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DISSERTATION

Molecular characterization of initial steps in carbohydrate metabolism in *Hypocrea jecorina* and their role in cellulase induction

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PREFACE

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This work is dedicated to my son Noah and to my deceased father, may he rest in peace.

but I found out the hard way, nothing is what it seems
(from “Duality” by Slipknot)

ABSTRACT

Sugar kinases play a central role in carbohydrate metabolism, since they phosphorylate sugars after uptake in order to trap them inside the cell and prepare them for further catabolism. The pyrenomycetous ascomycete *Hypocrea jecorina* features amongst other sugar kinases one galactokinase (*gal1*), one hexokinase (*hxk1*) and one glucokinase (*glk1*). The role of these enzymes in carbohydrate metabolism and signalling was investigated. The single galactokinase of *H. jecorina* is responsible for the first step of the Leloir pathway, the phosphorylation of D-galactose to D-galactose 1-phosphate. Induction of the other genes of the Leloir pathway is independent from the *H. jecorina* GAL1, which is in contrast to the situation in yeasts such as *Saccharomyces cerevisiae* or *Kluyveromyces lactis*, where the galactokinase acts as a regulatory protein and activates the transcription of the *GAL* pathway in the presence of D-galactose. We could show that galactokinase activity, but not the GAL1 protein itself is essential for induction of cellulases on lactose by replacing the *H. jecorina* GAL1 with the *Escherichia coli* galactokinase. Additionally we present evidence for the existence of a second D-galactose degrading pathway which proceeds via reduction of D-galactose to galactitol. The enzyme responsible for the second step in this pathway was identified as the L-arabinitol 4-dehydrogenase (*lad1*), since a Δ*gal1*Δ*lad1* strain was unable to grow on galactitol or D-galactose as carbon source.

To facilitate successive gene knock-outs in *H. jecorina* we applied a blaster cassette approach. This cassette consisting of the *H. jecorina pyr4* gene flanked by two direct repeats was used to delete the single hexokinase and glucokinase in *H. jecorina*. The cassette was removed after deletion of the glucokinase encoding gene to construct a strain deleted in both hexo- and glucokinase. Strains deleted in both genes were unable to grow on D-glucose and also showed a defect on a number of other carbon source. Using three genetic systems, diagnostic for

carbon catabolite (de)repression (cellulase formation, *cbh1*; xylanase formation, *xyn1*; *β*-galactosidase formation, *bga1*), we demonstrated, that only a deletion of both kinases, *hxk1* and *glk1*, leads to derepression, suggesting a catalytic effect to be responsible. Interestingly the induction of *cbh1* on the cellulase inducer sophorose and *bga1* on D-galactose were impaired in the Δ*glk1*Δ*hxk1* strain, while the transcript levels of *xyn1* on D-xylose were increased in both single and the double deletion strains.

ZUSAMMENFASSUNG

Zuckerkinasen spielen eine zentrale Rolle im Kohlenhydratstoffwechsel, da sie aufgenommene Zucker phosphorylieren, um ein Entweichen aus der Zelle zu erschweren und sie für den weiteren Abbau vorzubereiten. Der zur Gattung der Ascomyceten gehörende Pyrenomycet *Hypocrea jecorina* besitzt eine Galaktokinase (*gal1*), eine Hexokinase (*hxk1*) und eine Glukokinase (*glk1*). Es wurde die Rolle dieser Enzyme im Kohlenhydratstoffwechsel allgemein und im Speziellen in Bezug auf die Signalwirkung untersucht.

Die Galaktokinase von *H. jecorina* (GAL1) ist verantwortlich für den ersten Schritt des Leloir Weges und phosphoryliert D-Galaktose zu D-Galaktose 1-phosphat. Im Unterschied zu Hefen wie *Saccharomyces cerevisiae* und *Kluyveromyces lactis*, in denen die Galaktokinase als Regulatorprotein fungiert und die Aktivierung der Transkription der *GAL* Gene in Gegenwart von D-Galaktose bewirkt, ist in *H. jecorina* die Induktion der weiteren Gene des Leloir Weges unabhängig von GAL1. Durch das Ersetzen der *H. jecorina* GAL1 durch die Galaktokinase von *Escherichia coli* konnten wir zeigen, dass Galaktokinaseaktivität, aber nicht das GAL1-Protein essentiell für die Induktion der Zellulasen auf Laktose ist. Zusätzlich präsentieren wir Belege für die Existenz eines zweiten D-Galaktose Abbauweges, der über die Reduktion von D-Galaktose zu Galaktitol verläuft. L-Arabinitol 4-Dehydrogenase (*lad1*) ist für den zweiten Schritt in diesem Weg verantwortlich, weil ein Δ*gal1*Δ*lad1* Stamm weder auf Galaktitol, noch auf D-Galaktose wachsen konnte.

Um eine wiederholte Gendeletion zu erleichtern, wurde die so genannte Blaster Methode verwendet. Die entsprechende Blaster Kassette besteht aus dem *H. jecorina pyr4* Gen, flankiert von zwei identischen Sequenzen. Diese Kassette wurde verwendet, um die Gene für die Glukokinase und die Hexokinase auszuschalten. Nach der Deletion des Glukokinasegens wurde sie wieder entfernt, um sie ein zweites Mal für das Hexokinasegen zu verwenden.

Stämme, in denen beide Gene deletiert sind, konnten auf D-Glukose, aber auch auf einer Reihe von anderen C-Quellen nicht mehr wachsen. Unter Verwendung von drei Genen, die der Carbon Catabolit (De)repression unterliegen (Zellulase Bildung, *cbh1*; Xylanase Bildung, *xyn1*; *β*-Galaktosidase Bildung, *bga1*), haben wir gezeigt, dass nur eine Deletion von beiden Genen, *hxk1* und *glk1*, zur Derepression führt, was einen katalytischen Effekt nahe legt. Interessanterweise war auch die Induktion von *cbh1* auf Sophorose und von *bga1* auf D-Galaktose im doppelt deletierten Stamm beeinträchtigt, während die Transkription von *xyn1* auf D-Xylose sowohl in den einzeln deletierten sowie im doppelt deletierten Stamm erhöht war.

CONTENTS

Chapter 1

General Introduction

Carbon, being essential for all known living systems, is present in the biosphere, geosphere, hydrosphere and atmosphere of the earth and exchanged between these pools by the carbon cycle. Carbon dioxide is released into the atmosphere e.g. by respiration performed by plants and animals, by decay of animal and plant matter, and by combustion of organic material, to name a few. It is the role of plants to bind carbon dioxide and to convert it into carbohydrates by performing photosynthesis, releasing oxygen during this process. These carbohydrates, mostly polymeric sugars, represent a rich source of nutrition for various other organisms, ranging from herbivores down to microorganisms like bacteria and fungi. Enzymes for their degradation are secreted into the extracellular environment, and only mono- and disaccharides are usually taken up by the microbial cells (Aro et al., 2005).

The three sugar kinase families and their enzymatic properties

Microbes do not accumulate high amounts of free sugars as their reducing ability, which can give rise to the formation of Schiff's bases with protein amino groups, presents a potential threat to the cell (Szwergold, 2005). To this end, and in order to trap them inside the cell, they are quickly phosphorylated during or after entrance (the negatively charged phosphate group prevents passing the plasma membrane). Phosphorylation also serves as a tagging for further chemical reactions. This phosphorylation can be achieved by two different means: by the bacterial PEP-transferase system, which transfers phosphate to sugars and polyols during their transit through the cell membrane (Deutscher et al., 2006); and by sugar kinases, which have a high affinity for their respective substrates, thus ensuring that the stationary concentration of the sugar remains low. These sugar kinases can be divided into at least three distinct nonhomologous families, each of them having a specific three-dimensional structure (reviewed by Bork et al., 1993).

The hexokinase family

The first is the hexokinase family. It contains many prokaryotic and eukaryotic sugar kinases differing in their substrate specificities, but all of them using D-glucose as a substrate. Hexokinase is an important enzyme that catalyzes the ATP-dependent conversion of aldo- and keto-hexose sugars to the hexose 6-phosphate. It can catalyze this reaction on D-glucose, D-fructose, D-mannose, sorbitol and D-glucosamine, which makes it the first enzyme in a number of metabolic pathways (Griffin et al., 1991).

The enzyme is widely distributed in eukaryotes. In *Saccharomyces cerevisiae* there are three isoenzymes of hexokinase (Hxk1p, Hxk2p and Glk1p) (Rodriguez et al., 2001). The isoenzymes Hxk1p and Hxk2p can phosphorylate aldo- and keto-sugars, while Glk1p is specific for aldo-hexoses. All three enzymes exhibit two domains (Griffin et al., 1991). Structural studies of Hxk2p revealed a well-defined catalytic pocket which binds both, ATP and hexose, and therefore allows an easy transfer of the phosphate from ATP to the sugar (Kuser et al., 2000). Vertebrates feature four hexokinase isoenzymes, termed I to IV, where types I to III contain a duplication of the two-domain yeast-type hexokinases. Both the N- and C-terminal halves bind hexose and hexose 6-phosphate, though in types I an III only the C-terminal half supports catalysis, while both halves support catalysis in type II. The N-terminal half is the regulatory region. Type IV hexokinase is similar to the yeast enzyme in containing only the two domains, and is sometimes incorrectly referred to as glucokinase.

The different vertebrate isoenzymes differ in their catalysis, localization and regulation, thereby contributing to the different patterns of D-glucose metabolism in different tissues (Wilson, 2003). Whereas types I to III can phosphorylate a variety of hexose sugars and are inhibited by glucose 6-phosphate, type IV is specific for D-glucose and shows no glucose 6-phosphate inhibition. The type I enzyme may have a catabolic function, producing hexose 6-phosphate for energy production in glycolysis; it is bound to the mitochondrial membrane, which enables the coordination of glycolysis with the TCA cycle. The type II and

III enzymes may have anabolic functions, providing hexose 6-phosphate for glycogen or lipid synthesis. The type IV enzyme is found in the liver and pancreatic β-cells, where it is controlled by insulin (activation) and glucagon (inhibition). In pancreatic β-cells, the type IV enzyme acts as a glucose sensor to modify insulin secretion. Mutations in type IV hexokinase have been associated with diabetes mellitus.

The ribokinase family

The second is the ribokinase family. Ribokinases phosphorylate ribose, which is essential in nucleotide synthesis, to ribose 5-phosphate in the presence of ATP and magnesium. The phosphorylated sugar can then enter the pentose phosphate pathway or be used in the synthesis of amino acids (histidine and tryptophan). Ribokinases share an α/β-fold core consisting of a central eight-stranded β-sheet and eight flanking α-helices. Most members of the ribokinase family feature a distinct β-sheet that forms a lid over the ribose-binding site. This lid has been suggested to be a morphological marker of evolution within this family (Sigrell et al., 1998; Li et al., 2002; Arnfors et al., 2006).

The GHMP family

The third family contains several bacterial and yeast galactokinases, homoserine kinases and eukaryotic mevalonate and phosphomevalonate kinases and is therefore called the GHMP family. Galactokinase catalyzes the first reaction in the D-galactose metabolism pathway, the ATP-dependent phosphorylation of D-galactose, yielding galactose 1-phosphate (Bajwa et al., 1988; Lee et al., 1992). Galactokinase deficiency causes the disease galactosemia, which is responsible for the formation of cataracts in newborn babies, and is possibly responsible for presenile cataracts in adults (Lee et al., 1992). In *S. cerevisiae* Gal3p is required for the Gal4p-mediated induction of other enzymes involved in D-galactose metabolism. The induction of Gal1p production then reinforces this process, increasing the expression of other

galactose-inducible genes. Gal3p has been shown to be similar to the Gal1p (Bajwa et al., 1988).

Regulatory roles of sugar kinases – the hexokinases

Most early investigations of glycolytic enzymes have focused on their catalytic functions only. However, recent studies have provided evidence that some glycolytic enzymes, including hexokinases, play more complicated roles, rather than just being simple components of the glycolytic pathway (reviewed by Kim and Dang, 2005). These newly found functions include transcriptional regulation, apoptosis and cell motility. Considering these non enzymatic features makes it easy to look at these proteins from a different angle and to re-evaluate the roles for glycolytic enzymes in other biological processes. Here we will focus on the newly found functions of hexokinases in other organisms in order to underline the various possibilities of this enzyme family.

Hexokinase and its role in transcriptional regulation in *Saccharomyces cerevisiae*

Hexokinase catalyzes the phosphorylation of D-glucose to D-glucose 6-phosphate, which is the first step of glycolysis. *S. cerevisiae* can use different carbon sources, but is highly specialized to grow on D-glucose. D-glucose itself represses the expression of *HXK1* and *GLK1*, but not *HXK2* and is unable to repress *HXK1* or *GLK1* in a *HXK2* negative mutant, suggesting that Hxk2p plays a role in glucose repression (Niederacher and Entian, 1991; Herrero et al., 1995; Herrero et al., 1996; Diderich et al., 2001; Rodriguez et al., 2001). It has been shown that Hxk2p, while usually present in the cytosol, is translocated to the nucleus in the presence of D-glucose, where it associates with the specific transcription factors Mig1p and Med8p in order to impair the activation of transcription of the glucose-repressed genes *HXK1*, *GLK1* and *SUC2* and to activate the transcription of *HXK2* itself (Herrero et al., 1998; de la Cera et al., 2002; Ahuatzi et al., 2004; Ahuatzi et al., 2007). These findings demonstrate

the dual role of Hxk2p as a metabolic enzyme and a transcriptional regulator and its function is therefore comparable to that of Gal1p, which on one hand phosphorylates D-galactose to D-galactose 1-phosphate, and on the other hand binds the repressor Gal80p, hence releasing the transcriptional activator Gal4p from Gal80p-mediated inhibition. This will be dealt with in more detail in one of the following paragraphs. Hxk2p also regulates the activity of the protein kinase Snf1p, which is essential for metabolic regulation in response to stress e.g. D-glucose limitation (Vincent et al., 2001). Depending on D-glucose availability, Hxk2p regulates phosphorylation of Reg1p, which, in turn, affects Snf1p activity (Sanz et al., 2000). Lately it has also been shown, that Hxk2p regulates the phosphorylation of Mig1p by Snf1p and therefore its nucleocytoplasmic distribution (Ahuatzi et al., 2007). It is noteworthy, that a similar role of hexokinases in metabolic regulation could not be shown in mammalian cells.

A different role of hexokinases in mammals: apoptosis

Apoptosis, one of the main types of programmed cell deaths, is a highly conserved and finely regulated process for maintaining cellular homeostasis in metazoans. Obviously cellular homeostasis is partly dependent on energy status and therefore apoptosis might be dependent on D-glucose metabolism. It has been shown before, that D-glucose metabolism is related to cell death and survival, therefore a connection between glycolysis and apoptosis seems reasonable, but has not been truly understood until recently. Several isoforms of hexokinase and GAPD have been suggested to be involved. In this paragraph we will deal with the newly found activities of hexokinase in relation to apoptosis.

Most mammalian tumor cells display increased expression of *HXK2*, which might contribute to enhanced aerobic glycolysis (Arora et al., 1990; Rempel et al., 1996). Early studies demonstrated, that the type II hexokinase associates to the mitochondrial membrane by binding to the outer membrane voltage-dependent anion channels (VDAC) and uses intramitochondrial ATP to phosphorylate D-glucose. It therefore couples oxidative

phosphorylation with the glycolytic pathway (Golshani-Hebroni and Bessman, 1997; Pastorino and Hoek, 2003). Recently it has been shown, that type I and II hexokinases participate in the apoptotic pathway, being redistributed from the cytoplasm to the VADCs by Akt-protein kinase B signalling, which also increases mitochondrial hexokinase activity. Additionally mitochondrial hexokinase activity has been shown to inhibit apoptosis by preventing cytochrome *c* release from the mitochondria in various experimental systems (Gottlob et al., 2001; Bryson et al., 2002; Pastorino et al., 2002; Rathmell et al., 2003; Majewski et al., 2004). Also glucokinase, the type IV hexokinase expressed predominantly in liver, has been brought into connection with apoptosis, since it is present in a mitochondrial complex also containing BAD, which interacts with VDACs (Danial et al., 2003). BAD is a pro-apoptotic protein of the BCL2 family and induces apoptosis by inhibiting the anti-apoptotic molecule Bcl-XL.

Taken together, these findings point out the role of hexokinase as an apoptotic regulator, connecting oxidative phosphorylation, glycolysis and the apoptotic pathway.

Hexokinases as glucose sensors: from fungi to mammals

We have already described the complex role of the *S. cerevisiae* Hxk2p in D-glucose repression above. However, hexokinases also play an important role in filamentous fungi. In *Aspergillus nidulans*, carbon catabolite repression is mediated by the DNA-binding transcriptional repressor CreA, which inhibits activation of the catabolism of less preferred carbon sources if a more favourable growth substrate, for example D-glucose is available (Arst and Cove, 1973; Arst and Bailey, 1977; Felenbok and Kelly, 1996; Felenbok et al., 2001). The function of A. nidulans CreA is similar to Mig1p, the main mediator of D-glucose repression in S. cerevisiae (Nehlin and Ronne, 1990; Gancedo, 1998).

In order to investigate the role of hexose phosphorylating enzymes (HxkA1, GlkA4) in carbon catabolite repression of *A. nidulans*, Flipphi et al. (2003) have tested the effect of

deficiency in one and both hexokinases with three repressible diagnostic systems: ethanol utilization (*alcA* and *alcR* genes), xylan degradation (*xlnA*), and acetate catabolism (*facA*). Transcriptional repression by D-glucose did not change in the two single kinase mutants, whereas the hexokinase mutant was partially derepressed for D-fructose. Thus, hexokinase A and glucokinase A compensate each other for carbon catabolite repression by D-glucose in the single mutants. In contrast, both D-glucose and D-fructose repression were severely impaired for all three diagnostic systems in the double mutant. Unlike the situation in *S. cerevisiae*, where only Hxk2p has a function in transcriptional regulation, in *A. nidulans* the hexose phosphorylating enzymes play parallel roles in glucose repression.

A strong difference between unicellular yeasts and filamentous fungi on one side and multicellular plants on the other is the differentiation of the organism into specific organs, e.g. source tissues for sugar production and sink tissues for sugar storage. Therefore sugar sensing and regulation in plants is generally considered to be a more complex phenomenon than in fungi.

It was shown by Sheen et al. (1999), that hexokinases are involved in glucose repression of photosynthetic genes. 2-Deoxyglucose (but not 6-deoxyglucose or 3-O-methylglucose, which are no substrate for hexokinase) is able to trigger repression of photosynthetic maize genes in a protoplast transient expression system. Addition of mannoheptulose, a hexokinase inhibitor, lead to a reversion of this repressive effect. Only D-glucose and not D-glucose 6-phosphate or any other metabolite further downstream in glycolysis (introduced directly into the protoplasts by electroporation) was able to trigger this repression, pointing out an involvement of hexokinase. This is consistent with the idea that hexokinase serves a catalytic and a regulatory function (Jang and Sheen, 1994). Transgenic *Arabidopsis thaliana* plants overexpressing hexokinase showed sugar hypersensitivity whereas antisense expression caused sugar hyposensitivity (Jang and Sheen, 1997). Interestingly, the contrary effect was obtained by overexpressing yeast hexokinase in

transgenic plants. These plants, despite enhanced total hexokinase activity, displayed sugar hyposensitivity. This result underlines the expected different regulatory role of hexokinase in fungi and plants, which has been mentioned before. Hexokinase-mediated sugar sensing is also involved in germination of *A. thaliana* seeds. Mannose and 2-deoxyglucose, but not 3-O-methylglucose nor 6-deoxyglucose, inhibit germination, and this inhibition is relieved by the addition of mannoheptulose to the growth medium (Pego et al., 1999).

The situation is again different when looking at mammal cells. Here the concentration of D-glucose in the blood is precisely maintained within certain limits by the interplay of several endocrine and neural glucostatic systems. Impaired regulation of blood glucose levels causes severe disorders such as diabetes. The pancreatic β-cell, which is involved in the monitoring of blood sugar concentrations, has been extensively studied (Efrat et al., 1994; Matschinsky et al., 1998). In response to high levels of D-glucose, these cells secrete insulin, which stimulates fat and muscle cells to take up excess D-glucose from the blood.

Glucokinase is generally regarded as the primary β-cell D-glucose sensor, because phosphorylation of D-glucose by glucokinase is the rate-limiting step in D-glucose catabolism in β-cells (Matschinsky et al., 1998). Therefore, this function is considered to be only depending on the catalytic activity of glucokinase. The kinetic properties of glucokinase, which features an unusually high K_m value in the range of the physiological levels of blood glucose, support this theory. Glucokinase activity in β-cells appears to be regulated by its subcellular localization (Noma et al., 1996; Vanhoutte and Malaisse, 1998), an effect first studied in hepatocytes, where glucokinase is also the predominant D-glucose phosphorylating enzyme and where there is evidence for its involvement in the control of D-glucose homeostasis (Postic et al., 1999). At low D-glucose concentrations, glucokinase is sequestered in an inactive state in the nucleus by a glucokinase regulatory protein (GKRP). At elevated D-glucose levels, glucokinase translocates to the cytoplasm, resulting in its activation (de la Iglesia et al., 2000).

All the examples given above emphasize the potential of gluco- and hexokinases to be involved in processes much more complex than implied by their catalytic activity alone.

Regulatory roles of sugar kinases – the galactokinase of yeast

Yeast galactokinase is not only known for its catalytic properties, but also for its role as an inducer protein of the *GAL* genes. Similar to most other organisms, the conversion of D-galactose to D-glucose 1-phosphate is accomplished by the action of three enzymes, namely galactokinase, galactose 1-phosphate uridylyltransferase, and UDP-galactose 4-epimerase, that constitute the Leloir pathway (reviewed by Holden et al., 2003). These enzymes are encoded by *GAL1*, *GAL7* and *GAL10* respectively. In the first step intracellular *α*-D-galactose is converted to *α*-D-galactose 1-phosphate by galactokinase through ATP-dependent phosphorylation. The second enzyme in the pathway, galactose 1-phosphate uridylyltransferase, catalyzes the transfer of a UMP group from UDP-glucose to D-galactose 1-phosphate, thereby generating D-glucose 1-phosphate and UDP-galactose. Finally UDP-galactose is converted to UDP-glucose by UDP-galactose 4-epimerase to complete the pathway. *GAL2*, encoding galactose permease, *MEL1*, encoding α-galactosidase, *GAL1*, *GAL7*, and *GAL10* are the *GAL* structural genes.

D-galactose is the inducer of *GAL* gene transcription, leading from low basal levels to high levels. The complex interplay between Gal4p, a DNA-binding transcriptional activator, Gal80p, a repressor present in the nucleus and the cytosol, and Gal3p, a ligand sensor/inducer only present in the cytosol, determines the transcriptional status of the *GAL* genes. Gal4p binds to upstream activating sequences in the *GAL* promoter region. If D-galactose is absent, Gal4p is unable to activate transcription because Gal80p binds to it in the nucleus and thereby inhibits the transcription of the *GAL* genes. After binding D-galactose and ATP, which causes a conformational change, Gal3p binds to Gal80p in the cytosol, leading to a displacement of

Gal80p from the nucleus to the cytosol, releasing Gal4p from Gal80p-mediated inhibition and allowing it to activate *GAL* gene transcription (reviewed by Bhat and Murthy, 2001).

Although itself lacking galactokinase activity, Gal3p has a 73% identity and 92% similarity to Gal1p at the amino acid level. In fact, the insertion of just two amino acids (SA) in the ATP binding motif can restore galactokinase activity of Gal3p (Platt et al., 2000). Gal1p itself is also able to induce the *GAL* genes, however, due to its weaker ligand sensor activity, induction is considerably delayed in mutants lacking Gal3p. Still, in later stages of induction, when Gal1p becomes available at higher levels, it replaces Gal3p.

The regulation of transcription induction of the *GAL* regulon in *Kluyveromyces lactis* is similar (reviewed by Rubio-Texeira, 2005). One of the main differences is that Gal3 is not present in *K. lactis*, and that its function is performed by the bifunctional KlGal1, which in addition to its galactokinase activity acts also as inducer.

***Hypocrea jecorina*, the subject at hand**

Based on the findings explained above, we can conclude, that sugar kinases often play a more complex role than only participating in metabolism.

