Regulation of cytochrome *c* expression in the aerobic respiratory yeast *Kluyveromyces lactis*

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Abstract **Transcriptional regulation of the *KlCYC1* gene from the aerobic respiratory yeast *Kluyveromyces lactis* has been studied. The *KlCYC1* gene produces two transcripts of different sizes, in contrast with the single transcripts found for *CYC1* and *CYC7* from *Saccharomyces cerevisiae*, and for the *CYC* gene from *Schwanniomyces occidentalis*. Both *KlCYC1* transcripts respond in the same way to the regulatory signals studied here. The transcription of *KlCYC1* is regulated by oxygen and this control is mediated by heme. The *KlCYC1* gene is also subject to catabolite repression. Heterologous expression in *S. cerevisiae* mutants reveals that the factors HAP1 and HAP2 take part in the regulatory mechanism.**

Key words: Cytochrome *c* gene; *Kluyveromyces lactis*; Transcriptional regulation

1. Introduction

In *Saccharomyces cerevisiae*, the expression of genes related to electron transport functions and oxidative damage prevention is activated by the presence of oxygen in a process induced by heme. The *ScCYC1* gene, which encodes for the more abundant isoform 1 of cytochrome *c*, is oxygen-activated by a mechanism that implies the existence of two upstream activation sites, UAS1 and UAS2, to which the activator HAP1 and the complex HAP2/3/4 bind, respectively (for a review see [1]). These genes are also subject to catabolite repression, and derepression is mediated by a series of regulatory factors including the HAP2/3/4 complex. The pool of protein factors necessary for repression is very extensive and two of these proteins, TUP1 and SSN6, modulate the expression of *ScCYC1* (for a review see [2]).

Kluyveromyces lactis and *S. cerevisiae* differ in their respiratory metabolism; *K. lactis* is an aerobic respirative yeast, while *S. cerevisiae* is a fermentative yeast, for which under aerobic conditions fermentation is the principal energy source [3]. It has been shown recently that the *petite negative* phenotype in *K. lactis* is not a result of the inability to use the fermentative metabolic pathways but of the existence of nuclear genes, *MGI1* and *MGI2*, the products of which are necessary for mitochondrial integrity [4].

The upstream region of the *KlCYC1* gene of *K. lactis* reveals some homologies to regulatory regions present in the 5′ regions of *ScCYC1* and *ScCYC7* and other yeast genes related to respiratory functions [5]. Furthermore, we have reported that, in glycerol media, levels of *KlCYC1* mRNA are higher than those corresponding to the *ScCYC1* gene [6]. In this context an interesting question arises: are the genes related to respiratory functions controlled by the same signals in respiratory and fermentative yeasts, or are higher levels of expression due to a constitutive expression that does not change in the switch from aerobic to anaerobic conditions? We have studied the transcriptional regulation of the *KlCYC1* gene in *S. cerevisiae* as a first approach to answer this question.

2. Materials and methods

2.1. Yeast strains and growth conditions

K. lactis strains NRRL-Y1140 (*MATa*, wild-type) and MW98-8c (*MATa rag1 rag2 uraA metA1 argA*) were kindly provided by M. Wésolowski-Louvel (Paris-Cedex, France). *S. cerevisiae* strains ZW13 (*MATa trp1-1 leu2-3 leu2-112 his4-519 cyc1-1 cyp3-1 gal cyc1-1 cyc7*) and ZW10 (*MATa trp1-1 leu2-3 leu2-112 his4-519 cyp3-1 gal cyc1-1 CYP1-16*) are described in [7]. Strains BWG1-7a (*MATa ade1-100 his 4-519 leu2-2 leu2-112 ura3-52*), LPY22 (*MATa ade1-100 his4-519 leu2-2 leu2-112 ura3-52 Δhap1::LEU2 CYC1-ΔUAS2*) and JO1-1a (*MATa ade1-100 his4-519 leu2-2 leu2-112 ura3-52 Δhap2*) were provided by L. Guarente.

