The Inactivation of \textit{KINOT4}, a \textit{Kluyveromyces lactis} Gene Encoding a Component of the CCR4-NOT Complex, Reveals New Regulatory Functions

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Manuscript received February 9, 2005
Accepted for publication April 1, 2005

ABSTRACT

We have isolated the \textit{KINOT4} gene of the yeast \textit{Kluyveromyces lactis}, which encodes a component of the evolutionarily conserved CCR4-NOT complex. We show that inactivation of the gene leads to pleiotropic defects that were differentially suppressed by the \textit{NOT4} gene of \textit{S. cerevisiae}, indicating that these genes have overlapping, but not identical, functions. \textit{K. lactis} strains lacking Not4p are defective in fermentation and show reduced transcription of glucoспорase and glycolytic genes, which are phenotypes that are not found in the corresponding mutant of \textit{S. cerevisiae}. We also show that Not4 proteins control the respiratory pathway in both yeasts, although with some differences. They activate transcription of \textit{KICG2} and \textit{KICYC1}, but repress \textit{KICL1, ScICL1, ScACS1}, and \textit{ScCYC1}. Altogether, our results indicate that Not4p is a pivotal factor involved in the regulation of carbon metabolism in yeast.

The CCR4-NOT complex is an evolutionarily conserved protein complex mostly involved in positive and negative regulation of gene transcription (Collart and Struhl 1994; Liu et al. 2001). In \textit{Saccharomyces cerevisiae}, this complex exists in two forms, one that is 1.9 MDa and one that is 1 MDa in size. The smaller form represents the core complex. As shown by mass spectroscopic analysis, it contains Ccr4p, Caf1p, Caf40p, Caf130p, five Not proteins (Not1-5p), and other unidentified components, each with a specific function (Draper et al. 1994; Liu et al. 1998; Chen et al. 2001). In past years, the CCR4-NOT complex was shown to play roles in multiple cellular processes, including mRNA deadenylation (Daugeron et al. 2001; Tucker et al. 2001; Chen et al. 2002), transcriptional repression, and, through contacts with TFIID, the initiation and elongation of mRNA (Badarinarayana et al. 2000; Lemaire and Collart 2000; Denis et al. 2001). The isolation and characterization of each component of the yeast CCR4-NOT complex has been recently reviewed (Collart 2003).

In \textit{S. cerevisiae}, \textit{NOT4} was originally isolated as a gene coding for a transcription factor involved in the repression of the \textit{TATA}-less promoter of the \textit{HIS3} gene (Collart and Struhl 1994). It was isolated independently as a negative regulator of several groups of genes, including the mating-pheromone-responsive genes (Cade and Errede 1994; Irie et al. 1994; Leberer et al. 1994), and was assigned different names (\textit{MOT2} and \textit{SIG1}).

Not4p is a putative RNA-binding protein, and genetic interaction studies also revealed a link between the CCR4-NOT complex and the DEAD-RNA-helicase Dhh1p, a protein that promotes mRNA decapping (Hata et al. 1998; Albert et al. 2000; Maillet and Collart 2002). Recently it was demonstrated that the human Not4 protein has a ubiquitin-ligase activity \textit{in vitro}, indicating a new function of this protein that suggests that it could also act as an E3 ligase enzyme (Albert et al. 2002).

\textit{Kluyveromyces lactis} is a yeast that differs mainly from the related \textit{Saccharomyces cerevisiae} in that respiration is dominant over fermentation. Although most structural and regulatory genes have been evolutionarily conserved, some factors show marked differences in the regulation of cellular pathways in the two organisms. One important difference is that, unlike in \textit{S. cerevisiae}, in \textit{K. lactis} cells glucose repression on respiratory enzymes is absent or less severe (Mulder et al. 1995; Wesołowski-Louvel et al. 1996) and, as a consequence, respiration and fermentation can coexist (Fukuhara 2003). Moreover, the activation of gluconeogenesis in \textit{K. lactis} is not dependent on KlCat8p, a regulatory transcription factor that in \textit{S. cerevisiae} is required for derepression of the gluconeogenic genes \textit{FBP1} (fructose 1,6 biphosphatase), \textit{PKC1} (pyruvate carboxykinase), and \textit{ICL1} (isocitrate lyase) (Georius et al. 2000). Another difference between the two organisms is that the HAP complex is necessary for cell growth on respiratory carbon sources in \textit{S. cerevisiae}, but not in \textit{K. lactis} (Bourgarel et al. 1999; Breunig et al. 2000).

