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Differences in regulation of yeast gluconeogenesis revealed by Cat8p-independent activation of *PCK1* and *FBP1* genes in *Kluyveromyces lactis*

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Abstract The yeast *Kluyveromyces lactis* is can utilise a wide range of non-fermentable carbon compounds as sole sources of carbon and energy, and differs from *Saccharomyces cerevisiae* in being able to carry out oxidative and fermentative metabolism simultaneously. In *S. cerevisiae*, growth on all non-fermentable carbon sources requires Cat8p, a transcriptional activator that controls the expression of gluconeogenic and glyoxylate cycle genes via CSREs (Carbon Source Responsive Elements). The down-regulation of Cat8p by fermentable carbon sources is the primary factor responsible for the tight repression of gluconeogenesis by glucose in *S. cerevisiae*. To analyse the regulation of gluconeogenesis in *K. lactis*, we have cloned and characterised the *K. lactis* homologue of *CAT8* (*KlCAT8*). The gene was isolated by multicopy suppression of a *fog2/klsnf1* mutation, indicating a similar epistatic relationship between *KlSNF1* and *KlCAT8* as in the case of the *S. cerevisiae* homologues. *KlCAT8* encodes a protein of 1445 amino acids that is 40% identical to ScCat8p. The most highly conserved block is the putative $Zn(II)_2Cys_6$ DNA-binding domain, but additional conserved regions shared with members of the zinc-cluster family from *Aspergillus* define a subfamily of Cat8p-related proteins. *KlCAT8* complements the growth defect of a *Sccat8* mutant on non-fermentable carbon sources. In *K. lactis*, deletion of *KlCAT8* severely impairs growth on ethanol, acetate and lactate, but not on glycerol. Derepression of enzymes of the glyoxylate cycle – malate synthase and particularly isocitrate lyase – was impaired in a *Klcat8* mutant, whereas Northern analysis revealed that derepression of *KlFBP1* and *KlPCK1* does not require KlCat8p. Taken together, our results indicate that in *K. lactis* gluconeogenesis is not co-regulated with the glyoxylate cycle, and only the latter is controlled by KlCat8p.

Key words *Kluyveromyces lactis* · Carbon source regulation · *CAT8*

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Introduction

All organisms have evolved elaborate regulatory mechanisms for strict control of the opposing activities of glycolysis and gluconeogenesis, in order to prevent the simultaneous degradation and biosynthesis of carbohydrates. Depending on the level and the nature of the available carbon source(s), either glycolysis or gluconeogenesis is favoured. The key enzymes at which this control is exerted are the same from bacteria to man, and their allosteric regulation has been studied at great detail. Regulation of carbon metabolism also operates at the transcriptional level.

The molecular details of the transcriptional control of carbon metabolism have been studied most extensively in the yeast *Saccharomyces cerevisiae* (see Gancedo 1998 for a review). Recently, the Cat8 protein has been identified as a key transcription factor that regulates gluconeogenesis (Hedges et al. 1995). *cat8* mutants are unable to utilise non-fermentable carbon sources because a number of genes encoding enzymes of gluconeogenesis and the glyoxylate cycle are not activated. Cat8p belongs to a family of DNA-binding factors with a conserved $Zn(II)_2Cys_6$ DNA-binding motif (zinc cluster). Co-ordinate transcriptional control by Cat8p is mediated by *cis*-acting regulatory sequences called CSREs (consensus: 5′-CGGNBNVMHGGM-3′; Bojunga and Entian 1999), and CSRE-driven reporter genes are not activated in a *cat8* mutant (Schöler and

Schüller 1994; Vincent and Gancedo 1995). CSREs have been identified in the promoter regions of *FBP1*, encoding fructose-1,6-bisphosphatase (Mercado and Gancedo 1992; Vincent and Gancedo 1995; de Mesquita et al. 1998), *PCK1* (phosphoenolpyruvate carboxykinase; Mercado and Gancedo 1992; Proft et al. 1995), *MLS1* (malate synthase; Caspary et al. 1997), *ICL1* (isocitrate lyase; Schöler and Schüller 1994; Kratzer and Schüller 1995), *ACS1* (acetyl-CoA synthase; Kratzer and Schüller 1995), *ACR1* (succinate-fumarate transporter; Bojunga et al. 1998), *IDP2* and *JEN1* (encoding isocitrate dehydrogenase and lactate permease, respectively; Bojunga and Entian 1999).

Cat8p has been shown to bind to the CSRE in vitro (Rahner et al. 1999). In addition, a second CSRE-binding factor, Sip4p, has been identified, the expression of which is under the control of Cat8p (Vincent and Carlson 1998). Thus, whether or not Cat8p binds directly to CSREs in vivo is not yet settled.

A major player in the signal transduction pathway leading to the activation of gluconeogenic genes is the product of the *SNF1* gene, an evolutionarily conserved serine/threonine protein kinase related to the mammalian AMP-activated kinase (AMPK; Woods et al. 1994). *snf1* mutants are unable to utilise non-fermentable carbon sources because (1) they are unable to derepress glucose-repressed genes, one of which is *FBP1*; and (2) they are unable to activate the genes for gluconeogenic and glyoxylate cycle enzymes, which are under the transcriptional control of *CAT8*. Derepression requires the Snf1p function to inactivate the Mig1 protein, a DNA-binding protein that targets the general repressors Ssn6p and Tup1p to glucose-repressible genes (Treitel and Carlson 1995). Inactivation of Mig1p is achieved by its exclusion from the nucleus through Snf1p-dependent phosphorylation (De Vit et al. 1997).

The link between Snf1p and Cat8p is complex and is not completely understood. Cat8p-activated gene expression correlates with phosphorylation of Cat8p, which is partially dependent on Snf1p (Rahner et al. 1996; Randez-Gil et al. 1997). In addition, the expression of the *CAT8* gene is itself repressed by glucose via Mig1p, which, as mentioned above, is controlled by Snf1p. Thus, it appears that Cat8p activation requires release from glucose repression as well as post-translational modification.

