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Review

Lactose metabolism in filamentous fungi: how to deal with an unknown substrate

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ABSTRACT

The disaccharide lactose accumulates as a cheap by-product of the dairy industry and represents therefore a biotechnologically important carbon source for microbial fermentations. Lactose induces cellulolytic enzymes in *Hypocrea jecorina* (*Trichoderma reesei*) and is therefore used to produce cellulases and recombinant proteins. This review summarizes our current knowledge on lactose assimilation and regulation with special emphasis on the degradation of the D-galactose moiety in *Hypocrea jecorina* and *Aspergillus nidulans*. The *GAL*(*LAC*) regulon of the two yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis* has become a paradigm for transcriptional control in lower eukaryotes and a model system for gene regulation. Our results illustrate that in fungi the *gal* pathway genes are in general differentially organized and regulated and that fungi degrade D-galactose by at least two different pathways including the classical Leloir pathway and a novel pathway composed of different enzymes of the fungal L-arabinose and D-xylose catabolic pathway. The importance of these pathways for induction of lactose metabolism and cellulases in *H. jecorina* is discussed.

1. Introduction

The heterodisaccharide lactose (1,4-*O*-*β*-D-galactopyranosyl-D-glucose) is the major carbohydrate of milk and is synthesized in the Golgi organelle of the mammary glands. The lactose synthetase, composed of *α*-lactalbumin and galactosyltransferase, transfers the galactosyl unit from UDP-galactose to D-glucose. Lactose can be considered as a renewable and thus biotechnologically important carbon source which accumulates primarily as a by-product from cheese manufacture or whey processing industries to about 300.000 tons per year worldwide (Roelfsema *et al.* 1990). Yet lactose itself has only limited application in food products because it can cause lactose intolerance or galactosemia (Novelli & Reichardt 2000; Campbell *et al.* 2005).

Still biotechnological use of lactose is limited. This is due to the low concentration of lactose in whey, which needs concentration first; but also because not all microorganisms can readily utilize lactose. This is intriguing because the regulation of lactose utilization by the *lac* operon in *Escherichia coli* has become a paradigm of prokaryotic gene regulation, but in nature several bacteria lack this trait. In addition, only a few yeasts such as *K. lactis* but not e.g. *S. cerevisiae* can utilize lactose. Filamentous fungi often utilize lactose only at very low rates which were used e.g. for the industrial penicillin

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production by *Pencillium chrysogenum* in the 60's and 70's (Swartz 1985). Yet there are also some industrial important fungi such as *Aspergillus niger* which are unable to metabolise lactose. Today, lactose is an important carbon source for (hemi)cellulolytic enzyme fermentations with the ascomycete *H. jecorina* (anamorph *Trichoderma reesei*) on a technical scale. This fungus produces cellulases up to 100 g/l (Cherry & Fidantsef 2003) and the strong cellulase promoters are used to drive recombinant protein production (Kubicek & Harman 1998; Penttilä *et al.* 2004). The formation of cellulases is dependent on the induction by cellulose derived di-saccharides, but also other carbohydrates such as lactose or L-sorbose induce cellulase expression. When a fermentation medium without solids is preferred or required - such as in the case of recombinant protein production - lactose is virtually the only inducing carbon source that can be used economically. However, lactose metabolism is slow and cellulase yields produced on lactose are lower compared to cellulose (Andreotti *et al.* 1980).

To overcome these bottlenecks in the protein production and to enhance the employment of lactose in fungal fermentations, we have initiated a more detailed study on the physiology of lactose catabolism and cellulase formation in *H. jecorina*, which led to the discovery of several interesting features of this part of fungal carbon catabolism.

