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Kluyveromyces lactis HIS4 transcriptional regulation: similarities and differences to *Saccharomyces cerevisiae HIS4* gene

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Abstract Sequence analysis of the *Kluyveromyces lactis HIS4* (*KlHIS4*) gene promoter reveals relevant differences in comparison to the *Saccharomyces cerevisiae HIS4* homologous gene. Among them are the absence of a Rap1 binding site and the presence of only three putative Gcn4 binding consensus sites instead of the five described in the *S. cerevisiae* promoter. Since these factors are implicated in the general control, we investigated the transcriptional regulation of the *KlHIS4* gene under conditions of amino acid starvation and discovered that the mechanisms previously described for *S. cerevisiae HIS4* regulation and related to general control are not functional in *K. lactis*. The expression analysis of the *KlHIS4* gene under phosphate starvation or high adenine supply shows that factors, such as Bas1 or Bas2, involved in the basal control may also operate in a different way in *K. lactis*. Interestingly, and also in contrast to the *HIS4* regulation in *S. cerevisiae*, we found domains for Nit2-like and yeast-Ap1-like binding sequences. Northern analyses showed transcriptional activation under ammonia starvation and oxidative stress. The EMBL accession number for the *KlHIS4* promoter is AJ238494.

Key words: Transcriptional regulation; Histidine; Amino acid; *HIS4*; *Kluyveromyces lactis*

1. Introduction

The transcriptional regulation of the *Saccharomyces cerevisiae HIS4* gene has been studied extensively [1–3] and is currently probably one of the best characterised yeast genes of the amino acid biosynthetic pathways. Its expression is carried out under two well known mechanisms: 'general control' ensuring the activation of the gene during amino acid starvation, and 'basal control'.

S. cerevisiae adapts to conditions of amino acid starvation by increasing the transcriptional rate of at least 30 genes related to amino acid biosynthesis. This response, known as general control, is mediated by the Gcn4 transcriptional activator and can be invoked by starvation of even a single amino acid [4]. The *S. cerevisiae HIS4* promoter has five binding sites for the transcriptional activator Gcn4. When there is no amino acid starvation, the 'basal control' driven by factors Bas1 and Bas2(Pho2/Grf10) is responsible for *HIS4* transcriptional activation. Bas1 and Bas2 are able to bind independently to the *HIS4* promoter [1,5] and they also regulate the transcriptional activation of the *ADE* genes (*ADE2, 5* and *7*) when cells grow in an adenine-free medium. This cross-control is a consequence of the interplay between the histidine and purine biosynthetic pathways, which share common precursors and intermediates [6–8]. Bas2(Pho2), independent of Bas1, is also responsible for transcriptional regulation under phosphate limitation, as described in the *PHO5* promoter [9]. The *S. cerevisiae HIS4* gene, containing the Bas2 binding site, also responds to phosphate limitation [8].

Rap1 is a regulatory factor which has multiple roles in yeast cells, such as controlling telomere size [10,11], meiotic recombination [12], mating [13], Gcr1-mediated transcriptional activation of glycolytic genes [14,15] and also acting upon general and basal control of *HIS4* [2,16]. In the latter case, it is believed to allow the binding of other specific factors by maintaining an accessible chromatin structure [3].

Transcriptional regulation of amino acid biosynthetic genes from other yeast species, under conditions similar to those described above, is not as well documented. Although a similar regulation was first assumed, based only on the high homology of proteins encoded by homologous genes and on the assumption of a well conserved regulatory mechanism for a well conserved function, the small amount of experimental data available is in contrast with this simple model. The general control of amino acid biosynthetic enzymes is not commonly distributed within 15 yeast species [17]. Even this work did not include *Kluyveromyces lactis*, and was only based on enzymatic activities without including transcriptional studies on mRNA levels or promoter characterisations.

After cloning the *KlHIS4* gene by complementation, it was found to encode a protein with a 69% identity and 81% similarity to the multi-enzymatic protein encoded by the *S. cerevisiae HIS4* gene [18]. Herein we report the sequence of the promoter region which is responsible for its transcriptional regulation and necessary in order to allow histidine auxotrophy complementation in *S. cerevisiae*. Analyses of consensus sequences and quantification of mRNA levels show a divergent regulation of amino acid biosynthetic genes in *S. cerevisiae* and *K. lactis*.