My own interest in this subject comes from studies on the ascomycete *Hypocrea jecorina.* Its anamorph *Trichoderma reesei* is used for the production of (hemi)cellulolytic enzymes on a technical scale due to its capability to produce cellulases up to 100 g/l (Cherry and Fidantsef, 2003) and the strong cellulase promoters are used to drive recombinant enzyme and protein production (Persson et al., 1991; Kubicek and Harman, 1998; Penttila et al., 2004). The formation of cellulases and hemicellulases is regulated by induction, which can be achieved by cellulose, hemicelluloses, as well as di- and monosaccharides (e.g. xylose, cellobiose, xylobiose and sophorose) derived from them. However, also other sugars such as lactose or L-sorbose can act as inducers. When a soluble fermentation medium is preferred, lactose is virtually the only inducing carbon source that can be used economically on a

technical scale. The drawback, however, is that lactose metabolism is slow and cellulase yields produced on lactose are lower than on cellulose (Andreotti et al., 1980).

Lactose metabolism in *H. jecorina* occurs exclusively by extracellular hydrolysis and uptake of the two resulting disaccharides D-glucose and D-galactose (Seiboth et al., 2007). This implies that the metabolism of D-glucose and D-galactose under these conditions must be relevant to cellulase induction by lactose. Recent studies in this direction led to the discovery of an alternative pathway for D-galactose catabolism in *H. jecorina* which proceeds via galactitol, sorbitol and D-fructose, which is then phosphorylated by hexokinase. Consequently, lactose metabolism - in addition to galactokinase - also involves both glucokinase as well as hexokinase.

In this thesis, I have therefore investigated to which extent these three sugar kinases, known to be of regulatory importance in other cell systems, impact cellulase formation on lactose in *H. jecorina*. In addition, since almost nothing is known about these three kinases from filamentous fungi, I have also attempted to identify general regulatory roles for these three kinases in the fungus. While I contributed to the results presented in chapter 2 by creating the Δ*gal1*Δ*lad1* strain and working on the northern analyses, all experiments described in the following chapters were done by me under the scientific guidance of Univ.Prof.Dr. Christian P. Kubicek and Univ.Ass.Dr. Bernhard Seiboth.

Chapter 2

The galactokinase of *Hypocrea jecorina* is essential for cellulase induction by lactose but dispensable for growth on D-galactose

The results presented in this chapter have been published:
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ABSTRACT

Lactose is the only soluble carbon source which can be used economically for the production of cellulases or heterologous proteins under cellulase expression signals by *Hypocrea jecorina* (=*Trichoderma reesei*). Towards an understanding of lactose metabolism and its role in cellulase formation, we have cloned and characterized the *gal1* (galactokinase) gene of *H. jecorina*, which catalyzes the first step in D-galactose catabolism. It exhibits a calculated M_r of 57 kDa, and shows moderate identity (about 40%) to its putative homologues of *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. GAL1 is a member of the GHMP family, shows conservation of a Gly/Ser rich region involved in ATP binding and of amino acids (Arg 51, Glu 57, Asp 60, Asp 214, Tyr 270) responsible for galactose binding. A single transcript was formed constitutively during the rapid growth phase on all carbon sources investigated and accumulated to about twice this level during growth on D-galactose, L-arabinose and their corresponding polyols. Deletion of *gal1* reduces growth on D-galactose but does only slightly affect growth on lactose. This is due to the operation of a second pathway for D-galactose catabolism, which involves galactitol as an intermediate, whose transient concentration is strongly enhanced in the Δ*gal1* strain. In this pathway, galactitol is catabolized by the *lad1*-encoded L-arabinitol-4-dehydrogenase, because a Δ*gal1*Δ*lad1* strain failed to grow on D-galactose. In the Δ*gal1* strain, induction of the Leloir pathway gene *gal7* (encoding galactose 1-phosphate uridylyltransferase) by D-galactose, but not by L-arabinose, is impaired. Induction of cellulase gene expression by lactose is also impaired in a *gal1* deleted strain, while their induction by sophorose (the putative cellulose-derived inducer) was shown to be normal, thus demonstrating that galactokinase is a key enzyme for cellulase induction during growth on lactose, and that induction by lactose and sophorose involves different mechanisms.

INTRODUCTION

The disaccharide lactose (1,4-O-*β*-D-galactopyranosyl-D-glucose), the major carbohydrate in milk, is a carbon source which is catabolized only slowly by most eukaryotes. In biotechnology, it is a preferred carbon source for some fermentations (Roelfsema et al., 1990), and virtually is the only economic soluble carbon source for cellulase production or formation of heterologous proteins under the control of cellulase promoters by the ascomycete *Hypocrea jecorina* (anamorph: *Trichoderma reesei*; Persson et al., 1991; Penttilä, 1998). However, due to its slow metabolism, the cellulase yields on lactose are lower than on cellulose (Andreotti et al., 1980). Therefore knowledge of the mechanism by which lactose triggers cellulase formation would be helpful to improve yields for the production of recombinant proteins in fungal fermentations by genetic engineering of the pathway.

In fungi and other eukaryotes, lactose is cleaved to D-glucose and D-galactose, and while D-glucose enters the glycolytic pathway directly, D-galactose is converted into D-glucose 6-phosphate by a series of enzymatic reactions known as the Leloir pathway. As a first step towards a characterization of lactose and galactose metabolism in *H. jecorina* we have previously cloned *H. jecorina gal7* and *gal10* which encode the galactose 1-phosphate uridylyltransferase (EC 2.7.7.12; Seiboth et al., 2002a) and UDP-galactose 4-epimerase (EC 5.1.3.2; Seiboth et al., 2002b), respectively. We have shown that GAL7, which catalyzes the transfer of the uridine 5-phosphoryl from UDP-glucose to galactose 1-phosphate, thereby yielding glucose 1-phosphate and UDP-galactose, is essential for galactose catabolism, but not for induction of cellulase formation on lactose, thus implying that the components inducing cellulase formation are located at earlier steps of lactose catabolism.

In *S. cerevisiae*, the Leloir pathway genes *GAL1, GAL7* and *GAL10* are co-ordinately regulated by D-galactose, thereby involving the functions of the activator protein Gal4p, the negative regulator Gal80p and the inducer protein Gal3p (Bhat and Murthy, 2001). In the

absence of D-galactose, Gal4p is rendered inactive by the binding of Gal80p (Johnston et al., 1987; Lue et al., 1987; Ma and Ptashne, 1987). Recently, Peng and Hopper (2002) showed that the interaction between Gal3p-Gal80p occurs in the cytoplasm, and concurrently, Gal4p is activated by removal of Gal80p, resulting in transcription of the *GAL* pathway genes. Gal3p shows high similarity to Gal1p – the galactokinase (EC 2.7.1.6) of *S. cerevisiae* - which catalyzes the specific phosphorylation of D-galactose to D-galactose 1-phosphate, the essential difference being a SA-doublet, which is lacking in Gal3p and whose deletion from Gal1p renders it enzymatically inactive (Platt et al., 2000). Gal3p may be unique to *S. cerevisiae*, as *Kluyveromyces lactis* lacks a *GAL3* homologue, and its function presumable being carried out by *GAL1* as a *S. cerevisiae gal3* mutation can be complemented by *KlGAL1* (Meyer et al., 1990; Meyer et al., 1991).

The aim of the present work was to find out whether galactokinase of *H. jecorina* may be essential for cellulase formation and galactose utilization. To this end, we cloned and functionally characterized the gene encoding galactokinase (*gal1*) of *H. jecorina* responsible for the first step in galactose catabolism. We provide evidence for the existence of a novel alternative pathway for galactose utilization in *H. jecorina*, and that the galactokinase is essential for induction of cellulase formation by lactose in *H. jecorina*, and for the induction of the Leloir pathway gene *gal7* by D-galactose.

RESULTS

Cloning of a *H. jecorina* galactokinase

We identified conserved amino acid regions in different Gal1 proteins present in the NCBI database by alignment of the corresponding aa-sequences from *S. cerevisiae, K. lactis*, *S. pombe*, *H. sapiens, A. thaliana* and *E. coli*. The regions were subsequently used to design

gene specific degenerate primers, which successfully amplified a single 1.2-kb amplicon by RT-PCR from galactose grown *H. jecorina* RNA, whose deduced aa-sequence exhibited high identity to published galactokinases. With this PCR-amplicon as a probe, a genomic clone was isolated from a *H. jecorina* λBlueSTAR library and sequenced. It contained an open reading frame of 1996-bp, interrupted by a single intron of 415 bp, respectively, the position of which was verified by sequencing of the corresponding cDNA clone. Southern analysis of differentially digested chromosomal DNA suggests that *gal1* occurs as a single copy in the *H. jecorina* genome (data not shown). This is consistent with the fact that a BLAST search in the available genome databases of other filamentous fungi (*N. crassa* (http://www.genome.wi.mit.edu/annotation/fungi/neurospora/) *A. fumigatus* (http://www.tigr.org/tdb/fungal/)) also yielded only a single galactokinase-encoding gene (e-values e^{-177} and $5.3e^{-115}$ respectively).

The deduced polypeptide comprises 526 aa's with a calculated M_r of 57,03 kDa, which shows moderate identity across the entire aa sequence to Gal1 proteins from yeasts, mammals and prokaryotes (Fig. 1). *H. jecorina* GAL1 belongs to the family of GHMP-kinases (Tsay and Robinson, 1991) which originally included *g*alactokinases, *h*omoserine kinase, *m*evalonate kinase and *p*hosphomevalonate kinase (hence GHMP), and which are a unique class of metabolite kinases. This family is characterized by three well conserved motifs (Fig. 1), the first of which contains the galactokinase signature (G-R-x-N-[LIV]-I-G-[DE]-H-x-D-Y) and the second of which is characterized by a novel glycine-rich loop used in phosphate binding resulting in an unusual *syn* conformation of the bound nucleotide (motif 2 in Fig. 1; cf. Zhou et al., 2000). Motif 2 comprises also the SA-doublet which is the essential difference between the *S. cerevisiae* Gal1 and Gal3 proteins and responsible for the galactokinase enzyme activity in Gal1 (Platt et al., 2000). The amino acid side chains responsible for anchoring the sugar ligand to the protein as recently determined by

Thoden and Holden (2003) are strictly conserved in the galactokinase of *H. jecorina* (Arg 51, Glu 57, Asp 60, Asp 214, Tyr 270).

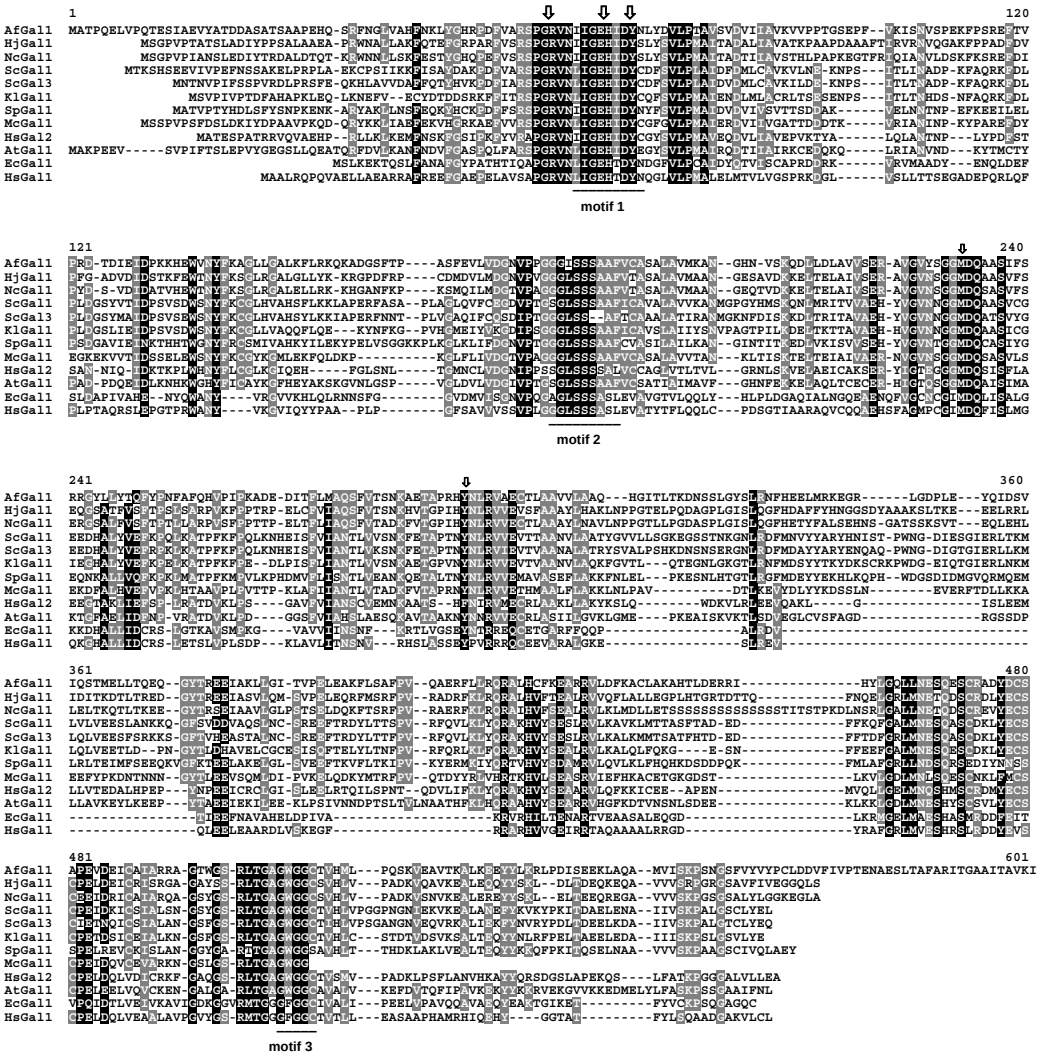

Fig. 1. Similarity in amino acid sequence of *H. jecorina* galactokinase and corresponding proteins from other eu- and prokaryotes including the *S. cerevisiae* Gal3 and *H. sapiens* Gal2 which encodes an N-acetylgalactosamine kinase (Pastuszak et al., 1996). Residues in white on a black background are conserved in at least 90% of the Gal proteins, residues in white on grey in 40%. Abbreviations and accession numbers: AfGal1 (*Aspergillus fumigatus*, http://www.tigr.org/tdb/e2k1/afu1/ contig:4897), HjGal1 (*H. jecorina;* AY249022), NcGal1 (*N. crassa;* http://www-genome.wi.mit.edu/annotation/fungi/neurospora/ contig:3.538), ScGal1 (*S. cerevisiae*, P04385), ScGal3 (*S. cerevisiae*; NP_010292.1), KlGal1 (*Kluyveromyces marxianus var. lactis*; P09608), SpGal1 (*Schizosaccharomyces pombe* NP_596859.1), McGal1 (*Mucor circinelloides*; CAD27346.1), HsGal2 (*H. sapiens*, Q01415), AtGal1 (*Arabidopsis thaliana*; T51592); EcGal1 (*E. coli*; P06976), HsGal1 (*H. sapiens*; P51570). *In silico* translation of the *N. crassa* and *A. fumigatus* Gal1 was done manually. In the case of the NcGal1 the C-terminal part is different from the hypothetical protein NCU08687.1 found on the *Neurospora* homepage. Regions typical for nucleotide binding by GHMP-kinases, as identified by (Bork et al., 1993) and refined by (Zhou et al., 2000) (motif 1, 2 and 3) are underlined. The amino acids responsible for anchoring the galactose to the galactokinase as identified by (Thoden and Holden, 2003) are marked by arrows.

Regulation of *gal1* gene expression

Northern analyses of total RNA from *H. jecorina* grown on different carbon sources showed that the *gal1* transcript was most abundant in young cultures, and sharply decreased during further cultivation (data not shown). It was most abundant during growth on D-galactose, galactitol, L-arabinose and L-arabinitol, but a lower amount of transcript was virtually present on every carbon source tested, indicating that expression of *gal1* occurs at a basic, constitutive level which can be further induced by the carbon sources mentioned above. The *gal1* transcript was equally abundant on pH 4.5 and 7 during growth on D-galactose and the simultaneous incubation with D-glucose and D-galactose did not increase *gal1* transcript levels, indicating that D-glucose prevents an upregulation of *gal1* transcript levels. This is in agreement with the constitutive transcript levels found during growth on lactose (Fig. 2). In accordance with an analysis of the *gal7* and *gal10* upstream regions (Seiboth et al., 2002a; Seiboth et al., 2002b) we were also not able to identify a *S. cerevisiae* Gal4p consensus binding site (Dhawale and Lane, 1993) in the *gal1* upstream region.

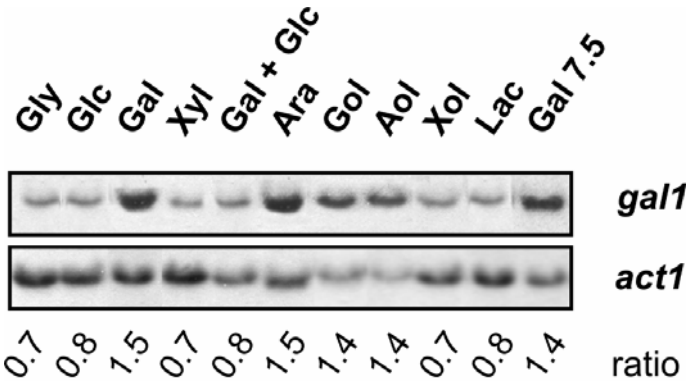

Fig. 2. Effect of various carbon sources on *gal1* gene transcription in *H. jecorina*. Abbreviations used are: Gly, glycerol; Glc, D-glucose; Gal, D-galactose; Xyl, D-xylose; Ara, L-arabinose; Gol, galactitol; Aol, L-arabinitol; Xol, xylitol; Lac, lactose. Samples shown were obtained after 6 h of growth in replacement medium as described in Materials and Methods. Mycelia were cultured at pH 4.5 and in the case of galactose also at pH 7.5 (Gal7.5). In the case of the carbon source lactose a sample was taken after 30 h of batch growth (conidial inoculum). The *act1* gene served as a loading control. The values indicate the ratio of the densitometric values obtained for the *gal1* transcript to that of the *act1* control.

GAL1 is not essential for growth on D-galactose or lactose

In order to investigate whether galactokinase is essential for growth of *H. jecorina* on D-galactose, we produced a *gal1* deletion strain by replacing the coding region of *gal1* with the *A. nidulans* acetamidase *(amdS)* gene (Fig. 3 A). The corresponding mutant strains displayed the same growth rate on D-glucose and glycerol as the wild-type strain, showed a slightly reduced growth rate on lactose (albeit resulting in a slightly higher final biomass yield), but a significantly reduced growth rate on D-galactose (Fig. 3 B). Despite of this reduction, however, it is obvious that the Δ*gal1* strain can grow on D-galactose. One possible explanation for this could be the occurrence of a second enzyme with galactokinase activity. However, cell-free extracts prepared from this strain showed only a very low level of galactokinase activity, which was essentially at the limit of detection (Fig. 3 C). Therefore, the residual growth on D-galactose must be due to the involvement of a second pathway of D-galactose catabolism.

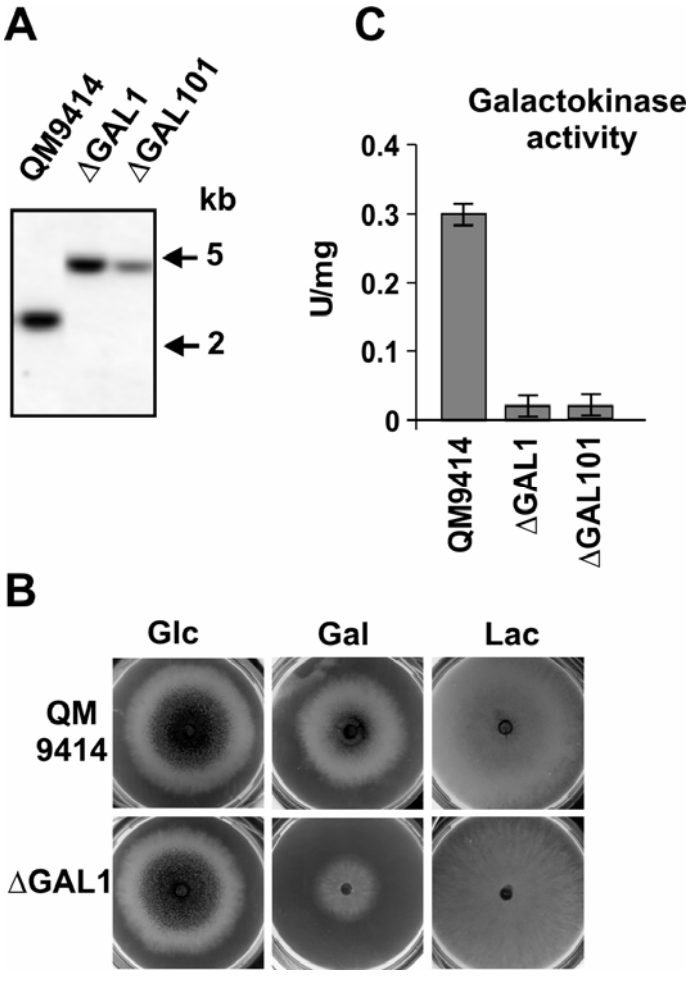

Fig. 3. Effect of *gal1* gene disruption on growth of *H. jecorina* on lactose and D-galactose. (A) Southern analysis of the wild-type strain QM9414 and the two Δ*gal1* strains, ΔGAL1 and ΔGAL101. Genomic DNA of the strains was digested with *Hind*III and probed with a 2.2-kb *Sac*II-*Hind*III fragment of the *gal1* gene. The insertion of the *A. nidulans amdS* gene results in an increase of about 2.1-kb of the hybridizing band in the strain. **(B)** Comparison of growth on plates of the parental strain QM9414 and the ΔGAL1 strain on D-glucose (Glc), D-galactose (Gal) and lactose (Lac). **(C)** Specific activity of galactokinase in cell free extracts after 24 hrs of growth on D-galactose. Activities are given in U/mg protein, where 1 Unit is defined as 1 µmol galactose 1-phosphate produced in one minute under the given conditions.

We should like to note at this place that strains retransformed with the *gal1* gene (see Materials and Methods) behaved essentially as the parent strain in these and the subsequent experiments (data not shown).

The alternative D-galactose catabolizing pathway of *H. jecorina* involves *lad1*-encoded L-arabinitol-4-dehydrogenase

In order to trace this putative second pathway of D-galactose metabolism, we first analyzed the mycelium of *H. jecorina* for the accumulation of D-galactose metabolites during growth on lactose. Thereby we found that both the wild-type strain as well as the Δ*gal1* strain transiently accumulated galactitol, but only the latter in significant amounts (Fig. 4 A and B). No accumulation of intermediates of an oxidative pathway (e.g. galactonic acid or galacturonic acid) as described for *A. niger* (Elshafei and Abdel-Fatah, 2001) could be detected under these conditions. We therefore considered it possible that D-galactose catabolism may proceed also via galactitol as an intermediate.

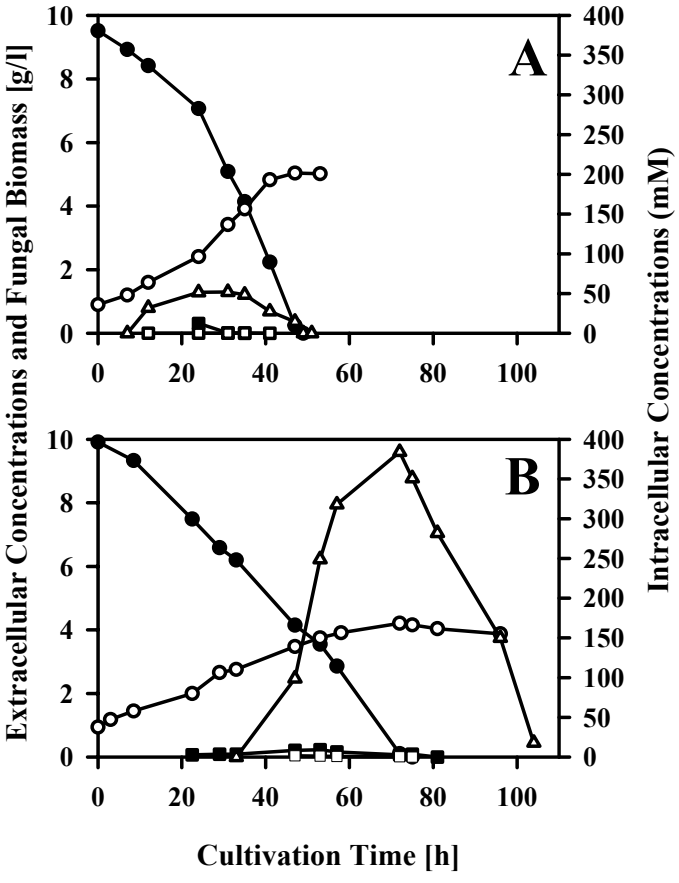

Fig. 4. D-galactose and galactitol accumulation in *H. jecorina* during growth on lactose. *H. jecorina* QM9414 **(A)** and ΔGAL1 **(B)** were cultivated in 2 l glass fermenters with lactose as carbon source and the accumulation of galactitol and D-galactose determined. (●),extracellular lactose; (○) dry weight; (■)extracellular D-galactose; (□) intracellular D-galactose; (▲) extracellular galactitol; (△) intracellular galactitol. For calculation of intracellular water, the ratio determined for *N. crassa* by (Slayman, 1964) was used (2.43 ml/g mycelial dry weight). Values given are means of two separate experiments, each with three different analyses.

