D-Galactose induces cellulase gene expression in *Hypocrea jecorina* at low growth rates

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Lactose (1,4-O-β-D-galactopyranosyl-D-glucose) is a soluble and economic carbon source for the industrial production of cellulases or recombinant proteins by *Hypocrea jecorina* (anamorph *Trichoderma reesei*). The mechanism by which lactose induces cellulase formation is not understood. Recent data showed that the galactokinase step is essential for cellulase induction by lactose, but growth on D-galactose alone does not induce cellulases. Consequently, the hypothesis was tested that D-galactose may be an inducer only at a low growth rate, which is typically observed when growing on lactose. Carbon-limited chemostat cultivations of *H. jecorina* were therefore performed at different dilution rates with D-galactose, lactose, galactitol and D-glucose. Cellulase gene expression was monitored by using a strain carrying a fusion between the cbh2 (encoding cellulobiohydrolase 2, Cel6A) promoter region and the *Aspergillus niger* glucose oxidase gene and by identification of the two major cellulobiohydrolases Cel7A and Cel6A. The results show that D-galactose indeed induces cbh2 gene transcription and leads to Cel7A and Cel6A accumulation at a low \( D = 0.015 \text{ h}^{-1} \) but not at higher dilution rates. At the same dilution rate, growth on D-glucose did not lead to cbh2 promoter activation or Cel6A formation but a basal level, lower than that observed on D-galactose, was detected for the carbon-catabolite-derepressible Cel7A. Lactose induced significantly higher cellulase levels at 0.015 h\(^{-1}\) than D-galactose and induced cellulases even at growth rates up to 0.042 h\(^{-1}\). Results of chemostats with an equimolar mixture of D-galactose and D-glucose essentially mimicked the behaviour on D-galactose alone, whereas an equimolar mixture of D-galactose and galactitol, the first intermediate of a recently described second pathway of D-galactose catabolism, led to cellulase induction at \( D = 0.030 \text{ h}^{-1} \). It is concluded that D-galactose indeed induces cellulases at low growth rate and that the operation of the alternative pathway further increases this induction. However, under those conditions lactose is still a superior inducer for which the mechanism remains to be clarified.

**INTRODUCTION**

The pantropical ascomycete *Hypocrea jecorina* (anamorph *Trichoderma reesei*) is used industrially to produce various extracellular enzymes including cellulases and hemicellulases. The major extracellular protein produced is cellulobiohydrolase I (Cel7A) and therefore its promoter is also used to drive heterologous protein production (Penttilä, 1998). Cellulases are usually formed on insoluble cellulose-containing materials, but their formation can also be induced by several mono- and disaccharides such as L-sorbose, sophorose and lactose (Aro et al., 2005). Lactose (1,4-O-β-D-galactopyranosyl-D-glucose) is a rather unusual inducer for cellulases as it is not expected to occur in the natural environment of *H. jecorina*. However, as a by-product of the dairy industry it represents an attractive renewable carbon source for industrial enzyme production with *H. jecorina*, but its slow metabolism and the fact that cellulase yields are lower than on cellulose still limit its use (Andreotti et al., 1980). Elucidating the mechanism by which lactose induces cellulase formation would therefore be helpful to improve its industrial use.

Lactose metabolism in *H. jecorina* is initiated by extracellular hydrolysis by β-galactosidases (Seiboth et al., 2005). The resulting D-glucose enters the glycolytic pathway directly whereas the D-galactose moiety can either be phosphorylated by galactokinase to galactose 1-phosphate and enter the Leloir pathway (Frey, 1996) or be reduced by an
aldose reductase to galactitol (Fig. 1; Seiboth et al., 2004). Deletion of the galactokinase-encoding gene gal1 drastically reduced cellulase expression during growth on lactose, while deletion of the galactose-1-phosphate uridylyltransferase gene (gal7), which encodes the next step in the Leloir pathway, had no effect on cellulase induction (Seiboth et al., 2002a, 2004). These results were interpreted such that the gene (gal7) is necessary for induction. In batch cultures, however, growth on D-galactose alone did not lead to cellulase induction, not even in the carbon-catabolite-repressed mutant strain Rut-C30 (Seiboth et al., 2004), and strains which overexpressed the main extracellular β-galactosidase showed faster growth on lactose but were unable to form cellulases (Seiboth et al., 2005). Thus, if D-galactose or D-galactose 1-phosphate is the actual inducer of cellulase formation, their action is limited to the slow growth on lactose which is due to the low extracellular β-galactosidase activity.