Yeast cells were grown at 30°C in rich media, YP (2% bacto-peptone, 1% yeast extract), supplemented with a carbon source (0.5% glucose, 2% glucose, or 2% glycerol) and in complete media, CM, prepared as previously described [7] and supplemented with the corresponding carbon source (CMG, 2% glycerol; CMD, 2% glucose). Anaerobic conditions were obtained by bubbling in N_2; for anaerobic growth, media were supplemented with 20 μg/ml ergosterol and 0.2% Tween 80. In some experiments 11.7 μg/ml hemin were added to the media, as described by Sambrook et al. [8]. Yeast cells were transformed by the procedure of Klebe et al. [9] and transformants were grown on selective plates.

2.2. Plasmids and plasmid constructions

The plasmid pART10 was obtained by inserting a *SalI* 1.9 kb fragment containing the *KlCYC1* gene of *K. lactis* [5] into the multiple cloning site of YEplac112 [10]. Two centromeric plasmids, pCAF9 and pCAF10, were also obtained by inserting the same fragment in YCplac33 and YCplac22 [10]. The plasmid pYeCYC1 2.4 was kindly provided by R. Zitomer and has been described [11].

The bacterial strain JM101 [12] was used for all the constructions, and cells were transformed using the procedure of Hanahan [13]. Alkaline plasmid preparations were carried out as described by Sambrook et al. [8].

2.3. RNA analysis

Cells were harvested at a mid-log density of $A_{600\ nm}$ between 0.5 and 0.9. Isolation of total RNA was performed as described by Zitomer and Hall [7]. Poly(A) mRNA was obtained from total RNA with the kit from Pharmacia following the manufacturer's instructions.

Northern analysis was carried out as previously described [8] using Nytran NY-13N membranes from Schleicher & Schuell. The probes were labelled by the random primer method [17,18] using the digoxigenine-11-dUTP kit from Boehringer Mannheim or [α-^{32}P]dATP from

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Amersham. Blots were prehybridized with 20 ml of hybridization solution (10 mM maleic acid, 10 mM NaCl, pH 7.5, 5× SSC, 1% blocking reagent from Boehringer Mannheim, 0.02% SDS and 0.1 mg/ml of sonicated salmon sperm DNA). After removal of the solution, the denatured labelled probe was added to 2.5 ml of fresh solution and hybridization was carried out for 6 h at 61°C. Membranes were rinsed in 2× SSC and 0.1% SDS for 5 min, once at 61°C and twice at room temperature. Detection was performed by autoradiography when ^{32}P-labelled probes were used, and by immuno-colour reaction with DIG antibody, NBT (Nitro blue tetrazolium salt) and X-phosphate when digoxigening-dUTP-labelled probes were used. Densitometric quantitation and corrections of sample loading were performed by Image Analysis with the INTERDENS program from Microm [16].

2.4. Mapping 5′ and 3′ termini of mRNA

For the determination of 5′ termini, an *Eco*RV fragment from pART10, containing part of the open reading frame (ORF) and the upstream region of the *KlCYC1* gene, was 5′ labelled with [γ-^{32}P]dATP and polynucleotide kinase. Determination of 3′ termini was carried out using a *Hin*dIII–*Pst*I fragment from pART10, containing a fragment of the ORF and the downstream region of *KlCYC1*, labelled with [α-^{32}P]dATP in the presence of Klenow. After 1 h, the reactions were interrupted and the non-incorporated label was eluted in a Sephadex-G50 column as described by Sambrook et al. [8]. Labeled DNA was ethanol-precipitated in the presence of LiCl.

S1-mapping was carried out according to Favorolo et al. [17] using 50 μg RNA and the labelled DNA. The size of the protected fragment was calculated in a 7 M urea-8% acrylamide gel.

3. Results and discussion

3.1. The KlCYC1 *transcripts*

Northern analysis of the total RNA from *K. lactis* showed that transcription of the *KlCYC1* gene produced two transcripts, the sizes of which (1.6 and 1.3 Kb) were calculated by reference to RNA marker III from Boehringer-Mannheim and in relation to the positions of the rRNA bands (Fig. 1A). The two transcripts were also detected in heterologous expression experiments (see below). S1-mapping analysis revealed the existence of only one principal start site of transcription (Fig. 1B). Hybridisation of a 5′ end labeled DNA from pART10 (see section 2) containing the upstream region of the *KlCYC1* gene led to a protected fragment of 380 bp covering a region extending from the *Eco*RV site in the ORF to approximately the −81 position. This means that the major start point is located 3′ from the pyrimidine-rich element and 5′ from two of the TATA sequences located in the upstream region of the gene [8] and represented in Fig. 1B.