2. Materials and methods

2.1. Yeast strains

K. lactis wild-type strain is NRRL-Y1140. Strains MW190-9B (*MAT*α, *lac4, uraA*), pM5-3C (*MATa, uraA, Rag*+) were kindly provided by M. Wésolowski-Louvel (Lyon, France). The *S. cerevisiae* strain MC1093 (*MATa, his4-539, lys2-801, ura3-52*) was a gift from M. Carlson, and *S. cerevisiae* aGHI (*MATa, trp1-289, leu2-3, leu2-112, gal* Δ*5*) from R. Zitomer [19].

2.2. Media and culture conditions

Yeast synthetic complete (CM) and omission media CM−His (without histidine), CM−Ura (without uracil) or CM−Ade (without

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Abbreviations: ORF, open reading frame; R, purines; Y, pyrimidines; W, adenine or thymine

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adenine) were prepared, in addition to YPD and YPG (with 2% dextrose or glycerol, respectively) [20]. For ammonia and phosphate starvation conditions yeast nitrogen base without ammonium sulphate or sodium phosphate was prepared.

In order to test oxidative stress conditions, hydrogen peroxide was added to a final concentration of 2 mM [21]. Cadmium sulphate was added to the cultures to a final concentration of 3 mM. In both cases cells were grown in CM medium and shifted for 1 h to CM+H_2O_2 or for 2 h to CM+cadmium sulphate.

DNA manipulations were performed as described in [22].

2.3. Sequencing

A 472 bp fragment was sequenced on both strands using the Sequenase kit from USB. For this purpose, serial nested deletions were obtained by DNase I treatment [23]. The sequence of the complementary strand was obtained by primer walking using the following oligonucleotides: oMFH19: 5′-AACACGGTACAACAGGCAAC-3′, oMFH21: 5′-GATTTTTGCAGTGGATTGTGA-3′, oMFH22: 5′-GACTGGATAAGTAAAGGTAA-3′, oMFH23: 5′-TTTCCTTGCATCGTCTA-3′ and oMFH24: 5′-CATTTTTAAGTCCGAGAA-3′.

2.4. Sequence analysis

Computer searches of binding sites for transcriptional factors were performed with the bioinformatic facilities of: YEAST-TOOLS (http:/alize.ulb.ac.be/~YRT), TFSEARCH (http:/pdap1.tcr.rwcp.or.jp/nph-tf) and TRANSFAC (http://padap1.tcr.rwcp.or.jp/nph-tf)

2.5. Northern analyses

Northern analyses were performed as previously described [24]. To facilitate the preparation of the *KlHIS4* probes, an internal *Hin*dIII–*Hin*dIII fragment of 0.9 kb from *KlHIS4* was cloned into pRS316 [25] as pM758. The *KlHIS4* probes were prepared either by PCR using *universal* and *reverse* primers and pM758 as a template or by *Hin*dIII digestion of the clone pM758.

For the loading correction, ribosomal RNA oligo-nucleotides RPL-5′-AGTTGGCTAGAGC and RPL-3′-GATTAAATGTAACCG, which hybridise with the *S. cerevisiae* 25S rRNA (RpL25), and also cross-hybridise with the *K. lactis* ribosomal RNA were used.

3. Results and discussion

3.1. Promoter sequence and consensus search

To define the upstream region capable of allowing the expression and regulation of the *KlHIS4* gene, the original clone PM756 was subcloned into the YCplac33 vector [26]. Plasmids containing the open reading frame and promoters of different sizes were obtained (Fig. 1). The *S. cerevisiae* strain MC1093 was used to test the complementation ability of these clones. Transformants were selected on CM−Ura medium and individual transformants replated in CM−Ura and CM−His. Cells transformed with the plasmid pKH4Pst, containing the smallest promoter, grew as well as the cells transformed with constructions containing longer promoters. Taking this result into account, we sequenced only the 472 bp upstream region of *KlHIS4* extending from the ATG start to the 5′ *Pst*I site (Figs. 1 and 2). This sequence is deposited in the data base (EMBL accession number AJ238494).

Computer searches on the *KlHIS4* promoter revealed the presence of consensus binding sequences for transcriptional activators involved in *S. cerevisiae HIS4* basal control (under non-limited amino acid conditions) (Fig. 2A). The sequence GACAAA for Bas1 binding [27] is present at position −396 and a sequence partially matching TGACTC, also defined as a Bas1 binding site [27], located at −382. Although the consensus for Bas2 (Pho2) binding was initially proposed as (T/C)TAA(T/A)T(T/G)AAT [16] or as AAATTAGTTAATTAATT [3], it does not have a very well defined consensus, and is generally assumed to be an A/T rich sequence [28]. A putative Bas2 binding site, AAATTcGTcccTTttaa, is located at position −365 in the promoter sequence and contiguous to the Bas1 site at position −382.

