A comparative analysis of the GAL genetic switch between not-so-distant cousins: *Saccharomyces cerevisiae* versus *Kluyveromyces lactis*

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Abstract

Despite their close phylogenetic relationship, *Kluyveromyces lactis* and *Saccharomyces cerevisiae* have adapted their carbon utilization systems to different environments. Although they share identities in the arrangement, sequence and functionality of their GAL gene set, both yeasts have evolved important differences in the GAL genetic switch in accordance to their relative preference for the utilization of galactose as a carbon source. This review provides a comparative overview of the GAL-specific regulatory network in *S. cerevisiae* and *K. lactis*, discusses the latest models proposed to explain the transduction of the galactose signal, and describes some of the particularities that both microorganisms display in their regulatory response to different carbon sources. Emphasis is placed on the potential for improved strategies in biotechnological applications using yeasts.

1. Introduction

The introduction of the most modern molecular technologies in taxonomic analysis has unveiled the unarguable origin of *Kluyveromyces* and *Saccharomyces* genera from an ancient common ancestor [1]. Most of the *Kluyveromyces lactis* genes were isolated precisely because of their capacity to functionally complement mutations in *Saccharomyces cerevisiae* and vice versa [2]. It is therefore not surprising to find that entire sets of genes devoted to specific metabolic tasks have maintained an extensive degree of conservation between these two yeasts, as is the case for the group of regulatory and structural genes involved in the utilization of galactose (*GAL/LAC* regulon in *K. lactis*, or *GAL/MEL* in *S. cerevisiae*). Yet differences have arisen as a result of their further evolution in different environmental conditions. While glucose is the main carbon and energy source for *S. cerevisiae* within its ecological niche, *K. lactis* has instead adapted to the utilization of the main sugar present in the milk, the disaccharide lactose (O-β-D-galactopyranosyl-(1-4)-β-D-glucose).

The molecular basis of the GAL gene switch is perhaps one of the most-documented and best-characterized signaling pathways in *Saccharomyces* and *Kluyveromyces* (extensively reviewed in [3–6]). However, uncertainties about specific aspects of this regulatory network still remain. Several models have been proposed to explain one of the biggest and yet unsolved enigmas: the nature of the signal transfer from the initial sensor and inducer, *S. cerevisiae* (Sc)Gal3p (or *K. lactis* (Kl)Gal1p), to Gal80 repressor protein for transcriptional activation in the presence of galactose. Regulation...
by subcellular compartmentalization, the synthetic rate, posttranslational modification, and the turnover of these and other regulatory factors, are just a few examples of the yet missing pieces of this puzzle. The application of new technologies such as high-throughput genomic screens, biocomputational, proteomic, and microarray analyses will indeed help discover additional functions that complete the entire picture.

The purpose of this review is first to establish an updated comparison of the most controversial parts of the signaling pathways controlling galactose utilization in *S. cerevisiae* and *K. lactis*, emphasizing the newest findings and contrasting the latest theories. A second goal seeks to promote awareness that gaining a better understanding of the overall yeast metabolism contains inherent potential for developing new successful molecular and genetic strategies controlling yeast metabolic properties for the future improvement of biotechnological processes, such as cheese-whey conversion into high value-added products. Additional differences displayed by *Kluyveromyces* and *Saccharomyces* concerning their general carbon metabolism will not be discussed here but can be found reviewed in great detail in [5,7–9].

2. Catabolic genes of the GAL regulon

*S. cerevisiae* and *K. lactis* have developed specific mechanisms to obtain galactose from the extracellular or intracellular hydrolysis of galactose-containing disaccharides that are naturally found in their corresponding environments. *S. cerevisiae* may find galactose as such or in the form of melibiose (6-α-D-galactopyranosyl-D-glucopyranose). Melibiose is hydrolyzed to glucose and galactose through the action of a secreted α-galactosidase encoded by *MEL1* (Table 1; [10]). Galactose enters *Saccharomyces* cells through the galactose permease, Gal2p [11]. *K. lactis* usually finds galactose as such or in the form of lactose. The ability of *K. lactis* to assimilate lactose is determined by the presence of *LAC12* and *LAC4*, which respectively encode a lactose/galactose permease and a β-galactosidase that hydrolyzes internalized lactose into glucose and galactose [12]. *LAC12* and *LAC4* are divergently transcribed and coregulated by an intergenic promoter [13].

Once inside the cells, catabolism of galactose involves its conversion into the glycolytic intermediate, glucose-6-phosphate. In a broad spectrum of organisms, from bacteria to vertebrates, this conversion occurs through the concerted action of four catalytic steps, which constitute the highly evolutionarily conserved Leloir pathway (Table 1; [14]). The first step, carried out by the galactokinase Gal1p (EC 2.7.1.6), consists of the ATP-dependent phosphorylation of galactose to yield galactose-1-phosphate. The second step involves the action of a uridine diphosphoglucone 4-epimerase, Gal10p (EC 5.1.3.2), which exchanges galactose with the glucose group in UDP-glucose to create UDP-galactose, and then changes the stereochemistry of C4 in UDP-galactose to yield galactose-1-phosphate. The third step is catalyzed by the galactose-1-phosphate uridylyltransferase, Gal7p (EC 2.7.7.12), which uses the glucose released in the second step to transform galactose-1-phosphate into glucose-1-phosphate. Glucose-1-phosphate is converted