2. Fungi have two different strategies for initiating lactose utilization

In fungi two principal strategies for catabolism of lactose are realised: (i) extracellular hydrolysis and subsequent uptake of the resulting monomers; and (ii) uptake of the disaccharide and subsequent intracellular hydrolysis (Fig. 1): in *K. lactis* lactose is taken up by the *LAC12* encoded lactose permease and subsequently hydrolysed by the intracellular *LAC4* encoded β-galactosidase to D-glucose and D-galactose. *LAC12* and *LAC4* are clustered and share an intergenic promoter region (Godecke *et al.* 1991). *In silico* analysis of available fungal genome databases (e.g. *Aspergillus nidulans*, *Neurospora crassa*, or *Fusarium graminearum*; http://www.broad.mit.edu/annotation/fgi/) suggests that these fungi follow the *K. lactis* strategy for lactose assimilation which is in *A. nidulans* supported by the lack of an extracellular but the presence of an intracellular β-galactosidase activity during growth on lactose (Fantes & Roberts 1973; Fekete *et al.* 2002). In contrast, *H. jecorina* hydrolyses lactose by an extracellular β-galactosidase and only takes up the monomers. Evidence for this is based on: (i) no orthologues of the *K. lactis* lactose permease or intracellular β-galactosidase are found in the *H. jecorina* genome database (http://genome.jgi-psf.org/); (ii) germinated spores of *H. jecorina* grown on lactose are able to take up D-glucose and D-galactose but not lactose; and (iii) no activity of an intracellular β-galactosidase can be detected in *H. jecorina* grown on lactose (Seiboth *et al.* 2005). The *H. jeorina* extracellular β-galactosidase Bga1 (EC 3.2.1.23) is active with several β-galactoside disaccharides including lactose but also acts as an exo-enzyme on polymeric substrates by releasing D-galactose from lupin- and potatoe-β-galactan. As lactose does not occur in the natural environment of *H. jecorina*, it is likely that the latter activity reveals at least part of the actual function of this β-galactosidase, which is the hydrolysis of terminal non-reducing β-D-galactose residues in plant cell wall components including hemicelluloses or pectins (Gamauf *et al.* 2007).

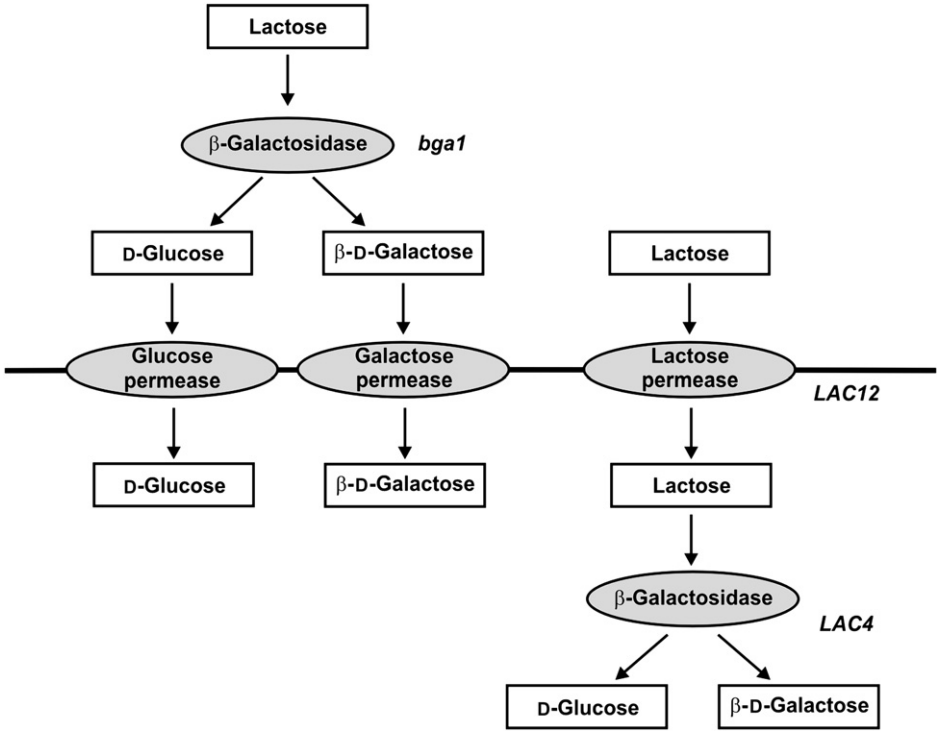

Fig. 1 – The *H. jecorina* (left) and *K. lactis* (right) model of lactose utilization.

