Lack of aldose 1-epimerase in *Hypocrea jecorina* (anamorph *Trichoderma reesei*): A key to cellulase gene expression on lactose

Erzsébet Fekete*, Bernhard Seiboth†, Christian P. Kubicek†, Attila Szentirmai*, and Levente Karaffa*‡

*Department of Genetics and Applied Microbiology, Faculty of Science and Technology, University of Debrecen, P.O. Box 56, H-4010, Debrecen, Hungary; and †Research Area Gene Technology and Applied Biochemistry, Institute of Chemical Engineering, Vienna University of Technology, Getreidemarkt 9/1665, A-1060 Vienna, Austria

The heterodisaccharide lactose (1,4-O-β-D-galactopyranosyl-D-glucose) induces cellulase formation in the ascomycete *Hypocrea jecorina* (= *Trichoderma reesei*). Lactose assimilation is slow, and the assimilation of its β-D-galactose moiety depends mainly on the operation of a recently described reductive pathway and depends less on the Leloir pathway, which accepts only α-D-galactose. We therefore reasoned whether galactomutarotase [aldose 1-epimerase (AEP)] activity might limit lactose assimilation and thus influence cellulase formation. We identified three putative AEP-encoding genes (*aep1, aep2, aep3*) in *H. jecorina*, of which two encoded intracellular protein (AEP1 and AEP2) and one encoded an extracellular protein (AEP3). Although all three were transcribed, only the *aep3* transcript was detected on lactose. However, no mutarotase activity was detected in the mycelia, their cell walls, or the extracellular medium during growth on lactose. Therefore, the effect of galactomutarotase activity on lactose assimilation was studied with *H. jecorina* strains expressing the C-terminal galactose mutarotase part of the *Saccharomyces cerevisiae* Gal10. These strains showed increased growth on lactose in a gene copy number-dependent manner, although their formation of extracellular β-galactosidase activity and transcription of the genes encoding the first steps in the Leloir and the reductive pathway was similar to the parental strain QM9414. Cellulase gene transcription on lactose dramatically decreased in these strains, but remained unaffected during growth on cellulose. Our data show that cellulase induction in *H. jecorina* by lactose requires the β-anomer of D-galactose and reveal the lack of mutarotase activity during growth on lactose as an important key for cellulase formation on this sugar.

mutarotase | Leloir pathway | reductive D-galactose catabolism

The heterodisaccharide lactose (1,4-*O*-β-D-galactopyranosyl-D-glucose) occurs mainly in mammalian milk where it makes up 2–8% of the dry weight. Its hydrolysis by β-galactosidases (= lactases) yields D-glucose and β-D-galactose. In prokaryotes, yeasts, and mammals, the latter is then catabolized via the Leloir pathway (1). However, the first enzyme in this pathway [galactokinase (EC 2.7.1.6)] accepts only α-D-galactose and cannot act on the β-anomer (2). Although the interconversion of the sugar anomers occurs spontaneously in pure water *in vitro*, efficient *in vivo* formation of α-D-galactopyranose from β-D-galactopyranose has been shown to depend on the presence of a mutarotase [aldose 1-epimerase (AEP); EC 5.1.3.3], suggesting that the activity of intracellular water is not high enough to permit mutarotation (3).

Galactose mutarotase activity has subsequently been observed in a wide range of organisms, including bacteria (3–5), plants (6, 7), fungi (8), and mammals (9–11) and is present in the cytoplasm of most of these cells. An intriguing case is the *Saccharomyces cerevisiae* Gal10 where mutarotase occurs as a fusion protein to another enzyme of the Leloir pathway, UDP-glucose 4-epimerase (EC 5.1.3.2) (12). All of these mutarotases have a broad specificity for aldoses, yet, at least in *S. cerevisiae*, exhibiting highest activity with D-galactose (13). In most bacteria, the enzyme is secreted from the cells (14), whereas the eukaryotic enzyme is mostly intracellular (12).