### Table 1

Specific genes of the yeast GAL regulon

| Gene | Category | Function | UASG | URSG |
|------|----------|----------|------|------|
| **(A) GAL/MEL regulon from S. cerevisiae** | | | |
| *MEL1* | C | α-Galactosidase; EC 3.2.1.22 | 1 | 1 |
| *GAL2* | C | Galactose permease | 2 | 0 |
| *GAL1* | C/R | Bifunctional sensor inducer/galactokinase; EC 2.7.1.6 | 4 | 1 |
| *GAL7* | C | Galactose-1-phosphate uridylyltransferase; EC 2.7.7.12 | 2 | 0 |
| *GAL10* | C | Uridine diphosphoglucone 4-epimerase; EC 5.1.3.2 | 4 | 0<sup>a</sup> |
| *GAL4* | R | Transcriptional activator | 0 | 1 |
| *GAL8* | R | Gal4p repressor | 1 | 0 |
| *GAL3* | R | Gal80 repressor (sensor/inducer) | 1 | 1 |
| **(B) GAL/LAC regulon from K. lactis** | | | |
| *LAC12* | C | Lactose/galactose permease | 4 | 0 |
| *LAC4* | C | β-Galactosidase; EC 3.2.1.23 | 4 | 0 |
| *KIGAL1* | C/R | Bifunctional sensor inducer/galactokinase; EC 2.7.1.6 | 4 | 1 |
| *KIGAL7* | C | Galactose-1-phosphate uridylyltransferase; EC 2.7.7.12 | 2 | 0 |
| *KIGAL10* | C | Uridine diphosphoglucone 4-epimerase; EC 5.1.3.2 | 4 | 0<sup>a</sup> |
| *KIGAL4* | R | Transcriptional activator | 1 | 0 |
| *KIGAL80* | R | Gal4p repressor | 2 | 0 |

Because of a more generalized role of *PGM2/GAL5* in carbon metabolism, this gene has not been included in this table.

* Catabolic (C) or regulatory (R) function.
* Confirmed Gal4p-binding sites (upstream activating sequence, UASG) are described in [4,13,20,23,26,30].
* Confirmed Mig1p-binding sites (upstream repressor sequence, URSG) are described in [77,78,85,91,94,96].
* URSG located in the *GAL1–GAL10* intergenic region is closest to *GAL1*. 
in a fourth step into glucose-6-phosphate through the action of the phosphoglucomutase, Gal5p (EC 2.7.5.1). In S. cerevisiae, the phosphoglucomutase function is in fact encoded by two different genes, PGMI and PGM2, which produce two electrophoretically separable isozyme forms [15]. PGM2 encodes the major isozyme form, since total variations in cellular phosphoglucomutase activity are primarily the result of regulation of this gene and its genetic product by different carbon sources [16,17]. The phosphoglucomutase reaction is not specific of the galactose pathway but instead plays a broader role in carbon metabolism [18]. Detailed schematic representations of the Leloir pathway can be found in [3,5].

In both S. cerevisiae and K. lactis, the Leloir genes are clustered on one chromosome, with a tight linkage between GAL7 and GAL10, and divergent transcription of GAL1 and GAL10 from an intergenic region [4]. PGM2/GAL5 is located on a different chromosome. As we shall see in the next sections, GAL1, GAL7, and GAL10, are subjected to a tighter regulation by galactose than PGM2/GAL5. Despite the strong similarity in these genes and their arrangement, S. cerevisiae and K. lactis display differences in their mode of regulation, which have important consequences in their overall response to carbon sources.

3. The rulers of the GAL game: interplay between inducer, repressor, and activator

In both S. cerevisiae and K. lactis, the induction of the GAL genes is determined by the interplay between three main GAL-specific regulatory proteins: a transcriptional activator, Gal4p; a repressor, Gal80p; and a ligand sensor, ScGal3p (or KlGal1), which activates GAL gene expression after binding galactose and ATP. ScGAL4 and its Kluyveromyces ortholog, KIGAL4/ LAC9, encode the GAL gene-specific transcriptional activator, Gal4p. In both organisms, Gal4p binds as homodimer to specific upstream activating sequences (UASG) in the GAL promoters (Table 1; [19,20]). Putative Gal4p binding sequences can be found in parts of the genome in which Gal4p binding is not actually detected. Additional factors, such as chromatin structure, must therefore contribute to the specificity of this interaction in vivo [21,22]. Gal4p seems to remain bound to the UASG sites of the GAL promoters in every condition, although different affinities depending on intrinsic features of the sequences and the available carbon source determine the relative strength of binding and proportion of occupied UASG sites [23,24]. ScGAL4 gene lacks Gal4p-binding sites in its promoter. Its expression is, therefore, constitutive and its activity is autoregulated only indirectly, through a direct control of the expression of its repressor, Gal80p [4]. KIGAL4 gene has a weak UASG binding site that allows an autoregulatory circuit of expression. This positive feedback loop is responsible for the two- to three-fold higher Gal4p concentration (compared to S. cerevisiae) observed in K. lactis in noninducing conditions [25,26]. In spite of their equivalent roles, ScGal4p and KgGal4p share only three regions of significant homology. These are involved in nuclear localization, DNA binding, oligomerization, and transcriptional activation [27,28]. The absence of a higher level of homology suggests that additional regulation specifically evolved by each of these yeasts may be contributing to differences in their Gal4p function by yet uncharacterized mechanisms.