In this article we report the isolation and characterization of \textit{KINOT4}, the \textit{K. lactis} gene orthologous to the
**TABLE 1**

Yeast strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW98-SC</td>
<td>MATa ura3 lys bg1 arg1 rgi1 gpl1 adh3</td>
<td>Bianchi et al. (1987)</td>
</tr>
<tr>
<td>CMK29</td>
<td>MATa/α ura3/ura3 leu2/LEU2 ade/ade met trp/TRP lac4-8/lac4-8</td>
<td>MAZZONI et al. (1994)</td>
</tr>
<tr>
<td>CMK37</td>
<td>Isogenic to CMK29 except Klnot4Δ1::kanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>CMK37-10B</td>
<td>MATa ura3 ade met lac4-8 Klnot4Δ1::kanMX4</td>
<td>This study</td>
</tr>
<tr>
<td>CMK37-10Bw</td>
<td>Isogenic to CMK37-10B, Kep6/KINOT4</td>
<td>This study</td>
</tr>
<tr>
<td>CMK37-10Bc</td>
<td>Isogenic to CMK37-10B, pCXJ3/, KINOT4Δ2</td>
<td>This study</td>
</tr>
<tr>
<td>CMK37-10Bsc</td>
<td>Isogenic to CMK37-10B, pCXJ18/ScNOT4</td>
<td>This study</td>
</tr>
<tr>
<td>BY4741</td>
<td>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</td>
<td>Brachmann et al. (1998)</td>
</tr>
<tr>
<td>Y00207</td>
<td>Isogenic to BY4741 except YER068vac::kanMX4</td>
<td>EUROSCARF collection</td>
</tr>
<tr>
<td>Y00207Kln</td>
<td>Isogenic to Y00207, pRS416/KINOT4</td>
<td>This study</td>
</tr>
<tr>
<td>Y00207Kln2</td>
<td>Isogenic to Y00207, pRS416/Knot4Δ2</td>
<td>This study</td>
</tr>
</tbody>
</table>

**NOT4** gene of *S. cerevisiae*. Cells deleted of **KINOT4** showed a pleiotropic phenotype similar to the one observed in **not4** mutants of *S. cerevisiae*. However, unexpected roles of **CCR4**-NOT complex were found in the regulation of **K. lactis** genes involved in the uptake of glucose, in glycolysis and in the utilization of respiratory carbon sources.

**MATERIALS AND METHODS**

**Yeast strains and growth conditions:** Yeast strains, listed in Table 1, were grown in YP medium (2% peptone, 1% yeast extract) containing 2% glucose (YPD) or different carbon sources as specified in the text. Yeast transformants were selected on minimal medium (0.67% yeast nitrogen base, 2% glucose) supplemented with 10 μg of the appropriate nutritional requirements, according to the genotype of strains. When indicated, G418 (Geneticin; Sigma, St. Louis) at the concentration of 100 μg/ml and antitoxin A (Sigma) at the concentration of 5 μg/ml, were added to plates. Solid media were supplemented with 2% Bactoagar (Difco, Detroit).