As regards putative sites for Gcn4 defined by the sequences TGACTC or TGAATCCAT [1] and more recently by the consensus RRTGACTCTTT [29], only three Gcn4 binding sites were found at positions: −415, −208 and −301 on the *KlHIS4* promoter, and only one is placed relatively close 3′ to the Bas1-Bas2 targets. In *S. cerevisiae* (Fig. 2B) there are five Gcn4 binding sites on the *HIS4* promoter but only one (denoted as sequence C, at position −194) is used preferentially; this C sequence partially overlaps with a Rap1 binding site. The latter factor is absolutely required for *HIS4* transcriptional activation in *S. cerevisiae* by both Gcn4 and Bas1-Bas2 systems. Although Rap1 binding sites may be found in the *K. lactis* promoter, they are highly degenerated. The *KlRAP1* homologue was cloned [30]; its binding sequence was defined as the consensus 5′-AYCYRTRCAYYW-3′. This consensus does not appear on the *KlHIS4* promoter.

Surprisingly, the consensus search provided information about the existence of putative binding sites for YAp1 (consensus TGASTCAG, TFSEARCH), a transcriptional activator in response to oxidative stress, at positions −419 and −206. The *YAP1* and *YAP2* genes play a major role in *S. cerevisiae* in regulating the adaptive response to oxidative stress [32]. A *YAP1* homologue has been cloned in *K. lactis* and its role in transcriptional activation through the Ap1-response element (ARE) when cells are exposed to H_2O_2 has been demonstrated [21]. This regulatory system also protects the *K. lactis* cells from cadmium damage since the Δ*KlAp1* mutant strain is hyper sensitive to this metal [21]. It is also interesting to take notice of the overlapping of the YAp1-like and the putative Gcn4 binding site at position −206. At positions −81 and −359 on the *KlHIS4* promoter we also found sequences similar to the *Neurospora* Nit2p and *S. cerevisiae* NIT family element (GATAAG) [29] binding sites related to nitrogen starvation (Fig. 2A). Since regulation by oxidative stress or nitrogen starvation has not been described for the *S. cerevisiae HIS4* gene, a search for the related factors was carried out in the promoter of the *S. cerevisiae* homologous

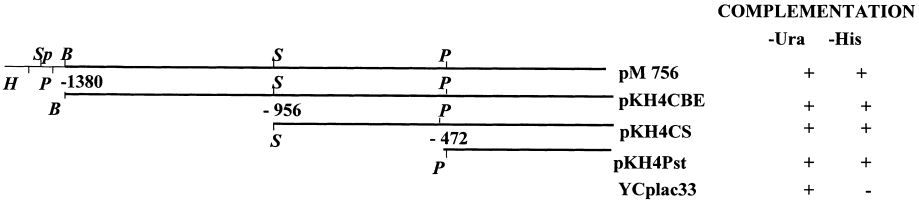

Fig. 1. Histidine auxotrophic complementation analysis of different *KlHIS4* size promoters. B, *Bam*HI; S, *Sal*I; Sp, *Sph*I; H, *Hin*dIII; P, *Pst*I.