For the ascomycete *Hypocrea jecorina* (the teleomorph of the filamentous fungus *Trichoderma reesei* that is used for the industrial production of cellulases and hemicellulases), lactose, although not being a natural substrate, serves as an important inducer of cellulase formation in industry (15). Interestingly, assimilation of lactose by this fungus depends less on the Leloir pathway of D-galactose catabolism that accepts only α-D-galactose (16, 17), but strongly depends on an alternative pathway in which the D-galactose moiety is first reduced to galactitol, and by a series of subsequent oxidation and reduction steps finally converted to D-fructose (18). The GAL10 protein of *H. jecorina* and its orthologues in other multicellular ascomycetes have only the UDP-glucose 4-epimerase domain but lack the mutarotase domain (19). Taken together, these data suggest that β-D-galactose mutarotation in *H. jecorina* (and maybe other filamentous fungi) is absent or inefficient. Direct proof for this hypothesis is lacking, however.

Here, we present evidence that *H. jecorina* contains three putative mutarotase genes, but indeed lacks mutarotase activity during growth on lactose. Moreover we will show that introduction of mutarotase activity into *H. jecorina* impairs cellulase induction by lactose. The lack of mutarotase activity is therefore an important trait for industrial cellulase production on lactose.

Results

H. jecorina Has Three *aep* Genes. As a prerequisite for this work, we have screened the genome database of *H. jecorina* (http://genome.jgi-psf.org/Trire2/Trire2.home.html) for genes potentially encoding AEPs. Three loci were found: tre42544 [Protein Data Bank (PDB) ID code 121661], tre44592 (PDB ID code 120784), and tre19341 (PDB ID code 22415). tre43544 encodes a protein with the highest amino acid similarity (35%) to the C terminus of the *S. cerevisiae* bifunctional Gal10 and exhibits 64% amino acid identity to a hypothetical protein from *Gibberella zeae*. Similarity of the other two putative AEPs (tre44592 and tre19341) was much weaker (Table 1). We found ESTs for all three genes in the mixed mRNA samples of (20), indicating that none of them is a pseudogene.

We have named the three genes *aep1*, *aep2*, and *aep3*, following established nomenclature rules for isoenzymes and starting the numbering with the one whose deduced protein has the lowest isoelectric point (Table 1). Among them, AEP3 contains a predicted signal peptide (SignalP probability 1.00) that is

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Table 1. Characterization of the three deductive mutarotase proteins of *H. jecorina*

	Protein		
Characteristic	AEP1	AEP2	AEP3
Name in genome annotation	tre19341	tre42544	tre44592
PDB ID code	22415	121661	120784
EST no.	4	4	10
Amino acid similarity to *S. cerevisiae* Gal10, %	7	35	23
No. of amino acids	315	342	383
M_r	33,988.2	37,074.5	42,380.0
Isoelectric point	4.99	5.29	6.44
Predicted gene product location	Intracellular	Intracellular	Extracellular

removed with a probability of 0.394 between positions 23 and 24, and therefore constitutes a secreted protein. The location of the other two is predicted to be cytoplasmic.

The Three Mutarotase Genes Are Not Expressed During Growth on Lactose. To learn whether any of these three genes could be involved in D-galactose mutarotation during growth of *H. jecorina*, the fungus was cultivated on D-glucose, D-galactose, and lactose, and the transcripts of *aep1*, *aep2*, and *aep3* were assayed (Fig. 1). The mRNA of *aep3* was most abundant and detected on all three carbon sources during the phase of early growth (5 h), but only on D-galactose during the later stages (27 h) of growth. Traces of the *aep1* transcripts were detected on D-glucose at 5 h and D-galactose at 27 h, whereas a higher, but still very low, level of *aep2*-mRNA was present on D-galactose at 5 h (Fig. 1).

The above results would imply that there is no galactomutarotase activity present in *H. jecorina* when growing on lactose. To independently test this notion, we assayed for mutarotase activity in cell-free extracts, cell walls, and the extracellular culture filtrates. Activities were below the detection limit in all samples, whereas controls with cell-free extracts from *S. cerevisiae* clearly showed activity (data not shown). The situation was similar on D-galactose. We conclude that *H. jecorina*, while containing three putative mutarotase genes, does not express such mutarotase activity during growth on lactose.