3. D-Galactose catabolism

The Leloir pathway

The hydrolysis products of lactose are D-glucose and D-galactose. D-glucose catabolism has been the subject of several reviews and will not be dealt with here. D-galactose, on the other hand, is converted in most eukaryotes by the Leloir pathway (Frey 1996, Holden *et al.* 2003; Fig. 2) to D-glucose-1-phosphate before it is channelled into the glycolytic pathway. The Leloir pathway consists of a sequence of enzymatic steps catalysed by galactokinase (Gal1), galactose-1-phosphate uridylyltransferase (Gal7) and UDP-galactose 4-epimerase (Gal10). A physiologically important feature of galactokinases in general is that they are strictly specific for α-D-galactose which is produced by the aldose 1-epimerase (D-galactose mutarotase) from the β-anomer. In yeasts such as *S. cerevisiae* and *K. lactis* both epimerase domains are found in the bifunctional Gal10 while e.g. in prokaryotes such as *E. coli* the aldose 1-epimerase is encoded by a separate gene.

The Leloir pathway has extensively been dealt with in *S. cerevisiae* and *K. lactis* (Bhat & Murthy 2001; Rubio-Texeira 2005). Pioneering work for fungi was done by Roberts (1963, 1970) but since then only little information accumulated. A genome database search reveals the presence of orthologues for *S. cerevisiae* Gal1, Gal7 and Gal10 in all fungal genomes available, but a detailed characterization of the genes and proteins has only been performed in *H. jecorina* (Seiboth *et al.* 2002a; Seiboth *et al.* 2002b; Seiboth *et al.* 2004).

The *H. jecorina* Gal1 and Gal7 are conserved proteins with moderate overall sequence identity to their yeast orthologues whereas the Gal10 of *H. jecorina* and other fungi consist only of an UDP-galactose 4-epimerase domain but lack the C-terminal aldose 1-epimerase domain. The absence of this domain does not affect the ability of the *H. jecorina gal10* to functionally complement a *S. cerevisiae gal10* strain, but has implications on the mechanism for D-galactose breakdown of lactose hydrolysis. β-galactosidase cleaves lactose by a retaining mechanism and releases therefore only β-D-galactose which has to be converted by the aldose 1-epimerase to the α-anomer, to become a substrate for the galactokinase. An analysis of the *H. jecorina* genome databases identifies at least two genes whose deduced protein sequences show similarity to the *S. cerevisiae* aldose 1-epimerase part of Gal10. But there is at the moment no information available if these genes encode indeed enzymes with D-galactose 1-epimerase activity, and - if so -whether they are expressed on lactose at all. The absence or a low activity of such an enzyme could be a reason for the slow catabolism of lactose because of the time needed for non-enzymatic mutarotation, which at 30 °C and pH 6.5 – 7.0 takes several hours (Pettersson & Pettersson 2001). At least for *E. coli* Bouffard *et al.* (1994) showed that the aldose 1-epimerase is essential for fast growth on lactose.

The genomic organization of the Leloir pathway genes in filamentous fungi reveals a major difference to yeasts, as they are not clustered in fungi. On the other hand, the genomic locations of *H. jecorina gal10* and *gal7* have been identified as syntenic with those in *N. crassa*, and colinear over an area of 6 and 3.5 kilobases. This indicates that – despite of the structural conservation – genomic organization of the *gal* genes has diverged at an early time of evolution.

S. cerevisiae and *K. lactis* use the Leloir pathway exclusively for D-galactose catabolism and therefore all *GAL* genes are essential for growth on D-galactose. In *H. jecorina* only deletion of *gal7* but not *gal1* is essential for growth on D-galactose but both strains are still able to grow on lactose. This apparent discrepancy might be explained by the accumulation of toxic compounds such as galactose-1-phosphate (Slepak *et al.* 2005) in the *H. jecorina* Δ*gal7* strain. Revertants of the *S. cerevisiae gal7* mutants can regain their ability to grow on D-galactose medium by down-regulation of the *GAL* regulon and up-regulation of UDP-glucose pyrophosporylase expression, which transfers D-galactose-1-phosphate to UTP forming UDP-galactose (Lai & Elsas 2000).