GAL80 product has the main role of countering the activating function of Gal4p in the presence of carbon sources other than galactose [29]. The GAL80 genes of K. lactis and S. cerevisiae are both controlled by direct autoregulation. Two Gal4p-binding sites are located in the KIGAL80 promoter, whereas only one Gal4p-binding site is present in ScGAL80 (Table 1; [30]). Gal4p cannot activate transcription in the absence of galactose because of the particular nature of its association with Gal80p [3,4,31–33]. Both proteins bind stoichiometrically with high affinity [34–36]. Gal80p binds to a 28-amino-acid region inside the second activation domain of Gal4p, AR2, thereby physically blocking interactions of the activation domain with the transcriptional machinery [37]. For a complete inhibition, Gal80p dimerizes and interacts as such with the Gal4p dimer [38]. In fact, the extent of the repression seems to be dependent on the number of DNA binding sites for Gal4p. Adjacent sites allow for the stabilization of transient interactions between adjacent Gal80p dimers, causing a complete shielding of the activation domains of Gal4p. KgGal80p has a high degree of homology with ScGal80p, with an overall identity of 60% and two blocks with over 80% of identical residues [30]. An important difference with ScGal80p is that KgGal80p is regulated by phosphorylation, shifting to a KgGal1p-dependent, hypophosphorylated state upon induction [39]. While there is no evidence for a similar regulation occurring in ScGal80p, ScGal4p activity is indeed modulated by changes in its phosphorylation state, as discussed below [40,41].

In S. cerevisiae, Gal4p is released from Gal80p-mediated inhibition through the action of the ligand (galactose) sensor/inducer, Gal3p [42–44]. Induction can only take place when Gal3p binds to its two allosteric effectors, galactose and ATP. A viable structural model has recently been proposed to explain the activation of the sensor/inducer protein by galactose and ATP [45]. According to it, proteins from the galactokinase, homoserine kinase, mevalonate kinase and phosphomevalonate kinase (GHMP) family, are characterized by the presence of a deep cleft located between the N-terminal and the C-terminal domains. This cleft is formed by a flexible upper and lower lip and contains the active site.
In Gal3p, after binding to galactose and ATP, these lips would move towards each other creating a binding site for Gal80p.

*S. cerevisiae* mutant strains lacking Gal3p suffer a substantially delayed induction of the *GAL* genes in galactose (phenomenon known as ‘long-term adaptation’, reviewed in [6]). This delay is only temporary because Gal1p can replace Gal3p function. In fact, in conditions of induction, Gal80p can bind both Gal3p and Gal1p with 1:1 stoichiometry [46]. ScGal1p is bifunctional, presenting both galactokinase and ligand sensing activities [47,48]. Its ligand sensor activity is, however, weaker (40-fold less efficient) than that of Gal3p, replacing it only in later stages of the induction, when it becomes available at sufficient levels [49,50]. Although itself lacking galactokinase activity, ScGal3p has yet a 73% identity and 92% similarity to ScGal1p at the amino acid level [47,51]. In fact, the insertion of just two amino acids in a specific region can restore galactokinase activity in Gal3p [52]. Moreover, *K. lactis* lacks the *GAL3* gene. The ligand sensor is in this case also the bifunctional galactokinase-inducer, KIGal1p [48,53]. These facts suggest that *GAL1* and *GAL3* are paralog genes maintained through evolution with slight but significant differences in function.

An interesting hypothesis proposed to explain the replacement of Gal1p by Gal3p as a sensor/inducer in *S. cerevisiae* suggests that a simultaneous galactokinase activity could lead to a potentially toxic accumulation of galactose-1-phosphate at an early stage of induction in which the expression of the subsequent catabolic activities of the Leloir pathway has not been induced yet [48]. *K. lactis* would not face such a situation. In this case, *LAC4*, required for the hydrolysis of lactose into glucose and galactose, is coinduced simultaneously with the rest of *GAL* genes [53]. A low concentration of β-galactosidase at earlier stages of induction would therefore prevent the intracellular release of high levels of galactose and prevent the subsequent accumulation of a toxic excess of galactose-1-phosphate. Accumulation of galactose-1-phosphate, caused by the lack of functional galactose-1-phosphate uridylytransferase, leads to galactosemia in humans [54]. The exact mechanisms by which galactose-1-phosphate causes toxicity are still poorly understood, although a possible role of this molecule in the regulation of the inositol monophosphatase (IMPase) activity, which is critical for the sustenance of neurotransmitter functions in the brain, has been recently discussed [55].