Overexpression of the *S. cerevisiae* Gal10 AEP Domain in *H. jecorina* Increases the Rate of Lactose Utilization. Because of this apparent lack of galactose mutarotase activity in *H. jecorina* during growth

on lactose, we were interested in the effect of overexpression of a mutarotase in the fungus, particularly in its consequences for cellulase induction by lactose. To this end, we used the C-terminal part of the *S. cerevisiae* Gal10 that is responsible for the D-galactopyranose mutarotation activity (13). A gene fragment spanning from nucleotides 1068 to 2100 ($GAL10^{1068-2100}$) was fused under the expression signals of the constitutive *pki1* (pyruvate kinase) promoter and the *cbh2* (cellobiohydrolase 2) terminator region (Fig. 2A). Transformants were purified and checked by Southern analysis and PCR (Fig. 2B). Three of them, which exhibited different copy numbers of the construct (i.e., one copy in strains M1 and two copies in strains M2a and M2b), also accumulated correspondingly different amounts of $GAL10^{1068-2100}$ transcripts (Fig. 2C). To ensure that these three transformants in fact also express a functional mutarotase, we assayed cell-free extracts of cultures grown on lactose as a carbon source (Fig. 2D). As clearly seen, all of the mutants contained mutarotase activity, and the activity correlated with the $GAL10^{1068-2100}$ copy number. They were thus chosen for further investigation.

When the reference strain of *H. jecorina* and the three mutarotase-expressing strains were grown on D-glucose and D-galactose, they exhibited similar growth rates and rates of sugar consumption as the reference strain, indicating that AEP (and thus mutarotase activity in general) is dispensable for the assimilation of both monosaccharides (Fig. 3 *A* and *B*). Data consistent with this claim were also obtained for L-arabinose, glycerol, D-xylose, and D-fructose (data not shown). A clear difference, however, was seen on lactose: all of the mutarotase transformants exhibited an enhanced assimilation (Fig. 3C) and a subsequently increased biomass formation (Fig. 3D), and the increase was again positively correlated with the $GAL10^{1068-2100}$ copy number.

Overexpression of the *S. cerevisiae* Gal10 AEP Domain in *H. jecorina* Does Not Affect Extracellular β-Galactosidase Activity. The phenotype of the $GAL10^{1068-2100}$ transformants is reminiscent of that of *H. jecorina* strains overexpressing the extracellular β-galactosidase BGA1 (21). To test whether the effect of mutarotase overexpression is direct or indirect, we measured the total extracellular β-galactosidase activity and the concentration of free D-galactose in the medium during growth of the reference strain and the three $GAL10^{1068-2100}$-expressing strains on lactose. The data show that extracellular β-galactosidase activity is not different in any of the mutants (Fig. 4), whereas we could not detect any free D-galactose in the medium of any of the *H. jecorina* strains (data not shown).

Overexpression of the *S. cerevisiae* Gal10 AEP Domain in *H. jecorina* Does Not Affect the Expression of the Aldose Reductase. β-D-galactose arising from lactose can be catabolized by *H. jecorina* via two pathways, i.e., the reductive pathway and after mutarotation via the Leloir pathway (22). Consequently, we were
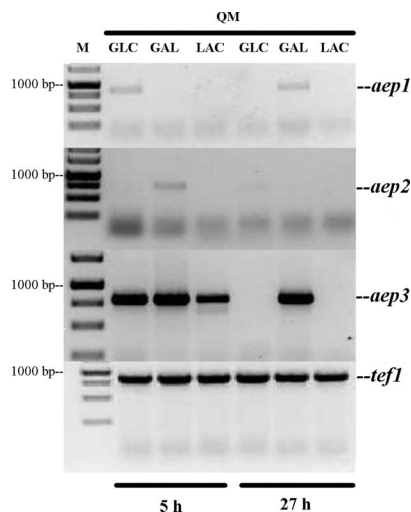