**General methods:** Restriction enzyme digestions, plasmid engineering, and other common techniques were performed according to standard procedures (Sambrook et al. 1989). *Escherichia coli* and yeast transformation was performed by electroporation with a Bio-Rad (Hercules, CA) Gene-Pulser apparatus following the procedures provided by the manufacturer. DNA sequence was determined by the enzymatic method of SANGER et al. (1977). The presence of coiled-coil and PEST domains in the protein sequence was verified by computer analysis at the sites http://www.ch.embnet.org/software/COILS_form.html and http://www.ch.embnet.or/embnet/tools/bio/PESTfind/, respectively.

**Plasmid construction and disruption of KINOT4:** The 2.2-kbp BamHI/BamHI fragment containing the first 1215 bp of the **KINOT4** gene and its promoter, obtained from plasmid Kep6-KINOT4, was cloned into the BamHI site of pKSII (Stratagene, La Jolla, CA) and the **K. lactis** multicopy plasmid pCXJ3 (CHEN 1996) to give plasmids pKS/KINOT4Δ2 and pCXJ3/KINOT4Δ2, respectively.

The pCXJ18/ScNOT4 plasmid, carrying the ScNOT4 gene and its promoter, was obtained by cloning the 3.5-kbp Xhol/EcoRI fragment from plasmid pYElac112-NOT4 (gift from Martine Collart) into the SalI/EcoRI sites of the **K. lactis** centromeric plasmid pCXJ18 (CHEN 1996). Plasmids pRS416/KINOT4 and pRS416/Knot4Δ2 were obtained by cloning the 4.0-kbp ClaI/BglII and the 2.1-kbp BamHI fragments, derived from Kep6-KINOT4, into the ClaI/BamHI and BamHI sites of the **S. cerevisiae** centromeric plasmid pRS416, respectively.

The 1.4-kbp HinII fragment derived from the pFa6a-KanMX4 plasmid (WACH et al. 1994) and containing the KanMX4 gene was cloned into the EcoRV sites of pKS/Knot4Δ2 to give plasmid pKS/KINOT4Δ1::KanMX4. In this way, the KINOT4 open reading frame was interrupted 174 bp after the ATG start codon. The linear fragment KINOT4Δ1::KanMX4 was amplified by PCR using the oligonucleotides 5'-gtttggagctgttaaatg-3' and 5'-ggtaagctgctgaattg-3' and then used to disrupt the **KINOT4** gene in the diploid strain CMK29 (MAZZONI and FALCON 2001).

After repeated growth on nonselective medium, stable transformants were selected on YPD plates containing G418, and the correct integration at the KINOT4 chromosomal locus was verified by Southern blots. Disruptants were then sporulated and small G418-resistant colonies bearing the not4 mutation appeared after 7 days of incubation at 24°C. From a four-spore ascus, we obtained the Knot4Δ1 strain used in this study. As a control, we used the Knot4Δ1 deletion mutant bearing the wild-type **KINOT4** gene on a multicopy plasmid.

**Fluorescence microscopy:** Cells were grown in YPD to late logarithmic phase, fixed in 3.7% formaldehyde, and stained with calcofluor white (Fluorescent Brightener 28; Sigma) to detect chitin localization, as described in PRINGLE (1991).

**RNA preparation and analysis:** Fifteen-microgram samples were loaded onto 1% agarose-MOPS gels containing 2% glucose (YPD) or different carbon sources. RNA preparation and analysis at the sites http://www.ch.embnet.org/software/COILS_form.html and http://www.ch.embnet.or/embnet/tools/bio/PESTfind/, respectively.
**RESULTS**

Isolation and sequence analysis of *KINOT4*: *KINOT4* was found by sequencing chromosome III next to the gene *ARG5,6* (Wesołowski-Louvel *et al.* 1988). *KINOT4* encodes a putative protein of 574 amino acids (KlNot4p) showing 52% identity to Not4p of *S. cerevisiae* (ScNOT4) and 32% identity to Not4p of *Homo sapiens* (HsNOT4). The human protein exists in three different forms de-
Figure 2.—Pleiotropic phenotypes of Klnot4Δ mutants. Cells were grown in YPD to saturation and 5 μl of 10-fold dilutions were spotted on complete medium in the absence (YPD) or in the presence of the osmotic stabilizer sorbitol (1MS), caffeine, and calcofluor. Plates were incubated at the indicated temperatures for 3 days.