To find out whether the 3′ region of the gene is implicated in the generation of these two transcripts, S1-mapping of the mRNA 3′ ends was carried out as described in section 2. The results (Fig. 1C) show the existence of two end-points which explain the existence and size of the two transcripts detected in Northern analysis.

The existence of two transcripts is in contrast to the single transcript found for *ScCYC1* [7] and *ScCYC7* [18] from *S. cerevisiae*, or for the *CYC* gene from *Schwanniomyces occidentalis* [19]. It is interesting to note that both *KlCYC1* transcripts are larger than the transcript corresponding to the *ScCYC1* gene, although the proteins differ in only one amino acid. It would be tempting to speculate that this increased length could be responsible for increased stability of the transcripts, leading to the higher levels of cytochrome *c* mRNA which have been observed in *S. cerevisiae* for the *KlCYC1* gene in comparison to the *CYC1* gene [6].

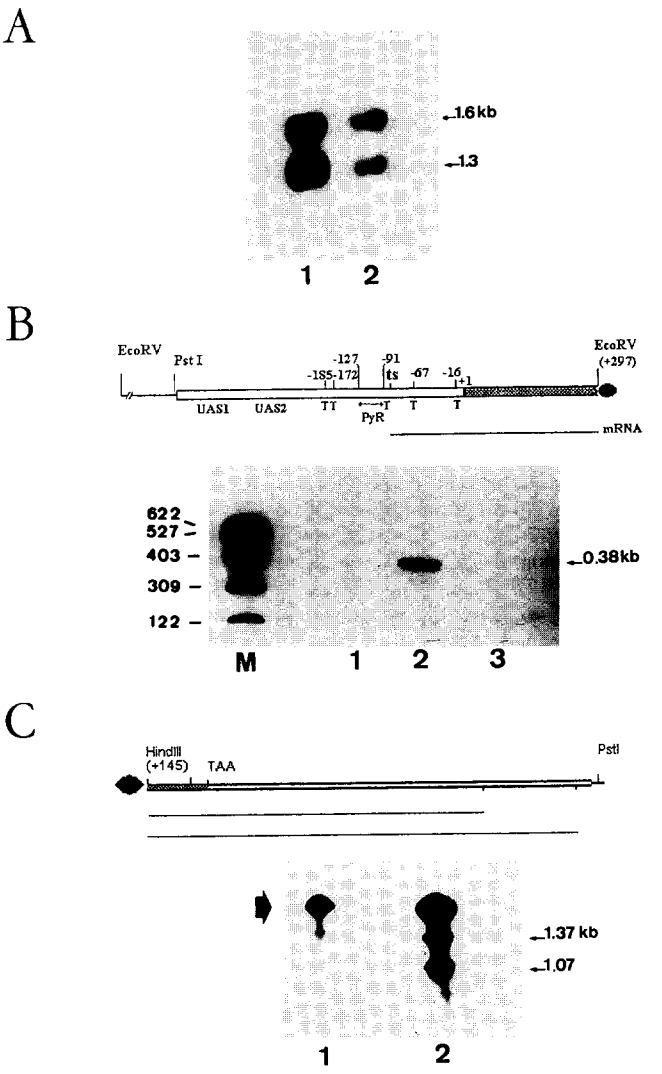

Fig. 1. Characterization of *KlCYC1* transcripts. (A) Northern detection of *KlCYC1* transcripts. Lane 1, total RNA; lane 2, poly(A) RNA. The RNA was extracted from Y1140 *K. lactis* cells cultured in YPG. The probe, a 1.9 kb *Pst*I fragment from pART10 containing the *KlCYC1* gene, was labeled with [α-^{32}P]dATP by the random primer method. (B) 5′ terminus of mRNA. At the top, schematic representation of the *KlCYC1* promoter. T, TATA-like regions; PyR, pyrimidine-rich element; hatched box, ORF; solid circle, 5′ labeled end; ts, transcription start site. In the lower part, autoradiography of the protected fragments. M, labelled size marker (pBR322 digested with *Hpa*II); lane 1, t-RNA (negative control); lane 2, RNA from cells cultured in glycerol; lane 3, RNA from cells in glucose. The arrow indicates the position of the 380 bp protected band. (C) 3′ termini of mRNA. At the top, schematic representation of the DNA fragment and the extension of protected areas; hatched box, ORF; solid circle, 5′ labeled end; lane 1, control DNA-RNA hybrid not digested with S1. Lane 2, S1-mapping of 3′ termini using RNA extracted from cells cultured in glycerol. The arrows indicate the positions of two protected bands, the sizes of which (1.37 kb and 1.07 kb) were calculated by their relative position to DNA size marker III from Boehringer-Mannheim.

3.2. Regulation by oxygen and carbon source

Northern blot analysis of RNA extracted from *K. lactis* cells grown under different conditions revealed that the level of *KlCYC1* mRNA was very low in anaerobiosis and that transcription was induced in the presence of oxygen (Fig. 2A). Under anaerobic conditions the cells were able to grow in