To gain further insight into the evolutionarily conserved aspects of gluconeogenic control, we have analysed a related yeast, *Kluyveromyces lactis*. The regulation of carbon metabolism in this yeast species reflects its more pronounced respiratory metabolism compared to *S. cerevisiae*. On non-fermentable carbon sources *K. lactis* can grow at rates similar to those attainable on media containing high levels of glucose, and genes encoding components of the respiratory chain are not subject to glucose repression in *K. lactis*, although the phenomenon of glucose repression also exists in this yeast (De Deken 1966; Ferrero et al. 1978; Breunig 1989; Lodi et al. 1994; Goffrini et al. 1995; Mulder et al. 1995; Weirich et al. 1997). Some regulators of the glucose repression/derepression pathway have been cloned from *K. lactis* and shown to complement the corresponding *S. cerevisiae* mutations (Wésolowski-Louvel et al. 1992; Cassart et al. 1995; Goffrini et al. 1995, 1996), indicating that differences in repression are not due to functional divergence of central regulatory proteins. Rather it seems that these regulators do not target the same genes. For example, glucose repression of *K. lactis* invertase does not require KlMig1p (Georis et al. 1999) and glucose repression of lactose and galactose metabolism is only weakly affected by a *mig1* mutation (Dong and Dickson 1997).

As in *S. cerevisiae*, the Snf1p protein kinase is a major regulator of gluconeogenic growth in *K. lactis*. The *KlSNF1* gene has been isolated on the basis of its ability to restore growth of the *fog2* mutant on non-fermentable carbon sources (Goffrini et al. 1996). Here we report the isolation of the *KlCAT8* gene as a high-copy-number suppressor of the *fog2* mutation. The analysis of a *Klcat8* mutant reveals that regulation of key gluconeogenic genes, like *KlFBP1* and *KlPCK1*, is independent of Cat8p in *K. lactis*, and growth on glycerol is not affected, indicating that the role of Cat8p as a general regulator of gluconeogenesis is not conserved. However, KlCat8p is required for efficient utilisation of C_2 carbon sources – like the FacB proteins of *Aspergillus*, which belong to the same family of Cat8-related proteins.

Materials and methods

Strains, media and yeast techniques

Escherichia coli strain DH10B (Gibco-BRL) was used for all cloning procedures and grown on standard LB medium. The *S. cerevisiae* strains CEN.NB1-1A (*MAT***a**, *ura3*, *his3*, *leu2*, *trp1*, *cat8::LEU2*) and CEN.PK2-1C (*MAT***a**, *ura3*, *his3*, *leu2*, *trp1*) are congenic and have been described previously (Randez-Gil et al. 1997). The *K. lactis* strain yIG8 is congenic to JA6 (Breunig and Kuger 1987) and was obtained by two-step gene replacement. The first step (integration of the disrupted gene) was carried out by transformation of JA6 with pGICAT8-2.1 (see below) linearised by digestion with *Eco*RI (which cleaves within the *KlCAT8* ORF, 3′ to the deleted region). 40 Ura^+ transformants were subjected to PCR amplification in order to distinguish locus-specific from ectopic integration events. One of the clones yielded the PCR signal expected in the case of homologous integration, and this was verified by Southern analysis. Eviction of the integrated plasmid sequences was achieved by selecting for the loss of the vector's *ScURA3* marker on minimal medium supplemented with 1 mg/ml 5-fluoroorotic acid (FOA) and 50 mg/l uracil (Boeke et al. 1987). Among 40 FOA-resistant clones, the two expected recombination events between repeated sequences, 5′ or 3′ to the deleted region, were distinguished by PCR amplification. yIG8 contained the *klcat8*Δ locus as verified by Southern analysis. The *K. lactis* strain JA6/207 is a *fog2* (*snf1*) derivative of JA6 (Goffrini et al. 1996). The *K. lactis* strain JSD1 is congenic to JA6 and carries a deletion/disruption of *KlSNF1* (Dong and Dickson 1997). A Ura^- derivative, JSD1R4, was isolated in a screen for FOA-resistant clones.

Multicopy suppressors of the $EtOH^-$ phenotype of JA6/207 were isolated by transformation with a KEp6-based genomic library (Wésolowski-Louvel et al. 1988) and replica-plating Ura^+ transformants onto solid YP (1% Bacto-yeast extract and 2% Bacto-peptone) medium containing 2% ethanol. Phenotypic

characterization of *K. lactis* yIG8 and heterologous complementation were carried out on solid minimal medium (MM: 0.67% Difco yeast nitrogen base without amino acids supplemented with the required amino acids and bases) containing 2% glucose (w/v) or 3% (v/v) glycerol or ethanol. The same liquid minimal medium was used for construction of growth curves, RNA isolation and enzyme activity measurements.

Transformation of *S. cerevisiae* was performed according to Gietz et al. (1995). Transformation of *K. lactis* was done following the same protocol, but the duration of heat shock was reduced to 10 min. Total RNA was isolated from *K. lactis* by the hot acidic phenol technique, as described in Ausubel et al. (1994).

Sequence analysis algorithms

Database searches were carried out with BlastP (Altschul et al. 1997) using the Blosum62 matrix and the default parameters. ProDom (Corpet et al. 1998) analysis of the KlCat8p sequence was performed by homology searches in the ProDom 98 protein domain database with BlastP.

Plasmids

The plasmids pMSN1 and pGID1 were isolated as multicopy suppressors of JA6/207 and harbour *KlSNF1* and *KlCAT8*, respectively.

The 233-bp segment of *ScCAT8* encoding the zinc-cluster, DNA-binding, domain of the protein was subcloned into the pGEMt vector (Promega) after PCR amplification with the primer pair pCAT8up (5′-CAAGCTTGTGACAGGTGT-3′) and pCAT8 down (5′-GAAACAAGGCTTATTTGC-3′) to give plasmid p230.

The plasmid pGICAT8-5 used for testing the heterologous complementation ability of *KlCAT8* in *S. cerevisiae* was constructed by inserting a 5.8-kb *Sal*I-*Stu*I fragment of pGID1 (containing *KlCAT8* and flanking regions) into the multiple cloning site of the pRS313 vector (Sikorski and Hieter 1981). The pGICAT8-2.1 plasmid used for deletion of *KlCAT8* consists of 5′ end and 3′ end fragments of *KlCAT8* [*Sal*I-*Nco*I (–745 to +48) and *Nco*I-*Hin*dIII (+3405 to +5140)] cloned into pRS306 (Sikorski and Hieter 1981) after removal of the *Eco*RI site present in the multiple cloning site of the vector.

The construction of pGICL1, harbouring *KlACT1*, has been described previously (Georis et al. 1999). Plasmid BS-PEPCK is a derivative of pBluescript containing *KlPCK1* (Kitamoto et al. 1998).