Figure 3.—Chitin distribution in not4 mutants. Calcofluor staining of Klnot4Δ1 cells (A) and of the same mutant expressing the wild-type protein (B), Klnot4Δ2p (C), and ScNot4p (D).

The analysis of KlNot4p also showed a putative PEST sequence (residues 351–396), which is a signal for proteolytic degradation (Rechsteiner and Rogers 1996). The PEST sequence is also present in ScNot4p and HsNot4p but it is not conserved in sequence and position (Figure 1B).

Construction and analysis of KINOT4 mutants: Two types of mutated Klnot4 alleles were constructed (Figure 1C): KlnotΔ1, interrupted within the RING-finger motif, and KlnotΔ2, lacking the C terminus and the glutamine stretch. It has been shown that the S. cerevisiae not4 deleterants cannot grow at 37° and that thermo-sensitivity can be rescued by the presence in the medium of an osmotic stabilizer such as 1 m sorbitol (Cade and Errede 1994; Irie et al. 1994; Leberer et al. 1994). The KlnotΔ1 mutant also showed thermo-sensitivity, but at a lower temperature (28°), and it was only partially suppressed in the presence of sorbitol (Figure 2). Interestingly, cells expressing the truncated allele KlnotΔ2 were able to grow at 28° but not at 37°, and the addition of sorbitol to the medium only partially restored cell viability.

To further examine the phenotypes of the KlnotΔ1 and KlnotΔ2 mutations, we also tested the ability of these mutants to grow in the presence of drugs. The results of this analysis (presented in Figure 2) revealed that KlnotΔ1 cells were partially sensitive to caffeine, a phenotype often associated with defects in the protein-kinase-C-mitogen-activated protein kinase pathway, indicating possible defects in the synthesis of the cell wall. This was also indicated by the sensitivity of KlnotΔ1 to calcofluor white, a compound that specifically binds to chitin and interferes with yeast cell-wall deposition (Heinisch et al. 1999). In contrast, cells expressing KlnotΔ2 showed a sensitivity to these drugs similar to that of the wild type. Microscopic observation of the KlnotΔ1 mutant revealed the presence of cells with an increased size and, as can be seen in Figure 3A, calcofluor white staining showed that chitin was delocalized all around the cell wall. The correct localization of chitin at the bud scars and at the septum was observed after transformation of this mutant with the wild type (Figure 3B) and the KlnotΔ2 (Figure 3C) alleles of K. lactis, as well as in cells transformed with the S. cerevisiae NOT4 gene (see below and Figure 3D).

Cross-complementation of S. cerevisiae and K. lactis not4 mutants: We performed cross-complementation ex-
periments to determine whether the NOT4 genes of S. cerevisiae and K. lactis are interchangeable. The introduction of the S. cerevisiae NOT4 gene on a centromeric plasmid into the Knout4ΔI mutant substantially suppressed all growth defects, although at different extents (Figure 4A). Furthermore, ScNOT4 restored growth at 28°C but not at 37°C, unless sorbitol was added to the medium. Normal growth was also restored in the presence of calcofluor and, in addition, calcofluor white staining revealed a correct cellular localization of chitin (Figure 3D).

We also found that ScNOT4 was able to restore the growth of Knout4ΔI in the presence of antimycin A, an inhibitor of mitochondrial respiration (see below). Unexpectedly, the ScNOT4 gene failed to restore growth in the presence of caffeine, suggesting that the products of the ScNOT4 and Knout4ΔI genes have similar, but not identical, functions. The wild-type gene from K. lactis fully suppressed all growth defects of the S. cerevisiae not4 mutant whereas the truncated allele Knout4Δ2 complemented temperature sensitivity and the utilization of respiratory carbon sources (see below) but only partially restored growth in the presence of caffeine and calcofluor (Figure 4B).