To test the hypothesis that D-galactose or D-galactose 1-phosphate would act as inducers of cellulase gene expression when D-galactose catabolism occurs at a low rate, we performed chemostat cultures of H. jecorina on D-galactose at different dilution rates.

**METHODS**

**Fungal strains and cultivation conditions.** H. jecorina QM9414 (ATCC 26921) and the uridine-auxotrophic, pyr4-negative mutant TU-6 (Gruber et al., 1990b) were maintained on malt extract agar supplemented with uridine (10 mM) when required. For chemostat cultivations, cultures were pre-grown in 250 ml Erlenmeyer flasks containing, per litre: 250 mg FeSO4·7H2O, 80 mg MnSO4·H2O, 70 mg ZnSO4·7H2O, 85 mg CoCl2 with 10 g glycerol l⁻¹ and 0·1 g peptone l⁻¹ as a carbon source. Mycelia were harvested by filtration after 36 h, washed with cold tap water and then transferred into a fresh medium with the respective carbon source without peptone. Chemostat cultivations were carried out in a 2·5 l glass bioreactor (Inel) with a working volume of 2 l, equipped with one six-blade Rushton disc turbine impeller. Operating conditions were pH 5·0, 30°C, 300 r.p.m. and 0·3vvm (volumes of air per volume of liquid per minute). To minimize water and substrate losses, the waste gas was cooled in a reflux condenser connected to an external cooling bath (4°C). The feeding medium contained carbon source at 3 g l⁻¹, a concentration low enough to make the culture carbon-limited. Prior to cultivations, glass parts of the reactor were treated with the anti-adhesive agent Sigmaclot (Sigma) to avoid fungal wall growth. Polypropylene glycol 2000 (Union Carbide Chemicals & Plastics) was used as antifoam agent; a few drops were injected into the reactor once a day through a membrane filter (Millipore). Onset of steady-state in the culture was established when no changes in the reactor once a day through a membrane filter (Millipore). Onset of steady-state in the culture was established when no changes in the reactor biomass dry weight were observed in three successive samples taken over a period of three residence times (defined as the reciprocal of the dilution rate, e.g. 20 h for = 0:050 h⁻¹). To exclude any regulatory effects on cellulase gene expression by light as observed recently (Schmoll et al., 2005), cultivations were performed in constant darkness.

**Construction of a cbh2::gxaA reporter strain.** A 621 bp region of the H. jecorina cbh2 upstream region was amplified from H. jecorina QM9414 genomic DNA by PCR using the primers cbh2XhoFw1 (5'-CAGTCTCGAGGAATTCTAGGCTAGGTATGCGAG-3') and cbh2XbaRv (5'-GGACTTCTAGAGGTGCAATACACAGAGGGTG-3') to introduce XhoI and XbaI restriction sites. The amplification protocol consisted of an initial denaturation step of 1 min at 94°C, followed by 30 cycles with a denaturation (1 min, 94°C), an annealing (45 s, 57°C) and an elongation step (1 min, 72°C), followed by

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**Fig. 1.** Pathways of D-galactose catabolism in H. jecorina. The classical Leloir pathway (right) and a second pathway (left) which was recently described for H. jecorina (Seiboth et al., 2004; Pail et al., 2004) and A. nidulans (Fekete et al., 2004) are shown. Results from these studies were summarized to draft this second hypothetical pathway. ? indicates steps which have still to be verified.
a final 7 min elongation step at 72 °C. The amplicon was cloned upstream of the *Aspergillus niger* glucose oxidase gene (goxA) into pSJ3 (Mach et al., 1999) using the Xhol and XhoI restriction sites and sequenced. Then the *H. jecorina* pyr4 gene was inserted as a 2.7 kb *SalI* fragment (Gruber et al., 1990a) into the XhoI site, resulting in pCHG1. Transformation of *H. jecorina* TU-6 with pCHG1 was performed via the protoplast method described by Gruber et al. (1990b). Single integration of the reporter cassette was verified by Southern blotting and hybridization with the goxA gene (data not shown). The reporter strain was designated CHG1. We have tested two other reporter strains with single reporter gene integration under selected conditions which essentially confirmed the cellulosome gene expression results obtained for CHG1.