An enzyme catabolizing galactitol in fungi is unknown, but we found recently that *lad1*-encoded L-arabinitol-4-dehydrogenase of *H. jecorina* (Richard et al., 2001) catalyzes the oxidation of galactitol *in vitro* (Pail, M., Peterbauer, T., Seiboth, B., Kubicek, C.P., unpublished data). In order to test the role of this enzyme in D-galactose metabolism *in vivo*, we constructed a *H. jecorina* double deletion strain in both *gal1* and *lad1*. To this end, a strain deleted in the *lad1* gene (Seiboth et al., 2003), was transformed with the *gal1* deletion cassette, resulting in strains deleted in both genes. These Δ*gal1*Δ*lad1* strains (Fig. 5 B) were investigated for their growth on D-galactose and galactitol. As shown in Fig. 5 B, this strain was now unable to grow on D-galactose or galactitol, and exhibited strongly reduced growth on lactose, whereas it displayed growth comparable to its parental ΔLAD1 strain on the other carbon sources tested. Therefore, we conclude that the second pathway of D-galactose catabolism proceeds via galactitol as an intermediate and involves L-arabinitol-4-dehydrogenase.

Retransformation of the

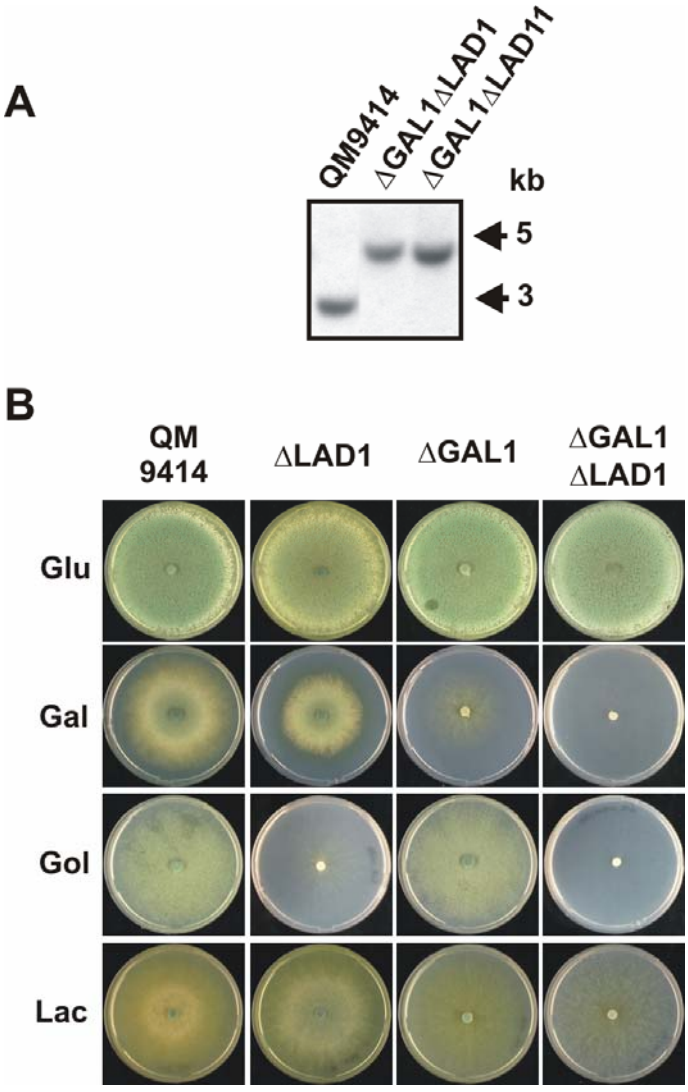

Fig. 5. A *gal1/lad1* double deletion strain cannot grow on D-galactose.
(A) Southern analysis of the Δ*gal1*Δ*lad1* strains. Genomic DNA of the strains was digested with *Bam*HI and probed with a 1-kb *Bam*HI-*Mlu*I *lad1* fragment. The homologous recombination of the deletion fragment gene results in an increase of 1.7–kb of the hybridizing bands in the deletion mutants.
(B) Comparison of growth of the wild-type strain QM9414, the ΔGAL1, ΔLAD1 and ΔGAL1ΔLAD1 on D-glucose (Glc), D-galactose (Gal), galactitol (Gol) and lactose (Lac).

double-disruptant strain with *gal1* (see Materials and Methods) resulted in the restoration of the Δ*lad1* phenotype (data not shown).

gal1 is essential for *cbh1* and *cbh2* gene expression on lactose

The major reason for studying the *H. jecorina* galactokinase was to learn whether its activity is relevant for cellulase formation on lactose. Therefore, the accumulation of the transcripts of two major cellulase genes – *cbh1* and *cbh2* – was determined in *H. jecorina* QM9414 and the Δ*gal1* strain during growth on lactose. Fig. 6 A clearly shows that the expression of both genes is strongly reduced in the *gal1* deletion strain, and – excluding the earliest cultivation phase when peptone is used as a carbon source (first 25 hrs; Kubicek et al., 1993) – was virtually zero. This correlated with an almost completely reduced accumulation of Cel7A, the gene product of *cbh1*, in the culture medium (Fig. 6 B).

Induction by sophorose – a *β*-1.2-diglucoside which is believed to be derived from cellulose and thus constitutes the actual mediator of cellulase induction (Kubicek and Penttilä, 1998) – was similar in the *H. jecorina* QM9414 and the Δ*gal1* strain (Fig. 6 C). This indicates that sophorose and lactose, despite of both being *β*-linked disaccharides, induce cellulases by different mechanisms.

The fact that a loss of *gal1* function impairs cellulase induction by lactose led us to wonder whether D-galactose would also induce cellulase gene transcription in *H. jecorina*. To this end, *H. jecorina* QM9414 was either grown on D-galactose as a carbon source, or pregrown on glycerol and then transferred to fresh medium containing D-galactose. However, *cbh1* or *cbh2* transcripts did not accumulate under these conditions (Fig. 6 D). Essentially the same findings were obtained with the *cre1*-loss of function strain *H. jecorina* RutC30 (Fig. 6 D). This indicates that the lack of induction of *cbh1* and *cbh2* by D-galactose is not due to carbon catabolite repression mediated by *cre1*.

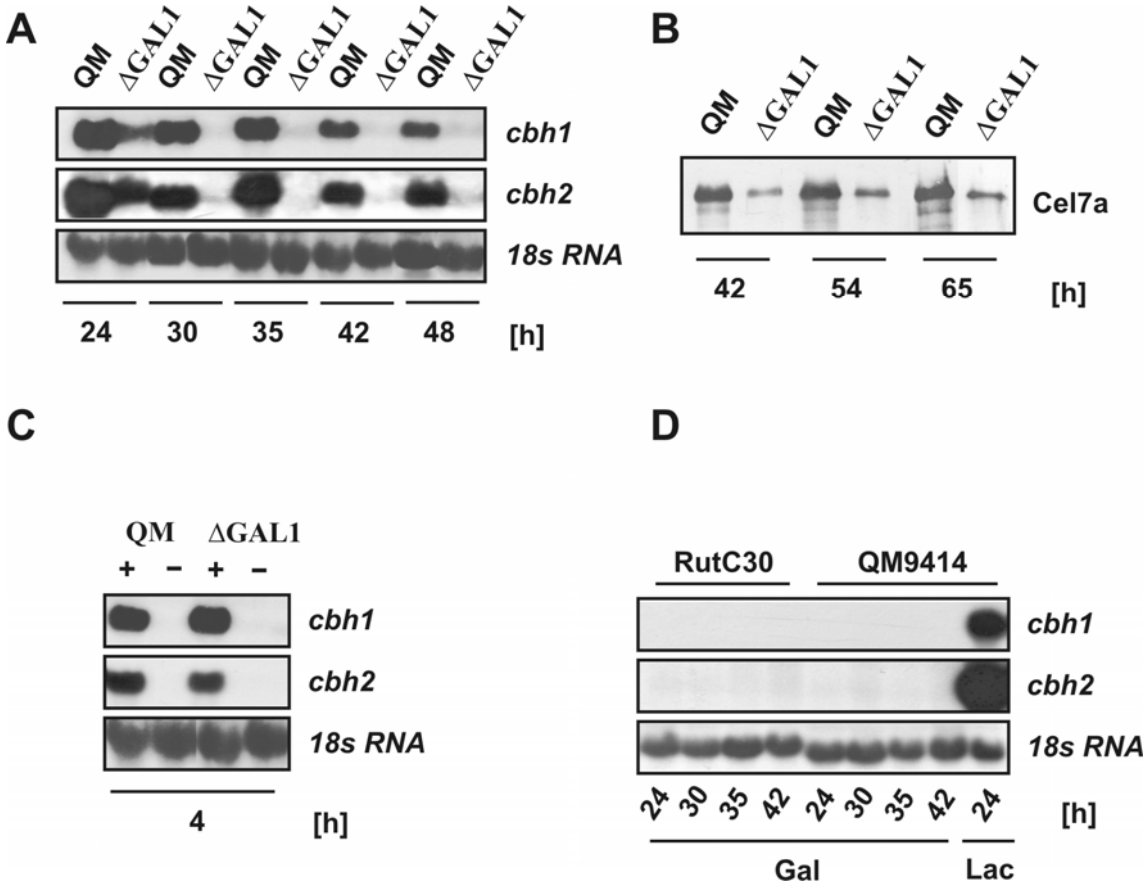

Fig. 6. Effect of *gal1* gene deletion on induction of *cbh1* and *cbh2* gene transcription by lactose and sophorose in *H. jecorina*. (A) Northern analysis of *cbh1* and *cbh2* transcript accumulation in *H. jecorina* QM9414 and strain ΔGAL1 on lactose. The 18s RNA was used as loading control. **(B)** Extracellular accumulation of the *cbh1*-encoded Cel7A protein under the same conditions. **(C)** Northern analysis of *cbh1* and *cbh2* transcript accumulation in *H. jecorina* QM9414 and strain ΔGAL1 upon induction by sophorose (1.5 mM). (+) and (-) indicate with and without sophorose addition; **(D)** Northern analysis of *cbh1* and *cbh2* transcript accumulation in *H. jecorina* QM9414 and the carbon catabolite derepressed mutant strain RutC30 on D-galactose. Total RNA from *H. jecorina* QM9414 grown for 24 h was used as a positive control.

gal1 is essential for galactose induction of *gal7*

To learn whether *gal1* also has a regulatory effect on the induction of the Leloir pathway, we examined the induction of transcription of the galactose 1-phosphate uridylyltransferase gene *gal7* by D-galactose in the Δ*gal1* strain (the UDP-galactose-4-epimerase encoding gene *gal10* was not studied as it is formed constitutively in *H. jecorina*; Seiboth et al., 2002b). Fig. 7 shows that the induction of *gal7* expression by D-galactose indeed does not occur in the Δ*gal1* strain, but the constitutive level of expression is unaffected. Interestingly, L-arabinose – which

induced the accumulation of the *gal7* transcript with equal efficacy as D-galactose (Seiboth et al., 2002a) – still induces *gal7* transcript accumulation in the Δ*gal1* strain (Fig. 7). Hence, galactokinase is involved in the induction of galactose 1-phosphate uridylyltransferase in *H. jecorina* by D-galactose, whereas induction by L-arabinose, involves a different, independent mechanism.

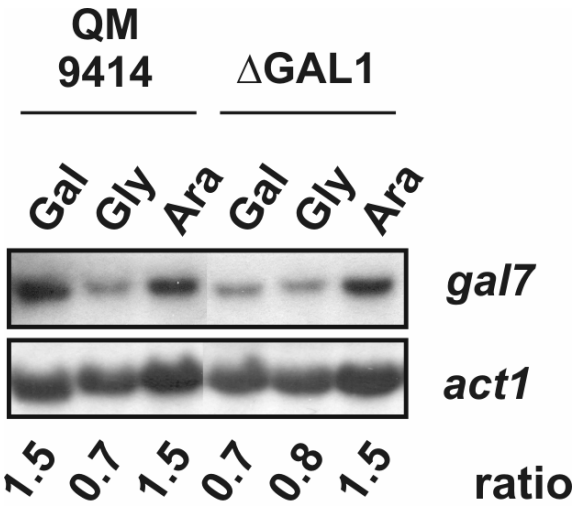

Fig. 7. Effect of *gal1* gene deletion on induction of *gal7* gene transcription by D-galactose and L-arabinose. Northern analysis of *gal7* transcript accumulation in *H. jecorina* QM9414 and the ΔGAL1 strain upon replacement to D-galactose (Gal), glycerol (Gly) or L-arabinose (Ara). The values indicate the ratio of the densitometric values obtained for the *gal7* transcript to that of the *act1* control.

DISCUSSION

In this paper, we have described the cloning and characterization of the galactokinase-encoding gene *gal1* from *H. jecorina*. Although the encoded galactokinase protein is highly similar to the well-characterized protein from yeasts, we show that the regulation of its expression (basic constitutive level, inducibility by D-galactose and L-arabinose, lack of catabolite repression of basal level) is significantly different from yeast (Lohr et al., 1995). Essentially the same pattern of regulation was also observed for the *gal7* (galactose 1-phosphate uridylyltransferase) gene (Seiboth et al., 2002a), whereas expression of the *gal10* (UDP-galactose-4-epimerase) gene occurs merely at a constitutive level (Seiboth et al.,

2002b). The regulation of the Leloir pathway genes in *H. jecorina* is thus strongly different from that in yeast, where it is essentially adaptive and carbon catabolite repressed, suggesting that D-galactose metabolism has different importance for yeast and *H. jecorina*. This may be due to the more frequent occurrence of D-galactose as a substrate in the natural environment of this necrotrophic fungus, e.g. in softwood polymers such as arabinogalactan (Dey and Brinson, 1984). This would also explain the coinduction of *gal1* and *gal7* by L-arabinose.

The present study further demonstrates that the galactokinase of *H. jecorina* has a regulatory role in D-galactose metabolism, being essential both for the induction of the Leloir pathway gene *gal7* as well as induction of cellulase formation by lactose. An involvement of Gal1 in the induction of the Leloir pathway is already known from *K. lactis*, where it replaces the function of *S. cerevisiae* Gal3p which is absent from *K. lactis* (Bhat and Murthy, 2001). Gal3 is also most likely not present in *H. jecorina*, as Southern hybridization experiments did not detect a second gene with high homology to *gal1*. However, the mere requirement of *gal1* for *gal7* induction does not necessarily imply that induction by D-galactose in *H. jecorina* acts by the same molecular mechanism as in *K. lactis,* namely that activated Gal1p inactivates a Gal80p homologue which would then allow Gal4p to bind to its target sequence. In fact, several findings suggest that this is not the case: (a) upstream non-coding regions of *gal1* or *gal7* do not contain consensus binding sites for Gal4p (b) we have failed to demonstrate the presence of a *GAL4* homologue in *H. jecorina* by Southern hybridization and PCR amplification (unpublished data), and (c) neither the *N. crassa* nor the *A. fumigatus* genome databases (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/, http://www.tigr.org/tdb/fungal/) contain genes encoding a Gal4p orthologue. The finding that induction by L-arabinose is not affected in the Δ*gal1* strain is an additional indication that the mechanism of induction of *gal7* is different from that in yeast.

In further contrast to the situation in yeasts, a loss of function of *H. jecorina* galactokinase still allowed the fungus to grow on D-galactose and had only a small effect on

growth on lactose. A similar finding has recently been reported for a galactokinase mutant (*galE*) of *A. nidulans* (Fekete et al., 2002). Since neither the *H. jecorina* Δ*gal1* strain nor the *A. nidulans galE* mutant display any remaining galactokinase activity, these results are not due to the existence of a second D-galactose phosphorylating enzyme in filamentous fungi, and can therefore only be explained by the existence of a second pathway of D-galactose catabolism in these two fungi. The existence of such an alternative pathway has been suggested to occur in *A. nidulans* during growth at pH > 7.0 by (Roberts, 1970), and more recently, an alternative, oxidative pathway has been demonstrated for *A. niger in vitro* (Elshafei and Abdel-Fatah, 2001). However, growth of the *gal1*-deletion strain of *H. jecorina* on D-galactose was not influenced by the pH (unpublished data), and we also were unable to detect the accumulation of intermediates of such an oxidative pathway (e.g. galactonic acid or galacturonic acid) during growth of *H. jecorina* on D-galactose. In contrast, the transient accumulation of galactitol in mycelia of *H. jecorina*, and its increased concentration in the Δ*gal1* strain suggested the presence of a pathway of D-galactose metabolism which involves reduction to the corresponding polyol and subsequent oxidation to a ketose. Such pathways are already known for catabolism of D-xylose and L-arabinose in filamentous fungi and yeasts (Jeffries and Shi, 1999; Richard et al., 2001; Richard et al., 2002), and the enzymes involved in these pathways display affinity for a wide range of substrates including D-galactose (viz. aldose reductase; (Verduyn et al., 1985) and galactitol (viz. *lad1*-encoded L-arabinitol-4-dehydrogenase; Pail, M., Peterbauer, T., Seiboth, B., Kubicek, C.P., unpublished data). The enzyme initiating this pathway with D-galactose likely is aldose reductase, which has also been demonstrated to be responsible for galactitol accumulation in eye lens (Lee *et al.*, 1995), and which is present in *H. jecorina* (unpublished data). Experimental verification of its involvement in D-galactose metabolism is currently in progress in our laboratory. However, with respect to the second step, we could show here that a Δ*gal1*Δ*lad1* strain completely fails to grow on D-galactose and galactitol, thus proving that L-arabinitol-4-dehydrogenase is

essential for D-galactose metabolism in a *gal1* negative background. This is also consistent with findings that an *A. nidulans* (*araA1*) mutant in L-arabinitol-4-dehydrogenase (de Vries et al., 1994) and a *H. jecorina* Δ*lad1* strain exhibit poor growth on D-galactose and while the *Aspergillus araA1* completely failed to grow on galactitol, the corresponding *H. jecorina* strain showed only a faint mycelial growth on plates containing D-galactose as sole carbon source. This alternative pathway of D-galactose metabolism appears to be not only a rescue bypass, but apparently operates also in parallel to a functioning Leloir pathway, as Δ*lad1* strain alone already display reduced growth on D-galactose. This pathway must therefore have an as yet unknown physiological function.

With respect to triggering of cellulase formation by lactose, we showed here that the **Δ***gal1* strain, although able to grow on lactose, failed to induce cellulase gene transcription, thus proving that *gal1* is essential for cellulase gene transcription. On a first glance, this suggests that the cellulases are subject to the same regulation as the Leloir pathway genes. However, unlike the latter, neither *cbh1* nor *cbh2* were induced by D-galactose, and this finding was also obtained in a carbon catabolite repressor (CRE1) negative background thus arguing against D-galactose as an inducer. How then does lactose induce cellulase formation? A possible explanation would be that D-galactose may act as an inducer only at a much lower growth rate (as occurs during cultivation on lactose) or at a very narrow range of steady-state concentrations which do not occur during growth on D-galactose. Some support for this assumption comes from our recent observation that the overexpression of the *bga1*-encoded extracellular *β*-galactosidase increases the growth rate on lactose and thereby impairs cellulase induction (unpublished data). It must be noted that this assumption would similarly be applicable to galactose 1-phosphate as an inducer. In fact, our previous findings that the half-life of *cbh1*-mRNA is increased in *gal7* deletion strains of *H. jecorina* (Seiboth et al., 2002a) would be indicative that the critical parameter rather is the intracellular galactose 1-phosphate concentration. However, accumulation of galactose 1-phosphate is toxic in many

organisms. Riley and Dickson (1984) and Cardinali et al. (1997) showed for *K. lactis* - where a deletion in *GAL7* resulted in a constitutive expression of *β*-galactosidase - that the actual inducer is indeed D-galactose and not D-galactose 1-phosphate. Finally, we should mention that our present experimental evidence does theoretically not rule out that lactose or a transglycosylation products derived from lactose may act as an inducer for cellulase induction, although we have so far been unable to detect a lactose permease gene in this fungus (Seiboth, B., unpublished data).

In conclusion, both the numerous differences in the regulation of the Leloir pathway between yeast and *H. jecorina*, as well as its clear involvement in the induction of cellulases (which may also be relevant to the mechanism by which plant pathogenic fungi attack plants), render D-galactose metabolism by *H. jecorina* an intriguing topic for further investigations. Further, an understanding of the linkage of the Leloir pathway to the formation and catabolism of galactitol may provide a model for studying sugar cataract in eye lens (Lee et al., 1995).

MATERIALS AND METHODS:

Strains and culture conditions

H. jecorina strain used in this study was QM9414 (ATCC 26921) and was maintained on malt extract agar. Strains were grown in 250-ml flasks on a rotary shaker (250 rpm) at 30°C in the medium described by (Mandels and Andreotti, 1978) with the respective carbon source at a final concentration of 10 g/l.

For the transcript analysis of *gal1 H. jecorina* QM9414 was pregrown on glycerol (1% w/v) for 22 h, mycelia harvested by filtration, washed with tap water, equal amounts of mycelia transferred to flasks containing the respective carbon source (1% w/v), and

cultivation continued for 6 h. In case of lactose, expression was analyzed after 24 h without pregrowth on glycerol.

Larger scale submerged cultivations were performed in 2 l glass fermenters, using a working volume of 1.75 l and 0.30 vvm aeration at an impeller speed of 600 rpm. pH was externally controlled with the automatic addition of 3M NaOH or 3M HCl. All incubations were done at 30 °C and pH 5.

Escherichia coli strains ER1647 and BM25.8 (Novagen, Madison, WI) were used for library screening, and strain JM109 (Promega, Madison, WI) for plasmid propagation.

Cloning of the *H. jecorina gal1* and sequence analysis

To amplify a *H. jecorina gal1* fragment by PCR, the degenerate primers galkinfor1 (5´-GTIAA(CT)CT(GCT)AT(CT)GGIGA(AG)CA(CT)AT(CT)AT(CT)AT(CT)GA(CT)-3‘) and galkinrev1 (5‘-G(CT)(AG)CA(AGT)CC(AGT)CCCCA(AGT)CCIGC-3‘) corresponding to the conserved aa-sequences VNLIGEHIDY and AGWGGCT/A, were derived from an alignment of different galactokinases of the NCBI database. A potential *gal1* cDNA clone was amplified by RT-PCR using the Reverse Transcription System, together with poly(A)-mRNA from galactose grown mycelia isolated by the aid of the PolyATtract mRNA Isolation System (Promega, Madison, WI). For *gal1* amplification the following conditions were chosen: 1 min initial denaturation (94°C), was followed by 30 cycles of amplification (1 min 94°C, 1 min 50°C, 2 min 72°C), and a final extension period of 7 min at 72°C. The amplified 1.2-kb fragment was cloned into pGEM-T (Promega, Madison, WI) and sequenced.

The *gal1* cDNA clone was used to screen a genomic λBlueSTAR library (Novagen, Madison, WI) of *H. jecorina* QM9414. The *gal1* gene was located on a 6.5-kb *Sac*II-*Apa*I subclone and ligated into pBluescript SK(+) and sequenced, using a LI-COR 4200 (LI-COR Inc., Lincoln) automatic sequencer. The genomic subclones and the deduced protein were analyzed using BLAST programs (Altschul et al., 1990) and Expasy (http://us.expasy.org/)

and multiple sequence alignment was done with MultiAlin (Corpet, 1988). Consensus binding sequences in the *gal1* 5′ region were identified manually. The nucleotide sequence reported in this paper has been deposited in the GeneBank database under GenBankAccession Number AY249022.