General DNA and RNA techniques

Standard DNA manipulations were performed as described in Ausubel et al. (1994). The dot-blot screen for multicopy suppressors was performed by spotting five different dilutions (1 pg–10 ng) of the candidate plasmid DNAs onto a nylon membrane (Macherey-Nagel) and hybridizing with the ^{32}P-labelled *Hin*dIII-*Pst*I insert from p230 as described (Georis et al. 1999). The final washing buffer was 2 × SSC/0.1% SDS. Sequencing was performed with the ThermoSequenase primer cycle sequencing kit (Amersham) using synthetic oligonucleotides and ^{33}P-labelled dideoxynucleotides. The procedure for Northern blot analysis has been described previously (Georis et al. 1999). The ^{32}P-labelled *KlACT1* (a 1.1-kb *Eco*RI fragment of pGICL1), *KlFBP1* [a 1.1-kb fragment of *KlFBP1* obtained by PCR amplification of *K. lactis* genomic DNA with the primers pKlFBP1F (5′-CCGAAGCTTCGCATATCCCG-3′) and pKLFBP1R (5′-GATGGGAGGTTCGTATACG-3′)] and *KlPCK1* (a 2-kb *Bgl*II fragment of BS-PEPCK) were obtained using the Random Prime labelling kit (Gibco-BRL).

Enzyme activity measurements

Crude extracts were prepared with glass beads in 40 mM TRIS-HCl pH 7, 5 mM $MgCl_2$, and the protein concentration was determined based on the absorbance at 280 nm using BSA as standard. Isocitrate lyase and malate synthase activities were determined using the protocol described by Dixon and Kornberg (1959).

Results

Isolation of *KlCAT8* as a multicopy suppressor of the *Klfog2* (*Klsnf1*) mutation

Our initial attempts to clone the *CAT8* gene of *K. lactis* by heterologous complementation of a *Sccat8::LEU2* strain (CEN.NB1-1A) were unsuccessful, possibly because the average insert size of the *K. lactis* genomic library in the *S. cerevisiae* vector was too small (*KlCAT8* is more than 4 kb long, see below). We therefore screened another library known to contain large inserts, which was based on the *K. lactis* vector KEp6 (Wésolowski-Louvel et al. 1988). Since no *K. lactis cat8* mutant was available, the strategy used to screen this library was inspired by the observation that, in *S. cerevisiae*, multicopy expression of *ScCAT8* restores growth of *snf1* mutants on ethanol (Hedges et al. 1995). The *K. lactis* strain JA6/207 (Goffrini et al. 1996), which carries a *fog2* (*snf1*) mutation that renders it deficient for growth on non-fermentable carbon sources, was transformed with the library and 10^5 Ura$^+$ transformants were screened by replica-plating onto YP medium containing ethanol as the sole carbon source. 22 clones that grew on ethanol were isolated; two of these were revertants and nine others were shown to carry a plasmid harbouring *KlSNF1* (Genbank Accession No. X87975). The remaining 11 clones were screened by hybridization with the sequence encoding the zinc-cluster region from the ScCat8p DNA-binding domain. This region is highly conserved in the zinc cluster protein family (Todd and Andrianopoulos 1997) and was expected to be similar in KlCat8p. One of the 11 clones, pGID1, hybridised to the probe (data not shown). The remaining 10 clones are currently under analysis.

The suppression of the *fog2* mutant strain by the pGID1 plasmid is shown in Fig. 1. Growth on ethanol and glycerol was partly restored by pGID1.

The nucleotide sequence of the 5.8-kb insert in the pGID1 clone revealed a single 4335-bp ORF (Genbank Accession No. AF070974).

KlCat8p is a member of the Gal4p family and is very closely related to ScCat8p

A BlastP search performed with the predicted amino acid sequence of KlCat8p retrieved six highly significant matches; these are, in descending order of homology: the *S. cerevisiae* and *Candida albicans* Cat8 proteins, ACU15 from *Neurospora crassa*, the FacB proteins from *Emericella nidulans*, *Aspergillus oryzae* and *A. niger*. The KlCat8p protein is about 40% identical to ScCat8p and has approximately the same molecular weight

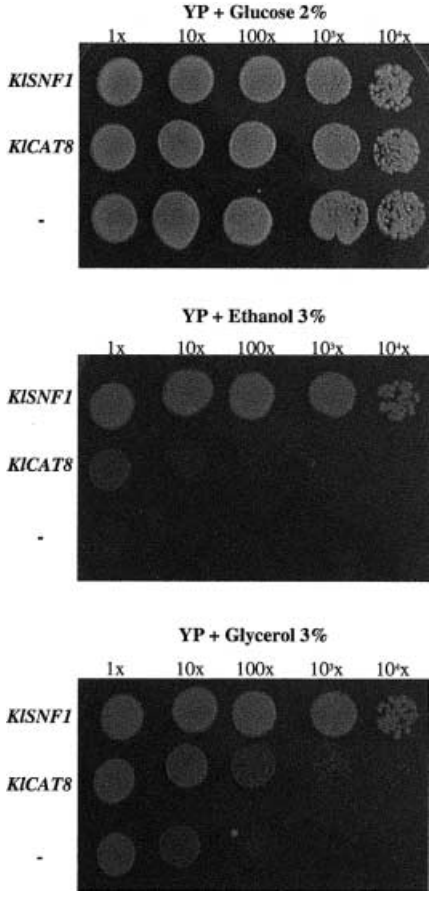

Fig. 1 Gluconeogenic growth phenotype of the suppressed JA6/207 strain. The JA6/207 *K. lactis* strain was transformed with three different plasmids [KEp6 as a negative control (–), pMSN1 as the positive control (*KlSNF1*) and pGID1 as the test plasmid (*KlCAT8*)], and spotted in five different dilutions onto YP medium containing 2% glucose, 3% ethanol or glycerol as the carbon source. The plates were incubated at 30 °C for 2 (glucose) or 4 days (ethanol or glycerol)

(160 kDa). The CaCat8p protein is smaller (120 kDa) and shows only 25% identity to the other two yeast Cat8p homologues. Based on this sequence homology and on the complementation of a *Sccat8* mutant (see below), we concluded that we had indeed isolated the *K. lactis* orthologue of *CAT8*.

Four large regions are conserved between the three yeast Cat8p sequences. The first (residues 176–258 in the KlCat8p sequence) contains the zinc-cluster, DNA-binding, domain plus a "coiled-coil" dimerisation domain. The zinc-cluster domain can be found in about 100 proteins in the database, all from fungi and belonging to the zinc-cluster protein family. The coiled-coil dimerisation region located immediately C-terminal to the zinc cluster, though structurally conserved, exhibits only weak similarity at the primary sequence level within the zinc-cluster protein family. However, this region is highly conserved between KlCat8p and six other proteins (Fig. 2a). These six proteins correspond to the six best matches retrieved by BlastP, and define a new subfamily of Cat8p-related proteins within the Gal4p family (Poch 1997).