**Enzyme assay.** For the determination of glucose oxidase (Gox) activity in the culture supernatants, samples were first dialysed using a Spectra/Por 3 dialysis membrane (Sera) with a MWCO of 3500 against the assay buffer for 24 h. Gox activity was quantified spectrophotometrically as described previously (Mach et al., 1999). One unit (1 U) of activity is defined as the amount of enzyme required to oxidize 1 μmol D-glucose min⁻¹ at pH 5.8 and 25°C. Protein concentration was determined by a modified Lowry method (Petersen, 1983), using BSA as protein standard.

**Electrophoretic and immunological techniques.** The protocols described by Ausubel et al. (2005) were used for SDS-PAGE with 10% polyacrylamide gels and subsequent Western blotting. CBHI/ Cel7A and CBHII/Cel6A were detected with the aid of monoclonal antibodies (Mischak et al., 1989).

**Analysis of monosaccharides and polyols.** The concentration of D-galactose, D-glucose, galactitol and lactose in the medium was determined by HPLC analysis, using the H⁺ exchange column Aminex HPX-H⁺ (Bio-Rad). As mobile phase 10 mM H₂SO₄ at 55 °C was employed with isocratic elution and refractive index detection.

**Determination of fungal dry weight.** Mycelial dry weight was determined by withdrawing two 20 ml aliquots from the medium, followed by suction filtration through a glass wool filter and drying to a constant weight at 80 °C. Data were averaged and deviated by not more than 14 %.

**Reproducibility.** Results are means of three to five measurements. The data were analysed by Sigmaplot (SPSS Inc.) and standard deviations for each procedure were determined. The SD values were always less than 14% of the means.

**RESULTS**

**Carbon-limited chemostat cultivation of *H. jecorina***

A series of constant-mass, carbon-limited, chemostat-type continuous cultures of the glucose oxidase reporter strain *H. jecorina* CHG1 were performed at five different dilution rates ranging from 0-075 h⁻¹ to 0-015 h⁻¹ with D-galactose or lactose as limiting carbon source. Table 1 shows that on D-galactose, cbh2 promoter activation, measured by glucose oxidase activity as reporter gene, did not occur at D between 0-075 and 0-030 h⁻¹, but was clearly detectable at D = 0-015 h⁻¹. These findings were further supported by the detection of the cbh2-encoded Cel6A protein in the culture fluid only at 0-015 h⁻¹ but not at higher dilution rates. Also the cbh1-encoded major cellulase Cel7A of *H. jecorina* was only detectable at this dilution rate (Fig. 2a).

Using lactose as a limiting carbon source, however, cbh2 promoter activation as well as the appearance of the Cel6A and Cel7A proteins (Fig. 2b) in the culture filtrates were detected already at higher dilution rates: both reporter gene expression and the two proteins were already detected at D = 0-042 h⁻¹. At higher dilution rates a washing out of the mycelia was observed, indicating that the maximal specific growth rate was exceeded. Levels of the two cellobiohydrolases increased when the dilution rate was further lowered. At D = 0-015 h⁻¹ cbh2 gene promoter activation was fourfold as high as on D-galactose.

**Effect of dilution rate on *H. jecorina* cbh2 gene expression on D-galactose and lactose**

Chemostat cultures of the cbh2::goxA reporter strain *H. jecorina* CHG1 were performed at five different dilution rates ranging from 0-075 h⁻¹ to 0-015 h⁻¹ with D-galactose or lactose as limiting carbon source. Table 1 shows that on D-galactose, cbh2 promoter activation, measured by glucose oxidase activity as reporter gene, did not occur at D between 0-075 and 0-030 h⁻¹, but was clearly detectable at D = 0-015 h⁻¹. These findings were further supported by the detection of the cbh2-encoded Cel6A protein in the culture fluid only at 0-015 h⁻¹ but not at higher dilution rates. Also the cbh1-encoded major cellulase Cel7A of *H. jecorina* was only detectable at this dilution rate (Fig. 2a).