4. Switching on and off the regulon

In spite of the obvious inability of *Saccharomyces* to use lactose as a carbon source, *S. cerevisiae* and *K. lactis* display no great differences in their respective growth rates when using separately glucose or galactose as the only carbon source (Table 2). Growth on glucose tends, in both yeasts, to be faster than growth on galactose. This is rather unsurprising since the conversion of galactose into a glycolytic intermediate needs energy and additional catabolic steps. As we shall see below, the induction of the *GAL* genes also involves a series of specific events that overall contribute to an elongated lag phase. *S. cerevisiae* shows a longer doubling time than *K. lactis* when growing in minimal medium with galactose as the only nitrogen source (Table 2). Such difference may partly arise as the result of different metabolisms, predominantly oxidative in *K. lactis* and fermentative in *S. cerevisiae* [7–9]. Differences in the growth rate on glucose may also be detected among different strains of *K. lactis*. These are in part caused by the inheritance of different sets of genes/alleles encoding hexose transporters, which not only determine the relative glucose uptake rate but also the ability of the different strains to repress genes in the presence of glucose [5].

From a regulatory point of view, three major states, according to the carbon source available, can be observed for the *GAL* genes in both *S. cerevisiae* and *K. lactis*: repressed in glucose, derepressed (noninduced) in respiratory carbon sources such as glycerol, and induced to high levels of expression in galactose (Fig. 1).

Table 2

| Strain | Medium | Lactose | Glucose | Galactose | References |
|--------|--------|---------|---------|-----------|------------|
| Sc W303-1A<sup>a</sup> | YP | – | 90 | 100 | This work |
| | SC | – | 105 | 279 | This work |
| Kl CBS 2359 | YP | 78 | 84 | 108 | [2] |
| Kl JA6 | SC | 98 | 102 | 103 | [78] |

<sup>a</sup> The doubling time is provided in minutes. Values obtained in this work represent the mean of three independent experiments.

<sup>b</sup> Growth cultures carried out in shake flasks at 28–30 °C in standard yeast extract-peptone (YP) or synthetic (SC) media supplemented with glucose, lactose, or galactose 2% (w/v) as the only carbon source.

<sup>c</sup> Genotype of W303-1A is *MAT α ura3-1 leu2-3 112 his3-11 15 trp1-1 ade2-1 can1-100*. Growth of the more standard S288C haploid lab strain is not shown because such strain carries a mutant *gal2* allele and therefore has a Gal<sup>–</sup> phenotype.
Fig. 1. A simplified model to explain the dynamics of *S. cerevisiae* GAL genetic switch in response to different carbon sources. The main effects of each carbon source are shown in red: (a) In the presence of respiratory carbon sources GAL/MEL regulon is uninduced. Low internal levels of the galactose permease Gal2p, the Leloir enzymes, Gal3p sensor/inducer, and Gal80p repressor, are present because of the low constitutive expression of the transcriptional activator, Gal4p (dimeric form represented as two orange rectangles). A certain production of internal galactose generated through the basal functioning of the Leloir pathway and the action of other uncharacterized enzymes (i.e. an UDP-galactose hydrolase and/or an UDP-galactose converting enzyme) may help to keep the system poised for induction. Levels of Gal3p are yet too low to cause a significant displacement of Gal80p equilibrium towards the cytosol, so the major effect preventing GAL induction is Gal80p repression exerted over Gal4p. Phosphorylation of the Mig1p repressor, mediated by Snf1p, is favored by the nuclear localization of this regulator kinase, which is positively controlled by its subunit, Gal83p. Mig1p phosphorylation disrupts its interaction with the co-repressor complex Cyc8-Tup1, which in turn favors the translocation of Mig1p back to the cytosol. (b) Galactose enters through the action of the basal levels of Gal2p. Galactose and ATP bind allosterically to Gal3p causing its activation, most likely through a conformational change. Gal3p binds Gal80p monomers in the cytosol, displacing Gal80p nuclear-cytosolic equilibrium towards the latter. Decreased amounts of nuclear Gal80p may hamper its dimerization and/or ability to interact with Gal4p bound to DNA. Gal4p bound to UASG (upstream-activating sequences) is then released from Gal80p repressor effect and can be accessed by the mediator complex/Gal11p and the RNA pol II transcriptional machinery. Srb10p phosphorylates Gal4p, causing its further activation and the GAL regulon is then fully induced. (c) Glucose entering the cells through the hexose transporters (Hxtp) causes inactivation of Snf1p. Snf1p inactivation allows interaction of the unphosphorylated Mig1p with Cyc8-Tup1. Mig1p, bound to this co-repressor complex and to specific upstream repressing sequences (URSG) in the GAL promoters, prevents Gal4p-directed activation by causing a decreased cooperative binding of Gal4p to UASG. Gal80p represses remaining DNA-bound Gal4p. The total of these overlapping effects causes a shut-off of the GAL regulon. Because of their high turnover, the levels of the Leloir enzymes are rapidly reduced. Gal6p could further enhance repression by controlling GAL mRNA levels and/or their translation rate.
4.1. Noninduced state

In respiratory carbon sources, such as glycerol or raffinose, only basal levels of the \textit{GAL} genes are being expressed. In this state the \textit{GAL} genes are poised for induction since they are not subjected to carbon catabolite repression. The basal levels of \textit{GAL} gene expression are more elevated in \textit{K. lactis} than in \textit{S. cerevisiae} because of higher levels of the protein KlGal4, caused by its positive feedback loop of autoregulatory expression [26]. Both \textit{S. cerevisiae} and \textit{K. lactis} gal80 mutants show constitutive expression of the \textit{GAL} genes to levels even higher than those of a fully-induced wild-type strain [4,26]. This fact indicates that under noninducing conditions, \textit{GAL80} expression is basically the only constraint preventing Gal4p-dependent induction of \textit{GAL} genes.