Fig. 1. Transcription profile of *aep1*, *aep2*, and *aep3* of *H. jecorina* QM9414 (QM) during growth on different carbon sources obtained by RT-PCR. Glc, glucose; Gal, galactose; Lac, lactose; M, marker.
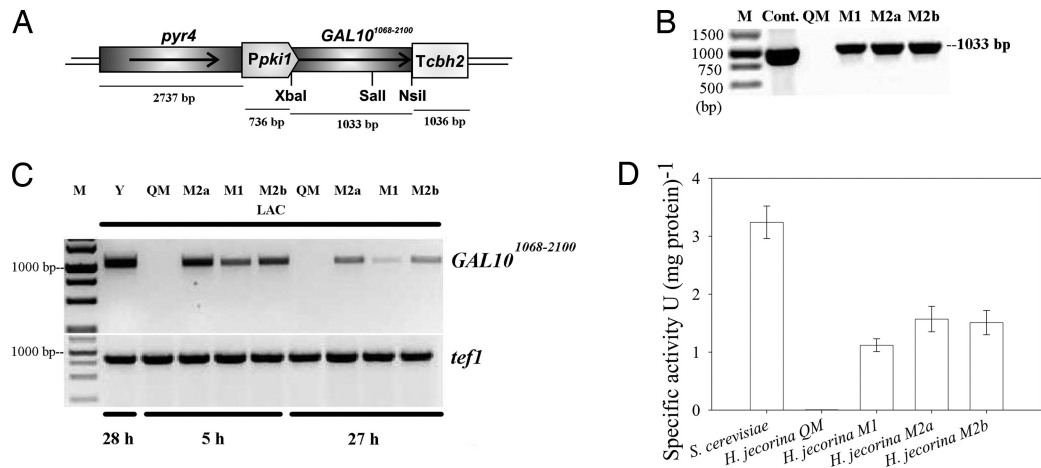
Fig. 2. Demonstration of the presence and expression of GAL10[1068-2100] in *H. jecorinh* mutants. (*A*) Schematic drawing of the *GAL10*[1068-2100] expression cassette used for the transformation of *H. jecorina* TU-6. (*B*) Demonstration of the presence of *S. cerevisiae GAL10*[1068-2100] in the *H. jecorina* mutant strains M1, M2a, and M2b by PCR and its absence in QM9414. M, marker; Cont., control (p*GAL10*[Kicsi] used as a template). (*C*) Transcription of the *S. cerevisae GAL10*[1068-2100] in *H. jecorina* strains M1, M2a, and M2b during growth on lactose at 5 and 27 h. Y, *S. cerevisiae* (positive control); QM, *H. jecorina* QM9414; M, marker; Lac, lactose. (*D*) Specific intracellular mutarotase activity of the QM9414- and *GAL10*[1068-2100]-expressing *H. jecorina* strains. Samples were taken 12 h after the transferring procedure.

interested to learn whether the activity of the reductive pathway would be reduced in the *H. jecorina GAL10*[1068-2100] transformants. Unfortunately, isotope labeling cannot be applied to this problem, because both pathways essentially conserve the individual carbon atoms in D-galactose. We therefore took an indirect means to test this hypothesis, i.e., we analyzed the transcripts of *gal1* (galactokinase, which is induced by D-galactose but formed constitutively during growth on lactose), and *xyl1* (D-xylose reductase, which is induced by lactose and to a lower level by D-galactose and galactitol and hence shows increased transcript levels when the pathway is in operation). The ratio of the relative transcript abundance of the two genes, however, was essentially similar (Fig. 5). Therefore, both path-

ways likely cooperate in a similar way in the parent strain and the *GAL10*[1068-2100]-expressing strains.

Overexpression of the *S. cerevisiae* Gal10 AEP Domain in *H. jecorina* Impairs Cellulase Gene Expression. Having established that the overexpression of the Gal10 AEP domain specifically alters the rates of lactose utilization, we have examined whether it would have an effect on cellulase gene expression. To this end, we used the *cbh1* and *cbh2* genes, which encode the two major cellobiohydrolases of *H. jecorina* (CEL7A and CEL6A, respectively) and are expressed coordinately with most other cellulases (20) as a model. Fig. 6*A* shows that transcript abundance of the two cellulases was much lower in the *GAL10*[1068-2100]-expressing strains and again inversely correlated with the abundance of the *GAL10*[1068-2100] transcript in them and their rate of growth on lactose.

To rule out that this effect may be unspecific, we also tested cellulase gene transcription by the transformants during growth on cellulose (Fig. 6*B*). Under these conditions, cellulase transcript formation by the reference strain and the three transformants was essentially indistinguishable, providing additional evidence that the effect is caused by the impact of the *S. cerevisiae* Gal10 C terminus on D-galactose mutarotation.