The deletion of Knout4 affects glycolysis: An unexpected finding was that the disruption of Knout4 resulted in a strong decrease in glycolytic activity. Wild-type K. lactis strains also can grow in glucose in the presence of antimycin A by fermentation of this carbon source (Goffrini et al. 1989). As mentioned above and shown in Figure 5A, the growth of the Knout4ΔI mutant on high and low concentrations of glucose was inhibited by antimycin A, indicating an impairment in the glycolytic pathway.

Since the this phenotype was more evident in cells growing in low glucose, we wondered if the absence of Knout4 could in some way affect glucose flux through the glycolytic pathway. To test this possibility, Northern blots of total RNA prepared from wild-type, Knout4Δ1, and Knout4Δ2 cultures grown on YM medium in the presence of 2 or 5% glucose were probed with sequences from KLRAG1 and KIPDC1, two glycolytic key genes encoding the low-affinity glucose carrier and the pyruvate decarboxylase activity, respectively (Chen et al. 1992; Wesolowski-Louvel et al. 1992; Bianchi et al. 1996).

As can be seen in Figure 5B, low levels of the KLRAG1 and KIPDC1 transcripts were detected in the presence of 2% glucose, and very low amounts of transcript of the KIPDC1 gene (lane 5) could be detected under the same conditions. Increased levels of the two transcripts were detected in Knout4Δ1 grown in 5% glucose (Figure 5B, lane 6), as expected on the basis of prior demonstrations that KLRAG1 and KIPDC1 are highly transcribed in fermenting cells (Chen et al. 1992; Bianchi et al. 1996).

In the case of Knout4Δ2, transcript levels of the two
tested genes were intermediate between those of the Klnot4Δ1 mutant and the wild type (Figure 5B, lanes 3 and 4). Since the Klnot4Δ2 mutant showed antimonycin A resistance similar to the wild type (Figure 5A), we could conclude that the transcription levels of KIRAG1 and KIPDC1 in this mutant were sufficient for normal cell growth. In contrast, in S. cerevisiae the deletion of Scnot4 had no effect on the transcription of HXT1 and PDC1 (Figure 5B, lane 8), the genes homologous to KIRAG1 and KIPDC1, respectively. According to this, not4 mutants were still able to grow in the presence of antimonycin A on 2% glucose (not shown).

To verify that KINOT4 is a general regulator of glycolysis in K. lactis, we analyzed the transcription of other glycolytic genes, namely KIPGI1, KIPGK1, and KlENO1. As shown in Figure 6, the transcription of all these genes in the Klnot4Δ1 mutant was significantly reduced, although to a lesser extent compared to KIPDC1 used as a control in this experiment.

**Role of KINOT4 in 2C and 3C carbon metabolism:** S. cerevisiae strains lacking NOT4 have been also isolated as respiratory-deficient mutants (Dimmer et al. 2002) showing poor growth in glycerol and ethanol. For this reason, we examined the growth of K. lactis mutants on different carbon sources and found that Klnot4Δ1 failed to grow on 2C respiratory carbon sources (ethanol and acetate), but it was still able to grow on 3C carbon sources (such as glycerol and lactate). This indicates that there are important differences between S. cerevisiae and K. lactis in the regulation of aerobic metabolism.

On the basis of these growth phenotypes, we analyzed the expression of the KlACS and KlICL1 genes, which are known to be transcriptionally regulated by KlCat8p. In K. lactis, two genes encode acetyl-CoA synthetase, which is required for the production of acetyl-CoA, an important fuel for the tricarboxylic acid (TCA) cycle during growth on acetate and ethanol. These genes are KlACS2, which is induced at the transcriptional level by ethanol, and KlACS1, which is induced by acetate and lactate (Lodt et al. 2001). To study the effect of Klnot4p on the expression of these K. lactis genes, we prepared total RNAs from wild-type and mutant cells grown in 2% glucose and shifted for 2 hr to 2% ethanol. As a control, we also analyzed RNAs from cells grown on 2% glycerol.