A certain level of internal galactose production seems to take place in noninducing conditions [56]. Mutants deficient in Gal7p have a semiconstitutive induced phenotype and increased internal levels of galactose. Based on this evidence, it has been suggested that the lack of Gal7p leads to an accumulation of UDP-glucose. A basal level of the epimerase Gal10p may convert this excess of UDP-glucose to UDP-galactose, which by the action of a yet-unknown enzyme would then be transformed to galactose (Fig. 1). This futile cycle of production/elimination of internal galactose could, in fact, be the key to a partial release from Gal80p repression that allows the system to remain poised for induction.

4.2. Induction by galactose

Since in noninducing conditions there is \textit{MELI} expression, \textit{Saccharomyces} cells are immediately able to generate galactose in the medium if in the presence of melibiose [4,10]. Galactose enters the cells through the presence of basal levels of Gal2p permease. In \textit{K. lactis}, the inducer galactose is mostly generated through the uptake and hydrolysis of lactose by basal levels of Lac12p and Lac4p [12,57,58]. Upon binding to galactose and ATP, ScGal3p (or KlGal1p) starts releasing Gal4p from Gal80-mediated inhibition through direct interaction with Gal80p.

Over the years, several models have been proposed to explain the mode of action of Gal3p. The idea that has prevailed for a long time was that Gal3p was involved in the synthesis of a small molecule (the co-inducer), which would allosterically interact with Gal80p to prevent Gal80p-mediated inhibition of Gal4p in the presence of galactose (‘catalytic model’, reviewed in [6]). Later on, it was demonstrated that a direct interaction between Gal3p and Gal80p is actually required for Gal4p activation [43,44]. The formation of a transcriptionally-active ternary complex Gal3-Gal80-Gal4p was then proposed [50]. It was also suggested that the interaction with Gal3p promotes Gal80p movement from the activation domain of Gal4p to a different part of the protein but not its dissociation [59]. Some of these models imply that Gal3p enters into the nucleus. However, further research has demonstrated that Gal3p is exclusively cytosolic and that even in constructs where it...
was tethered to the cytosolic face of the plasma and mitochondrial membranes it was still able to activate Gal4p [60,61], indicating that Gal3p-Gal80p interaction must occur in the cytosol. Interestingly, Gal80p is a nucleocytoplasmic protein and its Gal3p binding site overlaps with one of its putative nuclear localization signals [60,62]. Most recently, evidence has been provided to support a model in which the cytosolic binding of Gal3p to monomeric Gal80p competes with Gal80 self-association, thereby reducing nuclear levels of dimeric Gal80p, the form involved in the inhibition of Gal4p (Fig. 1; [33]). Similar conclusions have been reached through mathematical simulations and steady-state modeling [63,64]. These suggest that stochastic variations in the nuclear concentrations of the repressor Gal80p may account for the activation of the GAL genetic switch and that a Gal3p-dependent cytosolic sequestration of Gal80p may subsequently shift Gal80p equilibrium towards this compartment. Further supporting this possibility, Gal3p concentration exceeds Gal80p concentration by five-fold in conditions of induction [61]. It is likely that ScGal1p and KlGal1p act on Gal4p activation through a very similar process. In fact, the control of nuclear-cytosolic shuffling of transcriptional regulators through their sequestration into cytosolic complexes with inhibitory proteins is a very common mechanism to regulate gene expression, for which examples abound in the literature. Nuclear localization of the transcriptional factor Gln3p, necessary for activation of the nitrogen catabolite-repressed (NCR) genes, depends on its regulated release from an inhibitory complex with the repressor protein Ure2 [65]. Similarly, activation of the immune and inflammatory response in mammalian cells depends on the stimulated release of NFκB transcriptional factor from its cytosolic complex with the repressor IκB [66]. By whatever mechanism, the fact is that ScGal3p (KlGal1) interaction with Gal80p must provoke structural changes in Gal4p that increase its accessibility to proteins of the general transcriptional machinery. ScGal4p becomes phosphorylated on multiple serines while in its transcriptionally active state [4,67]. The phosphorylation is mediated by the cyclin-dependent protein kinases of the RNA Pol II holoenzyme [68]. In particular, the phosphorylation of S699 by the holoenzyme-associated cyclin-dependent kinase Srb10p is necessary for full GAL gene induction [69]. ScGal11p and its K. lactis homologue, as members of the mediator complex for transcriptional activation, have an additional subsidiary role. By interacting with Gal4p, the mediator helps to activate the transcription of at least some of the GAL genes [4,70].

Multiple signals and components intervene simultaneously in Gal4p-regulated expression upon induction. For instance, the recruitment of 19S proteasome subcomplex to the GAL1–10 promoter by Gal4p is a necessary step for RNA polymerase II-directed transcriptional elongation [71]. Later evidence has shown that Gal4p undergoes transcriptionally-dependent ubiquitination and that this step is necessary for the further activation of Gal4p target genes [72]. These findings support the hypothesis that transcriptional factors must be inactivated/degraded in situ, right after recruitment of transcriptional machinery, in order to allow later stages of transcription to proceed. Interaction of Gal4p with auxiliary factors also alters the chromatin structure in GAL gene promoters. The Swi/Snf global activators are involved in GAL expression [4]. It has recently been shown that Gal80p interaction with Gal4p prevents GAL gene activation by blocking interactions between Gal4p and the SAGA and Nua4 HAT complexes [73]. An exhaustive review of the nucleosomal changes and chromosomal architecture of the GAL promoters in response to carbon sources can be found in [74].