Discussion

Galactose mutarotase activity has been observed in a wide range of organisms, including bacteria (3–5), plants (6, 7), fungi (8),
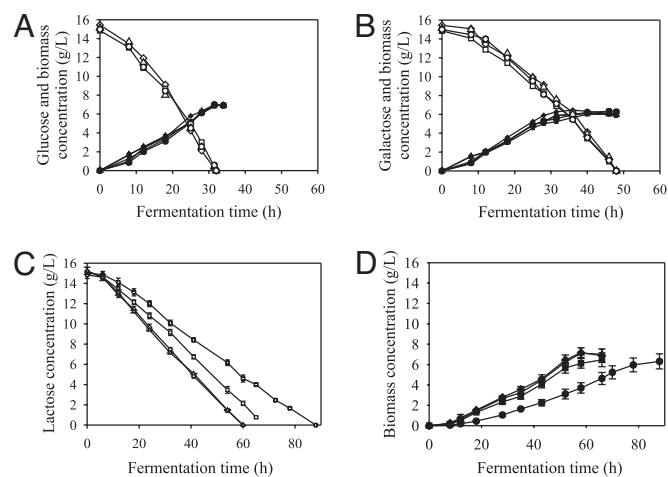

Fig. 3. Fermentation profiles of *H. jecorina* QM9414 and mutants M1, M2a, and M2b on different carbon sources. (*A*) Fermentation profile of *H. jecorina* QM9414 (circles) and the *GAL10*[1068-2100]-expressing strains M1 (squares), M2a (triangles), and M2b (diamonds) on glucose as a sole carbon source. Open symbols indicate glucose, and filled symbols indicate biomass time profiles. (*B*) Fermentation profile of *H. jecorina* QM9414 (circles) and strains M1 (squares) M2a (triangles), and M2b (diamonds) on galactose as a sole carbon source. Open symbols indicate galactose, and filled symbols indicate biomass time profiles. (*C*) Lactose uptake of *H. jecorina* QM9414 (○) and the *GAL10*[1068-2100]-expressing strains M1 (□) M2a (△), and M2b (◇). (*D*) Biomass formation of *H. jecorina* QM9414 (●) and the *GAL10*[1068-2100]-expressing strains M1 (■), M2a (▲), and M2b (◆) on lactose.
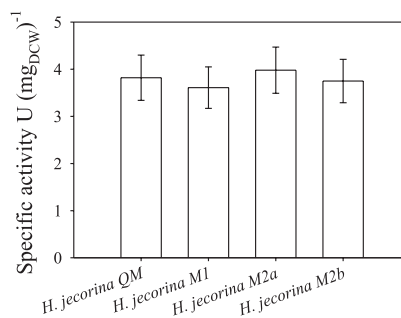

Fig. 4. Specific extracellular β-galactosidase activity of *H. jecorina* QM9414 and *GAL10*[1068-2100]-expressing strains M1, M2a, and M2b. Samples were taken 12 h after the transferring procedure.
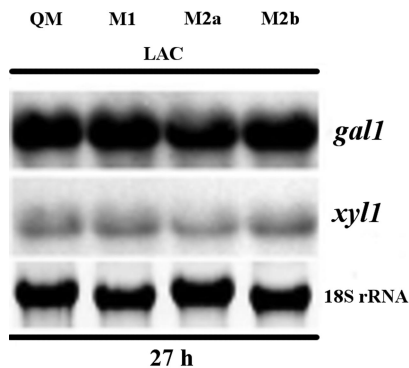
Fig. 5. Expression of the *H. jecorina* genes *gal1* (encoding a galactokinase) and *xyl1* (encoding an aldose reductase) on lactose in the QM9414 and *GAL10*[1068–2100]-expressing *H. jecorina* strains M1, M2a, and M2b.

and mammals (9–11). It is present in the cytoplasm of most cells, consistent with the fact that the *in vivo* rate of uncatalyzed mutarotation is insufficient for the metabolic needs of the organism as shown in *Escherichia coli* (3). In the present study, *H. jecorina* was shown to contain genes encoding three different putative mutarotases, of which two are in fact intracellular, whereas the third one (AEP3) is predicted to be a secreted protein. Interestingly, *aep3* also shows the highest number of ESTs of all three putative mutarotase genes and is most abundantly transcribed during the early phase of growth on lactose. Transcripts of the other two intracellular mutarotases were not detected on lactose. We therefore conclude that in *H. jecorina* enzyme catalyzed reaction should have a marginal role in the mutarotation of β-D-galactose. We should note in this context that the strain used throughout these studies is a mutant strain that was selected for enhanced cellulase formation from the WT strain QM6a after two mutagenesis steps. The lack of mutarotase activity may therefore not be valid for the WT strain, but *H. jecorina* QM6a was not investigated here because of its low level of cellulase formation.