Another previously described conserved region comprises amino acids 565–801 of the KlCat8p sequence. It overlaps with the known "Middle Homology Region", which is thought to be a regulatory region that is specific for the proteins of the $Zn(II)_2Cys_6$ family (Schjerling and Holmberg 1996; Poch 1997). Seven of the eight conserved motifs described by Poch (1997) are included in this region; 47 out of 53 residues of the KlCat8p sequence fit the consensus established for the eight motifs. Within this region, a subdomain previously identified by Chasman and Kornberg (1990) was detected by the ProDom algorithm (squared boxes; Fig. 2b). This subdomain has an approximate length of 70 amino acids and is conserved in 52 other proteins, all belonging to the zinc-cluster family, except for two predicted *S. pombe* proteins of unknown function (Fig. 2b). This region overlaps with the most conserved 5th and 6th motifs described by Poch (1997).

Besides these previously identified regions, two additional motifs are conserved only between the yeast Cat8p sequences. In one (amino acids 403–450 in the

Fig. 2a–d Sequence analysis of *KlCAT8* (Genbank Accession No. AF070974). **a** Multiple alignment of the coiled-coil region of seven Cat8p-related proteins (NcAcu-15, ACU15 of *N. crassa*; EnFacB, AoFacB and AnFacB, FacB proteins of *E. nidulans*, *A. oryzae* and *niger*, respectively). The position of the last residue of each sequence in the corresponding complete sequence is indicated by the number to the *right* of the sequence. The *inverted commas* and *asterisks above* the aligned sequences indicate residues of the linker region and the coiled coil, respectively. Residues indicated in *underlined italics* are conserved in all six sequences. The leucine residues indicated in *bold italics* define the heptad repeats. In the consensus, + indicates positively charged residues (R, K, H), – negatively charged residues (D, E, Q), "h" bulky aliphatic or aromatic residues (I, L, M, V, F, Y, W) and "s" small residues (P, A, G, S, T). **b** Conservation of the Chasman and Kornberg box in 52 zinc-cluster proteins. The *hatched boxes* at the N-terminal ends of the sequences correspond to the zinc-cluster region, the *squared boxes* represent the Chasman and Kornberg box. Other boxes are also outlined but are not discussed here. **c** Detailed view of the regions conserved only in the yeast Cat8p homologues. **d** Conservation of two small motifs between Kl, Ca and ScCat8p. The position of each sequence in the corresponding complete sequence is indicated by the numbers on each side of the sequences. Residues that are conserved in at least two of the three sequences are *shaded*

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                .............**********************************
KlCat8p         KISDKLSRRAFPRGYTETLEERVRELEAENRRLVALCDLKEEQLH  255
ScCat8p         RISDKLLRKAYPKGYTESLEERVRELEAENKRLLALCDIKEQQIS  142
CaCat8p         IVSDRLTRKSYPKAYTETLEERVRQLEAENKKLAGLLDMRDEQLE  125
NcAcu-15        KTSDKLSRRAFPRGYTESLEERVRALEAEIRELKDLLDEKDEKLD   96
AnuFacB         KTSDKLSRRALPRGYTESLEERVRALESEVRDLKNLLDEKDEKID   96
AouFacB         KTSDKLSRRAFPRGYTESLEERVRALEAEVRDLKNLLDEKDEKID   96
EnuFacB         KTSDKLSRRAFPRGYTESLEERVRTLEAEVRELKSLLDEKDEKID   96
                + SD+L R+shP+sYTEsLEERVR LEsE + L  L D +-- h  Consensus
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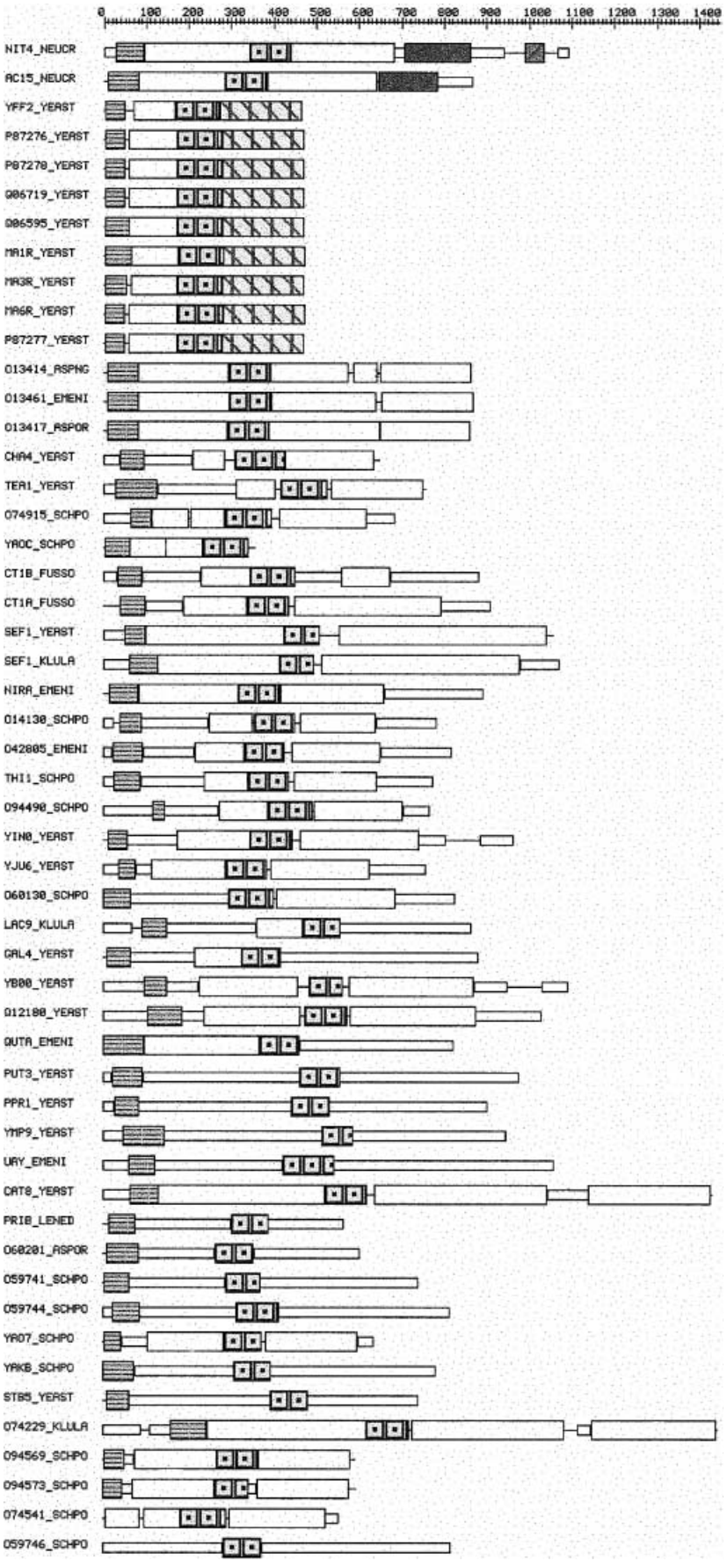
Fig. 2b