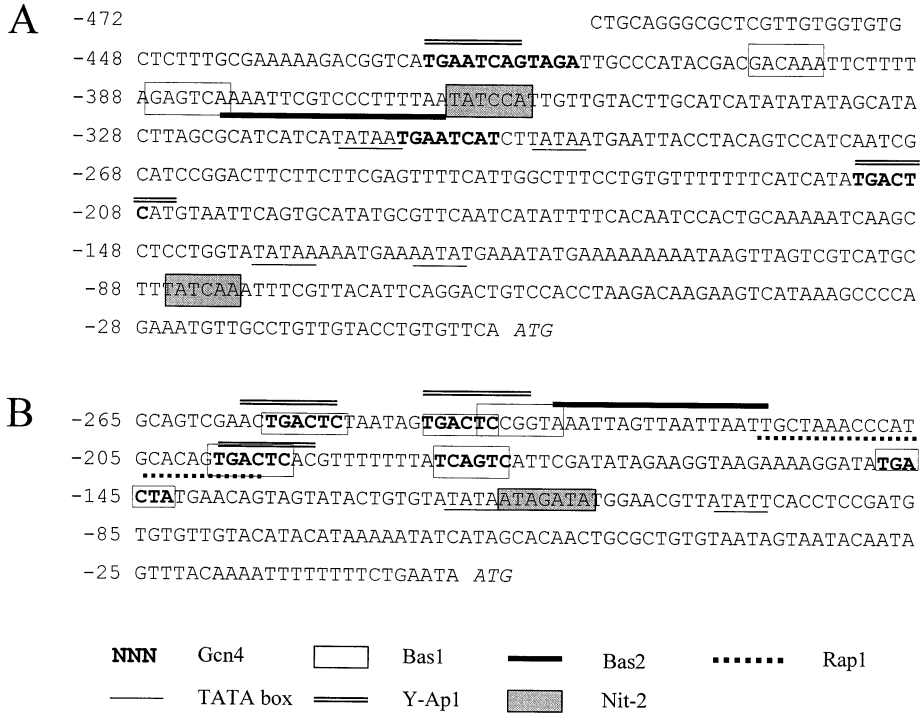

Fig. 2. A: Sequence of *KlHIS4* promoter and relevant consensus for transcriptional factors. B: *S. cerevisiae HIS4* promoter (EMBL J01331) [31] showing relevant *HIS4* regulatory elements [3,8] and positions for Nit-2-like and YAp1 consensus.

gene. The search revealed three YAp1 binding sites at positions −192, −235 and −248 and a single Nit2-like binding site at position −110 (Fig. 2B).

3.2. *Regulation of genes related to amino acid biosynthesis in K. lactis: general and basal control*

K. lactis NRRLY-1140 cells were grown in synthetic complete medium or under amino acid starvation conditions and *KlHIS4* mRNA levels were determined by Northern analyses. At least four independent experiments were done to test each condition. An insignificant increase (an average of 1.2 times) in *KlHIS4* mRNA levels was detected when histidine was omitted from the culture (Fig. 3A, lanes 3 and 4). In order to see if this absence of activation in response to the general control was strain-dependent, other *K. lactis* strains, PM5-C and MW190-9B, were tested with similar results (an average increase of 1.5; Fig. 3C). In a control experiment under identical laboratory conditions, a 10-fold increase was detected for the homologous gene *HIS4* from *S. cerevisiae* in strain aGH1 (Fig. 3A, lanes 7 and 8).

In *S. cerevisiae* other amino acid biosynthetic genes, including those involved in tryptophan and leucine production, are

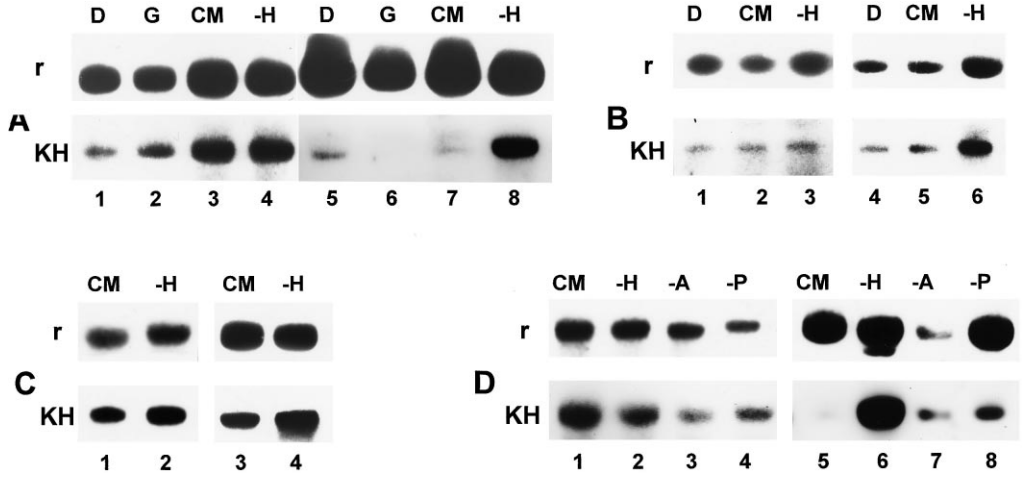

Fig. 3. Transcriptional analysis of general and basal control. A: Comparison of *K. lactis* NRRLY-1140 (lanes 1–4) and *S. cerevisiae* aGHI (lanes 5–8) response to carbon source and histidine starvation. B: Analysis of *KlHIS4* regulation in *S. cerevisiae his4* strain MC1093 (lanes 4–6), The *K. lactis* strain NRRL-Y1140 was included as control (lanes 1–3). C: Analysis of general control in other *K. lactis* strains: Mw190-9B (lanes 1,2), pM5-3C (lanes 3,4). D: Basal control in *K. lactis* NRRLY-1140 (lanes 1–4) and aGH1 strain (lanes 5–8). D = YPD; G = YPG; CM = complete medium; −H = CM−His; Cd = CM+cadmium; −P = CM phosphate-starved. Probes: r: ribosomal 25S; KH = *KlHIS4*.