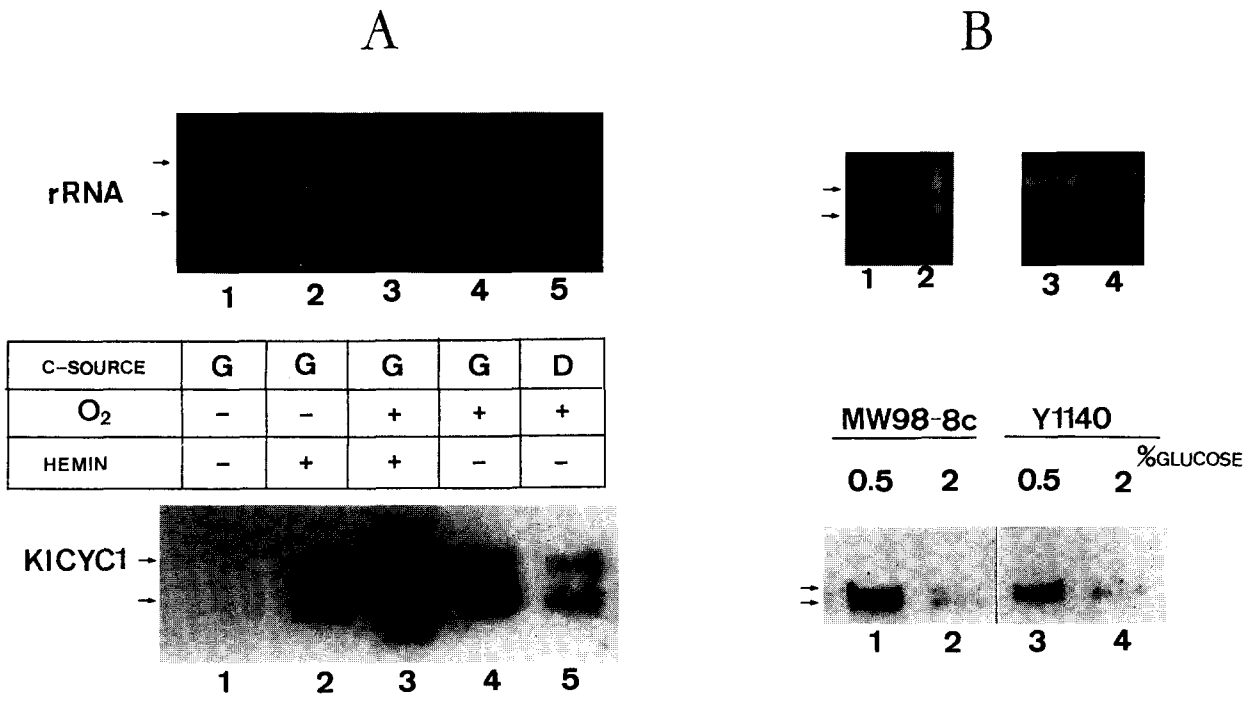

Fig. 2. Regulation of *KlCYC1* transcription by oxygen and carbon source. (A) Total RNA from Y1140 cells grown under different conditions was extracted and analysed by Northern blot using the *KlCYC1* probe described in Fig. 1. The upper part of the figure shows the intensity of ethidium bromide-stained ribosomal RNA bands used for loading correction by image analysis. Cells were pregrown in 2% glucose to $A_{600\ nm} = 0.6$, then washed and incubated for 2 h at the same density in the indicated media. The carbon source was 2%. G, glycerol; D, dextrose; O_2+, aerobic conditions; O_2−, anaerobic conditions; H+, addition of hemin to the media as described in section 2. (B) Northern analysis of total RNA extracted from *K. lactis* Y1140 (wt) and MW98-8c (*rag1, rag2*) cells grown aerobically. 2% glucose (repressed conditions) and 0.5% glucose (derepressed) were used in the cultures. The probe is described in Fig. 1.

glucose, although more slowly than aerobically (data not shown). Since oxygen was not present, this growth was due to fermentation which does not depend on cytochrome *c*. The addition of hemin to aerobic and anaerobic cultures caused a great increase in the level of the transcripts with respect to non-supplemented cells (Fig. 2A). Thus, in *K. lactis*, oxygen and hemin act as inducers of *KlCYC1* in the same way as has been described for the *ScCYC1* gene [20]. These results are remarkable in view of the fact that the mentioned yeast species show a difference in physiology with regard to fermentation and respiration; in comparison to *S. cerevisiae*, *K. lactis* has a much higher respiration rate on glucose under aerobic conditions, but both yeast species are able to induce cytochrome *c* expression in the presence of oxygen.

A comparison of the levels of mRNA in cells grown in glycerol vs. glucose (Fig. 2A) shows that the *KlCYC1* gene in strain Y1140 is subject to catabolite repression. However, the increase obtained after derepression is lower than the four-fold increase reported for the *ScCYC1* gene [7]; although a reliable comparison is difficult to establish since it is well known that catabolite repression is a strain-dependent feature in *K. lactis*, a lesser extent of glucose repression has been reported for other genes in this yeast [21].