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**Table 1**

<table>
<thead>
<tr>
<th>Dilution Rate (h⁻¹)</th>
<th>Initial Biomass (g l⁻¹)</th>
<th>Residual Biomass (g l⁻¹)</th>
<th>Initial Galactitol (g l⁻¹)</th>
<th>Residual Galactitol (g l⁻¹)</th>
<th>Initial D-Glucose (g l⁻¹)</th>
<th>Residual D-Glucose (g l⁻¹)</th>
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<tr>
<td>0.075</td>
<td>3.4</td>
<td>0.5</td>
<td>0.6</td>
<td>0.01</td>
<td>3.5</td>
<td>3.3</td>
</tr>
<tr>
<td>0.030</td>
<td>2.4</td>
<td>0.3</td>
<td>0.5</td>
<td>0.01</td>
<td>3.2</td>
<td>3.0</td>
</tr>
<tr>
<td>0.015</td>
<td>1.4</td>
<td>0.2</td>
<td>0.4</td>
<td>0.01</td>
<td>3.0</td>
<td>2.9</td>
</tr>
<tr>
<td>0.0075</td>
<td>0.4</td>
<td>0.0</td>
<td>0.3</td>
<td>0.01</td>
<td>3.0</td>
<td>2.9</td>
</tr>
<tr>
<td>0.0030</td>
<td>0.0</td>
<td>0.0</td>
<td>0.2</td>
<td>0.01</td>
<td>3.0</td>
<td>2.9</td>
</tr>
</tbody>
</table>

**Figure 2**

- **Figure 2a**: Western blot analysis of *H. jecorina* CBHII and CBHI proteins in triplicate chemostat cultures grown on D-galactose at different dilution rates. The cbh2::goxA reporter strain *H. jecorina* CHG1 displayed no detectable CBHII and CBHI, whereas the CBHII/Cel6A and CBHII/Cel6A proteins were detected at all dilution rates.
- **Figure 2b**: Western blot analysis of *H. jecorina* CBHII and CBHI proteins in triplicate chemostat cultures grown on lactose at different dilution rates. The cbh2::goxA reporter strain *H. jecorina* CHG1 displayed no detectable CBHII and CBHI, whereas the CBHII/Cel6A and CBHI/Cel6A proteins were detected at all dilution rates.
Effect of dilution rate on H. jecorina cbh2 gene expression on D-glucose

In theory, cbh2 gene expression at \( D = 0.015 \) h\(^{-1}\) could be the result of a specific D-galactose induction or due to a general derepression at the low growth rate. To discriminate between these two possibilities chemostat cultures were also performed with D-glucose as the limiting nutrient at low dilution rates. In contrast to the results obtained with D-galactose, no cbh2 promoter activation was detected under these conditions, which was also supported by the absence of the Cel6A protein. Cel7A – whose basal level is known to be under carbon catabolite repression by Cre1 (Zeilinger et al., 2003) – was detectable only at the lowest dilution rate, 0.015 h\(^{-1}\) (Table 1, Fig. 3a). These findings prove that the triggering of cbh2 gene expression by D-galactose at \( D = 0.015 \) h\(^{-1}\) is not due to a general carbon catabolite derepression but to a specific induction by D-galactose.

Influence of dilution rate on cellulase gene expression in chemostat cultures containing equimolar mixtures of D-glucose and D-galactose

The increased levels of formation of Cel7A and Cel6A during growth on lactose in comparison to D-galactose, and their occurrence at higher dilution rates on lactose, pointed to a clear difference between the induction by lactose and D-galactose. We tested whether this is due to lactose itself or to the presence of the two monomers D-glucose and D-galactose. Chemostat cultures were therefore performed with an equimolar mixture of the two monosaccharides. The results are shown in Table 1 and Fig. 3(b), and indicate that, identical to the culture on D-galactose alone, cbh2

Table 1. Glucose oxidase activity in the supernatant of carbon-limited chemostat cultures of H. jecorina CHG1 at different dilution rates

One unit (1 U) of glucose oxidase activity is defined as the amount of enzyme required to oxidize 1 \( \mu \)mol D-glucose min\(^{-1}\) at pH 5.8 and 25°C.