Construction of *H. jecorina* Δ*gal1* and Δ*gal1*Δ*lad1* strains

To construct a *gal1* deleted strain, the *H. jecorina gal1* coding region was replaced by the *A. nidulans amdS* (encoding acetamidase; Kelly and Hynes, 1985). To this end, a 1.7-kb *Hind*III/*Apa*I fragment of the *gal1* coding region was replaced by the *A. nidulans amdS Sal*I-*Acc*65I fragment. The final deletion vector pΔGAL1 comprises an about 2.3-kb *Eco*RV-*Hind*III of the *gal1* upstream region and 3.5-kb *Apa*I fragment of the *gal1* downstream region, interrupted by the *A. nidulans amdS* fragment. Transformation of *H. jecorina* was done according to (Gruber et al., 1990b). For gene replacement, the *gal1* deletion cassette was excised from pΔGAL1 with *Acc*65I and *Eco*RI, and the resulting 10.8-kb fragment used in transformation of strain QM9414. Transformants were selected for growth on acetamid as sole nitrogen source.

To construct a *gal1/lad1* double deletion strain, strain ΔLAD1 (Seiboth et al., 2003) which is deleted in the *lad1* gene was transformed essentially in the same way.

Retransformations of the Δ*gal1* and the Δ*gal1*Δ*lad1* strains was done by co-transformation with the Hygromycin B-resistance conferring expression cassette from pRLMex30 (Mach et al., 1994) and a *gal1* fragment comprising 1.5-kb upstream and 1.0-kb downstream of the *gal1*-coding region. The retransformants were checked for single integration of *gal1* into the genome, and strains carrying a single copy were used as controls in those experiments where the effect of *gal1* and *gal1-lad1* gene disruption was investigated.

Nucleic acid isolation and hybridization

Fungal mycelia were harvested by filtration, washed with tap water, frozen and ground in liquid nitrogen. For extraction of DNA, mycelial powder was suspended in buffer A (0.1 M Tris-HCl, pH 8.0, 1.2 M NaCl, 5 mM EDTA), incubated for 20 min at 65°C, cooled down on ice, mixed with 0.5 vol. phenol and 0.5 vol. chloroform and centrifuged (12.000 rpm, 15 min). Following an extraction with 1 vol. of chloroform, the DNA was precipitated with 1 vol. of isopropanol and washed with 70% (v/v) ethanol. Total RNA was isolated as described by (Chomczynski and Sacchi, 1987). Standard methods (Ausubel et al., 2005) were used for electrophoresis, blotting and hybridisation of nucleic acids.

Probes for hybridization were: a 1.4-kb *Bgl*I *cbh1* (cellobiohydrolase Cel7A encoding), a 1.3-kb *Hae*II *cbh2* (cellobiohydrolase Cel6A encoding), a 1.9-kb *Acc*65I *act1* (actin encoding) fragment and a 1.2-kb *gal1* cDNA fragment. Intensities were determined by densiometric measurements of autoradiographs derived from different exposure times (only values with linear correlation (r>0.9) were used).

Determination of fungal growth

To determine hyphal growth on agar plates, plates were inoculated with a small piece of agar in the centre of an 11-cm plate, and the increase in colony diameter measured daily twice. To measure growth in submerged cultures, the increase in biomass dry weight was recorded. Therefore, mycelia were harvested after appropriate times, washed extensively with distilled water and dried to constant weight in an oven at 80 °C. Data shown are the average of the two separate measurements, which deviated by not more than 15 %.

Extraction of fungal polyols

Aliquots of 2 x 5 ml were withdrawn from the cultures, mycelia were suction filtrated without washing to avoid losses of the intracellular polyols and were resuspended in 1 ml of distilled

water. Suspensions were shock-frozen at -75 °C for 1 h, boiled for 10 sec., and homogenized in a pre-cooled Potter-Elvehjem glass homogenizer. The suspension was spun down at 8.500 x *g*, for 5 min and the supernatant was used for analysis.

Galactokinase Assay

To prepare cell-free extracts, 10 ml of the culture broth were withdrawn, suction filtered, and the mycelial debris washed with 0.1 M sodium phosphate buffer, pH 7.6. The mycelia were then resuspended in 5 ml of the same buffer, and homogenized by one passage in a pre-cooled AB Biox Type X25 X-press (Göteborg, Sweden). The homogenate was centrifuged at 20.000 x *g* (20 min, 4 °C), and the supernatant was immediately used to assay galactokinase activity as described previously (Fekete et al., 2002).

Determination of monosaccharides, disaccharides and galactitol

Concentrations of D-galactose, lactose and galactitol in the medium or in the cell-free extracts were determined by HPLC analysis, using an H^+ exchange column (Bio-Rad Aminex HPX-H) thermostated at 55°C, and employing 10 mM H_2SO_4 as mobile phase with isocratic elution. Compounds were detected by means of a refractive index detector (Sándor et al., 1999). Data displayed are the average of the two separate measurements, which deviated by not more than 6%.

Biochemical analysis

The protocols described by (Ausubel et al., 2005) were used for SDS-PAGE (using 10% polyacrylamide gels), and Western blotting (using a monoclonal antibody to detect cellobiohydrolase Cel7A (Mischak et al., 1989).

ACKNOWLEDGEMENTS

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Chapter 3

Induction of the *gal* pathway and cellulase genes involves no transcriptional inducer function of the galactokinase in *Hypocrea jecorina*

The results presented in this chapter have been submitted for publication:
Hartl, L., Kubicek, C.P., Seiboth, B. (2007)
Induction of the *gal* pathway and cellulase genes involves no transcriptional inducer function of the galactokinase in *Hypocrea jecorina*.

ABSTRACT

The *Saccharomyces cerevisiae* galactokinase ScGal1 is a key enzyme of the Leloir pathway for D-galactose metabolism and catalyzes the conversion of D-galactose to D-galactose 1-phosphate, whereas the catalytically inactive galactokinase paralogue ScGal3 activates the transcription of the *GAL* pathway genes in the presence of D-galactose. In *Kluyveromyces lactis* the transcriptional inducer function and the galactokinase activity are encoded by a single bifunctional KlGal1. Here, we investigated the cellular function of the single galactokinase GAL1 in the multicellular ascomycete *Hypocrea jecorina* (=*Trichoderma reesei*) in the induction of the *gal* genes and of the galactokinase dependent induction of the cellulase genes by lactose (1,4-*O*-*β*-D-galactopyranosyl-D-glucose). Comparison of the transcriptional response of a strain deleted in the *gal1* gene (no putative transcriptional inducer and no galactokinase activity), a strain expressing a catalytically inactive GAL1 version (no galactokinase activity but a putative inducer function), and a strain expressing the *E. coli galK* (no putative transcriptional inducer but galactokinase activity) show that – in contrast to the two yeasts – both the GAL1 protein and the galactokinase activity are fully dispensable for induction of the Leloir pathway gene *gal7* by D-galactose and that only the galactokinase activity is required for cellulase induction by lactose. The data document a fundamental difference in the mechanisms by which yeasts and multicellular fungi respond to the presence of D-galactose, and show that the Gal1/Gal3-Gal4-Gal80 dependent regulatory circuit does not operate in multicellular fungi.

INTRODUCTION

The enzymes of the Leloir pathway are responsible for the conversion of D-galactose to D-glucose 1-phosphate and have been identified in all biological kingdoms. In the model organism *Saccharomyces cerevisiae*, the genes encoding the three main Leloir pathway enzymes *GAL1* (galactose kinase), *GAL7* (UDP-galactose uridylyltransferase) and *GAL10* (UDP-galactose epimerase and aldose 1-epimerase) are clustered and coordinately regulated at the level of transcription. The *S. cerevisiae GAL* genes are repressed by D-glucose, expressed only at a very low basal level on other respiratory carbon sources and highly induced (up to 1000-fold) when the cells are switched to a medium containing D-galactose. This transcriptional activation is mediated by the interplay of three proteins: in the presence of D-galactose and ATP, the transcriptional inducer ScGal3 associates with the ScGal80 repressor thereby alleviating its repressing effects. This allows the transcriptional activator ScGal4 to recruit the RNA polymerase II to each of the *GAL* genes (reviewed by Schaffrath and Breunig, 2000; Bhat and Murthy, 2001; Rubio-Texeira, 2005).

The *S. cerevisiae* galactokinase ScGal1 displays a 73% aa identity to the ScGal3, but a galactokinase activity is absent in ScGal3. However ScGal3 can be converted into a catalytically active galactokinase through the insertion of the two amino acids Ser and Ala (after Ser^{164}) into its sequence (Platt et al., 2000). Overexpression of ScGal1 or a phenomenon termed long term adaptation can recover a *gal3* phenotype in *S. cerevisiae* (Bhat et al., 1990). The presence of ScGal3 seems to be a unique trait of *S. cerevisiae*, and its role as a transcriptional inducer is fulfilled in other yeasts such as *Kluyveromyces lactis* by a single catalytically active galactokinase KlGal1. The regulatory function of KlGal1 does not require the galactokinase activity (Meyer et al., 1991) which is consistent with the recent demonstration that the enzymatic and the weak transcriptional inducer properties of ScGal1 are located in separate parts of the protein (Thoden et al., 2005).

In contrast to *S. cerevisiae* and *K. lactis*, the *gal* genes of filamentous fungi such as *Aspergillus nidulans, Hypocrea jecorina* and *Neurospora crassa*, are not clustered (http://www.broad.mit.edu/annotation/fgi/; http://genome.jgi-psf.org/), and an already high basal level of expression is found during growth on all carbon sources including D-glucose, indicating some major differences in the regulation of D-galactose metabolism between yeasts and filamentous fungi (Roberts, 1970; Seiboth et al., 2002a; Seiboth et al., 2002b; Seiboth et al., 2004). In *H. jecorina* only *gal1* and *gal7*, but not *gal10*, are further induced by D-galactose and in addition also by L-arabinose. The *H. jecorina gal1* has been shown to be essential for the induction of the cellulase genes by the D-galactose containing disaccharide lactose (1,4-*O*-*β*-D-galactopyranosyl-D-glucose), an important carbon source for many fungal fermentations including the production of cellulases and recombinant proteins in *H. jecorina* (Penttilä et al., 2004). A search in the *H. jecorina* genome database shows that this fungus does not possess a *GAL3* orthologue. Therefore, it would be possible that the regulation of the *gal* genes in *H. jecorina* follows the model of the *K. lactis GAL/LAC* regulon.

The objective of this paper was to test whether the induction of the Leloir pathway genes by D-galactose and of the cellulase genes by lactose depend on a putative transcriptional inducer function of *H. jecorina* GAL1. We will show that – in contrast to the situation in *S. cerevisiae* or *K. lactis* – induction by lactose indeed requires solely the galactokinase activity and that GAL1 is completely dispensable for the basal as well as the induced transcriptional level of the Leloir pathway genes. These data highlight a significant difference in D-galactose regulation between single and multicellular ascomycetes.

RESULTS

A catalytically inactive galactokinase is unable to induce cellulase formation by lactose

The necessity of the *gal1* gene for cellulase induction by lactose has been demonstrated by us before (Seiboth et al., 2004). In order to test whether cellulase induction by GAL1 depends on a putative transcriptional inducer function, we constructed a strain which expressed a catalytically inactive version of GAL1 (GAL1-SA) in a Δ*gal1* background. This recombinant GAL1-SA lacks the two amino acids Ser^{170} and Ala^{171} which are found in the conserved GLSSSAA motif of fungal galactokinases and are essential for their activity (Platt et al., 2000). Northern analysis confirmed that these strains had gained the ability to form a *gal1* (-SA) transcript on D-galactose (Fig. 1 A). In contrast to *H. jecorina* QM9414 or control strains which had been retransformed with the wild-type *gal1*, strains expressing the GAL1-SA fully lacked galactokinase activity and showed the same growth behavior as a

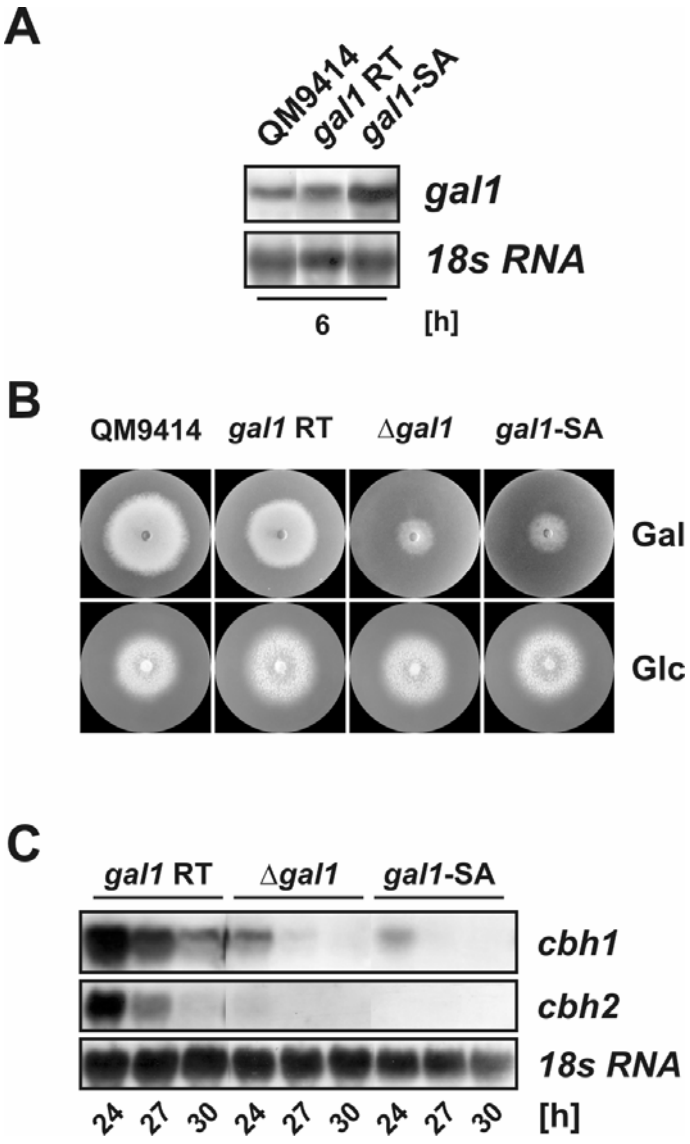


Fig. 1. Effect of the expression of the catalytically inactive GAL1-SA in an *H. jecorina* Δ*gal1* strain. (A) *gal1* transcript formation in *H. jecorina* QM9414, a *gal1* retransformed strain (*gal1* RT) and a *gal1*-SA transformed strain (*gal1*-SA) six hours after transfer to a D-galactose (1% w/v) containing medium. **(B)** Growth behaviour on minimal medium containing D-glucose (Glc) or D-galactose (Gal) after 3 days of incubation at 30°C. **(C)** Transcript formation of the two major cellobiohydrolase genes *cbh1* and *cbh2* during growth on lactose.

Δ*gal1* strain on D-galactose (Fig. 1 A), thus confirming that the deletion of the two amino acids leads to an inactive galactokinase in *H. jecorina*. Consequently, these strains were tested for their ability to induce transcription of the two major cellulase genes *cbh1* and *cbh2* by lactose.

Fig. 1 C shows that only strains expressing the wild-type *gal1* could fully restore *cbh1* and *cbh2* transcript formation, whereas strains expressing the Gal1-SA did not. From this we conclude that the presence of the SA amino acid doublet in the *H. jecorina* GAL1 is essential for the galactokinase activity and the induction of cellulases by lactose.

The *E. coli* galactokinase *galK* can rescue cellulase induction by lactose

The results described above suggest that a loss of galactokinase activity mimics the *H. jecorina* Δ*gal1* phenotype with respect to the induction of cellulases by lactose. In order to test if it is solely the galactokinase activity which is essential for cellulase induction, we examined whether the expression of a bacterial galactokinase, which possesses no transcriptional

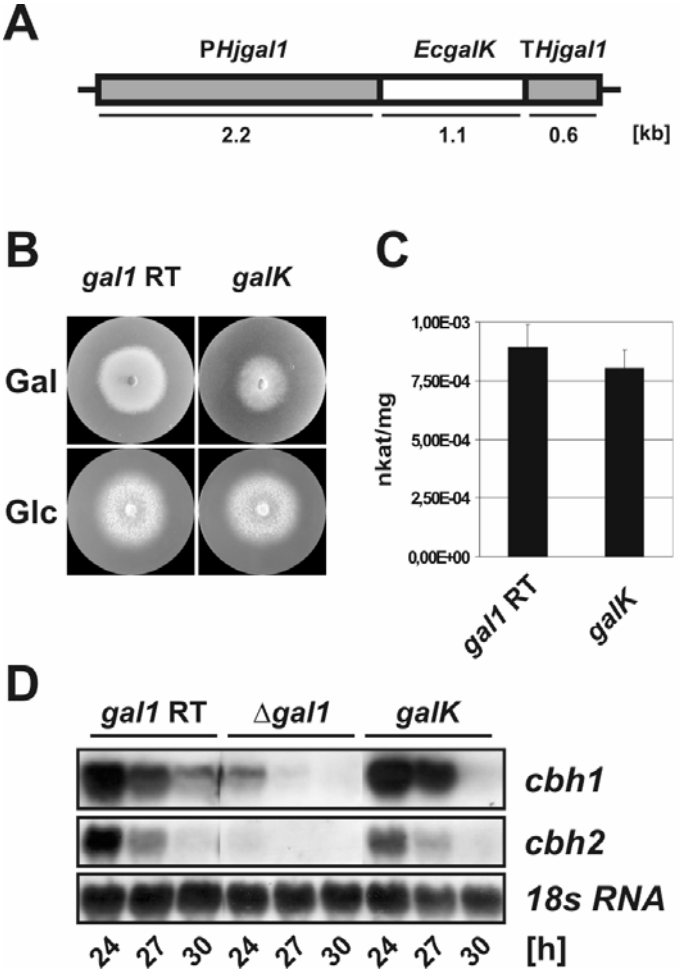


Fig. 2. Expression of the *E. coli* galactokinase *galK* in an *H. jecorina* Δ*gal1* strain (A) For the expression of the *E. coli galK* gene its coding region was placed between the *H. jecorina gal1* promoter and terminator region. **(B)** Comparison of the growth behaviour of a *gal1* retransformed (*gal1* RT) and a *galK* transformed *H. jecorina* Δ*gal1* strain. **(C)** Galactokinase activity formation after transfer to a D-galactose containing medium after 12 hours of growth. **(D)** Transcript levels of the two cellobiohydrolases *cbh1* and *cbh2* during growth on lactose.

inducer function, would be able to rescue the Δ*gal1* phenotype with respect to the inducibility of the cellulase genes by lactose. Therefore we used the *E. coli galK* gene, which is able to complement for the Sc*GAL1* but not the Sc*GAL3* in *S. cerevisiae* (Bhat et al., 1990). A *H. jecorina* Δ*gal1* strain was transformed with the *E. coli galK* gene under control of the *H. jecorina gal1* expression signals (Fig. 2 *A*). Integration and expression of the cassette was verified by Southern analysis and Northern analysis. The *galK* transformed Δ*gal1* strains grew better than the Δ*gal1* strains on D-galactose but were not able to reach the level of the parental strain QM9414 or *gal1* retransformed strains (Fig. 2 B). Measurements of galactokinase activity in these *galK* transformed strains showed that they produced somewhat lower enzyme activities (about 80-90 %) compared to QM9414 and *H. jecorina* retransformed strains (Fig. 2 C). But when grown on lactose, *galK* expressing strains showed that they had regained their ability to fully induce *cbh1* and *cbh2* transcription (Fig. 2 D), and thus prove that the galactokinase activity was sufficient for the induction of cellulases by lactose.

GAL1 is dispensable for induction of *gal7* by D-galactose

The results above have shown that galactokinase activity *per se* - but not a putative transcriptional inducer function of the Gal1 protein as found in the ScGal3 or KlGal1 - is necessary for cellulase induction by lactose. Consequently, we investigated whether the induction of the inducible Leloir genes would follow the same trend. Unlike in *S. cerevisiae* or *K. lactis* the *gal* genes in *H. jecorina* are constitutively transcribed during growth, and only the *H. jecorina gal1* and *gal7* (but not *gal10*) are induced to still higher levels by the addition of D-galactose. Therefore we compared the transcriptional level of *gal7* in our set of recombinant strains during growth on D-glucose and D-galactose. As can be seen from Fig. 3 induction by D-galactose led to an about threefold abundance of the *gal7* transcript in comparison to transfer to D-glucose, and this result was also obtained both with the parental strain, a Δ*gal1* strain, and Δ*gal1* strains which expressed the *E. coli* GalK or the *H. jecorina*

Gal1-SA. These results imply that the Gal1 protein as well as the galactokinase activity is dispensable for the basal as well as the inducible level of *gal7* transcription.

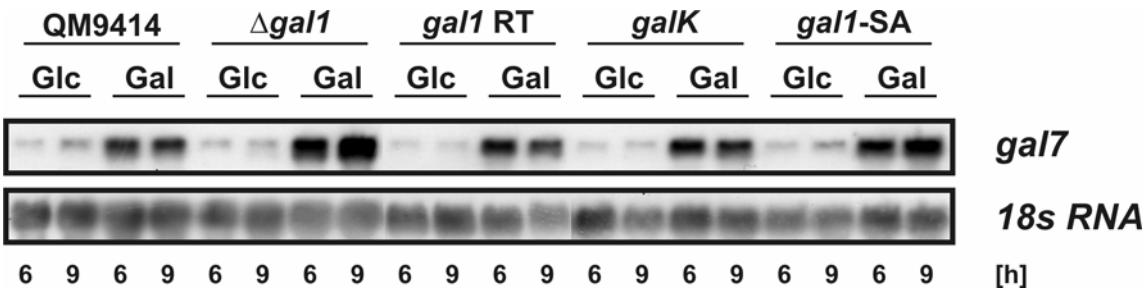
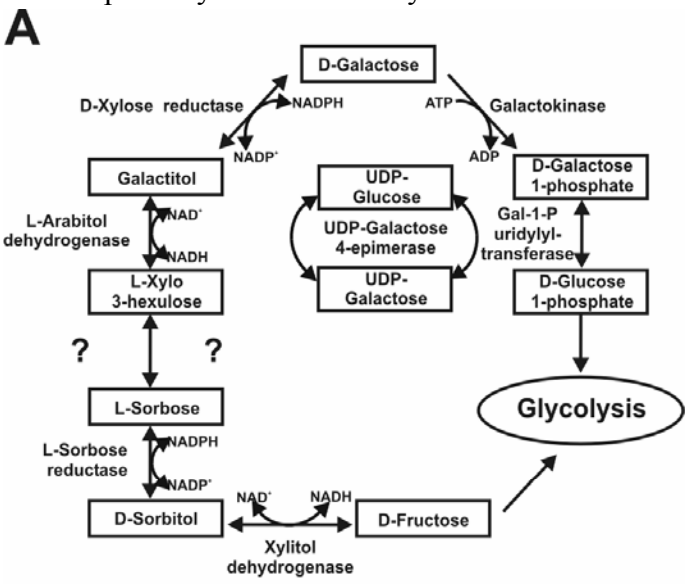


Fig. 3. Northern analysis of the Leloir pathway gene *gal7* in different *H. jecorina* strains. *gal7* transcript levels were determined after transfer to a minimal medium containing D-glucose (Glc) or D-galactose (Gal) in *H. jecorina* QM9414, a *gal1* deleted strain (Δ*gal1*), a *gal1* retransformed Δ*gal1* strain (*gal1* RT), an *E. coli galK* expressing Δ*gal1* strain (*galK*) and a *gal1*-SA expressing Δ*gal1* strain (*gal1*-SA).