The genes *RAG1* and *RAG2* encode for a low affinity glucose transporter and for phosphoglucose isomerase, respectively [22,23]. Expression of *KlCYC1* in the MW98-8c strain (*rag1, rag2*) showed no difference with respect to the wild-type (wt) strain Y1140; *KlCYC1* mRNA levels were lower in 2% glucose than in 0.5% glucose (Fig. 2B); therefore, we conclude that, although the low-affinity glucose transporter is blocked at the phosphoglucose isomerase step, as is glycolysis, the signal necessary for catabolite repression remains active.

3.3. *The effect of* trans *regulatory factors of* S. cerevisiae *on the expression of* KlCYC1

Two sequences, $CCG(N)_6CCG$ and TTGGTTTGTT, which show extensive homology with the consensus sequences for recognition of the HAP1 factor and the complex HAP2/3/4, have been found in the region upstream of the *KlCYC1* gene [5]. In order to verify their function, a DNA fragment containing the promoter and the *KlCYC1* gene was inserted in the centromeric plasmid YCplac22, giving rise to plasmid pCAF10, and used to transform several *hap1* and *hap2* mutant strains from *S. cerevisiae*. Levels of *KlCYC1* transcripts in the transformants were determined by Northern analysis (Fig. 3A). The strains ZW13 and ZW10 are isogenic, except that the first is wild-type for the gene *HAP1* and that ZW10 carries a mutant allele, *hap1-16*, which in *S. cerevisiae* causes a decrease in the transcription of *ScCYC1* and overexpression of *ScCYC7* under aerobic conditions. A similar result was observed for the *KlCYC1* gene that showed a decreased level of expression in the mutant strain (Fig. 3A). This result was confirmed in another *Δhap1* strain, LPY22 (which additionally contains a ΔUAS2), and its isogenic counterpart, BWG1-7a (Fig. 3B). These data support the hypothesis that the *KlCYC1* gene is regulated in *K. lactis* at the transcriptional level by the presence of oxygen through a factor similar to HAP1. In the *Δhap1* strain LPY22, there was no heme induction of the *ScCYC1* gene (Fig. 3B); this