S. cerevisiae GAL2, GAL1, GAL7, and GAL10, are induced by more than 1000-fold, and MEL1 by more than 100-fold [4]. PGM2/GAL5 shows an unusually high, GAL4-independent, basal level of transcription and its regulation by galactose is considerably weaker than that of the other GAL genes, being induced by only three to four-fold [16]. GAL3 and GAL80 are induced by only three to ten-fold, but these levels are enough for them to carry out their corresponding regulatory roles [4]. In K. lactis, the maximum levels of induction for LAC12, LAC4, and the Leloir genes are only 125–150 higher than the already high basal noninduced levels [57,58]. But, overall, both yeasts must reach similar levels of GAL proteins upon induction.

Maintaining induction requires the constant presence of galactose since Gal proteins usually are rapidly turned over [3]. Upon reaching stationary phase both yeasts experience a final shutting-off of the system. Galactose itself is responsible since, for instance, in K. lactis the induction has caused the levels of KlGal80p to increase by more than 70-fold. The reduction in galactose concentration progressively alleviates KlGal1p-mediated inhibition of KlGal80p, which in turn increases again repression over KlGal4p [30]. A similar effect switches off the GAL/MEL regulon in S. cerevisiae, although in this yeast the ScGal80p level has only been induced by five to ten-fold. This level is nevertheless sufficient to reduce the constitutive lower levels of active ScGal4p.

4.3. Glucose repression of the GAL genes

For S. cerevisiae, glucose is the favorite carbon source. Mechanisms have therefore evolved to shut off the expression of genes involved in the utilization of alternative carbon sources in the presence of this monosaccharide (most recently reviewed in [75,76]). The presence of glucose triggers a signaling cascade that, among other effects, results in Mig1p-dependent repression of
the GAL genes [77–79]. Carbon sources regulate the subcellular localization of the transcriptional repressor Mig1p, a Cys2-His2 zinc-finger DNA-binding protein, by controlling its nuclear-cytosolic localization. Mig1p nuclear concentration increases within minutes when exposed to glucose but rapidly decreases upon glucose depletion [80]. Nuclear export of Mig1p depends on the nuclear exportin, Msn5p [81]. Its nuclear import occurs by a poorly understood mechanism that may involve the importin Cse1p [75]. Increased intranuclear levels of phosphorylated Mig1p in the absence of glucose favor its cytosolic export by Msn5p [81]. The central regulator Snf1 kinase complex is largely responsible for the nuclear phosphorylation of Mig1p [82]. Snf1 is a heterotrimeric enzyme complex composed of a catalytic α-subunit, Snf1p, a γ-subunit, Snf4p, which may be required for the release of the kinase from autoinhibition, and one of the three related β-subunits controlling its intracellular localization, Gal83p, Sip1p, or Sip2p [75]. Gal83p is necessary for the nuclear localization of Snf1 in the presence of a nonfermentable carbon source, which explains the positive effect of this subunit in glucose derepression of the GAL genes [83]. Mig1p exerts its inhibitory action through binding to specific upstream repression sequences, (URS)G, present in the glucose-repressible promoters (Table 1; [77,84,85]). Mig1p-mediated transcriptional inhibition is also dependent upon its interaction with the general corepressor complex Cyc8/Ssn6-Tup1 [77,86,87]. It was initially believed that Mig1p was necessary for the recruitment of the Cyc8-Tup1 complex to DNA and that the relocation of Mig1p to the cytosol resulted in dissociation of Cyc8-Tup1 from the target promoter, which ultimately alleviated repression [77,86–88]. However, it has been recently demonstrated that Mig1 and Cyc8-Tup1 proteins remain tethered to the GAL1 promoter under both repressing and activating conditions, and that Cyc8-Tup1 tethering to DNA promoters is not dependent upon previous binding of Mig1p [89,90]. In mutants lacking Msn5p, Mig1p remains nuclear but GALI transcription is still inducible [81]. Altogether, these facts suggest that Mig1p translocation is not the key event by which Snf1-mediated phosphorylation of Mig1p causes derepression of GAL and other genes. Recent results indicate instead that Snf1-dependent phosphorylation of Mig1p causes derepression by disrupting Mig1p interaction with Cyc8-Tup1 [90]. By promoting its release from interaction with this DNA-tethered corepressor complex, phosphorylation of Mig1p may indirectly increase Mig1p chances to be exported back to the cytosol. This would explain the apparent dependence of Mig1p translocation upon previous Snf1-dependent phosphorylation of the repressor ([81]; Fig. 1).