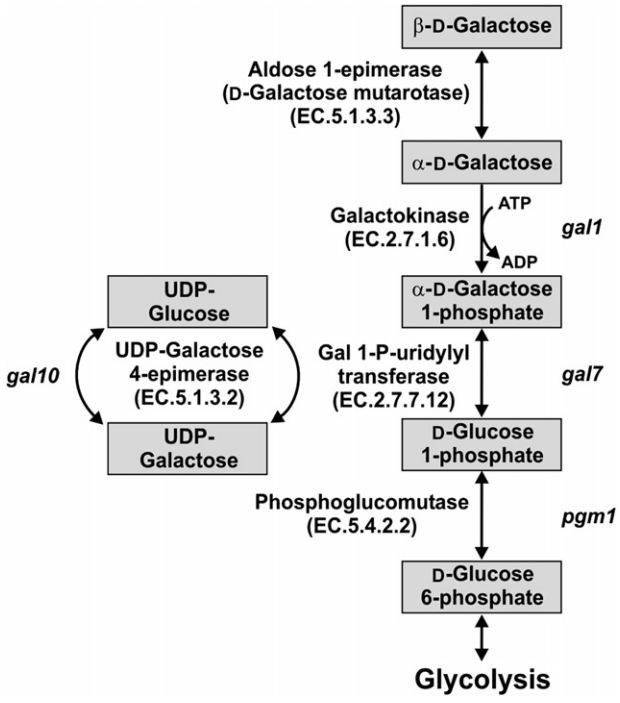

Fig. 2 – The Leloir pathway of D-galactose catabolism.

Filamentous Fungi contain a second pathway for D-galactose catabolism

H. jecorina Δ*gal1* strains are still capable of growing on D-galactose although no residual galactokinase activity is found (Seiboth *et al.* 2004). Roberts (1963, 1970) had already described an alternative D-galactose utilization in *A. nidulans*: Mutants lacking galactokinase or galactose-1-phosphate uridylyltransferase activity were unable to grow below pH < 7.0 but were capable of growing at higher pH values. Later Fekete *et al.* (2004) showed that these mutants were also able to grow on pH < 7.0 but only in the presence of ammonium but not nitrate as nitrogen source. Roberts speculated that the pathway found at pH > 7 starts with an oxidative step by a D-galactose oxidase. A nonphosphorolytic pathway for D-galactose utilization has been reported for *A. niger* (Elshafei & Abdel-Fatah 2001) but neither the genes nor the proteins have been identified.

In *H. jecorina*, we have now evidence for a second D-galactose pathway which is initiated by a reduction of D-galactose to galactitol. A first hint towards the existence of this pathway was obtained by the finding that *H. jecorina* Δ*gal1* strains, when grown on lactose as carbon source, transiently accumulated the polyol galactitol. We could further show that this pathway makes use of reactions and enzymes of the D-xylose and L-arabinose catabolism and in analogy to these pathways ends with a phosphorylation step of D-fructose (Fig. 3). This catabolic path is initiated in *H. jecorina* by the D-xylose reductase Xyl1 (Seiboth *et al.* submitted for publication) which catalyzes amongst others the reduction of D-xylose, L-arabinose and D-galactose with NADPH as cofactor. Δ*xyl1* strains show not only a strongly reduced growth on the two pentoses D-xylose and L-arabinose but also a decreased growth on D-galactose and consequently Δ*gal1*Δ*xyl1* strains are almost unable to grow on D-galactose. Growth on lactose is more impaired in Δ*xyl1* and Δ*gal1*Δ*xyl1* strains compared to the Δ*gal1* strain. This finding provides a hint for the understanding of how *H. jecorina* and other fungi can handle the β-D-galactose moiety of lactose: aldose reductases are able to reduce both anomers of D-galactose and make these fungi independent from the aldose 1-epimerase step of the Leloir pathway.

In analogy to the L-arabinose path, the second step in this pathway, i.e. the NAD^+ dependent oxidation of galactitol is catalysed by the *H. jecorina* L-arabinitol 4-dehydrogenase (Lad1). Δ*lad1* strains are severely affected in growth on galactitol and lactose but also growth on D-galactose is affected. Characterization of the *H. jecorina* Lad1 led to the unexpected finding that galactitol is oxidized to L-xylo-3-hexulose (Pail *et al.* 2004). This intermediate has not yet been identified in other fungi but also the *A. nidulans* orthologous LadA forms L-xylo-3-hexulose, indicating that this was not a peculiarity of the *H. jecorina* enzyme. The further fate of this metabolite is currently not known.