As shown in Figure 7, in the Klnot4Δ1 mutant we observed a strong reduction in the number of KlACS2 transcripts in glucose-grown cells and, most importantly, the induction of the gene by ethanol was prevented. In the case of KlACS1, the mRNA levels in the Klnot4Δ1 mutant were comparable to those of wild-type cells grown on all carbon sources, indicating that KINOT4 had no effect on the expression of this gene. We also examined the expression of KlICL1, the gene encoding isocitrate lyase, a key enzyme of the glyoxylate cycle. Surprisingly, in the Klnot4Δ1 mutant we observed, after the shift to ethanol, an overexpression of this gene compared to the wild type. We also observed a faster migrating band of hybridization that was present in both strains as respiratory-deficient mutants (Dimmer et al. 2002).
detected a very small number of transcripts in cells grown in glucose, and ACS2 was not induced after the ethanol shift, suggesting that respiration is lower in the Klnot4Δ1 mutant compared to the wild type.

In S. cerevisiae, ACS2 is constitutively expressed, while ACS1, ICL1, and CYC1 genes are repressed by glucose and activated on respiratory carbon sources (Schuller 2003). We examined the expression of the latter genes in the S. cerevisiae not4 mutant grown on glucose 2 hr after the shift to ethanol or glycerol. With both substrates (Figure 8), we observed an early induction of transcription of all these genes. However, it has been reported that expression of CYC1 is lowered in not4 mutants grown on these carbon sources (Liu et al. 1998).

To verify that the difference in our result was due to different experimental conditions, we analyzed the transcription of these genes after an overnight culture and found that the CYC1 transcripts were, in fact, significantly reduced, as were transcripts from ACS1.

**DISCUSSION**

In this article we describe a study of KlNOT4, the K. lactis ortholog of the S. cerevisiae NOT4 gene that encodes a component of the transcriptional regulatory complex, CCR4-NOT. Inactivation of NOT4 genes produces pleiotropic phenotypes in both yeasts, including thermo-sensitivity, defects in cell-wall biosynthesis, and defects in the utilization of respiratory carbon sources. Despite the 52% protein identity, KlNot4p differs from its S. cerevisiae counterpart in the C terminus, where a second coiled-coil domain rich in glutamines is present. Deletion of this motif caused less severe defects compared to those observed in the absence of the entire protein and could only partially restore the wild-type phenotypes when expressed in S. cerevisiae not4 mutants. Never-
Figure 8.—Northern blot analysis of wild-type and not4 null mutants of S. cerevisiae. Northern blots were prepared using total RNAs isolated from cells grown in 2% glucose, after a 2-hr shift to 2% ethanol, 2% glycerol, or grown overnight on 2% ethanol + 2% glycerol (EtOH/Gly). The blots were hybridized to the indicated probes specific for S. cerevisiae (see materials and methods). ND, not determined.

Figure 9.—Schematic of the Not4p role in the regulation of metabolic genes in K. lactis. Solid and open boxed genes are regulated by NOT4 negatively and positively, respectively. KlNot4p, as well as cells expressing ScNot4p that naturally lacks this part of the protein, are unable to grow at 37°C. This indicates that the second coiled-coil domain is necessary for K. lactis growth at higher temperatures.

We also found that cells lacking KlNot4p are unable to grow on glucose in the presence of antimycin A, an inhibitor of mitochondrial respiration. In addition, we determined that the absence of KlNot4p results in a significant reduction in transcription of all glycolytic genes, in particular of RAG1 and KLPDC1, indicating that KlNot4p is a general positive regulator of glycolysis. Recently, it was reported that the transcription of RAG1, the gene encoding the low-affinity glucose transporter in K. lactis, is strongly reduced in Kleno1, Klpgk1, and Klhxk1 mutant genes, but is not affected in Klpgi1 and Klpxc1 mutants, suggesting the existence of a complex regulatory network involving glucose transport and glycolytic genes (Lemaire and Wesołowsk-Louvel 2004). The fact that all these genes are less transcribed in the Knnot4Δ1 mutant places KlNot4p upstream of this regulatory network. As a consequence, the flux of glucose and the glycolytic pathway in the mutant are less efficient and cells cannot survive in the presence of mitochondrial inhibitors such as antimycin A.

