p kinase. This view is supported by the fact that glycerol metabolism also requires an active Snf1p complex [24]. Since *KlCAT8* is constitutively expressed ([23], and our unpublished results) and *KlSIP4* transcription seems to be controlled by KlCat8p, the glycerol effect could be exerted by regulating directly the activity KlCat8p. This view is consistent with published data showing that KlCat8p is less active in glycerol- than in ethanol-grown cells and that a single point mutation in *KlCAT8* results in a protein (KlCat8pS661E) which allows activation on glycerol [23].

What could be the trigger of glycerol regulation? In *S. cerevisiae*, a new signalling pathway which is independent of Snf1p, has been shown to control the glucose-regulated localization of the β-subunit Gal83 [35]. It was shown that phosphorylation of glucose is necessary and sufficient to produce this effect. Our data indicate that regulation by glycerol also only depends on its phosphorylation. Thus, a Snf1p independent mechanism which regulates KlCat8p or KlSip4p, could be in operation. Further work to investigate this hypothesis is currently in progress.

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