Fekete *et al.* (2004) showed that *A. nidulans* accumulated L-sorbose when grown on D-galactose, and this accumulation was eliminated in strains with a loss-of-activity of L-arabinitol dehydrogenase (*ara*A1), suggesting that L-sorbose is a further metabolite of this pathway. Further L-sorbose catabolism involved a hexokinase step, indicated by the inability of the *A. nidulans fr*A1 (fructose non-phosphorylating) mutant to grow on galactitol or L-sorbose and that a strain with an additional mutation in the galactokinase was unable to grow on D-galactose. Consistent results have been obtained with a hexokinase deletion strain of *H. jecorina*. The role of hexokinase implies that the metabolism of L-sorbose proceeds by reduction to D-sorbitol and subsequent oxidation to D-fructose. The D-sorbitol reaction can be carried out by the *H. jecorina* xylitol dehydrogenase Xdh1 and partially by the Lad1, while the L-sorbose step requires a reductase activity which in analogy to the L-arabinose pathway could be fulfilled by the L-xylulose reductase (Lxr1). The *H. jecorina* Lxr1 (Richard *et al.* 2002) shows L-sorbose reductase activity, but *lxr1* deletion did not have any effect on the growth on lactose, galactitol or D-galactose, and more surprisingly also not on L-arabinose as it would

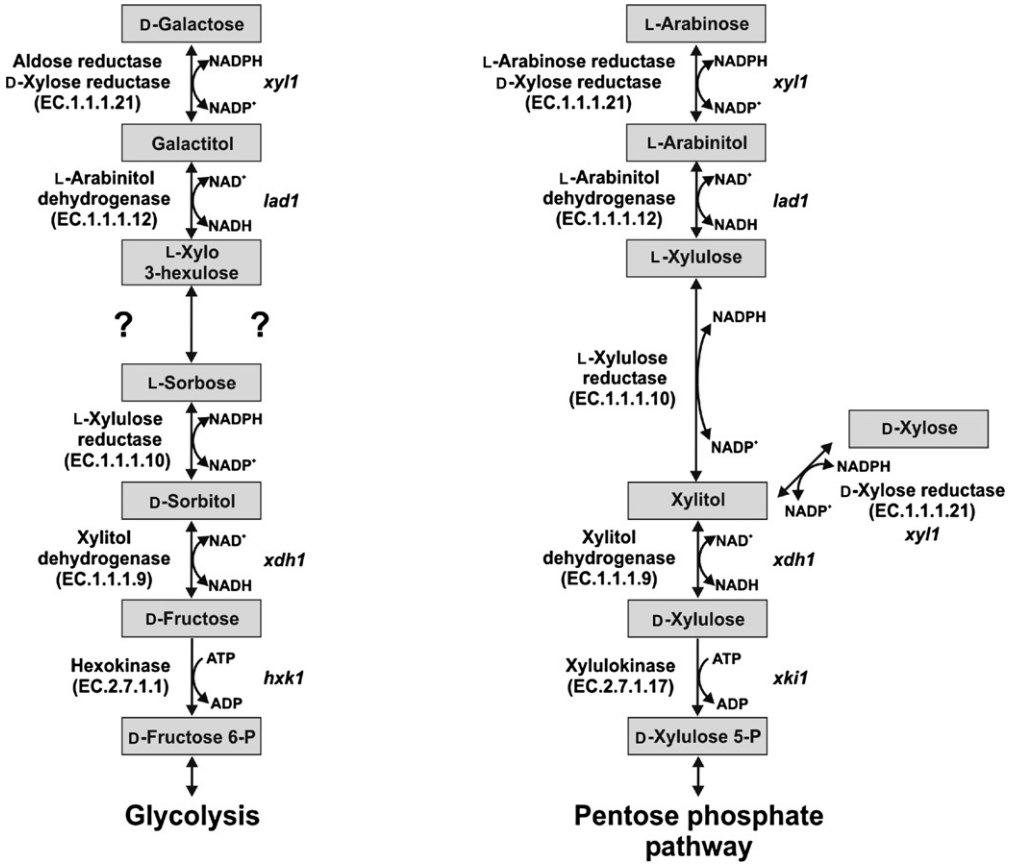

Fig. 3 – A comparison of the main steps of the fungal D-galactose catabolism (left) and the interconnected D-xylose and L-arabinose catabolism (right). Our results from *H. jecorina* and *A. nidulans* were summarized to draft this pathway.

be expected. In fact, we have obtained evidence that Lxr1 is actually a D-mannitol dehydrogenase. Therefore the gene encoding the ''true'' L-xylulose reductase involved in L-arabinose and D-galactose metabolism is still missing.