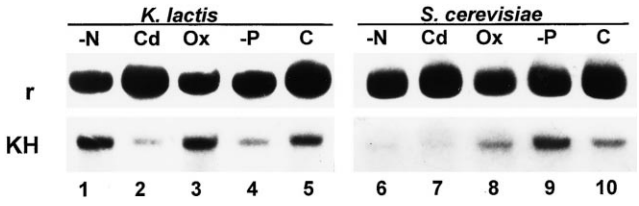

Fig. 4. Northern analysis showing *KlHIS4* and *ScHIS4* regulation under stress conditions and ammonia starvation. C = control culture (CM, complete medium); Ox = oxidative stress ($CM+H_2O_2$); Cd = CM+Cd; and −P = CM phosphate-starved. Probes: r: ribosomal 25S; KH = *KlHIS4*.

under regulation by general control and the corresponding promoters have Gcn4 binding sites. We selected two other *K. lactis* genes related to amino acid biosynthesis to check if they were activated during amino acid starvation but the results obtained (data not shown) indicated that the general control is not functional for these genes either. Computer analyses of *KlLEU2* [33] and *KlTRP1* [34] promoters showed only a Gcn4 binding site on each, probably too close to the ATG to be relevant. There are no Rap1 binding sites and several Nit2 sequences are distributed along both promoters.

As commented above (see Fig. 2A), only three Gcn4 binding sites were found at positions −415, −208 and −301 on the *KlHIS4* promoter, while in the *HIS4* gene from *S. cerevisiae* there are five. In order to see if the lack of regulation related to the general control could be attributed to the structure of the promoters or to a different action of *trans*-acting factors, the heterologous expression of the *KlHIS4* gene was determined in *S. cerevisiae* transformed cells. Strain MC1093 was transformed with the plasmid pKH4CBE, depicted in Fig. 1, containing a big (1380 bp) promoter driving *KlHIS4* expression. Transformants were selected on CM−Ura plates. Overnight cultures of independent transformants were done in CM−Ura and the next day cells were used to inoculate three different media, YPD, CM and CM−His and the cultures maintained at 30°C until A_{600} reached 0.6. Aliquots of each culture were plated on YPD and selective medium (CM−Ura) to verify that plasmid loss in non-selective (CM) medium was not significant. The results of the heterologous expression are shown in Fig. 3B and (after normalising values) clearly demonstrate that there is no significant transcriptional activation in CM−His medium (compare lanes 5 and 6). Therefore, although the *KlHIS4* gene is expressed in a yeast strain in which Gcn4 is functional, since aGHI is wild-type for this factor, the general control does not act on this promoter even in a *S. cerevisiae* genetic background. We might consider two possible explanations: one is the fact that there are only three Gcn4 putative binding sites in the *KlHIS4* promoter, however, although five sites exist in the *S. cerevisiae HIS4* promoter, only sequence C has a high affinity for Gcn4 binding and this site is responsible for the major activation [8]. Thus in *K. lactis* we would expect to see activation if any of the two sequences were functional. On the other hand, the fact that there is not a well conserved Rap1 binding site on the *KlHIS4* promoter may be an important point to take into account. In *S. cerevisiae* Rap1p has been shown to be necessary for basal and general control of the *HIS4* gene. In a wild-type background for Gcn4, Bas1 and Bas2, the presence of mutations in the Rap1 binding site cause poor growth on the medium without histidine and a drastic decrease in *HIS4* mRNA levels. Since the *RAP1* homologous gene has been cloned in *K. lactis*, we cannot attribute the lack of this factor in this species as an explanation for the absence of general control. On other hand, since the *K. lactis* gene, under regulation of its native promoter (without a well conserved Rap1 binding site), is expressed in *S. cerevisiae* conferring a His+ phenotype on these cells and maintaining its own transcriptional pattern (Fig. 3B), there are probably conserved mechanisms that would allow an adequate regulation. For instance, a different nucleosome positioning along the *K. lactis* promoter might allow its characteristic transcriptional expression.