We have also shown that the situation is different in S. cerevisiae not4 mutants in that the transcription levels of HXT1, the functional homolog of RAG1 encoding the low-affinity glucose transporter, were not affected, as was the case for the PDC1 gene as well. In this case, the glucose flux is normal and can be consumed through glycolysis and fermentation. Since ScNot4p can restore the growth of Knnot4Δ1 in the presence of antimycin A, we can conclude that the opposite response of K. lactis and S. cerevisiae not4 mutants to this drug probably reflects differences in the regulatory networks, and not in the Not4 proteins.

Remarkable differences were also observed when we compared the utilization of respiratory carbon sources in the two yeasts. In fact, not4 mutants of S. cerevisiae showed very limited growth in the presence of nonfermentable carbon sources, while K. lactis mutants were still able to grow on glycerol and lactate, indicating that the utilization of 3C carbon sources was not affected by mutation of KnNOT4 in K. lactis. The utilization of ethanol and acetate requires three separate pathways: production of acetyl-CoA, replenishment of oxaloacetate through the glyoxylate cycle, and gluconeogenesis. In S. cerevisiae, Cat8p is required for the activation of genes of these pathways, such as ACS1, ICL1, and FBP1 (Schuller 2003), whereas in K. lactis the regulation of gluconeogenesis is unlinked to that of the glyoxylate cycle and is not dependent on KICAT8 (Géoris et al. 2000; Lodì et al. 2001). In the Knnot4Δ1 mutant, the transcription of KICASI was not affected while KICAS2, a target of KiCat8p, was poorly induced on all carbon sources. Since KlICL1, which is also regulated by KICAT8, was induced even higher in the Knnot4Δ1 mutant after the shift to ethanol, we conclude that in K. lactis the induction of KICAS2 requires an additional pathway involving KnNot4p, independent of KiCat8p. Similarly to KlICL1, KlMLS1, another gene of the glyoxylate cycle encoding the malate synthase activity, showed increased transcription following the shift on ethanol.
(not shown). We can hypothesize that in the presence of ethanol, due to reduced AcS activity and an increase in Icl1 and Mls1 activities, acetyl-CoA is produced in lower amounts and preferentially channeled into the glyoxylate cycle. As a consequence, this molecule is removed from the TCA cycle, impairing respiration and the anaerobic reactions necessary for the biosynthesis of cellular components.

In *S. cerevisiae*, it has been reported that not4 mutants grow very poorly in ethanol/glycerol medium and show reduced transcription of *CYC1*. We found that shortly after a shift of *not4* mutant cells to a respiratory carbon source, ACS1, ICL1, and *CYC1* are transcriptionally induced, indicating that Not4p acts negatively and is located upstream from the specific activators of these genes, such as Cat8p, Adr1p, and Hap2p-5p (Schuller 2003).

In conclusion, our results indicate a role for Not4p in the utilization of respiratory carbon sources. Such a role seems to be more general in *K. lactis* in that Not4p also regulates fermentative metabolism (see Figure 9), although the mechanism and components of these regulatory pathways still need to be identified.

We thank M. Collart for kindly providing plasmid pYElac11-2 NOT4 containing the *SNOT4* gene. We are also grateful to B. Burhans for critical reading of the manuscript and Yvan Zivanovic for help in *K. lactis* genomic analysis. This work was supported by a grant "Cofin 2000" protocol MM05C03814.

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Communicating editor: A. Nicolas