4. Regulation of D-galactose and lactose metabolism

Regulation of ß-galactosidase formation

Analysis of the formation of the *bga1* transcript in *H. jecorina* showed that it was induced by L-arabinose, L-arabinitol, lactose, D-galactose, galactitol, D-xylose, and xylitol (Seiboth *et al.* 2005). Using carbon limited chemostat cultivations with D-galactose, galactitol and lactose as carbon source we could show that β-galactosidase induction levels correlated positively with the growth rate and that galactitol and D-galactose induced the highest activities (Karaffa *et al.* 2006). We could further show that the inducer for β-galactosidase expression is formed *via* the second D-galactose pathway and that galactitol is the true inducer. This correlates well with the major importance of this pathway for lactose utilization and β-D-galactose catabolism n *H. jecorina*. Induction by D-xylose and L-arabinose of the *bga1* orthologue in *A. niger* is dependent on the presence of the transcriptional activator XlnR (de Vries *et al.* 1999) but its role in the galactitol mediated induction remains to be determined.

A broad spectrum of inducers has been also observed for several other glycosidases acting on α- as well as the β-linked carbohydrates (Zeilinger *et al.* 1993; Nikolaev & Vinetski 1998; de Vries *et al.* 1999; de Vries *et al.* 2002). In view of our results, it is likely that the fungus favours to catabolize the monomeric components *via* the same i.e. the reductive pentose catabolic pathway and therefore ensures that already catabolism of one of the hemicellulose monomers induces the full array of activities needed to decompose polymeric substrates such as hemicelluloses.

In *A. nidulans* the formation of the intracellular β-galactosidase was rapidly induced during growth on lactose, D-galactose, L-arabinose, and D-xylose. Lactose uptake and β-galactosidase formation was inhibited by D-glucose, whereas D-galactose uptake and β-galactosidase formation remained unaffected. Chemostat cultures on D-glucose showed that formation of β-galactosidase activity was derepressed at low growth rates and that the carbon catabolite repressor CreA was responsible for the repression of the basal as well as the induced level of β-galactosidase expression (Fekete *et al.* 2002).

Regulation of the Leloir pathway

The *S. cerevisiae GAL* regulon is one of the best characterized eukaryotic systems for transcriptional regulation. The induction of the *GAL* genes is controlled by the interplay of three proteins: a transcriptional activator, Gal4, a repressor, Gal80, and an inducer, Gal3. Induction appears to occur as a result of a D-galactose- and ATP-dependent interaction between Gal3 and Gal80, which results in the formation of a transcriptionally active Gal4-Gal80-Gal3 complex (Bhat & Murthy 2001). Gal3 shares a high degree of sequence identity to the galactokinase Gal1, but has no galactokinase activity due to the lack of the amino acid doublet SA in the ATP binding domain (Platt *et al.* 2000). The regulation of transcriptional induction of the *GAL* regulon in *K. lactis* is similar (Rubio-Texeira 2005). One of the main differences is that Gal3 is not present in *K. lactis* and that both the inducer as well as the enzymatic function is performed by the *K. lactis* Gal1.

Unlike in yeasts, a high basal level of expression of the *gal* genes is already found under non-inducing and D-glucose repressing conditions in fungi. In *H. jecorina* all three genes were formed constitutively during rapid growth and *gal1* and *gal7*, but not *gal10*, could be further induced (about 2-3 fold) by D-galactose and L-arabinose but not by lactose (Seiboth *et al.* 2002a, 2002b, 2004). In *A. nidulans*, activities of the Leloir pathway enzymes were also found under non-induced conditions and these enzyme activities were induced by about 3-5 fold by D-galactose (Roberts 1970).

The differences to *S. cerevisiae* are further highlighted by the absence of orthologues of *GAL3* and *GAL4* in the fungal genomes of *H. jecorina* or *A. nidulans*. Further, Hartl *et al.* (submitted for publication) demonstrated that – in contrast to the two yeasts – neither the galactokinase activity nor the galactokinase protein is needed for the constitutive level or induced level of *gal7* transcription. These data document an important difference in D-galactose metabolism between yeasts and filamentous fungi, and show that the latter developed a fundamental different mechanism for D-galactose induction. These differences may originate from the abundance of D-galactose in the plant polysaccharides available in the natural habitats of filamentous fungi, and the role of D-galactose as an important component in the cell walls and glycoproteins of fungi. UDP-galactose, needed for the synthesis of both, is produced endogenously by Gal10, which may also explain its constitutive expression in *H. jecorina*.