It is also interesting to note the different behaviour of *KlHIS4* and *S. cerevisiae HIS4* in glucose or glycerol growing conditions. In *KlHIS4* there is a similar level in both carbon sources with an average increase of 1.5 times in glycerol growing cells (Fig. 3A, lanes 1 and 2). However, we find an average four-fold decrease in *S. cerevisiae HIS4* levels in glycerol growing cells (Fig. 3A, lanes 5 and 6). Even at this point it is not possible to offer an explanation based on our data or the sequences found or those on *S. cerevisiae* which we consider relevant to note.

The *KlHIS4* promoter contains Bas1- and Bas2-like binding sequences (Fig. 2A) and in *S. cerevisiae*, the existence of a transcriptional activation in the phosphate starvation medium which is mediated by *Bas2* [8] has been previously described. *Bas1* and *Bas2* acting together are common regulators of purine and histidine biosynthesis and a reduction of the protein encoded by *HIS4* was observed in cells grown in 0.15 mM adenine final concentration [6]. To test the importance of the basal control in the *KlHIS4* gene we also measured *KlHIS4* mRNA levels under these two conditions. For adenine repression 0.30 mM final adenine concentration was used in the CM medium. As shown in Fig. 3D, we did not find significant differences in mRNA levels as compared to the control medium in any of the conditions tested, just a slight decrease in adenine and phosphate starvation (compare lane 1 with lanes 3 and 4) (also for phosphate see Fig. 4, lanes 1 and 4), while a control carried out for the *S. cerevisiae* gene in the *S. cerevisiae* strain aGHI showed the expected regulatory pattern under these conditions (a three-fold increase under phosphate starvation and three-fold decrease for adenine repression) (compare lane 5 with lanes 7 and 8). Therefore, the basal control through the Bas factors previously described does not work for the *KlHIS4* gene.

3.3. The KlHIS4 gene is regulated by nitrogen source limitation and oxidative stress

The existence of putative binding sites for Nit2-like and YAp1 like factors in the *KlHIS4* promoter prompted us to check regulation by nitrogen source limitation, oxidative stress and Cd. Cells were grown in CM medium until A_{600} reached 0.5; they were then pelleted, washed and divided into four equivalent fractions to inoculate the CM medium (control), CM medium without ammonium sulphate (to check nitrogen limitation), CM medium plus cadmium or CM medium plus H_2O_2 (to test YAp1-like activation). Fig. 4 shows that there is a two-fold decrease in mRNA levels by cadmium (lane 2) and a significant increase (approximately two-fold) under nitrogen starvation and oxidative conditions (lanes 1 and 3) when compared with the control (lane 5). Since this regulation was not described in the literature for the *S. cere-*

visiae HIS4 gene, we carried out parallel experiments in the *S. cerevisiae* strain aGHI and found that any of these responses may be produced in this yeast (Fig. 4). And moreover, there is a 2.3-fold decrease under ammonia starvation.

The changes in mRNA levels observed in *K. lactis* are in agreement with the functional significance of the regulatory sites, Nit2-like and YAp1, found on the promoter. In *K. lactis* differences in the regulation of *KlHIS4* transcription are more relevant under nitrogen limitation or oxidative stress than under amino acid starvation. Since respiratory processes produce reactive oxygen species, in a respiratory yeast like *K. lactis* the utilisation of these molecules to activate the biosynthetic pathways and to allow rapid growth could be a useful regulatory mechanism. The extent to which it has physiological significance and could serve as a differential mechanism in respiratory versus fermentative yeast remains to be elucidated. However, in the course of this research we have observed a remarkable difference in the expression of the *HIS* genes in *K. lactis* and *S. cerevisiae* when comparing respiratory conditions (glycerol medium) and respiro-fermentative conditions (glucose medium). The expression of the gene from *S. cerevisiae* decreases four times in respiratory conditions, while in *K. lactis* there is some increase (1.6-fold) (Fig. 3A).

In summary, the expression of the *KlHIS4* gene differs notably from the one previously described for the *S. cerevisiae* homologue *HIS4*. First of all, the gene does not respond significantly to amino acid starvation (general control) adenine repression or low phosphate levels (basal control) and the absence of the general control may be extended to other genes from *K. lactis* related to amino acid biosynthesis. Secondly, the gene is activated by nitrogen limitation and oxidative stress, suggesting that the Nit-2-like and YAp1-like sites are functional, although further research needs to be carried out in order to characterise the key sites exerting this regulation. The different transcriptional regulations probably reflect the physiological differences in amino acid metabolism between the two organisms.

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