0
100
200
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1100
1200
1300
1400
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TEA1_YEAST
O74915_SCHPO
YAOC_SCHPO
CT1B_FUSSO
CT1A_FUSSO
SEF1_YEAST
SEF1_KLULA
NIRA_EMENI
O14130_SCHPO
O42805_EMENI
THI1_SCHPO
O94490_SCHPO
YIN0_YEAST
YJU6_YEAST
O60130_SCHPO
LAC9_KLULA
GAL4_YEAST
YB00_YEAST
Q12180_YEAST
QUTA_EMENI
PUT3_YEAST
PPR1_YEAST
YMP9_YEAST
UAY_EMENI
CAT8_YEAST
PRIB_LENED
O60201_ASPOR
O59741_SCHPO
O59744_SCHPO
YA07_SCHPO
YAK8_SCHPO
STB5_YEAST
O74229_KLULA
O94569_SCHPO
O94573_SCHPO
O74541_SCHPO
O59746_SCHPO

Fig. 2c

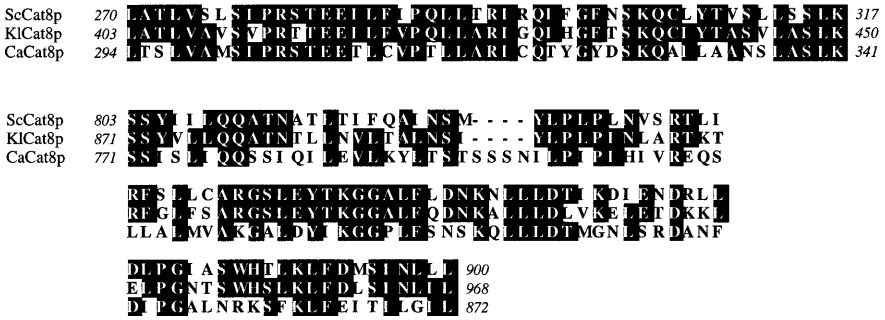
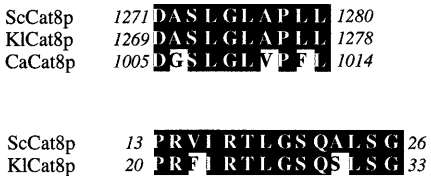


Fig. 2d



KlCat8p sequence; Fig. 2c) 50% of the residues are conserved in all three proteins, while the other (amino acids 871–968 in the KlCat8p sequence; Fig. 2c) shows 33% conservation.

Two additional, shorter sequence motifs were detected by BlastP. One, 14 amino acids in length, is located near the N-terminus of KlCat8p and ScCat8p but not CaCat8p, preceding an insertion of about 100 amino acids in KlCat8p (Fig. 2d). The other (10 amino acids) is found in the less conserved C-terminal part of the proteins, around position 1275 in the Kl and ScCat8p sequences and 1000 in CaCat8p (Fig. 2d). An Asn-rich region ($N_7TN_2TN_4$) was identified around amino acid 1110 in the KlCat8p sequence that has no equivalent in ScCat8p and CaCat8p, though the latter also contain high levels of Asn residues dispersed throughout the corresponding zone. Such Asn-rich sequences can be found in a large number of proteins, including yeast DNA-binding proteins (Met4p), but no function has yet been assigned to them.

KlCat8p is able functionally to substitute for ScCat8p in *S. cerevisiae*

Since the *KlCAT8* gene had not been cloned directly by heterologous complementation in *S. cerevisiae*, we tested the ability of the cloned *KlCAT8* gene to complement the growth defect of a *Sccat8* mutant. The entire *KlCAT8* ORF, together with 730 bp and 420 bp of 5′ and 3′ flanking regions, respectively, was cloned into a *S. cerevisiae* centromeric vector and introduced into the *cat8* deletion strain CEN.NB1-1A. His^+ transformants were selected on MM + glucose plates and subsequently tested for growth on minimal medium containing ethanol or glycerol as carbon sources.

The results presented in Fig. 3 clearly show that *KlCAT8*, under the control of its own promoter and carried on a centromeric vector, is able fully to restore the growth of a *cat8*Δ strain of *S. cerevisiae* on ethanol and glycerol, and can therefore be considered a functional homologue of *ScCAT8*.

In this heterologous complementation experiment, we also noticed that growth of the *ScCAT8* parent strain CEN.PK2-1C on glucose was weakly affected by transformation with a *S. cerevisiae* multicopy vector carrying *KlCAT8* (data not shown).

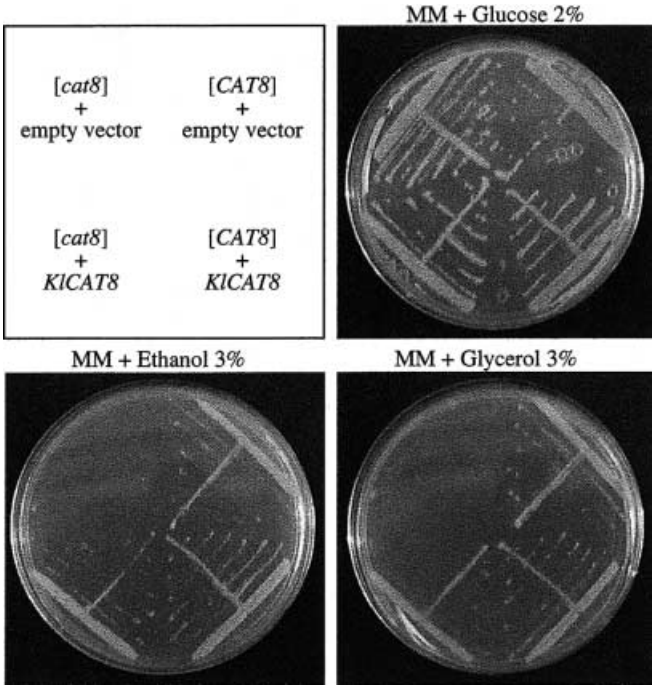


Fig. 3 Results of the heterologous complementation analysis. The *S. cerevisiae* strains CEN.PK2-1C (the positive control [*CAT8*]) and CEN.NB1-1A (the test *cat8*Δ strain [*cat8*]) were transformed with pRS313 (the empty vector, used as a negative control) or pGICAT8-5 (the test plasmid carrying *KlCAT8*), streaked onto selective minimal medium (lacking histidine) containing 2% glucose, 3% ethanol or glycerol as the carbon source, and incubated at 30 °C for 2 (glucose) or 4 days (ethanol or glycerol)