In any case, the present data provide clear evidence that the overexpression of a mutarotase in *H. jecorina* QM9414 enhances its growth rate on lactose. These findings are consistent with similar reports in *E. coli* (3) and indicate that the intracellular water activity is not high enough to permit efficient mutarotation in the absence of mutarotase enzyme activity. However, although the maintenance of the anomeric equilibrium of galactose is likely to be a major biochemical role for galactose mutarotase, it may also function in the metabolism of glucose and other sugars. Indeed, mutarotases have been shown to turn over various sugars, including D-glucose, D-fucose, D-quinovose, L-arabinose, and D-xylose (23, 24). It is therefore interesting that our finding of an enhanced rate of assimilation was obtained with

lactose, but not with any of the monosaccharides tested (D-glucose, D-galactose, and others). Apart from our finding that the putative mutarotase-encoding *aep1* is slightly transcribed on D-galactose, we suppose that in some cases this happens because these sugars already undergo chemical mutarotation before they are taken up by the cells. This notion seems to hold true also for D-galactose, whose β-anomer cannot be catabolized via the Leloir pathway, and yet the mutarotase-overproducing strains did not show any changes in growth on and assimilation of D-galactose. Alternatively, the catabolism of these sugars may proceed at a similar rate with both the α-anomer and β-anomer. In fact, fungal pentose metabolism is initiated by aldose reductase that acts on both sugars with the same affinity and rate (18). Hexokinase, on the other hand, has different affinities for α- and β-D-glucose (25), but both are still <50 μM and thus will unlikely influence the rate of glucose catabolism in a significant way. In the case of lactose, in contrast, β-galactosidase is rate limiting in the assimilation of lactose by *H. jecorina* (21), and therefore a higher portion of the released β-anomer would be transported into the cells, which is facilitated by the location of part of the β-galactosidase within the cell wall of the fungus and thus physically close to its transport system.

Remaining is the question of why the overexpression of mutarotase activity impairs cellulase gene expression. The fact that this phenomenon is observed on lactose, but not on cellulose, indicates that it is specific and confined to the presence of lactose. We have previously shown that the overexpression of the extracellular β-galactosidase gene *bga1* also increased the assimilation of lactose by *H. jecorina* and at the same time impairs cellulase formation on lactose (21). In the strain overexpressing *bga1*, however, D-galactose accumulated in the medium, and it is conceivable that this accumulation allowed chemical mutarotation to occur at an increased rate and thus channel D-galactose preferentially through the Leloir pathway. The result is consistent with our findings that cellulase induction on D-galactose can occur only at a low growth rate, but at the same growth rate lactose still induces much higher cellulase expression (26). In contrast, overexpression of the mutarotase in *H. jecorina* had no effect on extracellular β-galactosidase formation and only a negligible effect on the transcription of *gal1* and *xyl1* and thus the activity of the two D-galactose-degrading pathways. We consider it therefore unlikely that the growth rate *per se* is the reason for the impaired cellulase formation. This notion is substantiated by the fact that between 30 and 50 h of growth the transformant with the highest mutarotase activity exhibits a specific growth rate of 0.04 h^{-1}, which is well within the range of growth rates that support cellulase gene expression on lactose (26). We rather speculate that mutarotase specifically interferes with formation of the cellulase inducer from lactose. The chemical nature of this component is still unknown, but it is possible that this yet-to-be-identified molecule must contain D-galactose in the β-anomeric form.
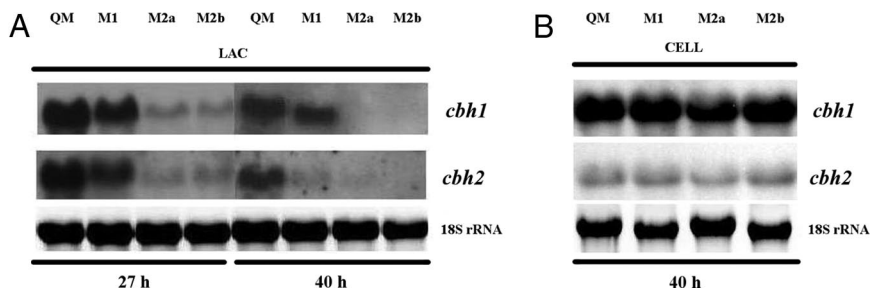

Fig. 6. Expression of *H. jecorina cbh1* and *cbh2* genes on different carbon sources. (*A*) Expression of the *H. jecorina* cellulase genes *cbh1* and *cbh2* on lactose at 27 h and at 40 h in the QM9414 and *GAL10*[1068–2100]-expressing *H. jecorina* strains M1, M2a, and M2b. (*B*) Expression of the *H. jecorina* cellulase genes *cbh1* and *cbh2* on cellulose at 40 h in the QM9414 and *GAL10*[1068–2100]-expressing *H. jecorina* strains M1, M2a, and M2b.