Galactitol formation is dispensable for *gal7* induction by D-galactose

D-galactose can be catabolized by filamentous fungi such as *H. jecorina* or *A. nidulans* via a second pathway absent in the yeasts *S. cerevisiae* and *K. lactis* (Fig. 4 A) (Fekete et al., 2004; Seiboth et al., 2004). The first step in this pathway is the reduction to galactitol by an aldose reductase which in the case of *H. jecorina* was identified as the D-xylose reductase XYL1. Galactitol formation by the D-



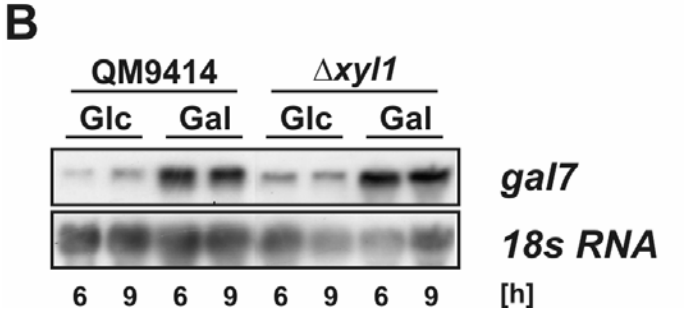


Fig. 4 (A) D-galactose catabolizing pathways in *H. jecorina*. The Leloir pathway and the second pathway for D-galactose catabolism in fungi (Seiboth et al., 2007). ? indicates steps which have to be still verified. **(B)** Effect of deletion of the D-xylose reductase gene (*xyl1*) on the induction of the *gal7* gene. Transcript formation was studied after six and nine hours of transfer to D-glucose and D-galactose containing medium.

xylose reductase in this pathway has been shown to be responsible for the induction of the extracellular *β*-galactosidase formation by D-galactose (Fekete et al., 2007). Since neither the GAL1 protein nor galactokinase activity is required for the induction of *gal7* by D-galactose, we tested whether catabolism of D-galactose via this second pathway would also create the inducer of the Leloir pathway. To this end, we tested the induction of *gal7* by D-galactose in a Δ*xyl1* strain. Results shown in Fig. 4 B document that *gal7* induction is not affected in this strain, implying that the alternative pathway and galactitol formation are not involved in induction. We conclude from these results that D-galactose induction of *gal7* in *H. jecorina* occurs independently of a blockage in the first step of either of the two pathways for D-galactose catabolism and is independent of galactitol formation.

Table 1 Oligonucleotides used in the present study

Name	Sequence*
GAL1mut1	5′-GACAAAGGCGGAGCTCAGGCC-3′
GAL1mut2	5′-GAGCTCCGCCTTTGTCACCGC-3′
GAL1mut3	5′-GAGGTGTGGGCTTGCTG-3′
EcGAL1fw (*Hin*dIII)	5′-GACTAAGCTTCGCCAACATGAGTCT GAAGGAAAAGACACAA-3′
EcGAL1rv (*Hin*dIII, *Acc*65I)	5′-GATCAAGCTTGGTACCTCAGCACTGTCCTGCTC-3′
GAL1termfw (*Acc*65I)	5′-GATCGGTACCAGGAATTAAGGGTGGGAAAG-3′
GAL1termrv (*Acc*65I)	5′-GCATGGTACCTTCCTTTGCCCGTGGGT-3′
Pgpdf (*Bam*HI)	5′-GATCGGATCCGAGAGCTACCTTACATCAA-3′
Pgpdr (*Xba*I)	5′-GATCTCTAGATTTGTATCTGCGAATTGAGCTT-3′
Tgpdf (*Nsi*I)	5′-GATCATGCATGTGCTGTGTTCCTCAGAAT-3′
Tgpdr (*Sal*I)	5′-GATCGTCGACTTTCTTGGATTTGCAGCACAG-3′
CBH1SF	5′-TCGGCCTGCACTCTCCAATC-3′
CBH1SR	5′-TGGAGTCCAGCCACAGCATG-3′
CBH2SF	5′-ATTCTCACCACGCTGGCTAC-3′
CBH2SR	5′-CGGCGTAGTTGATGCACTC-3′
18sRF	5′-GGTGGAGTGATTTGTCTG-3′
18sRR	5′-CTTACTAGGGATTCCTCG-3′
gal7for	5′-CCGATATCATGCCTGACAAGATCCTCGATG-3′
gal7rev	5′-GTCTAGCTCAACTTGTTCCGG-3′

* Respective restriction sites are underlined

DISCUSSION

The *S. cerevisiae GAL* regulon has become a paradigm for transcriptional control in lower eukaryotes, and a model system for gene regulation (Schaffrath and Breunig, 2000; Bhat and Murthy, 2001; Rubio-Texeira, 2005). While its importance is uncontested, the present results illustrate that this regulatory network may be restricted to yeasts and not representative for other ascomycetous fungi in general. The present data - that the Leloir pathway is regulated in a GAL1 independent way in *H. jecorina* – are consistent to previous findings such as non-clustered genomic organization, lack of carbon catabolite repression by D-glucose, induction of *gal7* and *gal1* by L-arabinose, existence of a second D-galactose catabolic pathway and lack of a mutarotase (aldose 1-epimerase) domain in GAL10 (reviewed by Seiboth et al., 2007). This and the fact that we were not able to detect orthologues of *GAL4* in the genome sequences of *H. jecorina* and other filamentous fungi (*N. crassa, Magnaporthe grisea, Fusarium graminearum, A. nidulans*) support the present data that the Leloir pathway is controlled in a different way. While the present study has only been performed with one ascomycetous species from the Pyrenomycetes, we also have preliminary data from *A. nidulans* and *Aspergillus niger* which are fully consistent with those obtained for *H. jecorina*. Consequently, we conclude that the *GAL* regulon is a yeast-specific trait but cannot be used to explain D-galactose regulation in filamentous fungi.

In a previous study we failed to detect the induced level of the *gal7* transcript in a Δ*gal1* strain of *H. jecorina*, however, we used only a single time point and different conditions (Seiboth et al., 2004). In contrast, here we showed that D-galactose clearly induces *gal7* in the Δ*gal1* strain. While this implies that D-galactose induces *gal7* without being metabolized through one of the two pathways, the underlying mechanism is unclear. It could be D-galactose itself, either bound to its permease (a Gal2 orthologue is present in the *H. jecorina* genome; scaffold 10: 1127812-1130067) or its intracellular steady state

concentration. It should be noted that the constitutive expression of the Leloir pathway genes could create a reverse pathway of galactose 1-phosphate formation, by the action of GAL10 (forming UDP-galactose from UDP-glucose), and its subsequent conversion to D-galactose 1-phosphate by GAL7. Therefore, we cannot exclude at the moment that the inducer could still be D-galactose 1-phosphate.

In contrast to *gal7* induction, galactokinase activity was required for the induction of the cellulase genes by lactose. This implies that the regulation of the Leloir pathway and that of cellulases is not linked, which is corroborated also by the findings that D-galactose does induce cellulase gene expression only at a low level at low growth rates (Karaffa et al., 2006). In theory, this would be consistent with a role for D-galactose 1-phosphate as an inducer of cellulase formation. However, such an assumption raises the question why galactokinase activity promotes high cellulase gene expression during growth on lactose. As emphasized previously, a major difference between growth on D-galactose and on lactose is that the latter is accompanied by a stronger participation of the alternative, reductive D-galactose degrading pathway (Karaffa et al., 2006; Seiboth et al., 2007). Blockage of the latter in a Δ*xyl1* also strongly impairs cellulase induction on lactose, and the effect is not additive with that in a Δ*gal1*, as seen in a Δ*gal1*Δ*xyl1* strain (Seiboth, B., Gamauf, C., Hartl, L., Pail, M., Kubicek, C.P., unpublished results; Fekete et al., 2007). In other words, D-galactose 1-phosphate as well as galactitol or catabolites thereof are needed for high induction of cellulase formation. Whether they both cooperate in the induction themselves, or first need to be converted to another metabolite which then acts as an inducer is currently investigated in our laboratory.

In *S. cerevisiae* the *GAL* genes are repressed by D-glucose, derepressed (non-induced) on other respiratory carbon sources and highly induced (1000 fold) in the presence of D-galactose. In comparison to *S. cerevisiae* the basal level of *GAL* gene expression is higher in other yeasts e.g. *K. lactis* (Zachariae and Breunig, 1993). In *H. jecorina* no repression by D-glucose was found, and transcription on D-glucose occurred to a similar extent as on other

non-inducing carbon sources, and only a relatively weak induction (three to fourfold) was found during growth on D-galactose. These differences in the transcriptional regulation raise the question why the Leloir pathway is regulated in a different way by filamentous fungi. An explanation for this may be derived from the different importance of D-galactose for fungal cell biology: D-galactose is an important component of the cell wall of many filamentous fungi, and also of their glycosylated proteins, whereas it does not occur in the cell walls of *S. cerevisiae* and is only a minor component of its glycoproteins (Kainuma et al., 2001). The importance of D-galactose for the cell wall and glycoproteins is higher in other yeasts including *K. lactis* or *Schizosaccharomyces pombe* than in *S. cerevisiae* (Klis et al., 2002; Grün et al., 2005; Lesage and Bussey, 2006). It seems therefore especially important for filamentous fungi to metabolize D-galactose which results from the normal turnover of the cell wall and glycoproteins, before it accumulates intracellularly and would interfere with the regulation of other pathways. This may also explain the constitutive expression of *gal10* whose gene product is needed for the production of the activated precursor UDP-galactose for the synthesis of the carbohydrates in cell wall components and glycoproteins. While there may still be other reasons, we speculate that these differences gave rise to the evolution of the different patterns of D-galactose regulation in fungi.

MATERIALS AND METHODS

Strains and culture conditions

H. jecorina strains QM9414 (ATCC 26921), Δ*gal1* (Seiboth et al., 2004) and Δ*xyl1* (Fekete et al., 2007) were maintained on malt extract agar (Merck, VWR International, Vienna, Austria). Strains were grown in 1 l Erlenmeyer flasks on a rotary shaker (250 rpm) at 30 °C in the medium described by (Mandels and Andreotti, 1978) with the respective carbon source at a

final concentration of 10 g l^{-1}. For transcript analysis and galactokinase activity determination, strains were pregrown in with glycerol (1% w/v) as carbon source for about 24 h, mycelia harvested by filtration, washed with medium without carbon source and equal amounts of mycelia transferred to 500 ml flasks containing the respective carbon source (1% w/v) and cultivation continued as indicated.

To determine hyphal growth on agar plates, plates were inoculated with a small piece of agar in the centre of an 11-cm plate.

Escherichia coli strain JM109 (Promega, Madison, WI) and K12 (ATCC 19215) were used for plasmid propagation and cloning of the *galK*.

Plasmids and plasmid constructions

pGAL1 contains a 4.8 kb *Eco*RV-*Hin*dIII clone of the *H. jecorina gal1* (Seiboth et al., 2004) in pUC19 (Yanisch-Perron et al., 1985) and pGALSH a 2.1 kb *Sac*II-*Hin*dIII *gal1* subclone in pBluescript SK(+) (Stratagene, La Jolla, CA).

pGAL1-SA: contains the 4.8 kb *H. jecorina gal1* clone in which 6 nucleotides encoding the aa doublet $Ser^{170}Ala^{171}$ were deleted by fusion PCR approach: First two *gal1* fragments were amplified using pGALSH as template and the universal primer M13 reverse and GAL1mut1 respectively GAL1mut2 and GAL1mut3. Both amplicons were joined in a second round using the outside/outward primers M13 reverse and GAL1mut3. The resulting PCR product was cut with *Sac*II/*Sfi*I to give a 140 bp fragment which was ligated in the *Sac*II/*Sfi*I restricted pGAL1 and designated pGAL1-SA.

pGALK: the *E. coli galK* coding region (1.1 kb) was placed under the promoter and terminator region of the *H. jecorina gal1*. *E. coli galK* was amplified from genomic *E. coli* K12 DNA with oligonucleotides EcGAL1fw inserting a *Hin*dIII site and EcGAL1rv inserting *Acc*65I/*Hin*dIII sites by PCR. The *Hin*dIII restricted *galK* fragment was ligated into the *Hin*dIII site of pGAL1. The introduced *Acc*65I site was used to insert a 0.6 kb fragment of the

terminator region which was amplified by PCR using oligonucleotides GAL1termfw and GAL1termrv.

pLH1hph: A plasmid expressing the *E. coli* hygromycin B phosphotransferase gene (*hph*) under the promoter and terminator region of *H. jecorina gpd1* (glyceraldehyde 3-phosphate dehydrogenase, http://genome.jgi-psf.org/Trire2/Trire2.home.html, scaffold 1: 1562760-1564086) was constructed. 800 bp of the *gpd1* promoter region were amplified using the primer pair Pgpdf and Pgpdr and 500 bp of the terminator region using the primer pair Tgpdf and Tgpdr. The *Xba*I/*Nsi*I fragment of the *E. coli hph* of pRLMex30 (Mach et al., 1994) and the *Nsi*I/*Sal*I *gpd1* terminator region were ligated into an *Xba*I/*Sal*I restricted pUC19. The *Bam*HI/*Xba*I sites of the resulting vector were used for the insertion of the promoter region. All constructs were verified by sequencing.

Construction of fungal strains

Protoplast preparation, DNA mediated transformation and purification of the conidia was described previously (Gruber et al., 1990b). Malt extract agar plates containing D-sorbitol (1 M) and hygromycin B (50 µg ml^{-1}) were used for selection of transformed protoplasts.

Transformations of the Δ*gal1* strain were done as co-transformations either with the circular pLH1hph and a 4.2 kb *Pvu*II fragment of pGALK or with the excised P*gpd1-hph*-T*gpd1* cassette of pLH1hph together with the circular pGAL1 or pGAL1-SA. The P*gpd1-hph*-T*gpd1* cassette was excised with *Bam*HI, *Sal*I and *Pdm*I. Fragments were purified from agarose gel using the QIAquick Gel Extraction Kit (QIAGEN Inc, Valencia, California).

Southern analysis was performed to verify the integration of the constructs in the different strains. Only strains showing a single ectopic integration of the construct were used for further analysis. Genomic DNA of the Δ*gal1* retransformed strains with pGAL1 or pGAL1-SA was digested with *Hin*dIII. Hybridization of these strains with a 2.6 kb *Hin*dIII *gal1* fragment, results in *H. jecorina* QM9414 in a 2.6 kb band, in the Δ*gal1* strain in a 4.7 kb

band and in retransformed strains in a 4.7 kb band and an additional band which represents either the *gal1* or *gal1*-SA fragment. Integration of the *E. coli galK* was verified by digestion with *Mlu*I, which cuts once within the *galK* coding region. For hybridization a 1.1 kb PCR fragment of *galK*, amplified with EcGAL1fw and EcGAL1rv was used. Strains which showed two hybridizing bands indicating the integration of one copy of the *E. coli galK* were used for further analysis.

Transcript analysis

DIG labeled probes for the Northern analyses of *cbh1*, *cbh2* and 18S rRNA with the respective sizes of 1.2 kb, 1 kb and 300 bp were amplified from chromosomal DNA using the primers CBH1SF, CBH1SR, CBH2SF, CBH2SR, 18sRF, and 18sRR. In the case of *gal7* the DIG labeled probe was amplified from a plasmid containing a 1.1 kb cDNA fragment of *gal7* using the primers gal7for and gal7rev.

Nucleic acid isolation and hybridization

DNA and RNA preparation from mycelia was described previously (Seiboth et al., 2004). Standard methods were used for nucleic acid electrophoresis and blotting (Sambrook and Russel, 2001). Target DNAs were detected with probes labeled with [α-^{32}P]dCTP by random priming. Northern analysis was performed with the DIG nonradioactive system from Roche Applied Science (Mannheim, Germany).

Galactokinase assay

Strains were grown as described above in transfer cultures and after transfer cultivation was continued for 12 h (D-galactose) or 20 h (lactose) respectively. Mycelia were harvested by filtration, washed extensively with cold sterile tap water, blotted dry with paper towels, and ground to a fine powder under liquid N_2 with mortar and pestle. About one gram (wet weight)

of mycelium was suspended in 3 ml of extraction buffer (0.1 M Tris-HCl (pH 7.5), 1 mM EDTA, 5 mM β-mercaptoethanol) and homogenized by sonication 10 times for 30 s at 2°C, with intermittent 1-min cooling periods. The resulting homogenate was centrifuged at 10.000 x *g* for 20 min at 4°C. The supernatant (average protein concentration 10 to 15 mg ml^{-1}) was used as a cell extract for measuring intracellular galactokinase activity as described previously (Fekete et al., 2002). Specific activities are reported as nkat per mg protein, which was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany).

ACKNOWLEDGEMENTS

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Chapter 4

Sequential gene deletions in *Hypocrea jecorina* using a single blaster cassette

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Sequential gene deletions in *Hypocrea jecorina* using a single blaster cassette.
Curr Genet **48**, 204-211

ABSTRACT

In *Hypocrea jecorina* (anamorph: *Trichoderma reesei*) multiple gene deletions are limited by the number of readily available selection markers. We have therefore constructed a blaster cassette which enables successive gene knock-outs in *H. jecorina*. This 3.5 kb *pyr4* blaster cassette contains the *H. jecorina pyr4* marker gene encoding orotidine-5'-monophosphate (OMP) decarboxylase flanked by two direct repeats of the *Streptoalloteichus hindustanus* bleomycin gene (*Sh ble*), which facilitate the excision of the blaster cassette by homologous recombination after each round of deletion. Functionality of this *pyr4* blaster cassette was demonstrated by deletion of the *glk1* encoding glucokinase and *hxk1* encoding hexokinase. 1.4 to 1.8 kb of the non-coding flanking regions of both target genes were cloned into the respective blaster cassettes and transformation of a *pyr4* negative *H. jecorina* strain with the two cassettes resulted in 10-13 % of the transformants in the deletion of one of the two kinase genes. For excision of the *pyr4* blaster cassettes, Δ*glk1* strains were selected for growth in the presence of 5-fluoroorotic acid. Recombination between the two *Sh ble* elements resulted in uridine auxotrophic strains which retained their respective glucokinase negative phenotype. Subsequent transformation of one of these auxotrophic Δ*glk1* strains with the hexokinase blaster cassette resulted in *pyr4* prototrophic strains deleted in both *glk1* and *hxk1*. Δ*glk1* strains showed reduced growth on D-glucose and D-fructose whereas Δ*hxkl* strains showed reduced compact growth on D-glucose but were unable to grow on D-fructose as carbon source. The double Δ*glk1*Δ*hxk1* deletion strain was completely unable to grow either D-glucose or D-fructose.

INTRODUCTION

The ascomycete *Hypocrea jecorina* (anamorph *Trichoderma reesei*) is industrially applied for the production of enzymes including a number of (hemi)cellulases and its strong cellobiohydrolase promoters are used for the expression of recombinant proteins (Penttilä, 1998). Although a sexual cycle of *H. jecorina* has been described (Kuhls et al., 1996), most of the research and all of the industrial application are performed almost exclusively with a single asexual isolate *H. jecorina* QM6a from the Solomon Islands and its derivatives (Kubicek and Harman, 1998). Functional genomic studies in *H. jecorina* depend on an efficient targeted gene manipulation system and the construction of defined strains for the investigation of gene function. DNA mediated transformation in *H. jecorina* is integrative and relies on a limited number of dominant markers and auxotrophic markers. These include the *Escherichia coli hph* (hygromycin B phosphotransferase), the *E. coli* and *Strepotalloteichus hindustanus ble* (bleo/phleomycin resistance), the *Aspergillus nidulans amdS* (acetamidase) or the *H. jecorina pyr4* (reviewed by Mach, 2004).

Traditional DNA mediated transformations are limited in terms of the number of marker genes which can be inserted. This fact restricts studies of e.g. the function of orthologous and paralogous genes or of whole gene families. Therefore the development of a versatile transformation system independent on the number of available markers would be beneficial. In yeasts, so called blaster cassettes were developed which allow the repeated use of the *URA3* (the yeast *pyr4* homologue) marker to construct multiple disrupted strains (Alani et al., 1987; Fonzi and Irwin, 1993). Such blaster cassettes consist of the *URA3* encoding the orotidine-5'-decarboxylase flanked by two direct repeats. Strains which are defective in *URA3* are auxotrophic for uridine (uracil), but are - in contrast to prototrophic strains - resistant to 5-fluoroorotic acid (5-FOA; (Boeke et al., 1984), which is converted by OMP-decarboxylase to the toxic intermediate 5-fluoro-UMP. Integration of the blaster is therefore selected via

Ura3 function and excision of the *URA3* marker is then forced in the presence of 5-FOA by recombination between the two direct repeats. As a consequence this blaster cassette can be reused for successive rounds of gene deletions, allowing multiple deletions with a single cassette. This cassette has permitted successive disruption of *C. albicans* alleles (reviewed by Pla et al., 1996) and even families of genes (Mio et al., 1996; Muhlschlegel and Fonzi, 1997; Sanglard et al., 1997) with a single auxotrophic marker. In filamentous fungi, a similar blaster cassette was successfully applied for the deletion of *rodA* in the opportunistic pathogen *Aspergillus fumigatus* (d'Enfert, 1996) and *aroC* in *Aspergillus nidulans* (Krappmann and Braus, 2003).

We developed a blaster cassette for multiple gene deletions in *H. jecorina* based on the *H. jecorina pyr4* flanked by direct repeats of the *S. hindustanus ble*. The functionality of the blaster cassette for successive gene deletion is demonstrated by the construction of stable *H. jecorina* strains deleted in the gluco- or hexokinase encoding genes and the reuse of the *pyr4* blaster to construct double knock-out strains.

RESULTS

Construction of a *pyr4* blaster cassette for sequential targeted gene deletions

A blaster cassette containing the *H. jecorina pyr4* gene flanked by two gene fragments of the *S. hindustanus Sh ble* was constructed (Fig. 1). The two *Sh ble* elements were orientated as direct repeats to facilitate the excision of the *pyr4* marker by loop-out between the homologous regions after a successful gene deletion. On each side of the blaster cassette unique restriction sites (*Eco*RI, *Bam*HI, *Xba*I, *Sal*I and *Hin*dIII) were located to enable the insertion of the up- and downstream regions of the target genes. Starting from this *pyr4* blaster cassette we constructed two different blaster cassettes for deletion of the *H. jecorina*

glucokinase (*glk1*) and hexokinase (*hxk1*) encoding genes by amplification of their noncoding regions by PCR. Appropriate restriction sites were introduced at the end of each fragment by PCR to facilitate the ligation of these fragments into the *pyr4* blaster cassette. The final glucokinase blaster p∆*glk1* contained 1.4 kb of each flanking regions of the *glk1* coding region, while the hexokinase blaster p∆*hxk1* contains 1.4 kb of the up- and 1.8 kb of the downstream region of the *hxk1* coding region. In addition, the final blaster cassettes for the two genes were constructed in such a way that they could be easily excised by a single *Eco*RI digest. Alternatively, the respective cassette can be amplified by PCR using the primer pair located in the up- and downstream regions.

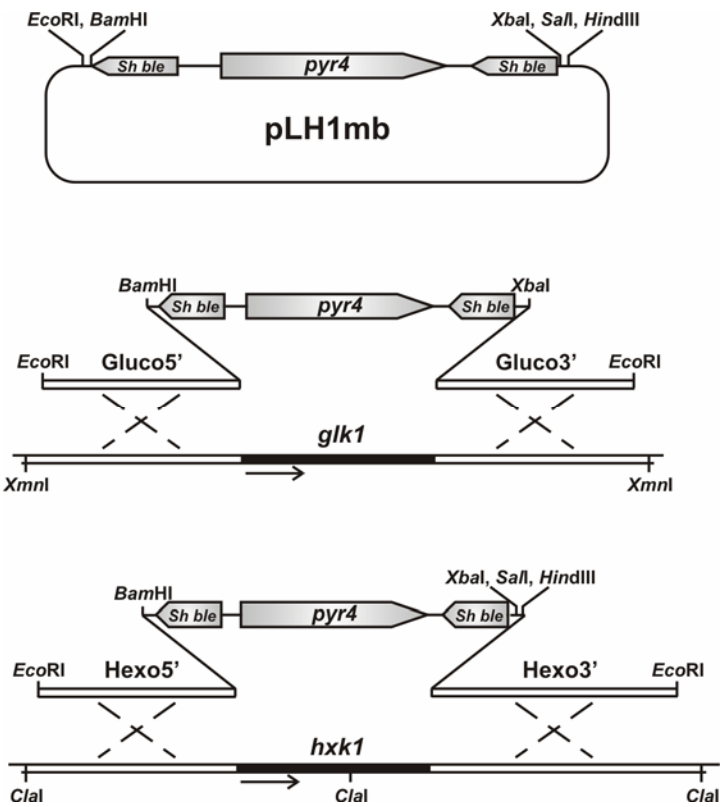

Fig. 1 Schematic representation of the *pyr4* blaster cassette pLH1mb and gene replacement at the *H. jecorina glk1* and *hxk1* loci. pLH1mb contains the *H. jecorina pyr4* gene flanked by two direct repeats of a *S. hindustanus ble* fragment. Orientation of the different genes is indicated by arrows. Important restriction enzyme sites which are useful for cloning of the up and downstream regions of the target genes into pLH1mb, for the release of the blaster cassettes from the vector or for the Southern analyses are also indicated. Positions of the probes for *glk1* and *hxk1* are indicated.