<table>
<thead>
<tr>
<th>( D (h^{-1}) )</th>
<th>Lactose</th>
<th>Glucose</th>
<th>Galactose</th>
<th>Galactose + glucose</th>
<th>Galactitol</th>
<th>Galactose + galactitol</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.075</td>
<td>WO</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>WO</td>
<td>WO</td>
</tr>
<tr>
<td>0.050</td>
<td>WO</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.042</td>
<td>1.68</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.030</td>
<td>2.71</td>
<td>0</td>
<td>0.62</td>
<td>0.55</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>0.015</td>
<td>1.68</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.51</td>
<td>0.51</td>
</tr>
</tbody>
</table>

WO, Washing out of the mycelia; NA, a stable steady state was not achievable even after prolonged cultivation.

![Fig. 2](https://example.com/fig2.jpg)

**Fig. 2.** Cellulase formation in carbon-limited chemostat cultures of H. jecorina CHG1 at different dilution rates: Western blots of Cel7A and Cel6A accumulation in the supernatant of chemostat cultures with D-galactose (a) and lactose (b) as carbon source. Equal volumes (corresponding to 300 µl supernatant) were loaded per lane.

![Fig. 3](https://example.com/fig3.jpg)

**Fig. 3.** Cellulase formation in carbon-limited chemostat cultures of H. jecorina CHG1 at different dilution rates: Western blots of Cel7A and Cel6A accumulation in the supernatant of chemostat cultures with D-glucose (a) and an equimolar mixture of D-galactose and D-glucose (b). For comparison the supernatants of the chemostat cultures with lactose (Lac) and D-galactose (Gal) as carbon source (\( D = 0.015 \)) were loaded. Equal volumes (corresponding to 300 µl supernatant) were loaded per lane.
promoter activation did not occur at $D=0.075-0.030 \text{ h}^{-1}$, but was clearly detectable at $D=0.015 \text{ h}^{-1}$. The accumulation of Cel7A and Cel6A was also consistent with this finding and paralleled the accumulation pattern seen on D-galactose alone. These data therefore indicate that it is the lactose disaccharide which is responsible for the observed difference in cellulase formation, and, moreover, that the simultaneous presence of D-glucose and D-galactose does not influence the induction of cellulases by D-galactose at low growth rates.

**Effect of dilution rate on *H. jecorina* cbh2 expression on equimolar mixtures of D-galactose and galactitol**

In *H. jecorina*, catabolism of D-galactose can proceed via at least two different pathways, i.e. the classical Leloir pathway and a pathway involving formation of galactitol as an intermediate (Seiboth et al., 2004). In order to find out whether this second pathway contributes to cellulase formation on lactose, we performed chemostat cultivations with galactitol and equimolar mixtures of D-galactose and galactitol as carbon source, thus forcing catabolism of at least half of the substrate through this pathway. Maximal growth rate was exceeded for both conditions at $D=0.075 \text{ h}^{-1}$, which resulted in a washing out of the mycelia. However, cbh2 promoter activation as well as expression of Cel7A and Cel6A was observed at $0.030 \text{ h}^{-1}$ on the mixture of D-galactose and galactitol, whereas no cellulases were formed at this dilution rate on D-galactose or galactitol alone (Fig. 4, Table 1). We could not achieve a steady state with the mixture of galactitol and D-galactose or galactitol alone at $D=0.015 \text{ h}^{-1}$, which was the lowest growth rate for lactose or D-galactose as limiting carbon source.

While these data are consistent with our hypothesis that D-galactose can act as an inducer at slow growth rates, the higher cbh2 gene expression levels and stronger accumulation of Cel7A and Cel6A in the culture fluid on lactose than on D-galactose indicate that lactose is nevertheless a far superior inducer of cellulase formation to D-galactose. The induction seen by D-galactose at $0.015 \text{ h}^{-1}$ is thus not sufficient to account for the efficient cellulase induction seen on lactose, and lactose must therefore transmit an additional signal to the fungus, which ultimately triggers cellulase gene expression at high levels. An obvious hypothesis to explain this finding would be to postulate a lactose-binding receptor which would then trigger a cellulase induction cascade. At this moment, we can neither prove nor reject this theory. However, we note that this theory would not explain why a mutant with a knockout in the galactokinase gene would be impaired in cellulase induction. Further work is needed to elucidate the role of carbohydrate catabolism in cellulase induction.