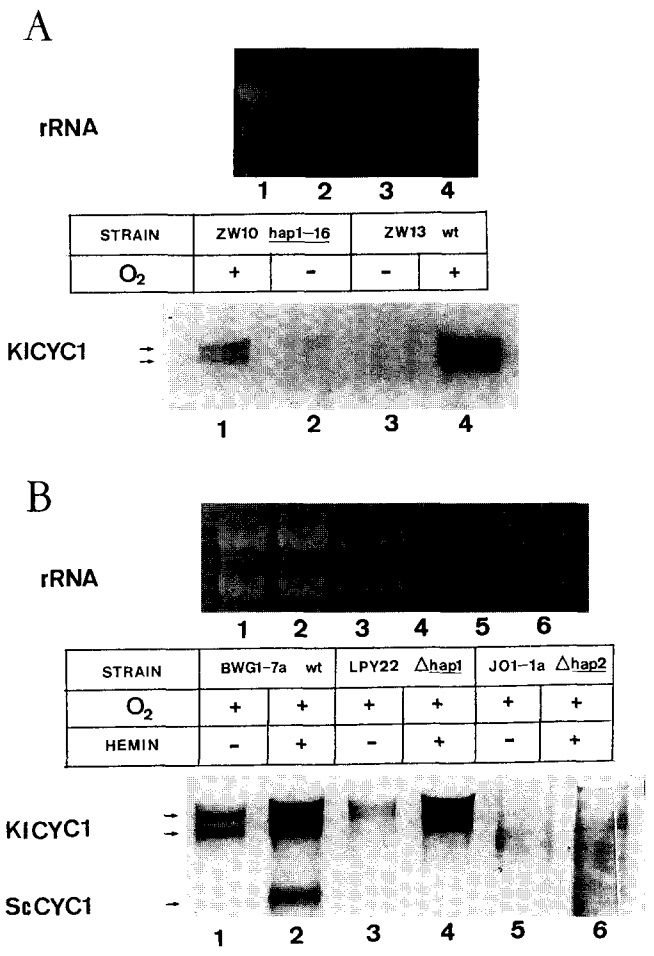

Fig. 3. *KlCYC1* heterologous expression in *S. cerevisiae hap* mutants. Total RNA was extracted from wild-type and mutant strains of *S. cerevisiae* transformed with centromeric plasmids containing the *KlCYC1* gene (pCAF10 for 4A and pCAF9 for 4B). Cultures were grown in 2% glycerol. (JO1-1a cells did not grow on YPG, they were grown in YPD, washed and maintained in YPG for 2 h.) The probe for the *CYC1* gene was a *Xho*I–*Hin*dIII fragment from plasmid pYeCYC1 2.4; the *KlCYC1* probe is described in Fig. 1. Abbreviations and controls are as described in Fig. 2. (A) Effect of the *hap1* mutation. Lane 1, ZW10 (*Hap 1-16*)/O_2+; lane 2, ZW10/O_2−; lane 3, ZW13 (wt)/O_2−; lane 4, ZW13/O_2+. (B) Effect of *hap1* and *hap2* deletions. Lane 1, BWG1-7a (wt)/O_2+; lane 3, LPY22 (*Δhap1*)/O_2+; lane 5, JO1-1a(*Δhap2*)/O_2+; lanes 2, 4 and 6 were, respectively, as 1, 3, 5, but with the addition of hemin to the cultures as described in section 2.

is due to the fact that this strain contains a deletion affecting UAS2. There is, however, induction of the *KlCYC1* transcript that probably occurs through the UAS2-like sequence present in the upstream region of *KlCYC1*.