Regulation of the second D-galactose degrading pathway

Less is known about the regulating factors of the second D-galactose catabolizing pathway. Because of their involvement in the metabolism of various carbohydrates and their respective polyols, induction of the three enzymes studied so far – *xyl1*, *lad1* and *xdh1* – can be triggered by D-xylose and L-arabinose but lower transcript levels were also induced by lactose and D-galactose. Transcription of *H. jecorina xdh1* and *lad1* follow a similar trend: both transcripts are found on D-galactose, galactitol and lactose as carbon sources beside their natural inducers (Seiboth *et al.* 2003). In *A. niger,* the transcriptional activator XlnR is essential for D-xylose reductase induction on D-xylose (Hasper *et al.* 2000) and similar results were found recently for *H. jecorina* (Stricker *et al.* 2006) but data on the role of XlnR orthologues on the induction of the D-xylose reductase by D-galactose or lactose are still missing. In *A. nidulans*, evidence has been obtained that the second pathway is only active in the presence of ammonium but not nitrate (Fekete *et al.* 2004). It is tempting to speculate that this is related to the high demand of NADPH for nitrate reduction, for which it may compete with aldose reductase. However, this speculation still needs experimental approval.

5. Lactose utilization and cellulase gene expression in *H. jecorina*

Cellulase expression during growth on lactose was shown to be a growth rate dependent process: Highest cellulase expression was found on low growth rates which are also used in the industrial cellulase production (Pakula *et al.* 2005; Karaffa *et al.* 2006). A low level of extracellular β–galactosidase activity is essential for cellulase expression whereas higher levels indeed improved growth on lactose but repressed cellulase formation (Seiboth *et al.* 2005). Deletion of the first gene of the Leloir pathway (*gal1*) drastically reduces cellulase gene expression during growth on lactose (Seiboth *et al.* 2004), while a deletion of the subsequent step encoded by *gal7* had no significant effect on cellulase induction (Seiboth *et al.* 2002a). Expression of a catalytically inactive galactokinase could not rescue cellulases expression, whereas the expression of the *E. coli* galactokinase fully restored cellulase expression (Hartl *et al.* submitted for publication). Therefore, in contrast to *gal7* induction on D-galactose, induction of cellulase gene transcription by lactose requires galactokinase activity. But D-galactose alone is not an inducer of cellulase gene expression, which was also valid for a carbon catabolite derepressed Cre1 mutant (Seiboth *et al.* 2004). D-galactose was only able to induce cellulase formation at low growth rates in chemostat cultures but the induction of cellulase formation by lactose was still superior, indicating that slow metabolism of D-galactose alone is not sufficient to account for the efficient cellulase induction seen on lactose (Karaffa *et al.* 2006).

The second D-galactose catabolizing pathway is also of major importance for lactose catabolism and we could show that induction of cellulases is also dependent on the presence of D-xylose reductase. The mechanism how Xyl1 contributes to the formation of the inducer of cellulase formation is not understood at the moment. However, a Δ*gal1*Δ*xyl1* strain reduces cellulase gene induction in the same way as either gene deletion alone, suggesting that both enzymes are necessary for full induction by lactose (Seiboth *et al.* submitted for publication). An indication for an explanation could come from a metabolomic study by van Werf and coworkers (personal communication) They demonstrated a correlation of galactooligosaccharides containing hexitols with the level of cellulase formation during growth of *H. jecorina* on lactose. Experiments are under way to test whether the accumulation of these oligosaccharides depends on the function of *gal1* and *xyl1*, and whether they indeed would trigger cellulase gene transcription.

6. Conclusions

Saprobic fungi have developed an arsenal of extracellular enzymes to efficiently degrade plant cell wall polymers such as cellulose, hemicellulose or pectin. For the catabolism of the hemicellulosic fraction fungi possess a unique pathway which is able to degrade both pentoses and hexoses. This pathway is not only responsible for D-galactose degradation but is essential for inducer formation of the extracellullar enzymes as highlighted for the *H. jecorina* β-galactosidase. This review also makes clear that we still do not know some of the main enzymatic and regulatory components which are necessary to degrade rather simple sugars like lactose, D-galactose or L-arabinose by fungi. To genetically and metabolically engineer these pathways for the generation of improved industrial strains, a detailed fundamental research is needed to decode these pathways.

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