**DISCUSSION**

In this study, we have tested the hypothesis whether the triggering of cellulase formation by lactose is due to induction by D-galactose (or a metabolite thereof) at slow growth rates. This hypothesis was created to explain the previous findings that a galactokinase knockout mutant of *H. jecorina* is impaired in cellulase induction, yet growth on D-galactose in batch culture does not induce cellulase formation (Seiboth et al., 2004). We therefore grew *H. jecorina* in chemostat cultures with D-galactose as limiting carbon source at various dilution rates between 0.075 and 0.015 h$^{-1}$. Experiments reported here indeed showed cellulase gene expression at a very low (0.015 h$^{-1}$) but not a higher (0.030 h$^{-1}$) dilution rate. Of course, this cellulase formation at a low growth rate does not necessarily prove induction but could also be due to carbon catabolite derepression (Ilyés et al., 2004). It is therefore essential to compare these results to those of chemostat cultures on D-glucose, and also to compare the formation of the two cellulases Cel7A and Cel6A. Cel7A is encoded by cbh1, whose basal expression level is under the control of the Cre1 carbon catabolite repressor. Therefore, a low level of Cel7A is formed under derepressing conditions even without the addition of an inducer, whereas formation of Cel6A is strictly dependent on the presence of an inducer (Zeilinger et al., 2003). This behaviour is also reflected in the present results, i.e. only Cel7A but not Cel6A was formed at 0.015 h$^{-1}$ on D-glucose. However, both proteins accumulated to a clearly higher level on D-galactose at 0.015 h$^{-1}$. Consequently, D-galactose indeed acts as an inducer at a low dilution rate and this effect is therefore not due to carbon catabolite derepression, although the two effects may overlap in the case of cbh1.
enzymically active galactokinase (Meyer et al., 1991). In S. cerevisiae these two functions are found on two independent but highly related proteins: Gal1 performs the galactokinase activity and Gal3 is responsible for the signal transduction of D-galactose to the transcriptional activator Gal4 (Bhat & Murthy, 2001).

Alternatively, and by analogy with cellulase induction by cellulose which is believed to be mediated by transglycosylation products, e.g. sophorose (Sternberg & Mandels, 1979; Vaheri et al., 1979), it could be postulated that lactose would enter the cells and, either directly or also after transglycosylation, act as inducer of cellulase formation. Such an assumption would however be in conflict with the fact that we have not been able to detect either a lactose permease or an intracellular β-galactosidase activity in H. jecorina (Seiboth et al., 2005), which would both be prerequisites for the operation of such a mechanism. In addition, this theory would not explain the need for galactokinase activity in cellulase induction.

Results from the present work show that, despite hydrolysing it before taking it up into the cell, H. jecorina recognizes the presence of hydrolysed lactose in a different way than the simultaneous presence of D-glucose and D-galactose. What is the difference between hydrolysed lactose and a mixture of D-glucose and D-galactose? D-Galactose is, because of mutarotation, a mixture of the α- and β-anomer whereas D-galactose is released from lactose as the β-anomer. The following enzymic step catalysed by galactokinase is strictly specific for α-D-galactose (Holden et al., 2003) and consequently the β-D-galactose has to be first converted by mutarotation to the α-D-galactose by an aldose 1-epimerase.

To this end S. cerevisiae and other yeasts possess an aldose 1-epimerase activity located on the bifunctional Gal10 protein, which also has a UDP-glucose 4-epimerase activity (Thoden & Holden, 2005). This bifunctional composition of Gal10 seems to be yeast specific while in other organisms, including H. jecorina, these enzymic functions are found on two different proteins (Seiboth et al., 2002b). A Blastx search identifies at least two genes encoding proteins with a high similarity to the yeast aldose 1-epimerase part of Gal10 in the H. jecorina genome sequence database (http://gsphere.lanl.gov/trire1/trire1.home.html). We do not know at the moment whether they indeed encode D-galactose mutarotases and are expressed on lactose. But the absence or only a low expression of such an enzyme would considerably delay catabolism of the lactose-derived β-D-galactose via the Leloir pathway because of the time needed for non-enzymic mutarotation, which at 30 °C and pH 6.5–7.0 takes several hours (Pettersson & Pettersson, 2001). This could eventually lead to an accumulation of β-D-galactose in the mycelia to toxic levels. It is therefore possible that this may be the reason why H. jecorina and other fungi have in addition to the Leloir pathway an alternative pathway for D-galactose catabolism (Fekete et al., 2004; Seiboth et al., 2004), which by analogy with the L-arabinose catabolic pathway uses reductive and oxidative reactions to convert D-galactose into fructose 6-phosphate. The first enzyme in this alternative pathway is an aldose reductase, which is able to use both D-galactose anomers as a substrate to form galactitol.