Deletion of *glk1* encoding glucokinase and *hxk1* encoding hexokinase in *H. jecorina*

The functionality of the blaster approach for *H. jecorina* was tested by deletion of two genes encoding hexose phosphorylating enzymes in the uridine auxotrophic *pyr4* negative strain TU-6. Although *A. nidulans* strains lacking hexokinase or glucokinase grew well on D-glucose containing media (Flipphi et al., 2003) we replaced D-glucose in the protoplast

regeneration plates by either glycerol or L-arabinose to avoid any possible negative selection for homologous integrated blaster cassettes. Glycerol was chosen because it is channelled into glycolysis after the hexose phosphorylation steps while the pentose L-arabinose is catabolized by a path not involving glycolytic enzymes (Chiang and Knight, 1961). *H. jecorina* TU-6 was transformed with the two blaster fragments and the resulting transformants were selected for uridine prototrophy on minimal medium. Purified transformants were tested for growth on a number of carbon sources including D-glucose, D-fructose, glycerol, and L-arabinose to select for putative gene knock-outs. Southern analysis confirmed the *glk1* or *hxk1* deletions (Fig. 2 A and B). 13 % of the total number of glucokinase and 10 % of the hexokinase transformants showed a deletion at the respective gene locus. Hybridization with the coding region of the respective genes confirmed their complete removal. Growth tests on a number of carbon sources showed that Δ*glk1* strains showed reduced growth on D-glucose and D-fructose, whereas Δ*hxk1* strains showed reduced compact growth on

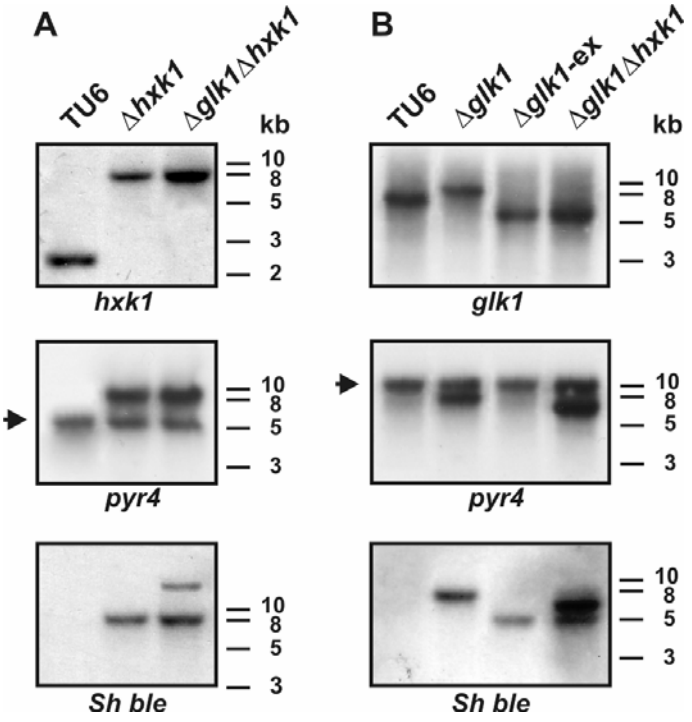

Fig. 2. Southern analyses of *H. jecorina* gluco- and hexokinase negative strains. The endogenous non-functional copy of the *pyr4* is marked by an arrow. **(A)** Genomic DNA of the parental strain TU-6, a Δ*hxk1* and a Δ*glk1*Δ*hxk1* strain were digested with *Cla*I and probed with *hxk1*, *pyr4* and *Sh ble* fragments. Insertion of the blaster cassette at the *hxk1* locus leads to an increase of the hybridizing band from 2.7 kb in strain TU-6 to 8.7 kb in the Δ*hxk1* and Δ*glk1*Δ*hxk1* using the *hxk1* fragment as probe. This 8.7 kb band is also detected with the *pyr4* or *Sh bl*e fragments as probe. In the Δ*glk1*Δ*hxk1* strain an additional weaker hybridizing fragment is found with the *Sh ble* fragment as probe which corresponds to a single *Sh ble* fragment resulting from the excision of the glucokinase blaster cassette. **(B)** Genomic DNA of strain TU-6, a Δ*glk1*, a glucokinase blaster excised strain Δ*glk1*-ex and a Δ*glk1*Δ*hxk1* strain were digested with *Xmn*I and probed with the respective fragments. Homologous insertion of the glucokinase blaster cassette leads to an increase of the hybridizing band from 7.1 kb in the TU-6 strain to 8.8 kb in the Δ*glk1* strain when probed with the *glk1* fragment. This 8.8 kb band is also detected with the *pyr4* or *Sh ble* probe. In strain Δ*glk1*-ex the *glk1* and *Sh ble* hybridizing band is reduced to 5.5 kb due the excision of the glucokinase blaster. In strain Δ*glk1*Δ*hxk1* the *glk1* band is also reduced to 5.5 kb, but an additional stronger *Sh ble* hybridizing band resulting from two *Sh ble* fragments of the hexokinase blaster and a *pyr4* hybridizing band are found.

D-glucose and were unable to grow on D-fructose (Fig. 3). However, growth of both deletion strains was also affected on glycerol or L-arabinose indicating a pleiotropic effect resulting from these deletions.

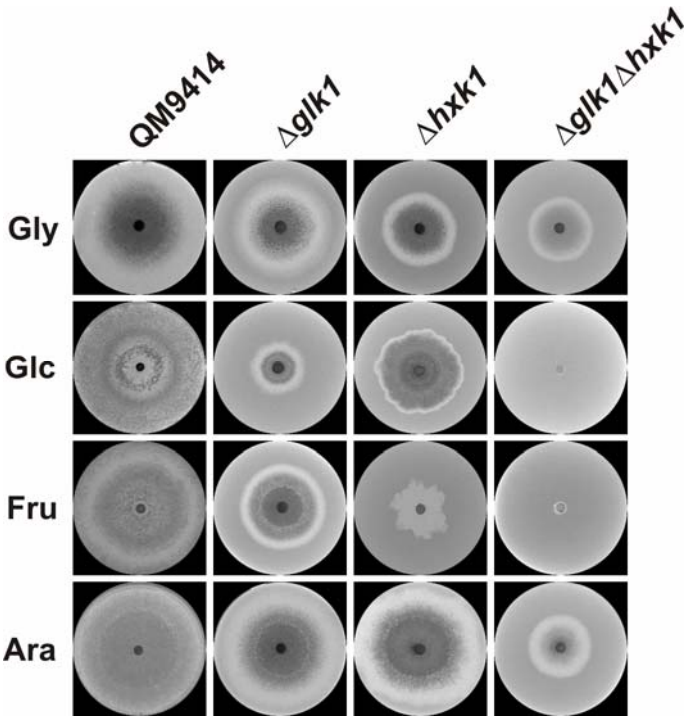

Fig. 3. Growth comparison of QM9414, a Δ*glk1*, a Δ*hxk1* and a Δ*glk1*Δ*hxk1* strain after 3.5 days on different carbon sources. The growth behaviour of QM9414 or TU-6 strains with an ectopically integrated hexokinase or glucokinase cassette was essentially the same. Abbreviations: Gly, glycerol; Glc, D-glucose; Fru, D-fructose and Ara, L-arabinose.

Excision of the *pyr4* blaster in the Δ*glk1* strain

A successful re-use of the blaster cassette depends on the excision of the *pyr4* marker by recombination between the flanking *Sh ble* direct repeats. We chose three Δ*glk1* strains and plated their conidiospores on 5-FOA plates to force and select for the excision of the *pyr4* blaster. 5-FOA resistant colonies appeared after 3-5 days of incubation with a frequency of about 1-2 x 10^{-4}. As these colonies did not sporulate, 5-FOA resistant colonies were transferred to minimal medium plates containing uridine which allowed also the growth of strains in which the blaster cassette did not loop out. Purified colonies were then tested for uridine auxotrophy on minimal medium. About 90% of the 5-FOA resistant strains picked were found to be uridine auxotroph while the remaining 10% were uridine prototroph. A Southern analysis showed that only a single copy of the *Sh ble* fragment was left in the auxotrophic strains and that the *pyr4* from the blaster cassette was completely removed, but

the Δ*glk1* genotype retained (Fig. 2 B). Sequencing of a PCR fragment comprising the disrupted *glk1* locus confirmed that only a single *Sh ble* fragment was left in these strains. The *Sh ble* fragment was bordered by two restriction enzyme sites for *Xba*I and *Bam*HI which could only originate from a recombination between the two original *Sh ble* fragments from the blaster cassette.

Table 1 Oligonucleotides used in the present study

Name	Sequence*
zeo1fw	5'-GATCTCTAGAACCATGGCCAAGTTGACCAG-3'
zeo1rv	5'-GATCCTCGAGTCAGTCCTGCTCCTCGG-3'
zeo2fw	5'-GATCCTCGAGACCATGGCCAAGTTGACCAG-3'
zeo2rv	5'-GATCGGATCCTCAGTCCTGCTCCTCGG-3'
Gluco5'F	5'-GATCGAATTCAAAGCAGCAGAACAACG-3'
Gluco5'R	5'-CTAGGGATCCTCCGAAAAGTCGAACTG-3'
Gluco3'F	5'-GATCTCTAGATAAGGTACTGACCATGTC-3'
Gluco3'R	5'-GATCTCTAGAGAATTCCAGCAGCACAACATATAC-3'
Hexo5'F	5'-GATCGAATTCTATGAGGTACGTATGTAG-3'
Hexo5'R	5'-GATCGGATCCATGGTGGTCAGTATTTTC-3'
Hexo3'F	5'-CTAGAAGCTTTAGATTTGGAACATGTTTGTC-3'
Hexo3'R	5'-GATCAAGCTTGAATTCAAGTTGGGCAG-3'

* Respective restriction sites are underlined

Deletion of *hxk1* in the Δ*glk1* strain

Following the successful excision of the blaster cassette we demonstrated its reuse in a second round of gene deletion. We chose to construct a Δ*glk1*Δ*hxk1* strain and transformed therefore five of the uridine auxotrophic Δ*glk1* strains - obtained after the blaster cassette excision - with the hexokinase blaster. All five Δ*glk1* strains could be transformed to uridine prototrophy, indicating that their auxotrophy was indeed a result of the excision of the *pyr4* blaster and not due to other mutations. L-arabinose was used in the protoplast regeneration medium since we expected the double deletion strain to be unable to grow on D-glucose. Transformants were purified and subjected to growth tests and Southern analyses (Fig. 2 and 3). About 12 % of the transformants turned out to be double deleted Δ*glk1*Δ*hxk1* strains. They were completely unable to grow on D-glucose and D-fructose, and showed a stronger reduced growth on glycerol and L-arabinose.

DISCUSSION

Complementation of uridine auxotrophic *pyr4* strains to prototrophy is likely the most successful strategy for gene manipulation in filamentous fungi but is limited by the one-time use of the *pyr4* as marker gene. Here, we successfully overcame this limitation by applying a blaster approach for successive gene knock-outs in *H. jecorina* using a single marker. The *pyr4* blaster cassette was successfully excised by selection for resistance to 5-FOA in Δ*glk1* strains and could be re-used to construct Δ*glk1*Δ*hxk1* strains. The frequency of recombination between the two 380 bp *Sh ble* fragments was with 1-2 x 10^{-4} in about the same range as reported for *A. fumigatus* (4 x 10^{-4}; (d'Enfert, 1996) and for *A. nidulans* (2 x 10^{-4}; (Krappmann and Braus, 2003), which allowed a straight forward selection of *pyr4* negative strains resulting from the looping out of the blaster cassette. 5-FOA resistance can in principle result from mutations in at least two genes: orotate phosphoribosyltransferase (*pyr2*) and orotidine-5'-monophosphate decarboxylase (*pyr4*). 90 % of the obtained 5-FOA resistant colonies were uridine auxotrophic, while the remaining 10% were prototrophic. This was most likely the result of the transfer of the colonies to non-selective medium which was done to facilitate their sporulation. Transformation of five randomly chosen auxotrophic strains with the hexokinase blaster showed that all five strains could be complemented with the *pyr4* gene proving that the uridine auxotrophy was the result of the *pyr4* excision.

In *H. jecorina* research is focused on its anamorph form *T. reesei*. The lack of research with the sexual form prevented characterization of auxotrophic strains, and therefore transformation strategies that involve the conversion of auxotrophic strains to prototrophy are only poorly developed in *H. jecorina*, while they are well established in other fungal species including *S. cerevisiae* or *A. nidulans*. The successful application of the blaster cassette system to the anamorph of *H. jecorina*, provides therefore an interesting opportunity to accelerate functional genomics in this fungus especially in the view of the recent release of a

draft version of the *H. jecorina* genome (http://gsphere.lanl.gov/trire1/trire1.home.html). Although it is at the moment illusive to target all putative genes in *H. jecorina*, we think that our approach is especially valuable for the investigation of the function of fungal, respectively *H. jecorina* specific genes. Our interest is directed towards paralogous genes which have developed during evolution from their ancestral genes by gene duplication and often tend to evolve toward functional diversification. It my also aid in the investigation of the function of whole gene families: *H. jecorina* is an excellent producer of extracellular enzymes secreting a high number of e.g. cellulases or xylanases most of which have not yet been functionally characterized. A search of the *H. jecorina* genome sequence database reveals the presence of a high number of additional biomass degrading enzymes including genes encoding for cellulases, xylanases, pectinases or chitinases.

The blaster system offers also an application for the construction of industrial *Hypocrea*/*Trichoderma* strains. As a producer of low cost enzymes and recombinant proteins for a number of applications, genetic transformation systems are desired which do not lead to the accumulation of antibiotic resistance marker. Although the strains used in this study still carry a single antibiotic resistance marker after excision of the *pyr4* blaster, it should be possible to replace the *Sh ble* direct repeat by an autochthonous *H. jecorina* sequence.

Here, we applied the blaster system to *H. jecorina*, but it can easily be adapted to other *Trichoderma* or fungal species in general, especially for those in which classical genetic approaches are not practicable. The range of organisms seems to be limited only by the availability of orotidine-5'-monophosphate decarboxylase negative strains. Such strains can be obtained by classical mutagenesis approaches and selection on 5-FOA (Gruber et al., 1990b). Fungal orotidine-5'-monophosphate decarboxylase genes are highly conserved and work therefore also in heterologous systems (cf. d'Enfert, 1996; Punt et al., 2001). It is therefore possible to construct multiple disrupted strains with this blaster cassette in any fungal species which is efficiently transformed by the *H. jecorina pyr4* gene.

In *A. nidulans,* glucose-, hexokinase mutants and double mutants were obtained by classical mutagenesis (Roberts, 1963; Flipphi et al., 2003). Although the growth phenotype of *A. nidulans hxk*A1 (hexokinase deficient, formerly designated *fr*A1 for fructose non-utilizing) mutant (Roberts, 1963; Ruijter et al., 1996) is comparable to the *H. jecorina* Δ*hxk1* by being unable to grow on D-fructose and that both *H. jecorina* and *A. nidulans* double mutants are unable to grow on D-glucose and D-fructose, we noted differences in the utilization of the other carbon sources tested. While the *A. nidulans* glucokinase and hexokinase single mutants exhibit no other nutritional deficiencies, we found that in *H. jecorina* both kinase genes are necessary for fast growth on a number of carbon sources in *H. jecorina* including L-arabinose or glycerol.

D-Fructose inhibited the growth of the *hxk*A1 mutant on other sugars (Roberts, 1963) and (Ruijter et al., 1996) showed that D-fructose and D-mannitol inhibited growth of this mutant on L-arabinose. This observation could be explained by repression of enzymes involved in L-arabinose catabolism by D-fructose and D-mannitol: Although both are not metabolized in the absence of hexokinase, their accumulation might be able to at least partially repress the synthesis of enzymes necessary for metabolism of other carbon sources.

The availability of the three isogenic mutants constructed in this study will also allow the study of the role of the two hexose phosphorylating enzymes in the signalling of carbon catabolite repression and in a second path of D-galactose utilization besides the classical Leloir pathway in *H. jecorina* (Seiboth et al., 2004). In *A. nidulans* only the double mutant is impaired in D-glucose and D-fructose repression for ethanol and acetate catabolism and xylan degradation (Flipphi et al., 2003). In *H. jecorina*, so far only a single carbon catabolite derepressed strain which has a truncated *cre1* gene was described (Ilmen et al., 1996b). With respect to D-galactose utilization in *A. nidulans* (Fekete et al., 2004) showed that a double mutant in the galactokinase (which catalyzes the first step in the Leloir pathway of D-galactose) and hexokinase is unable to grow on D-galactose as single carbon source.

Although differences in the catabolic pathways for D-galactose in these two fungi might exist, it is likely that the second pathway of D-galactose utilization in *H. jecorina* proceeds also via D-fructose involving hexokinase.

MATERIALS AND METHODS

Strains and culture conditions

H. jecorina strain QM9414 (ATCC 26921) and its uridine auxotrophic *pyr4* mutant TU-6 (ATCC MYA-256) (Gruber et al., 1990b) were maintained on malt extract agar (Merck, VWR International, Austria) or potato dextrose agar (Difco, BD Biosciences, Schwechat, Austria) supplemented with 10 mM uridine when necessary. Fungal cultures were grown on 30°C in a medium described by (Mandels and Andreotti, 1978). Fungal growth on different carbon sources was determined by placing a small piece of agar (d = 0.5 cm) in the centre of each agar plate. *Escherichia coli* strain JM109 (Promega, Madison, Wis.) was used for plasmid propagation.

Identification and sequence analysis of the *H. jecorina glk1* (encoding glucokinase) and *hxk1* (encoding hexokinase)

A tblastn search of the *Trichoderma reesei* / *H. jecorina* QM6a genome sequence (http://gsphere.lanl.gov/trire1/trire1.home.html) with the *Aspergillus niger* glucokinase (GenBank accession no. CAA67949) and hexokinase (GenBank accession no. CAA08922) proteins as query identified single orthologues for each gene. The deduced aa sequence of the *H. jecorina* glucokinase encoding gene (*glk1*) and the hexokinase encoding gene (*hxk1*) showed 58% sequence identity to the *A. niger* GlkA, respectively 73% to the *A. niger* HxkA. The two kinase genes were amplified by PCR with oligonucleotide pair Gluco5'F and

Gluco3'R, respectively Hexo5'F and Hexo3'R from *H. jecorina* QM9414 genomic DNA and sequenced.

Plasmid constructions

The *pyr4* blaster cassette was constructed by inserting the *H. jecorina pyr4* (Gruber et al., 1990a) gene between two *S. hindustanus Sh ble* fragments orientated as direct repeats. Therefore the *Sh ble* was amplified twice from the plasmid pPICZB (Invitrogen, Vienna, Austria) using two primer pairs and introducing the following restriction sites (given in brackets): zeo1fw (*Xba*I) and zeo1rv (*Xho*I), respectively zeo2fw (*Xho*I) and zeo2rv (*Bam*HI). The resulting 380 bp amplicons were digested with *Xba*I/*Xho*I and *Bam*HI/*Xho*I respectively and ligated into an *Xba*I/*Bam*HI digested pUC19 (Yanisch-Perron et al., 1985). The resulting vector containing the two *Sh ble* gene fragments as direct repeat was digested with *Xho*I to insert a 2.7 kb *Sal*I *H. jecorina pyr4* fragment resulting in the 6.2 kb blaster plasmid pLH1mb.

About 1.4 kb of the 5' and 3' region of *glk1* were amplified using the primer pairs which introduced the following restriction site: gluco5'F (*Eco*RI) and gluco5'R (*Bam*HI), gluco3'F (*Xba*I) and gluco3'R (*Xba*I and *Eco*RI). The *Eco*RI/*Bam*HI restricted 5' region fragment was ligated into the *Eco*RI/*Bam*HI sites of pLH1mb. Next, the *Xba*I restricted 3' region of *glk1* was inserted into the *Xba*I site resulting in the final vector pΔ*glk1*.

About 1.4 kb of the 5' and 1.8 kb of the 3' region of *hxk1* were amplified using the primers hexo5'F (*Eco*RI) and hexo5'R (*Bam*HI), respectively hexo3'F (*Hin*dIII) and hexo3'R (*Hin*dIII plus a natural *Eco*RI site). The *Eco*RI/*Bam*HI digested 5' region was ligated into the respective sites in pLH1mb following the insertion of the 3' region of *glk1* into the *Hin*dIII site resulting in pΔ*hxk1*.

Transformation of *H. jecorina*

Protoplast preparation and DNA mediated transformation was described by (Gruber et al., 1990b). For deletion of the *glk1* and *hxk1* the blaster cassettes (about 6,2 and 6,6 kb) were excised from p∆*glk1 and* p∆*hxk1* with *Eco*RI. Fragments were purified from agarose gels (QIAquick Gel Extraction Kit, VWR International, Vienna, Austria). After transformation protoplasts were stabilized and regenerated on minimal medium plates containing D-sorbitol (1M). D-glucose as carbon source was replaced by glycerol or L-arabinose to prevent a negative selection for strains deleted in one of the kinase genes. After 4-5 days colonies were transferred to minimal medium without D-sorbitol for sporulation. Conidia were usually obtained after 3-4 days and purified on minimal medium plates containing the colony restrictor Triton X-100 (0.1 % v/v) and peptone (0.1 % w/v) which accelerates germination. After 1.5 days single colonies were picked and transferred to minimal medium for sporulation.

Excision of the *pyr4* blaster cassette

2-3 day old spores were suspended in 0.9 % (w/v) NaCl and 0.05 % (w/v) Tween 80, filtered through glass wool to remove residual hyphae. $0.9x10^7$ to $1.5x10^7$ conidia were plated on minimal medium plates containing 5-FOA (1.5 g/l; Fermentas, St. Leon-Rot, Germany), peptone (0.1 g/l) and 10 mM uridine. 5-FOA resistant colonies were obtained after 3-4 days and transferred to minimal medium containing uridine for sporulation. Purified conidia were then tested for uridine auxotrophy on minimal medium plates.

Fungal DNA isolation and hybridization

DNA was prepared from *H. jecorina* strains grown for about 24-30 h in 100 ml flasks on a rotary shaker (250 rpm) at 30°C. Mycelia were harvested by filtration, washed with cold

sterile tap water, blotted dry between paper towels, and ground to a fine powder under liquid nitrogen. Powdered mycelia was suspended in buffer A (0.1 M Tris-HCl, pH 8.0, 1.2 M NaCl, 5 mM EDTA), incubated for 20 min at 65°C, cooled down on ice, mixed with 0.5 vol phenol and 0.5 vol chloroform and centrifuged (12.000 rpm., 15 min). Following a chloroform (1 vol) extraction, DNA was precipitated with 1 volume of isopropanol and washed with 70% (v/v) ethanol. Standard methods (Sambrook and Russel, 2001) were used for DNA electrophoresis, blotting, and hybridization of DNA. Probes labelled with [α^{32}P]dCTP by random priming were: a 1.4 kb *Xba*I *glk1*, a 1.8 kb *Hin*dIII *hxk1,* a 2.7 kb *Sal*I *pyr4* fragment and a 380 bp *Sh ble* amplicon.

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Chapter 5

Carbon signalling by hexose phosphorylating enzymes in *Hypocrea jecorina*

The results presented in this chapter will be submitted for publication:
Hartl, L., Kubicek, C.P., Seiboth, B. (2007)
Carbon signalling by hexose phosphorylating enzymes in *Hypocrea jecorina*.