Disruption of *KlCAT8* impairs growth on ethanol, acetate and lactate, but not on glycerol

In order to investigate the functions of KlCat8p in *K. lactis*, we deleted the *KlCAT8* locus in JA6. In the resulting strain, yIG8, more than 75% of the *KlCAT8* ORF was removed, including the regions encoding the zinc cluster, the coiled coil and the "Middle Homology Region" (Fig. 4a).

The phenotype of yIG8 was characterised on plates and in liquid cultures containing various non-fermentable carbon sources (Fig. 4b and c). We observed that growth of yIG8 was reduced on ethanol, lactate and acetate, indicating that KlCat8p function is required for efficient utilisation of these carbon sources. However, in contrast to the corresponding *S. cerevisiae* mutant (Hedges et al. 1995), the *K. lactis cat8* mutant grew on glycerol like the wild-type strain.

It should be noted that, as reported previously (Goffrini et al. 1996; Dong and Dickson 1997), a *Klsnf1*Δ strain (JSD1R4; Fig. 4b) is not able to grow on glycerol, indicating that the influence of the Snf1p

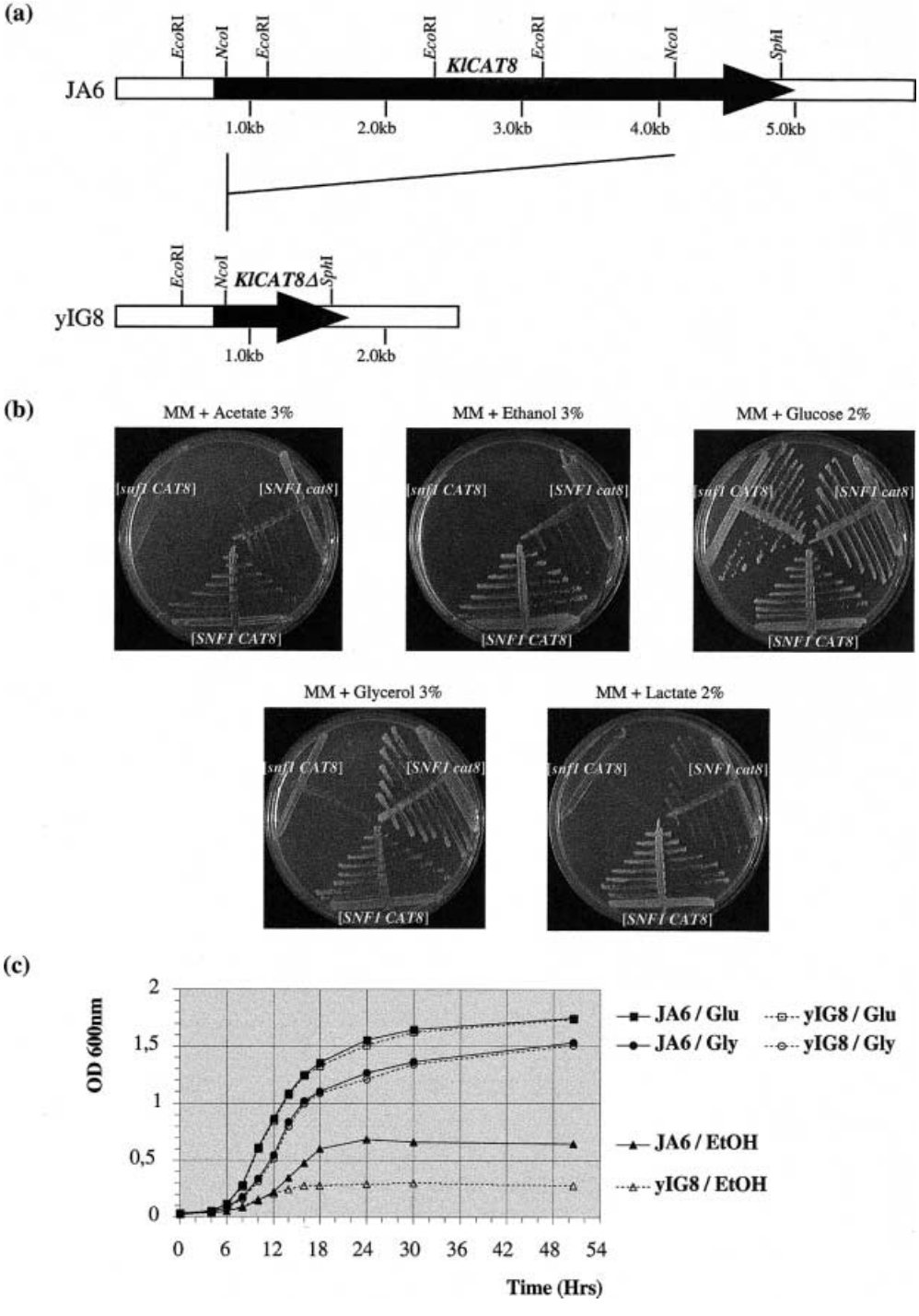


Fig. 4a–c Effects of *KlCAT8* disruption on gluconeogenic growth. **a** Schematic representation of the DNA regions deleted in yIG8. The *thick black arrow* represents the *KlCAT8* ORF, the *open boxes* on both sides indicate the sequenced regions of the *KlCAT8* locus. **b** Comparison of the growth phenotypes of yIG8 [*SNF1 cat8*], JA6 [*SNF1 CAT8*] and JSD1R4 [*snf1 CAT8*]. The different strains were streaked onto minimal medium containing 2% glucose, 3% ethanol or glycerol as the carbon source, and incubated at 30 °C for 2 (glucose) or 4 days (ethanol or glycerol). **c** Growth curves for the yIG8 strain (*cat8*Δ) and JA6 (*CAT8*). The cells were first grown in minimal medium containing 2% glucose, then diluted to an OD_{600} of 0.03 in minimal medium containing 2% glucose, 3% ethanol or glycerol as the carbon source

protein kinase on glycerol metabolism is not mediated by KlCat8p.