We have recently found that deletion of a general aldose reductase led to slower growth on lactose and D-galactose (unpublished data), although some residual D-galactose reductase activity was still present. This indicates that this pathway is operative under the conditions of this work. In order to test whether this pathway also contributes to the inducer formation from lactose, we have grown H. jecorina on an equimolar mixture of D-galactose and galactitol, which resulted in cellulase induction at a growth rate (0.030 h⁻¹) at which otherwise no cellulase formation was detectable on D-galactose alone. Unfortunately, we were unable to cultivate H. jecorina on D-galactose and galactitol at 0.015 h⁻¹, and are thus unable to provide quantitative data for the possible enhancement of cellulase formation over that on D-galactose alone. But we should like to note that a direct comparison of results obtained by growth on lactose and D-galactose/galactitol may be problematic in any case, because galactitol is more reduced than D-galactose, and its presence as a substrate creates a higher demand for maintenance energy. This fact is also reflected in our inability to cultivate H. jecorina on galactitol at D=0.015 h⁻¹ and in the slightly lower steady-state biomass concentration values at higher dilution rates. Nevertheless, these data show that catabolism of galactitol (= operation of the alternative D-galactose catabolic pathway) positively contributes to the induction of cellulase gene expression. Further investigations will be dedicated to finding out how galactokinase and this pathway interact in this process.

The dilution rates reported for lactose are in the same range as those reported for the H. jecorina Rut-C30 (Pakula et al., 2005), with the exception that the lowest dilution rate was 0.022 h⁻¹. Rut-C30 is defective in at least two genes: one encodes the wide-domain regulator Cre1 for carbon catabolite repression (Ilmen et al., 1996), the second the glucosidase II α subunit (Gls2α), a protein which is involved in endoplasmic reticulum quality control (Geyssens et al., 2005). In Aspergillus nidulans it was shown that carbon catabolite repression mediated by the orthologous CreA is growth rate dependent and that below a certain growth rate carbon catabolite derepression occurs (Ilyès et al., 2004). Nevertheless, in the carbon-catabolite-derepressed Rut-C30 most Cel7A is produced at a low specific growth rate, with the optimum at 0.031 h⁻¹. Cel7A production decreases with higher dilution rates but also at 0.022 h⁻¹ lower production is found. At low specific growth rates a limitation of the capacity of the secretory pathway was found which prevented an efficient transport of Cel7A through the secretory pathway. It has to be proven if this difference might be due to the truncated gls2α or to other effects. Here we could show that Cel7A and Cel6A gene expression increased with decreasing dilution rates on lactose in CHG1. A comparison of these results indicates that Cre1 is certainly not the only regulator which controls high cellobiohydrolase activity and Gal3 is responsible for the signal transduction of D-galactose to the transcriptional activator Gal4 (Bhat & Murthy, 2001).
production, especially during fast growth at high dilution rates.

An interesting side-observation of this study is the finding of much higher cellulase protein concentrations in culture filtrates from lactose- than from D-galactose-grown cultures, significantly exceeding the ratio which would be predictable from the ratio in gene expression measured by glucose oxidase activity. This indicates that induction is not the only mechanism by which lactose promotes cellulase production at high levels and that additional factors are necessary to assure a high production of cellobiohydrolases. The observed difference might be simply the result of the fact that (although of fungal origin) glucose oxidase is a heterologous protein, while the two cellobiohydrolases are homologous proteins. It remains to be tested at which level of gene expression this regulation takes place.

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