ABSTRACT

The role of hexose phosphorylating enzymes in the signalling of carbon compounds (specific induction, general carbon catabolite repression) was investigated in the pyrenomycetous ascomycete *Hypocrea jecorina*. By gene deletion we demonstrate that the fungus has only one glucokinase GLK1 and hexokinase HXK1, and a knock out in both of them renders the fungus unable to phosphorylate D-glucose, D-fructose and D-mannose, and consequently to grow on any of these carbon sources. The *hxk1* knock out also impairs growth on galactitol and D-sorbitol, and on D-mannitol. However, a Δ*glk1*Δ*hxk1* strain fails to grow on numerous carbohydrates and exhibits only very slow growth on glycerol, D-galactose, D-xylose and L-arabinose. Three gene systems, diagnostic for carbon catabolite (de)repression (cellulase formation, *cbh1*; xylanase formation, *xyn1*; and *β*-galactosidase formation, *bga1*) were analyzed in the Δ*glk1,* Δ*hxk1* and Δ*glk1*Δ*hxk1* strains at the transcript level. Carbon catabolite repression is retained in both single deletion strains, whereas Δ*glk1*Δ*hxk1* strains are carbon catabolite derepressed. This derepression is faster than in a mutant with a defect in the carbon catabolite repressor protein CRE1. Induction by specific carbon compounds is differently affected: induction of *cbh1* by sophorose and of *bga1* by D-galactose is impaired in Δ*glk1*Δ*hxk1* strains, whereas induction of *xyn1* by D-xylose is strongly increased. Normal transcript levels were found for the induction of *cbh1* and *bga1* in the single deletion strains whereas already higher transcript levels were found in both single deletion strains for the *xyn1* gene. Our data show that the two hexose phosphorylating enzymes of *H. jecorina* exert a general influence on carbohydrate metabolism in this fungus which goes beyond phenomena which can be explained by their catalytic action.

INTRODUCTION

Mitosporic fungi dominate various ecosystems by their amazing versatility for assimilation of a wide range of carbon sources. While there is still a hierarchy in the assimilation of a mixture of carbon sources, which is mainly energy yield-driven, adaptation of fungi to different habitats has resulted in the existence of different priorities. The respective regulatory mechanisms e.g. in *Saccharomyces cerevisiae* can be explained by specialization of this yeast for D-glucose (Santangelo, 2006). On the other hand, saprobic or plant pathogenic fungi have developed different carbon source priorities (Tanzer et al., 2003; Druzhinina et al., 2006). The molecular mechanisms guaranteeing this hierarchy include carbon sensing and signalling, leading to specific induction and general carbon catabolite repression. Unfortunately, the former two are poorly documented in mitosporic fungi and the majority of the data are most exclusively related to the repressing effects of the monosaccharide D-glucose (Ruijter and Visser, 1997; Rolland et al., 2001).

Much more detailed knowledge is available for *S. cerevisiae*, but for reasons given above the resulting findings are in many cases different from those in mitosporic fungi and are thus of limited use (Rolland et al., 2002). We have recently documented this for D-galactose assimilation (Hartl et al., submitted). Another example for this is the role of the D-glucose and D-fructose phosphorylating enzyme hexokinase (EC 2.7.1.1.) Hxk2p, in glucose-sensing: *S. cerevisiae* has three hexose phosphorylating enzymes (Hxk1p, Hxk2p, and the glucokinase (EC 2.7.1.2) Glk1p. Each of them enables the yeast to grow on D-glucose, but Hxk2p is responsible for the main activity for D-glucose phosphorylation and loss-of-function *hxk2* mutants are defective in D-glucose repression mediated by the carbon catabolite repressor Mig1p. The mechanism by which Hxk2p contributes to D-glucose repression has not yet been fully elucidated, but its catalytic activity of Hxk2p seems to be dispensable and thus signal transmission may rather be linked to substrate binding-induced conformational changes

in the Hxk2p protein (Niederacher and Entian, 1991; Herrero et al., 1995; Herrero et al., 1996; Rodriguez et al., 2001; Ahuatzi et al., 2007) . This mechanism appears to be unique in yeast, because it is not present in the mitosporic fungus *Aspergillus nidulans*. Here, the two hexose phosphorylating enzymes – one gluco- and one hexokinase – cooperate in D-glucose repression, and mutations in either single gene are without effect (Flipphi et al., 2003).

The anamorph of the ascomycete *H. jecorina, Trichoderma reesei*, is a pantropical fungus specialized on plant litter degradation in the soil. One of its isolates (*T. reesei* QM 6a, collected during the Second World War in the South Pacific (Reese, 1976) has become the progenor of a series of mutants for the industrial production of cellulases, hemicellulases and heterologous proteins under the cellulase promoters (Penttila et al., 2004). Cellulase and hemicellulase formation appears to be less stringently affected by carbon catabolite repression in *H. jecorina* than in *A. nidulans* (Schmoll and Kubicek, 2003), thus making this fungus an interesting complement to studies with the latter "model fungus". For this reason, we have studied the presence and role of hexose phosphorylating enzymes in carbon source assimilation and regulation in *T. reesei*. The data, described in this paper, in fact illustrate several important differences, notably a role of the two hexose phosphorylating enzymes in the assimilation and signalling of carbohydrates on which they do not act enzymatically.

RESULTS

glk1 and *hxk1* are the only hexose phosphorylating enzymes in *H. jecorina*

We have recently identified putative genes encoding a glucokinase and a hexokinase in *H. jecorina*, and showed that double deletion strains are completely unable to grow on D-glucose (Hartl and Seiboth, 2005), suggesting that these two genes are the only D-glucose phosphorylating genes in the fungus. In order to provide direct evidence for this claim, we

compared the hexose phosphorylating activities in cell free extracts from the parental strain, the Δ*glk1*, Δ*hxk1* and Δ*glk1*Δ*hxk1* strain (Table 1). As clearly shown, knock out of *glk1* only affects the ability to phosphorylate D-glucose, whereas a knock-out in *hxk1* affects both D-glucose and D-fructose phosphorylation. The Δ*glk1*Δ*hxk1* strain had no measurable phosphorylating activity on any of the two substrates, thus proving that GLK1 and HXK1 fully account for gluco- and hexokinase activity in *H. jecorina*, and that their terming as *glk1* and *hxk1* is correct. Their identity as orthologues of fungal hexo- and glucokinases, respectively, was also confirmed by a phylogenetic analysis of their amino acid sequences, which shows that the two enzymes cluster in two different, strongly supported clades in the vicinity of already confirmed gluco- and hexokinases (Fig. 1). Finally, the physicochemical properties of the two enzymes (GLK1: 59.7 kDa, IP 5.2; HXK1: 54.1 kDa, IP 5.2) are also

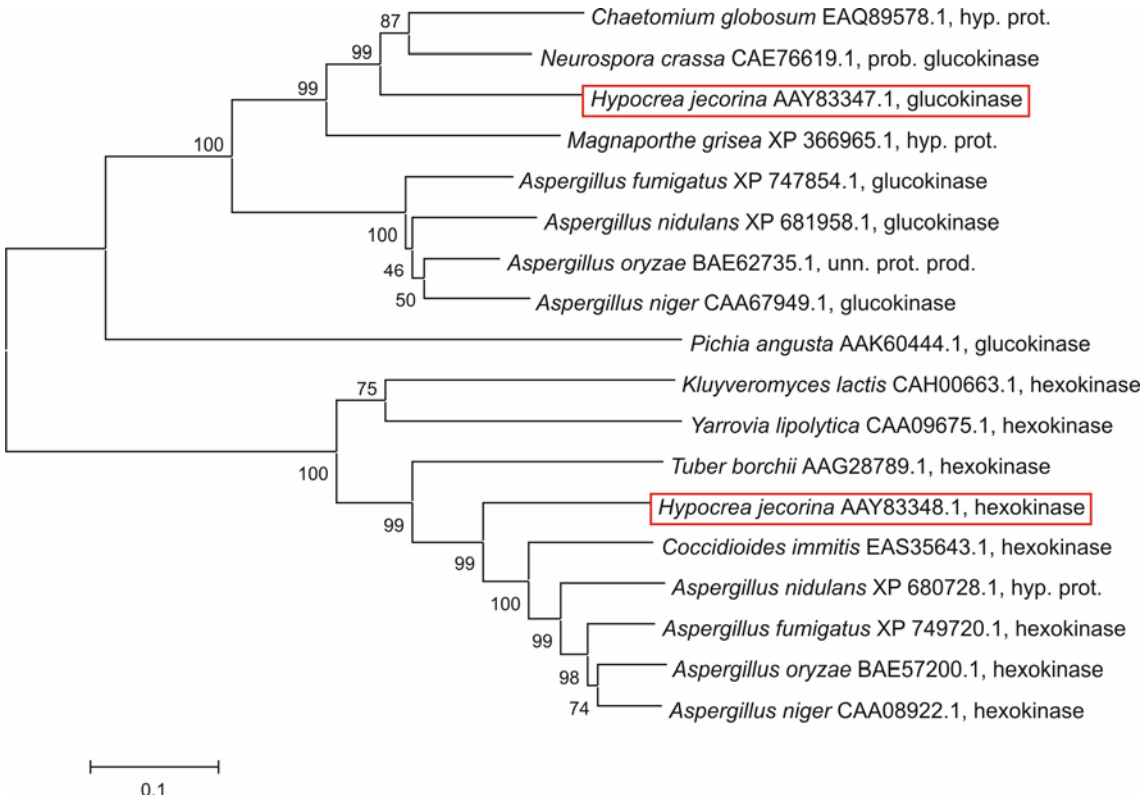

Fig. 1. Phylogenetic analysis of GLK1 and HXK1. Proteins similar to GLK1 and HXK1 were identified by a BLASTP search of the NCBI database and their sequences analyzed using the neighbour joining method. Clearly two clades in the vicinity of confirmed gluco- and hexokinases are formed, supporting the suggested identities of the two H. jecorina enzymes.

well comparable to those known from other fungal gluco- and hexokinases (Panneman et al., 1996; Panneman et al., 1998). Together, these data provide evidence that *H. jecorina* has a single glucokinase and hexokinase, and that both together are exclusively responsible for D-glucose phosphorylation.

Table 1 Phosphorylation of D-glucose and D-fructose by cell free extracts of various *H. jecorina* strains, grown on D-glucose and D-fructose.

Strain	Carbon source	Activity with D-glucose*	Activity with D-fructose*
QM9414	D-glucose	2.50 ± 0.38	2.48 ± 0.86
	D-fructose	3.62 ± 0.11	5.59 ± 0.49
Δ*glk1*	D-glucose	2.31 ± 0.00	7.50 ± 0.27
	D-fructose	3.16 ± 0.00	6.00 ± 0.63
Δ*hxk1*	D-glucose	0.63 ± 0.30	0.32 ± 0.35
	D-fructose	0.70 ± 0.26	0.37 ± 0.10

*Relative activities are given in nkat/mg protein. Activities of the strain Δ*glk1*Δ*hxk1* were below the detection limit.

Expression of *glk1* and *hxk1* is modulated by the carbon source

Expression of fungal glycolytic genes is generally believed to be constitutive. However, while the transcripts of both genes can indeed be always detected during growth on several different carbon sources, *H. jecorina glk1* displays some carbon source specific variation of expression (Fig. 2). Highest expression levels were observed on D-glucose, D-galactose and D-xylose. In contrast, *hxk1* gene expression was more uniform, but also stronger on D-glucose, D-fructose, glycerol, D-mannitol and D-xylitol.

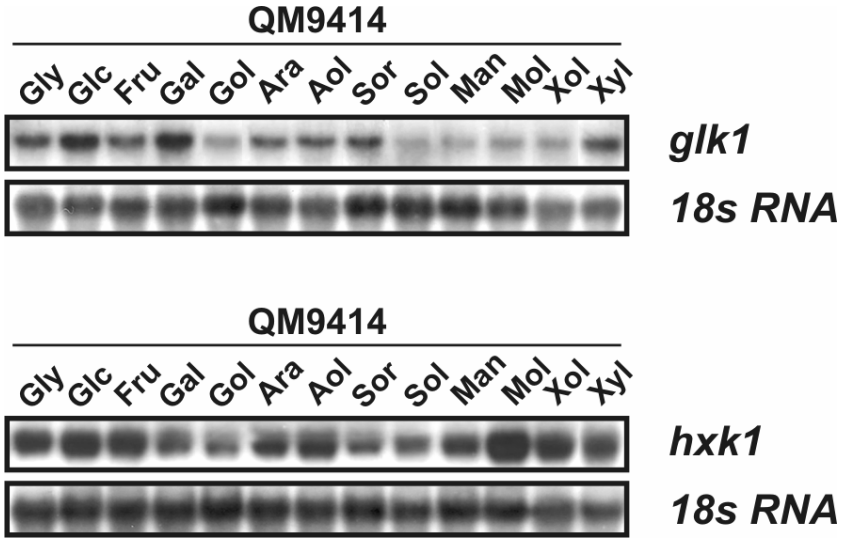

Fig. 2. Northern analysis of *glk1* and *hxk1* in QM9414 on various carbon sources. Transcript formation was studied six hours after transfer from medium containing glycerol (1% w/v) to media containing the respective carbon sources (1% w/v): glycerol (Gly), D-glucose (Glc), D-fructose (Fru), D-galactose (Gal), galactitol (Gol), L-arabinose (Ara), L-arabinitol (Aol), L-sorbose (Sor), D-sorbitol (Sol), D-mannose (Man), D-mannitol (Mol), D-xylitol (Xol) , D-xylose (Xyl).

We also investigated whether a loss-of-function in one of the two genes would affect the expression of the other, and therefore tested this during growth on D-glucose, D-fructose or D-mannose. No differences in transcript levels of the two single deletion strains compared to the parent strain were observed, however (data not shown).

glk1 and *hxk1* are necessary but not essential for growth of *H. jecorina* on several carbon sources

In order to test whether *glk1* and *hxk1* may also be involved in the assimilation of other carbohydrates than D-glucose and D-fructose, we investigated the growth of the recombinant strains on a number of other carbon sources (Fig. 3). In order to rule out any pleiotropic effects of the transformation procedure and gene deletion, data were compared to respective retransformant strains. The rationale for selection of the compounds was that we assumed GLK1 or HXK1 to be necessary for catabolism of some of them (e.g. D-mannose, D-mannitol; lactose and D-galactose; L-sorbose and D-sorbitol), whereas not for the others (glycerol, L-arabinose, D-xylose). HXK1 was shown to be necessary for growth on the carbon sources D-fructose (cf. Hartl and Seiboth, 2005), D-mannitol and D-sorbitol which are catabolized via D-fructose and galactitol which is an intermediate of a second D-galactose catabolic pathway and catabolized also via D-fructose. Growth of both the Δ*hxk1* and Δ*glk1* strains was also slower on D-mannose, which can be explained by the catalytic activity of both HXK1 and GLK1 for D-mannose. Moreover, Δ*glk1* and Δ*hxk1* strains, while showing the expected decreased growth on D-glucose, showed also impaired growth rates on L-arabinose, glycerol, D-xylose and lactose.

This growth defects were found to be more severe in the double deletion strains, which were virtually unable to grow on any carbon source except of slow growth on D-xylose, and even slower on L-arabinose, D-galactose and glycerol. Interestingly, the double deletion strain was also unable to grow on lactose.

We conclude from these data that GLK1 and HXK1, besides being catalytically involved in the catabolism of several other carbon sources than D-glucose and D-fructose have also a general effect on the assimilation of several other carbon sources.

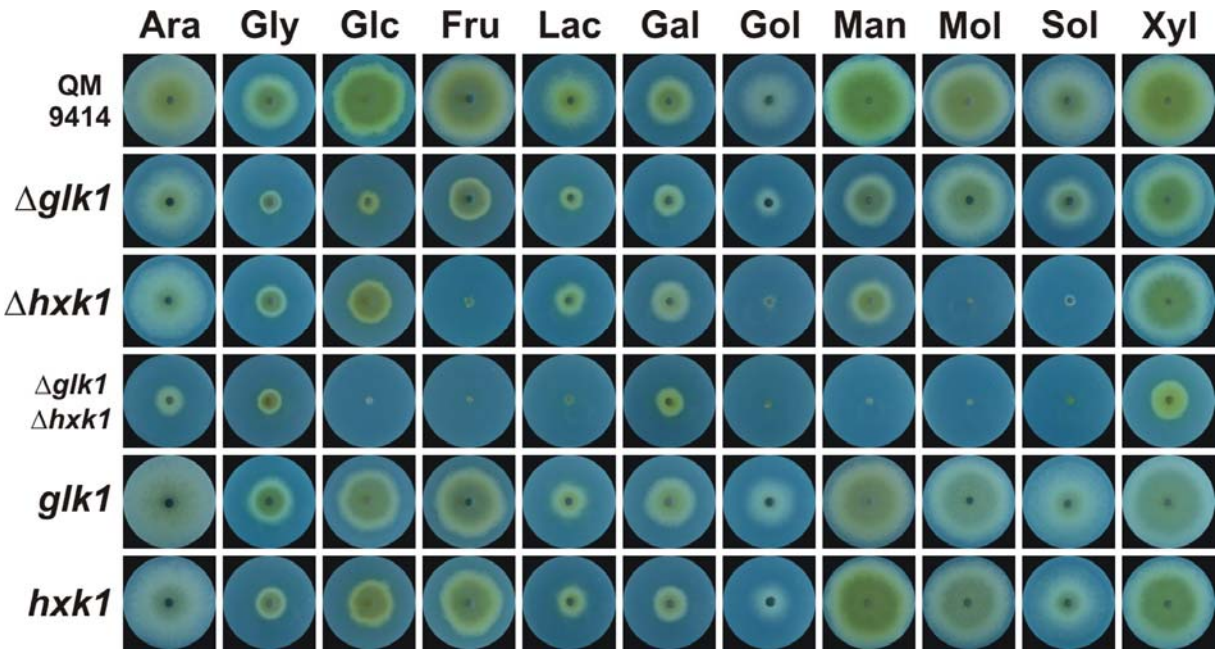

Fig. 3. Growth test on various carbon sources. The wild type strain QM9414, the single deletion strains Δ*glk1* and Δ*hxk1*, the double deletion strain Δ*glk1*Δ*hxk1* and retransformants of the single deletion strains (*glk1* and *hxk1*) were grown on plates containing L-arabinose (Ara), glycerol (Gly), D-glucose (Glc), D-fructose (Fru), lactose (Lac), D-galactose (Gal), galactitol (Gol), D-mannose (Man), D-mannitol (Mol), D-sorbitol (Sol), and D-xylose (Xyl) as carbon sources for 72h.

The double knock out strain Δ*glk1*Δ*hxk1* of *H. jecorina* is impaired in conidiation

During working with the delta-strains as described above, we observed that the Δ*glk1*Δ*hxk1* strain – but not Δ*glk1* or Δ*hxk1* – did not sporulate, even after very prolonged cultivation. In order to test this in more detail, we applied two triggers of *Trichoderma* sporulation – illumination and mechanical injury (Casas-Flores et al., 2004) – to rescue conidiation in these strains. None of these treatments had an effect. We conclude that the lack of hexose phosphorylating enzymes impairs conidiation in *H. jecorina*.

Deletion of both *glk1* and *hxk1* is necessary to evoke carbon catabolite derepression in *H. jecorina*

In contrast to the model organism *S. cerevisiae*, Flipphi et al. (2003) have recently reported that in *A. nidulans* not the hexokinase protein but mutations in both gluco- and hexokinase are necessary to exert carbon catabolite derepression in this fungus. In order to test whether this is also true for *H. jecorina*, we first selected appropriate model systems. An application of two of the test systems used for *A. nidulans – alcA* and *facA* - was avoided because *H. jecorina* does not show a regulation of alcohol catabolism, typical for *alcA* (Hodits, R. and Kubicek, C.P., unpublished data) and regulation of *facA* is not yet been studied. We therefore used carbon catabolite repression of the basal, non-induced level of expression the cellulase cellobiohydrolase 1 (CEL7A) gene *cbh1*, the xylanase 1 encoding gene *xyn1,* and the *β*-galactosidase gene *bga1* (Mach et al., 1996; Zeilinger et al., 2003; Seiboth et al., 2005) as test systems. The *cre1*-loss of function mutant RutC30 was used as a positive control.

Fig. 4 shows that when mycelia of QM9414, the *cre1* mutant, the Δ*glk1*, the Δ*hxk1* and the Δ*glk1*Δ*hxk1* strains were pregrown on glycerol and then transferred to D-glucose for up to 9 hrs, accumulation of the *cbh1, xyn1* and *bga1* transcripts only occurred in the Δ*glk1*Δ*hxk1* strain but not in any of the others. Prolonged incubation led to transcript accumulation also in the *cre1* mutant RutC30 (data not shown; see also Mach et al., 1996; Zeilinger et al., 2003; Seiboth et al., 2005). These data basically confirm similar findings in *A. nidulans*, but exhibit a major difference i.e. that carbon catabolite derepression is faster than in a *cre1* loss-of-function mutant.

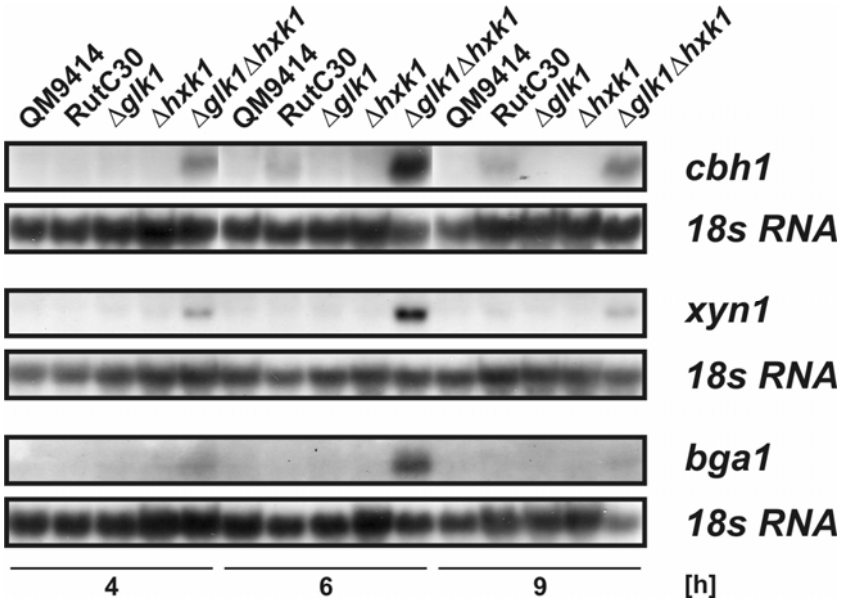

Fig. 4. Northern analysis of *cbh1*, *xyn1* and *bga1* on D-glucose. Transcript formation of *cbh1*, *xyn1* and *bga1* in the parental strain QM9414, the *cre1* loss of function mutant RutC30, the single deletion strains Δ*glk1* and Δ*hxk1*, and the double deletion strain Δ*glk1*Δ*hxk1* after transfer to medium containing D-glucose (1%w/v).

Presence of either *glk1* or *hxk1* is essential for induction of *cbh1* and *bga1* gene expression in *H. jecorina*

Carbon catabolite repression affects the expression of *cbh1*, *bga1* and *xyn1* not only at the basal level of transcription, but also at the level of induction. Owing to the inability of the Δ*glk1*Δ*hxk1* strains to grow on cellulose and lactose, these two inducing carbon sources could not be used to induce *cbh1*, and the *β*-1,2-linked disaccharide sophorose (Sternberg and Mandels, 1979) was used instead. Induction in QM9414 took place within 5 hrs, and a comparable level and kinetic of induction was observed in the Δ*glk1* strain (Fig. 5 A). In accordance with induction being subject to carbon catabolite repression, enhanced abundance of the *cbh1* transcript was observed in the *cre1* mutant strain, but interestingly also in the Δ*hxk1* strain. In contrast, in Δ*glk1*Δ*hxk1* strains the *cbh1* transcript remained at the level of basal derepression throughout, and was not induced anymore by sophorose.

Similar findings were made with D-galactose induction of *bga1* (Fig. 5 B): while the kinetic pattern of induction varied in the different strains (probably due to the different rates of assimilation of D-galactose), induction clearly took place in QM9414, in the *cre1* mutant

and in the Δ*glk1* and Δ*hxk1* strains. No induction took place, however, in the Δ*glk1*Δ*hxk1* strain.

We conclude from these data that either the *glk1* or the *hxk1* gene is needed to enable *cbh1* and *bga1* gene induction in *H. jecorina*.