A *Klcat8* mutation has no influence on regulation of the gluconeogenic genes *KlFBP1* and *KlPCK1*

To identify possible target genes of KlCat8p, we compared the expression of two genes specifically required for gluconeogenesis, *KlFBP1* (encoding fructose-1,6-bisphosphatase) and *KlPCK1* (encoding phosphoenolpyruvate carboxykinase) in wild-type *K. lactis* and the *Klcat8*Δ disruptant strain. Transcription of the *S. cerevisiae* homologues of these two genes is known to be under the control of ScCat8p (Niederacher et al. 1992; Proft et al. 1995). Northern analysis (Fig. 5) revealed that the level of *KlFBP1*-specific mRNA in glucose-grown wild-type cells is reduced compared to that in glycerol- and ethanol-grown cells. The repression observed in strain JA6 is stronger than that described previously for strain 2UV21 (Zaror et al. 1993). Similarly, the amount of *KlPCK1* mRNA expressed in glucose medium is lower than that in ethanol. Notably, *KlPCK1* mRNA remains very low in glycerol compared to ethanol whereas the *KlFBP1* mRNA reaches the same level on both non-fermentable carbon sources.

No difference in *KlPCK1* and *KlFBP1* mRNA concentrations was observed between the *Klcat8* strain yIG8 and wild-type JA6 under any of the conditions tested. We therefore conclude that KlCat8p is not required for the regulation of these genes in *K. lactis*.

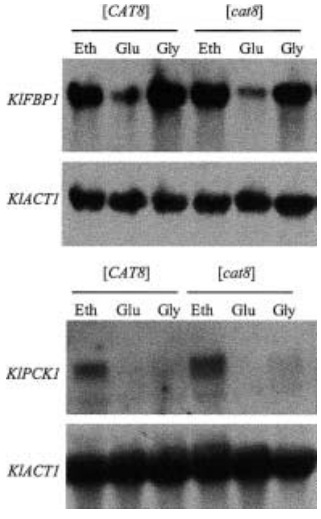


Fig. 5 Effects of *KlCAT8* disruption on gluconeogenic gene expression. Total RNA was extracted from the wild-type (JA6, [*CAT8*]) and the *Klcat8*Δ (yIG8, [*cat8*]) strains grown in minimal medium containing 2% glucose (Glu), 3% glycerol (Gly) or ethanol (Eth) as the carbon source. Each RNA sample (50 μg) was electrophoresed on an agarose gel in the presence of formaldehyde, blotted onto a nylon membrane and hybridised to *KlFBP1*, *KlPCK1* and *KlACT1* probes

Derepression of glyoxylate cycle enzymes requires KlCat8p

In order to ascertain the role of KlCat8p in non-fermentable carbon source utilisation, we tested whether it is required for derepression of two key enzymes of the glyoxylate cycle – isocitrate lyase (ICL) and malate synthase (MS). Transcription of the *S. cerevisiae* genes encoding these enzymes is under the control of Cat8p (Schöler and Schüller 1994; Kratzer and Schüller 1995; Caspary et al. 1997). Since the sequence of the homologous genes of *K. lactis* was not available yet, we assayed the influence of *KlCAT8* disruption on isocitrate lyase and malate synthase activities (Table 1).

For both enzymes an influence of the *Klcat8* mutation was apparent in ethanol-grown cells. ICL activity was reduced tenfold compared to the wild-type JA6 strain, MS activity was reduced only 2.5 fold (Table 1).

Remarkably, the enzyme levels on glycerol barely exceeded those measured on glucose, which were 28- and 8-fold lower than those on ethanol for ICL and MS, respectively (Table 1). Thus, the anaplerotic reactions that compensate for the withdrawal of citric cycle intermediates for anabolism under gluconeogenic growth conditions are not, or only slightly, up-regulated when *K. lactis* is grown on glycerol.

Discussion

Cat8-related proteins form a subfamily among the $Zn(II)_2Cys_6$ transcriptional activators

Sequence analysis indicates that KlCat8p is a true member of the zinc-cluster family of proteins – with the consensus sequence ([GASTPV]-C-x(2)-C-[RKH-STACW]-x(2)-[RKHQ]-x(2)-C-x(5,12)-C-x(2)-C-x(6,8)-C) described for this family (PROSITE Motif PS00463). The two other yeast Cat8p, from *S. cerevisiae* and *C. albicans*, are most closely related to KlCat8p, due to the

Table 1 Carbon source-dependent activities of glyoxylate enzymes in the wild-type strain JA6 and the yIG8 *Klcat8* disruptant

Strain	Malate synthase[a]			Isocitrate lyase[a]		
	Glucose	Glycerol	Ethanol	Glucose	Glycerol	Ethanol
JA6 [*CAT8*]	11.9 (1.2)	20.9 (1.5)	89.2 (1.8)	< 1	< 1	27.9 (0.5)
yIG8 [*cat8*]	12.3 (1.6)	18.2 (2.9)	35.9 (2.3)	< 1	< 1	1.2 (0.1)

[a] Enzyme activities are expressed as nanomoles of substrate converted per min per mg of protein. The values are the means of three measurements, standard deviations are indicated in *parentheses*

sequence conservation in the coiled-coil structure adjacent to the zinc cluster, the "Middle Homology Region" (region III, amino acids 562–803; Chasman and Kornberg 1990; Schjerling and Holmberg 1996; Poch 1997), and particularly regions II and IV, which are only found in the three yeast Cat8p homologues. Whether these last motifs indicate a Cat8p-specific function or are simply evolutionary relics remains to be determined.

Based on the similarity of the DNA-binding domains (region I), in particular the conservation of the primary sequence in the coiled-coil and linker regions, a group of seven Cat8p-related proteins (*K. lactis* Cat8p, *S. cerevisiae* Cat8p, *C. albicans* Cat8p, *A. niger* FacB, *A. oryzae* FacB, *E. nidulans* FacB and *N. crassa* ACU15) emerged. It is likely that these proteins recognise very similar sequences.

We note that ScSip4p, a Snf1p-interacting protein recently shown to be able to bind the CSRE (Vincent and Carlson 1998), is not part of this Cat8p-related subgroup, due to some divergence within the amino acid sequence of the coiled coil.