It was now of interest to determine the influence of *Δhap2* on *KlCYC1* expression. Cells from the strain JO1-1a (*Δhap2*) were unable to grow in lactate or glycerol media even when they were transformed with a plasmid containing the *KlCYC1* gene. To ascertain the absence of *KlCYC1* and *ScCYC1* transcription in this strain, cells were grown in 2% glucose media up to $A_{600\,nm}$ 0.5, washed, and transferred to selective glycerol medium for 2 h. Northern blot analysis of the RNA extracted from these cells revealed that both *CYC1* genes were not transcribed in this strain (Fig. 3B). The HAP2 factor from *S. cerevisiae* is essential for the expression of *KlCYC1*, as it is for *ScCYC1*. This observation, as well as our previous observation of the existence of a consensus sequence for the recognition of the complex HAP2/3/4 [5], support the function of HAP2 in *KlCYC1* expression. A *KlHAP2* gene has been recently cloned and used to construct a null mutant *K. lactis* strain that, surprisingly, showed a capacity for growth in glycerol (M. Bolotin-Fukuhara, personal communication). Therefore, despite the presence of similar *cis* and probably *trans* acting signals in *K. lactis* and *S. cerevisiae*, there are additional controls that modulate cytochrome *c* levels differently. These factors are probably specific to the yeast species since respiratory and fermentative yeasts might have distinct strategies for modulating the general regulation performed by the HAP2/3/4 complex. Future studies combining metabolic and gene expression analysis will be necessary to clarify this point.

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References

[1] Zitomer, R.S. and Lowry, C.V. (1992) Microbiol. Rev. 56, 1–11.
[2] Gancedo, J.M. (1992) Eur. J. Biochem. 206, 297–313.
[3] Gancedo, C. and Serrano, R. (1989) in: The Yeasts III (Rose, A.H. and Harrison, J.S. eds.) pp. 205–259, Academic Press.
[4] Chen, X.J. and Clark-Walker, D. (1993) Genetics 133, 517–525.
[5] Freire-Picos, M.A., Rodríguez-Torres, A.M., Ramil, E., Cerdán, M.E., Breuning, K.D., Hollenberg, C.P. and Zitomer, R.S. (1993) Yeast 9, 201–204.
[6] Freire-Picos, M.A., González-Siso, M.I., Rodríguez-Belmonte, E., Rodríguez-Torres, A.M., Ramil, E. and Cerdán, M.E. (1994) Gene 139, 35–41.
[7] Zitomer, R.S. and Hall, B.D. (1976) J. Biol. Chem. 251, 6320–6326.
[8] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY.
[9] Klebe, R.S., Harris, J.V., Sharp, Z.D. and Douglas, M.G. (1983) Gene 25, 333–341.
[10] Gietz, R.D. and Sugino, A. (1988) Gene 74, 527–534.
[11] Lowry, C.V., Weiss, J.L., Walthall, A. and Zitomer, R.S. (1983) Proc. Natl. Acad. Sci. USA 80, 151–155.
[12] Messing, J. (1979) Recombinant DNA Tech. Bull. 2, 43.
[13] Hanahan, H. (1983) J. Mol. Biol. 166, 557–780.
[14] Feinberg, A.P. and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13.
[15] Feinberg, A.P. and Vogelstein, B. (1984) Addendum Anal. Biochem. 137, 266–267.
[16] Freire-Picos, M.A., Rodríguez-Belmonte, E., Ramil, E., Rodríguez-Torres, A.M., González-Siso, M.I. and Cerdán, M.E. (1994) Biomed. Lett. 49, 241–246.
[17] Favorolo, J., Treisman, R. and Kamen, R. (1980) Methods Enzymol. 65, 718–749.
[18] Zitomer, R.S., Sellers, J.W., McCarter, D.W., Hastings, P.A., Wick, P. and Lowry, C.V. (1987) Mol. Cell Biol. 7, 2212–2220.
[19] Amegadzie, B.Y., Zitomer, R.S. and Hollenberg, C.P. (1990) Yeast 6, 429–440.
[20] Guarente, L. and Mason, T. (1983) Cell 32, 1279–1286.
[21] Zaror, I., Marcus, F., Moyer, D.L., Tung, J. and Shuster, J. (1993) Eur. J. Biochem. 212, 193–199.
[22] Goffrini, P., Wésolowski-Louvel, M., Ferrero, I. and Fukuhara, H. (1990) Nucleic Acids Res. 18, 5294.
[23] Wésolowski-Louvel, M., Goffrini, P. and Ferrero, I. (1988) Nucleic Acids Res. 16, 8714.