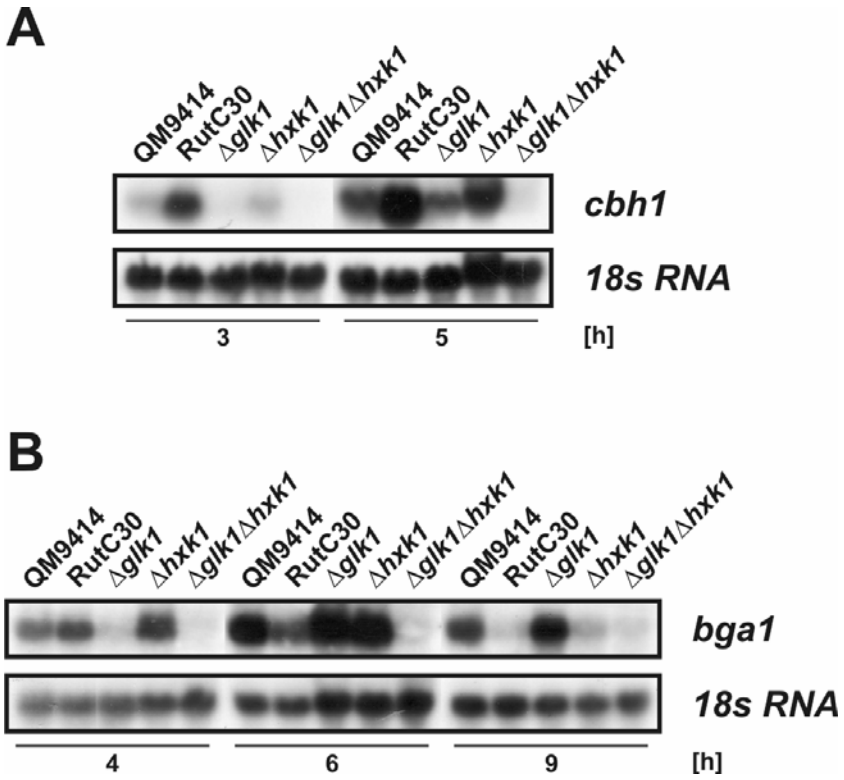

Fig. 5. Northern analysis of *cbh1* on sophorose and *bga1* on D-galactose. Transcript formation of *cbh1* **(A)** and *bga1* **(B)** in the parental strain QM9414, the *cre1* loss of function mutant RutC30, the single deletion strains Δ*glk1* and Δ*hxk1*, and the double deletion strain Δ*glk1*Δ*hxk1* after transfer to media containing sophorose and D-galactose respectively (1% w/v).

Deletion of both *glk1* and *hxk1* leads to enhanced *xyn1* induction in *H. jecorina*

We also investigated the effect of *glk1* and *hxk1* on the induction of *xyn1* by D-xylose, and obtained essentially different findings than with *cbh1* and *bga1* above (Fig. 6): induction by D-xylose is slow, but clearly enhanced in the *cre1* mutant strain. It was also enhanced to a lower level in the two single deletion strains. In the double deletion strain, however, the *xyn1* transcript was most abundant, clearly exceeding the transcript level found in the *cre1* mutant. We conclude that the induction of *xyn1* by D-xylose is strongly enhanced in the absence of glucose phosphorylating enzymes, and this exceeds the level seen in a mutant in the carbon

catabolite repressor CRE1. This is essentially the opposite what has been reported for expression of the xylanase gene *xlnA* in *A. nidulans* (Flipphi et al., 2003).

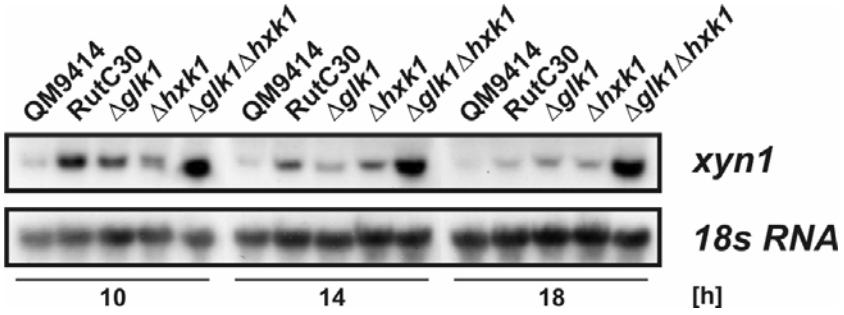

Fig. 6. Northern analysis of *xyn1* on D-xylose. Transcript formation of *xyn1* in the parental strain QM9414, the *cre1* loss of function mutant RutC30, the single deletion strains Δ*glk1* and Δ*hxk1*, and the double deletion strain Δ*glk1*Δ*hxk1* after transfer to medium containing D-xylose (1% w/v).

Table 2 Oligonucleotides used in the present study

Name	Sequence*
CBH1SF	5′-TCGGCCTGCACTCTCCAATC-3′
CBH1SR	5′-TGGAGTCCAGCCACAGCATG-3′
CBH2SF	5′-ATTCTCACCACGCTGGCTAC-3′
CBH2SR	5′-CGGCGTAGTTGATGCACTC-3′
XYN1SF	5′-CAACTGTCTTATAAAGGAGC-3′
XYN1SR	5′-GCCAGAGCTAAGTAACC-3′
XYN2SF	5′-GAGAGCAGTGTCAACGTC-3′
XYN2SR	5′-CCACTCCAAGTCAACATC-3′
18sRF	5′-GGTGGAGTGATTTGTCTG-3′
18sRR	5′-CTTACTAGGGATTCCTCG-3′
GlucoF (*Hin*dIII)	5′-GATCAAGCTTACAGGATGAGGAGGAGGGT-3′
GlucoR (*Hin*dIII)	5′-GATCAAGCTTCGTCTACCAGCCATCCCTT-3′
HexoF (*Hin*dIII)	5′-GATCAAGCTTGCATCGCCTTTGTAGGTGTG-3′
HexoR (*Hin*dIII)	5′-GATCAAGCTTTCTGGACGTGGAATGGGAG-3′

* Respective restriction sites are underlined

DISCUSSION

In this paper, we have investigated the roles of the single gluco- and hexokinase of *H. jecorina* in carbon catabolite repression and carbon mediated induction, using three unlinked model systems (*cbh1, xyn1, bga1*). Our data also show that a knock-out of both hexose kinases, but not in either of them alone, renders *H. jecorina* carbon catabolite derepressed. These findings are basically consistent with those obtained by Flipphi et al.

(2003) for *A. nidulans*. These authors concluded that glucokinase and hexokinase play a mere catalytic role in carbon catabolite repression, and – unlike *S. cerevisiae* Hxk2p – exhibit no general regulatory function in it. Our data confirm this. This fundamental difference might be related to the lack of similarity between the interacting domains of the respective CCR-mediating repressor proteins, Mig1p and CreA/CRE1 (Dowzer and Kelly, 1991; Strauss et al., 1995; Cziferszky et al., 2002). This difference is also reflected in the involvement of the SNF1 kinase in glucose regulation in *H. jecorina* and other mitosporic fungi: in contrast to yeast, which regulates both gluconeogenesis and alternative carbon catabolism via Snf1p, *H. jecorina* does not transmit the signal to CreA/CRE1 via SNF1 (Cziferszky et al., 2003).

Our findings, together with the data obtained for *A. nidulans*, stress the difference in the regulatory mechanisms found in multicellular fungi and the yeast *S. cerevisiae*. However, there are also striking differences between these two multicellular ascomycetes. One of them concerns the level of derepression in the hexose phosphorylation mutants in relation to a CRE1 loss-of-function mutant: the levels of derepression of the three model systems used are higher in the glucose-derepressed Δ*glk1*Δ*hxk1* strain than those seen in the RutC30 *cre1* mutant strain. This is essentially the contrary what has been observed in similar experiments with *A. nidulans* (Flipphi et al., 2003). These authors concluded that in the hexose kinase double mutant, the CreA protein still senses repression from other metabolites, irrespective of the sugar kinase lesions. In contrast to *A. nidulans*, we conclude from our findings that in *H. jecorina* the activity of the hexose phosphorylating enzymes is of more importance for carbon catabolite repression in *H. jecorina* than the function of the CRE1 protein. Tanzer et al. (Tanzer et al., 2003) have shown that carbon catabolite repression confers different carbon sources in *A. nidulans* and *Magnaporthe grisea*. For example dextrin had a derepressing effect in *A. nidulans* and a repressing effect in *M. grisea*. Moreover several other carbon sources, e.g. maltose, D-sorbitol and D-trehalose were significantly more repressing in

M. grisea. Our data indicate that the consequences of carbon catabolite repression in *H. jecorina* are also different from those in *A. nidulans*.

Adding to the differences between *A. nidulans* and *H. jecorina*, the GlkA mutant strains of *A. nidulans* did not show any obvious nutritional deficiencies while the Δ*glk1* *H. jecorina* strain exhibited impaired growth on various carbon sources, especially on D-glucose (Fig. 3). Also, while the enzyme activities with D-fructose were higher in the glucokinase deficient strains than in the parental strains in both organisms, the situation was different when D-glucose was used as a substrate. Here the activity was also higher in *A. nidulans* but lower in *H. jecorina*.

Significant differences were also noted with respect to the role of the two hexose kinases in the induction of the three model genes: Flipphi et al. (2003) have reported that the induction of the *xlnA* xylanase was considerably reduced in the single hexokinase mutant, as well as in a hexose kinase double mutant and in a triple (gluco-, hexokinase, CreA) mutant of *A. nidulans*, but not in the glucokinase mutant, suggesting that HxkA has a CreA-independent, positive role in D-xylose induction of *xlnA*. In contrast, essentially the opposite – i.e. an enhancement of D-xylose induction of the *H. jecorina* orthologue *xyn1* in the double deletion strain, and, to a lower extent, also in both single deletion strains – was detected in the present study. Clearly, hexose phosphorylation plays no positive role in *xyn1* induction in *H. jecorina*. We rather interpret these findings by the carbon catabolite repressing action of D-xylose on xylanase induction (Mach et al., 1996): the effect seen is best explained by the derepression occurring in the Δ*glk1*Δ*hxk1*, and/or the slow assimilation of D-xylose by this strain which may occur below the level required for full carbon catabolite repression, and thus enhance the induction by D-xylose.

On the other hand, the Δ*glk1*Δ*hxk1* strain displayed an unexpected defect in the induction of the cellulase gene *cbh1* by sophorose and *β*-galactosidase *bga1* by D-galactose, thereby extending its role from mediating carbon catabolite repression to signalling enzyme

induction. Unfortunately, the molecular mechanism of these two induction processes is not well enough understood to aid in explaining of this observation. Sophorose has frequently been claimed to arise by transglycosylation during the initial degradation of cellulose and thus being the natural inducer of cellulase formation (Vaheri et al., 1979; Kubicek, 1987), but more recent findings raise doubt on this hypothesis. Schmoll and Kubicek (2005) demonstrated the specific expression of 10 genes in *H. jecorina* when grown on cellulose but not induced by sophorose, using the RaSH (rapid subtraction hybridization) approach. The obligatory role of D-glucose phosphorylation for cellulase induction suggests that sophorose must be intracellularly hydrolysed and its products (D-glucose) be phosphorylated to trigger the induction. This seems to be a contradiction at first glance, because this process is exactly what would be assumed to lead to carbon catabolite repression. However, the rate of sophorose uptake by the hyphae is known to be critical for induction, and cellulase formation does not occur if the uptake is too fast (cf. Sternberg and Mandels, 1979; Sternberg and Mandels, 1980). Therefore, assuming that hydrolysis and phosphorylation indeed occur, the intracellular concentration of D-glucose 6-phosphate should be low. The further fate of glucose 6-phosphate and its role in promoting induction requires further investigations. We should note that there are alternative explanations possible as well, but the most simple one – that the inability to phosphorylate D-glucose deprives the fungus from the energy needed to perform transcription and translation of the cellulase genes – is unlikely because the same conditions clearly lead to cellulase formation on D-glucose (i.e. by carbon catabolite derepression).

While the lack of induction by sophorose may be explained by the catalytic action of hexo- and glucokinases, the impairment of *bga1* induction by D-galactose is even more difficult to explain. We have recently shown that the triggering of *bga1* gene expression by D-galactose depends on its conversion to galactitol by the XYL1 aldose reductase (Fekete et al., 2007). The formation of galactitol is mediated via a second pathway for D-galactose

degradation and in subsequent steps the activity of hexokinase is needed to channel the arising D-fructose into glycolysis (Fekete et al., 2004). One could speculate that the absence of hexokinase could create a feedback preventing the accumulation of galactitol. However, in this case also the Δ*hxk1* strain alone should have shown reduced *bga1* induction, which however was not the case. Also, a deletion in the *lad1* L-arabinitol dehydrogenase gene, the enzyme catabolizing galactitol in this pathway, has no effect on *bga1* expression (Fekete et al., 2007). One hypothesis, which has not been tested yet, would be that the major aldose reductase *xyl1* is not formed in the Δ*glk1*Δ*hxk1* strain, an explanation which would be consistent with the slow growth of this mutant on D-xylose and L-arabinose.

Hexokinases have in general been implied in sugar sensing in plants and mammalian tissues (Matschinsky et al., 1998; Avonce et al., 2004), while their role in fungi has so far been restricted to carbon catabolite repression (Moreno and Herrero, 2002; Flipphi et al., 2003). Results from this study emphasize that the two hexose phosphorylating enzymes of *H. jecorina*, i.e. one gluco- and one hexokinase, may play a general role in carbon sensing in mitosporic fungi too, for which each of them alone would be sufficient.

MATERIALS AND METHODS

Strains and culture conditions

Hypocrea jecorina strains QM9414 (ATCC 26921) and the *cre1* mutant RutC30 (Ilmen et al., 1996a) were maintained on malt extract agar (Merck, VWR International, Vienna, Austria), whereas the Δ*glk1*, Δ*hxk1* and the Δ*glk1*Δ*hxk1* strains were kept on minimal medium containing L-arabinose as a carbon source, since growth on L-arabinose was least affected in these strains. Also prolonged cultivation on full media like potato dextrose agar and malt extract agar led to partial revertance of the phenotypes of the single deletion strains. For

growth tests on plates a minimal medium was used in combination with various carbon sources.

Liquid cultures were performed in 1 l Erlenmeyer flasks on a rotary shaker (250 rpm) at 28 °C in the medium described by (Mandels and Andreotti, 1978) with the respective carbon source at a final concentration of 1 % (w/v). Due to the poor growth of the strain Δ*glk1* Δ*hxk1* and its lack of sporulation, liquid media were inoculated with filamentous mycelium grown on minimal medium plates covered with cellophane discs.

For transcript analysis and enzyme activity assays strains were pregrown in 1 l flasks with medium containing glycerol (1% w/v) as a carbon source. Precultivation times were adapted to the different growth rates of the strains: QM9414 and RutC30 were grown for 24 h, Δ*glk1* and Δ*hxk1* strains for 48 h and Δ*glk1*Δ*hxk1* strains for 62 h. The mycelia were then harvested by filtration, washed with medium without carbon source and equal amounts of mycelia were transferred to 500 ml flasks containing the respective carbon sources (1% w/v) and cultivation continued as indicated.

Escherichia coli strain JM109 (Promega, Madison, WI) was used for plasmid propagation.

Preparation of cell-free extracts

To obtain cell-free extracts for enzyme activity assays, strains were pregrown on glycerol (1% w/v) and replaced to media containing D-glucose and D-fructose (1% w/v) as described above. Mycelia were then harvested after 7 h by filtration through Miracloth (Calbiochem, VWR International, Vienna, Austria), washed with cold sterile tap water, blotted dry with paper towels, and ground to a fine powder under liquid nitrogen using a mortar and a pestle. One gram (wet weight) of ground mycelium was suspended in 3 ml of extraction buffer (0.1 M Tris-HCl, pH 7.5; containing 1 mM EDTA and 5 mM β-mercaptoethanol), and homogenized by sonicating ten times for 30 s at 2°C, with intermittent 2-min cooling periods.

The resulting homogenate was centrifuged at 10.000 x *g* for 20 min at 4°C. The supernatant (average protein concentration, 8 to 15 mg/ml) was used to measure hexose phosphorylating enzyme activities.

Enzyme assays

Glucokinase activity was determined by measuring the formation of glucose 6-phosphate by coupling it to glucose 6-phosphate dehydrogenase and monitoring the formation of NADPH. The assay mixture (1.0 ml) consisted of 0.1 M Tris.HCl (pH 7.5), 20 mM $MgCl_2$, 0.1 mM D-glucose, 10 mM ATP, 0.5 mM NADP, 0.5 U glucose 6-phosphate dehydrogenase and 10% (v/v) cell free extract. The reaction was initiated by the addition of the substrate, and an eventual blank was considered in the calculation.

Fructokinase activity was determined in a similar way, but converting the fructose 6-phosphate formed to glucose 6-phosphate by addition of phosphoglucose isomerase (1 U per ml assay). The reaction was initiated by the addition of the substrate, and an eventual blank was considered in the calculation

Specific activities are reported as nkat (=nmoles of product formed per s) per mg protein. Protein concentrations were determined by the Bio-Rad protein assay using BSA as standard (Bio-Rad Laboratories, Munich, Germany).

Nucleic acid isolation and hybridization

Fungal mycelia were harvested by filtration, washed with tap water, frozen and ground in liquid nitrogen. Total RNA was isolated as described by (Chomczynski and Sacchi, 1987). Standard methods (Ausubel et al., 2005) were used for electrophoresis, blotting and hybridization of nucleic acids. As probes for hybridization the following PCR fragments were amplified from genomic DNA: *cbh1* (1.2 kb), *cbh2* (1 kb), *xyn1* (0.9 kb), *xyn2* (0.7 kb), 18sRNA (300 bp). Primers used to amplify these fragments, and their locations within the

respective genes, are given in Table 1. A complete *bga1* cDNA fragment was amplified with M13forward and M13reverse primers from the respective plasmid (Seiboth et al., 2005). For the other probes, a 2.6 kb *Nhe*I/*Hin*dIII fragment of *glk1*, and a 2.2 kb *Xba*I fragment of *hxk1* were isolated from pGLK1 and pHXK1.

Construction of fungal recombinant strains

The construction of the Δ*glk1,* Δ*hxk1* and Δ*glk1*Δ*hxk1* strains has been described by Hartl and Seiboth (2005). To retransform the single deletion strains, the two vectors pGLK1 and pHXK1 were constructed. pGLK1 contains a 3.3 kb PCR fragment of *glk1* including 900 bps of the up- and 522 bp of the downstream noncoding sequence, amplified with the primers GlucoF and GlucoR. pHXK1 contains a 3.5 kb PCR fragment of *hxk1*which includes 856 bp and 389 bp of the up- and downstream noncoding sequences, and which was amplified with the primers HexoF and HexoR. Both fragments were inserted as *Hin*dIII fragments into pLH1hph (Hartl et al., submitted) which contains the *E. coli* hygromycin B phosphotransferase gene as a selection marker under the expression signals of the *gpd1* (encoding glyceraldehyde 3-phosphate dehydrogenase) gene.

ACKNOWLEDGEMENTS

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Chapter 6

Concluding Remarks

This thesis focuses on the characterization of three genes of *Hypocrea jecorina*, encoding the sugar phosphorylating enzymes galactokinase (*gal1*), glucokinase (*glk1*) and hexokinase (*hxk1*), and on the investigation of their possible regulatory functions. While little is known about regulatory mechanisms in *H. jecorina*, the well studied yeast *Saccharomyces cerevisiae* has become a model organism for many biochemical phenomena.

For example the *S. cerevisiae GAL* regulon has become an example for transcriptional control in lower eukaryotes, and a model system for gene regulation (Schaffrath and Breunig, 2000; Bhat and Murthy, 2001; Rubio-Texeira, 2005). However, the present results point out major differences between *H. jecorina* and *S. cerevisiae* regarding this regulatory network. We could clearly show, that GAL1 does not act as a regulatory protein for the expression of the Leloir pathway genes in *H. jecorina*, since *gal7* transcript was also detected in the *gal1* negative strain when replaced to D-galactose. Furthermore we did not observe a repression of the Leloir pathway genes by D-glucose, like it is the case with the *GAL* genes of *S. cerevisiae*. On the other hand the investigation of *gal1* revealed its essential role for cellulase gene transcription. Even though the Δ*gal1* strain was able to grow on lactose, no induction of *cbh1* and *cbh2* could be observed. Additionally, the deletion of *gal1* lead to the discovery of a second D-galactose degrading pathway, because although growth was heavily impaired, this strain was still able to grow on D-galactose without featuring any residual galactokinase activity. This and the finding that the Δ*gal1* strain accumulated galactitol suggested a reductive pathway with aldose reductase being the first enzyme.

Also the role of the *S. cerevisiae* Hxk2p in the regulation of *GLK1*, *HXK1* and *HXK2* expression and in glucose repression, including its ability to enter the nucleus to form a complex with Mig1p and to regulate the phosphorylation state of Mig1p, has been the subject of many studies (Rodriguez et al., 2001; Moreno and Herrero, 2002; Ahuatzi et al., 2007). Again our findings in *H. jecorina* disclose big discrepancies between these two organisms, which had been expected due to available results from *A. nidulans* (Flipphi et al., 2003).

Despite the fact, that *H. jecorina* features only one hexokinase and one glucokinase, none of the two acts as a regulatory protein like Hxk2p and only a deletion of both genes leads to a derepression, even stronger than in the carbon catabolite derepressed strain RutC30, indicating that both enzymes play a catalytic role in CCR. A possible cause for this fundamental difference might be the lack of similarity between the interacting domains of the CCR-mediating repressor proteins Mig1p and CRE1 (Cziferszky et al., 2002). Nevertheless some unexpected findings were made relating the induction of the three genes which were used to test the deletion strains for carbon catabolite derepression: *cbh1*, *bga1* and *xyn1*. While the induction of *cbh1* by sophorose and *bga1* by D-galactose were totally impaired in the Δ*glk1*Δ*hxk1*, induction of xyn1 by D-xylose was increased in the single deletion strains and even more enhanced in the double deletion strain, clearly exceeding the effect of derepression in the *cre1* lack of function strain RutC30. Since it has recently been shown, that the induction of *bga1* is mediated by galactitol (Fekete et al., 2007), which itself is formed by the aldose reductase XYL1, *xyn1* transcription in the Δ*glk1*Δ*hxk1* strain will be the subject of additional experiments. While all three effects need further investigation, these results suggest, that the two hexose phosphorylating enzymes play a general role in carbon sensing.

Taken together, both studies started with a well established yeast model and tested its applicability on the mitosporic fungus *H. jecorina*. Both times the yeast models failed to explain the situation in *H. jecorina*, stressing the difference between a unicellular yeast and multicellular filamentous fungus, resulting from specialization to the respective environments. While D-galactose is an important component of the cell wall and glycosylated proteins of filamentous fungi, it does not occur in the *S. cerevisiae* cell walls and is only a minor component of its glycoproteins. Therefore a constant turnover of D-galactose seems especially important for filamentous fungi in order to prevent intracellular accumulation, while the yeast, highly specialized on D-glucose, can allow the D-galactose catabolism to be repressed by D-glucose. The specialization on D-glucose might also be the reason for a more advanced

regulatory mechanism for glucose repression, including Hxk2p as a regulatory protein. As a final conclusion one could underline the limited use of yeast models for filamentous fungi, while they definitely serve as an inspiration for designing experiments, and stress the importance of further studies concerning industrially important mitosporic fungi like *H. jecorina*.

I would also like to underline the importance of a transformation system which enables fast successive gene deletions, especially in an era where the genomes of more and more organisms get sequenced and annotated. It has already been demonstrated by the work on *Neurospora crassa*, that the next logical step is to delete all annotated genes in order to reveal their functions. This, of course, can only be accomplished with the right tool at hand.

Chapter 7

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ABBREVIATIONS

ADP	adenosinediphosphate
ATP	adenosinetriphosphate
BGA	*β*-galactosidase
bga	gene encoding BGA
bp	base pairs
CBH	cellobiohydrolase
cbh	gene encoding CBH
EDTA	ethylenedinitrilotetraacetic acid
GLK	glucokinase
glk	gene encoding GLK
HPLC	high performance liquid chromatography
HXK	hexokinase
hxk	gene encoding HXK
kb	kilo base pairs
kDa	kilo Dalton
NADP	nicotinamide adenine dinucleotide phosphate
nkat	nanokatal
rpm	rounds per minute
SDS	sodim dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TCA	tricarboxylic
Tris	tris(hydroxymethyl)aminomethane
UDP	uridinediphosphate
UTP	uridinetriphosphate
v/v	volume per volume
w/v	weight per volume
XYN	xylanase
xyn	gene encoding XYN

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