Interestingly, five of the seven homologues forming the Cat8p subgroup appear to perform overlapping functions in vivo: the three FacB homologues are required for the induction of the acetate utilisation enzymes (acetyl-CoA synthase, isocitrate lyase and malate synthase; Todd et al. 1997) in *Aspergillus*, ScCat8p is necessary for the derepression of the gluconeogenic and glyoxylate enzymes (Hedges et al. 1995), and this work has provided evidence that KlCat8p is required for the derepression of glyoxylate genes in *K. lactis*. The functional roles of ACU15 and CaCat8p in *N. crassa* and *C. albicans*, respectively, remain to be determined.

KlCat8p regulates glyoxylate cycle but not gluconeogenesis in *K. lactis*

Whereas Cat8p is a key factor in the control of gluconeogenesis in *S. cerevisiae*, no evidence for its involvement in the regulation of key gluconeogenic genes in *K. lactis* has been obtained. Both *KlFBP1* and *KlPCK1* were shown to be induced under non-fermentative growth conditions in the *Klcat8* mutant as in the wild-type (Fig. 5). The KlCat8p-independent regulation of *KlFBP1* may be sufficient to explain why *K. lactis*, in contrast to *S. cerevisiae*, *cat8* mutants are able to utilise glycerol as a sole carbon source. In contrast to the other non-fermentable carbon sources tested, glycerol enters the glycolytic pathway upstream of phosphoenolpyruvate as dihydroxyacetonephosphate (either via glycerol phosphate as in *S. cerevisiae* or via dihydroxyacetone as in some other yeasts; Gancedo and Serrano 1989). Thus, fructose-1,6-bisphosphatase (FBPase) is the only gluconeogenic enzyme required. In agreement with this, *KlPCK1* mRNA and glyoxylate cycle enzymes remained low in glycerol grown cells.

During the utilisation of ethanol, lactate and acetate, oxaloacetate is consumed and anaplerotic reactions are required to replace it. Up-regulation of the phosphoenolpyruvate carboxykinase gene *KlPCK1* was shown here to be independent of Cat8p, but up-regulation of the glyoxylate cycle enzymes was partially under the control of KlCat8p. Isocitrate lyase levels in the *Klcat8* mutant strain were about ten-fold lower than in wild-type, whereas malate synthase was only moderately reduced. Whether or not the reduced levels of these enzymes are sufficient to explain the growth deficiencies of the *Klcat8* mutant remains to determined. The cloning of the corresponding genes will allow us to address this question and to analyse whether or not KlCat8p is involved in the transcriptional regulation of the expression of isocitrate lyase and malate synthase, as in *S. cerevisiae*.

The signalling cascade seems to be conserved between *K. lactis* and *S. cerevisiae*

The fact that KlCat8p is able to substitute for ScCat8p in *S. cerevisiae* suggests that, like ScCat8p, it regulates gene expression through CSREs. In agreement with this hypothesis, the Cat8p-independent regulation of *KlFBP1* is correlated with the absence of CSREs in the promoter region of this gene. (Only 390 bp of the *KlPCK1* promoter region have been sequenced). In a region of more than 1000 bp upstream of the *KlFBP1* ORF, no close match to the consensus sequence (5′-CGGNBNVMHGGM-3′) is found (J. J. Heinisch, personal communication). In *S. cerevisiae*, evidence for direct binding of ScCat8p to CSREs in vitro has been obtained (Rahner et al. 1999). In addition, the CSRE was shown to bind another protein, Sip4p (Vincent and Carlson 1998). The *ScSIP4* promoter is subject to regulation by *ScCAT8*, indicating that Sip4p also functions downstream of ScCat8p. We have recently cloned the *K. lactis* homologue of *SIP4* as a multicopy suppressor of the phenotype of the *Klcat8* deletion strain (Krijger et al., in preparation), supporting the view that Sip4p functions downstream of Cat8p in both yeasts.

The *KlCAT8* gene was cloned as a multicopy suppressor of a *fog2* (*snf1*) mutant. In *S. cerevisiae* Snf1p is known to control ScCat8p activity at two levels (Rahner et al. 1996; Randez-Gil et al. 1997): Firstly, it derepresses transcription, probably by decreasing the nuclear concentration of Mig1p (De Vit et al. 1997). Secondly, it directly or indirectly controls the Cat8p-dependent transactivator activity. This latter regulatory mechanism, as yet only poorly understood, is crucial for Cat8p-controlled gene expression – even under conditions of moderate ScCat8p overexpression. Since single-copy *KlCAT8* can complement the *S. cerevisiae cat8* mutant, it is likely that the Snf1p-dependent signalling pathway that activates Cat8p is conserved between both yeasts.

Thus, it appears that, despite the difference in *cat8* mutant phenotypes, the signalling cascade through which Cat8p controls gene expression and through which Cat8p itself is controlled is similar in *S. cerevisiae* and *K. lactis*. A number of other *K. lactis* regulatory

genes that are homologous to known *S. cerevisiae* genes were also found to function through similar regulatory DNA sequences and to be controlled by similar upstream regulators as in the case of *ScCAT8* and *KlCAT8* but to have strikingly different loss-of-function phenotypes. *KlMIG1*, although involved in regulating transcription of the *KlGAL1* gene through a Mig1p-binding site as in *S. cerevisiae* (Dong and Dickson 1997), has no detectable influence on glucose repression of the *K. lactis* invertase gene *KlINV1*, an orthologue of *SUC2*, which is a major target of Mig1p in *S. cerevisiae* (Georis et al. 1999). Moreover, no potential binding sites for Mig1p are found in the promoter regions of *KlCAT8* or *KlFBP1*, the orthologues of which are again targets of Mig1p in *S. cerevisiae* (Mercado et al. 1991; Hedges et al. 1995). Likewise, although three of the four *K. lactis* genes encoding components of the Hap2/3/4/5 complex, a regulator of respiration, have been cloned (Mulder et al. 1994; Nguyen et al. 1995; Bourgarel et al. 1999), none of the *K. lactis hap* mutants exhibit any growth deficiency on non-fermentable carbon sources.

The explanation for the difference in phenotypes that is emerging in all these cases is that the target gene sets differ, at least partially. Thus, in the two yeasts, in which carbon metabolism is regulated rather differently (Wésolowski-Louvel et al. 1996), the same regulators seem to control different sets of genes. Tools are now available to address the question of how these differences in regulatory patterns have evolved.

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