It is estimated that, in the presence of glucose, Mig1p-binding to the URSG site in the GAL4 promoter results in about five-fold decreased levels of Gal4p expression [84,91,92]. This causes decreased cooperative Gal4p binding to multiple UASG sites, an effect that accounts for a 40-fold repression of the GAL structural genes. Binding of Mig1p to URSG present in the promoters of genes such as GALI causes another four-fold repression. Gal80-mediated inhibition reduces expression by another five- to ten-fold [92,93]. Altogether, these effects account for the more than 1000-fold repression by glucose observed for most of the GAL genes. Although no URSG has been found near the promoters of GAL10 and GAL7, these are as strongly glucose-repressed as GALI [94,95]. This suggests that the presence of URSG in the promoters of regulatory genes like GALI, may affect Gal4p ability to activate the remaining GAL genes not only because of reducing GAL4 expression itself but also because Mig1p prevents further synthesis of the Gal80p-inhibitory functions, Gal1p and Gal3p. Binding of Mig1p to the GAL promoters seems to be required only at an early stage of repression, but not to maintain the repressed state [96]. As mentioned above, a fast turnover of the Gal proteins ensures a rapid shut-off of the system soon after their expression is repressed. For instance, glucose triggers Gal2p ubiquitination, endocytosis, and targeting to the vacuole for degradation, thereby reducing the entrance of the inducer galactose into the cells [97].

K. lactis is mostly adapted to the combined use of glucose and galactose, originating from lactose hydrolysis, as primary carbon sources. It is therefore not surprising to find that glucose represses expression of the GALIAC regulon in some, but not all, of the K. lactis strains and that even in the glucose-repressible strains the extent of the repression is less pronounced than in S. cerevisiae [98]. Part of the reason why strains with different degrees of glucose-sensitivity exist depends on differences in the KlGal4p binding sites. Some repressing and non-repressing strains differ by two bases in the KIGAL4 promoter [25]. The repressor allele possesses a promoter with lower affinity for KIGal4p binding. Glucose and galactose generated by the hydrolysis of lactose must compete in their repressing/inducing antagonizing effects. The overall output depends, at least partially, on the strength of the corresponding KIGAL4 allele to bind its own gene product in the autoregulatory circuit of expression [26]. In accordance, moderately increased dosages of KIGal4p expression are sufficient to override repression.

However, there are additional factors accounting for the relative response to glucose in different strains of K. lactis. Although K. lactis has homologues of the S. cerevisiae SNF1 (KISNF1/FOG2), MIG1 (KIMIG1), and GAL83 (FOG1) genes, the way in which their genetic products are integrated into the regulatory circuits is different for each regulon [78,79,99]. Although Fog1p and KlSnf1/ Fog2p are necessary for full induction and KlMig1p for full glucose repression...
of the GAL/LAC regulon, pathways operating independently of these functions must also exist in *Kluyveromyces* [78, 99]. After screening all GAL promoters only one KIMiglp-binding site has been found in *KIGAL1* (Table 1; [78]). This fact additionally contributes to a weaker regulation by glucose in *K. lactis* compared to *S. cerevisiae*.

Recent studies have made evident that the overall affinity in the transport of glucose determined by different alleles of the hexose transporter genes *KHT1, KHT2*, and *RAG1*, also plays a key role in the relative sensitivity of the different *K. lactis* strains to glucose repression (reviewed in [5, 9]). Strains that are most responsive to glucose repression have the hexose transporter genes, *KHT1* and *KHT2*, which are closely linked and tandemly transcribed [98, 100]. Strains with lower sensitivity to glucose repression only have the gene *RAG1*, which encodes a low-affinity glucose transporter and maps to the same chromosomal locus as *KHT1* and *KHT2* [101]. *RAG1* is almost identical to *KHT1* and evidence indicates that *RAG1* arose by recombination between *KHT1* and *KHT2* [100]. A natural isolate of *K. lactis* has been found which is totally insensitive to glucose repression and it has been shown to carry a defective ragl allele [102].

The existence of glucose transporters with an important effect in glucose repression obviously reminds us that *Kluyveromyces* must obtain glucose by sources other than the intracellular hydrolysis of lactose. Within its ecological niche this yeast must coexist with other microorganisms and developing systems for the transport of glucose increases its chances to metabolize the carbon sources existent in the medium (e.g. by taking profit of the glucose resulting from the extracellular hydrolysis of lactose carried out by other microorganisms). In fact, the more efficient uptake and utilization of glucose displayed by some strains of *Kluyveromyces* is one of the main reasons that make this organism a major causative of contaminations in industrial cultures of *S. cerevisiae* [7].

New functions with roles in the GAL gene switch are now starting to emerge. For instance, MRG19 seems to be necessary to induce GAL gene expression in the presence of low concentrations of galactose [103]. GAL6 encodes a cysteine peptidase of poorly characterized function with a role in the downregulation of the GAL system. This gene seems to be regulated in a similar manner as GAL80, since its promoter contains an UASG [104]. Gal6p exhibits sequence-independent binding to nucleic acids and its cytosolic localization suggests a role in the regulation of GAL mRNA levels [105, 106]. Imp2p is another new example of a transcriptional factor with a Mig1 and Nrglp negative repressors-dependent and Gal6p-independent positive effect on glucose derepression of GAL genes [107, 108].

5. How to apply the lessons learned

The characterization of the GAL genetic switch in yeast has unveiled an extensive number of possibilities to take advantage of this particular genetic system. The best example is indeed reflected in the discovery of the possibility to separately express active DNA binding and activating domains of the transcriptional factor Gal4p, which inspired the design of one of the most powerful technologies to detect protein–protein interactions: the two-hybrid analysis [109]. The tight control by carbon source to which GAL promoters are subjected also makes them invaluable tools for heterologous protein expression in biological studies and in high-scale production of proteins of industrial interest [110].

Some of the recent discoveries concerning the functioning of the GAL regulon have led to the development of interesting strategies that significantly improve the efficiency in the use of GAL promoter-based systems for heterologous expression. Vectors have been created that allow the amplified expression of the *S. cerevisiae* Gal3, Gal80, and Gal4 proteins [111]. Their utilization has increased the levels of GAL promoter-directed protein expression by fifteen- to thirty-fold without causing significant disturbances in the log-phase growth or the kinetics of galactose induction of the chromosomal GAL genes. Another approach, consisting of knocking out the genes encoding for the three most important negative regulators, Gal6p, Gal80p, and Mig1p, has enabled a 41% increased flux through the galactose utilization pathway towards ethanol formation [112]. The need to add high concentrations of galactose, a rather expensive carbon source, into the medium can now be avoided through the utilization of mutant strains able to induce the GAL genes but unable to metabolize galactose [48, 113, 114]. Additional information regarding the quantitative effect that deleting/overexpressing different genes may have in GAL promoter-inducible expression can now be extracted from the combined use of reporter genes such as *GFP*, which will further help to design new strategies based on metabolic engineering [115].

Both *K. lactis* and *S. cerevisiae* are classified as GRAS (generally regarded as safe) organisms by the United States Food and Drug Administration, and are therefore applicable in food- and pharmaceutical-related industrial processes [116]. Their ability to assimilate lactose and galactose has therefore provided additional usefulness for the GAL genes in the elimination of hazardous industrial residues such as cheese- whey, a byproduct from cheese manufacturing produced in vast amounts worldwide which owes most of its biological oxygen demand (BOD) to a high content in lactose [117, 118]. Different species of *Kluyveromyces* are widely used for the conversion of lactose into commercially useful products [119, 120]. Nevertheless, strategies have been developed to transfer the ability to use lactose to *Saccharomyces*,
with the aim to combine some of the most useful properties from both yeasts into just one organism. Generation of hybrids between *K. lactis* and *S. cerevisiae* by protoplast fusion was first approached to meet this end [121]. Later advances in genetic engineering technologies have allowed the construction of *S. cerevisiae* Lac+ strains through genetic transformation.

Attempts to generate lactose-assimilating strains through genetic engineering can be categorized into two main strategies depending on whether they involve the extracellular or intracellular hydrolysis of lactose. Methods based in the extracellular hydrolysis of lactose involve the generation of autolytic strains that release intracellular β-galactosidase to the medium [122–124], or the construction of strains expressing heterologous secretable β-galactosidase [125,126]. Methods based in the second strategy involve the expression in *S. cerevisiae* of genes that provide it with a system for the permeabilization and intracellular hydrolysis of lactose. The earliest successful attempt consisted of the expression of genes from an endogenous lactose-assimilating strain has made possible the construction of *S. cerevisiae* Lac+ strains [129,130], and their insertion in multiple copies within non-coding regions of the *RDN1* locus to obtain high levels of expression and a mitotically stable phenotype [128]. Additional properties of industrial interest, such as flocculence, have been coupled to the use of *LAC4–LAC12* genes to further improve the performance of the resulting strains [129,130].

The transfer of the genetic system developed in [128] into a baker’s *S. cerevisiae* strain has made possible the production of bread by yeasts grown on cheese-whey, enabling not only the simultaneous elimination of this hazardous residue but also providing industries with an alternative growth substrate [131]. The use of more traditional substrates like molasses, which contain complex mixtures of carbohydrates, often has undesirable effects in fermentation processes (e.g. slow or incomplete fermentation, ‘off flavors’, poor maintenance of yeast vitality, etc.) because of the combined effect that these carbohydrates have in different cellular pathways (reviewed in [76]). For instance, the presence of high concentrations of glucose represses the expression of genes for the use of alternative carbon sources and these are not therefore used until glucose in the medium is depleted. This produces diauxic growth and lower yields in ethanol and biomass. For similar reasons, the utilization of the intracellular lactose-hydrolysis strategy for the generation of *S. cerevisiae* lactose-assimilating strains is, in general, more advantageous than the extracellular approach since glucose generated intracellularly is rapidly assimilated, never reaching concentrations as high as to cause total repression of the galactose utilization pathway. These strains are generally able to grow faster, producing higher yields in biomass [128] and ethanol [126,130].

Additional studies derived from the utilization of the most modern technologies, such as two-hybrid, microarray analysis, and mass spectrometry, coupled to bioinformatic analysis, are now further helping to elucidate not just the role of novel gene functions in the GAL genetic switch, but also how perturbations of this system globally affect the expression and protein levels of components from other metabolic pathways [24,132–134]. Integrated genomic and proteomic analysis in yeasts like *S. cerevisiae* and *K. lactis* will indeed facilitate behavioral predictions of different regulatory networks in response to determined stimuli, which in turn will help to improve the cost and efficiency of their utilization for biotechnological purposes.

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