Table 2. Primers used for the RT amplification of the three putative *H. jecorina* mutarotase genes and *tef1* (translation elongation factor 1α)

Gene	Oligonucleotide	Oligonucleotide sequence	Amplicon size, bp
aep1	tre19341forw	5′-ATACAACCTCTCTCTCTCTCTG-3′	942
	tre19341rev	5′-ACGGTAGATTCACACACATAG-3′	
aep2	tre42544 forw	5′-TCACCTTCTCATCTTCACCAC-3′	740
	tre42544 rev	5′-TGGTGGTTGTGAAGCAGC-3′	
aep3	tre44592forw	5′-CTGGCTGTCTTTGCTCTG-3′	790
	tre44592rev	5′-TCCCACTCTGCTCAAACC-3′	
tef1	tef1forw	5′-TCATCGTCGCCATCAACAAG-3′	877
	tef1rev	5′-TCGACGGCCTTGATGACAC-3′	

Materials and Methods

Strains. *H. jecorina* QM9414 (ATCC 26921) and the uridine-auxotrophic *pyr4*-negative mutant of it, TU-6 (27), were maintained on malt extract agar supplemented with uridine (10 mM) when required. Strains were grown in 500-ml Erlenmeyer flasks containing 100 ml of medium on a rotary shaker New Brunswick Scientific Co., (250 rpm; gyrotory shaker model G-10) at 30°C in the medium as described (28) with the appropriate carbon source at a final concentration of 15 g/liter.

For transcript analysis (except on cellulose), strains were pregrown on glycerol (1%, wt/vol) for 24 h. Mycelia were then harvested by filtration and washed with autoclaved tap water. Equal amounts of mycelia were transferred to flasks containing the appropriate carbon source (1.5%, wt/vol), and cultivation was continued for the periods indicated. For transcript analysis on cellulose, direct cultivation was used.

For the amplification of yeast mutarotase gene, *S. cerevisiae* strain S288C (ATCC 204508) was used. Cells were grown in YPD medium (1 g/liter yeast extract, 1 g/liter peptone, and 20 g/liter glucose) for 14 h at 30°C.

E. coli strain JM109 (Promega) was used for plasmid propagation.

Nucleic Acid Isolation and Hybridization. Fungal mycelia were harvested by filtration, washed with distilled cold water, frozen, and ground under liquid nitrogen. For extraction of genomic DNA, plasmid DNA, and RNA, purification kits (Wizard Genomic DNA Purification Kit, PureYield Plasmid Midiprep System and SV Total RNA Isolation System, respectively; all from Promega) were used according to the manufacturer's protocol. Standard methods were used for electrophoresis, blotting, and hybridization of nucleic acids. A kit was used for performing Southern and Northern analysis (PCR DIG Probe Synthesis kit; Roche), following the manufacturer's protocol. For RT-PCR analysis, first-strand cDNA was synthesized by a RevertAid H minus First Strand cDNA Synthesis Kit (Fermentas) according to the manufacturer's protocol. cDNA was thereafter used as a template for PCR using gene-specific primers (Table 2). Translation-elongation factor-α (*tef1*) was used as a control.

Construction of a Mutarotase Expression Vector. The *S. cerevisiae GAL10* part (nucleotides 1068–2100 relative to the ATG) encoding the mutarotase was expressed under the expression signals of the *H. jecorina pki1* (pyruvate kinase) promoter and the *cbh2* (cellobiohydrolase 2) terminator region. Therefore the *GAL10* part was amplified from *S. cerevisiae* genomic DNA by PCR using the primers MRfw (5′-GTTATCTAGAATGGAGGCCAGATTTTCC-3′) and MRrev (5′-AGCTATGCATTCAGGAAAATCTGTAGAC-3′), introducing a START codon (ATG) plus an XbaI restriction site, and an NsiI restriction site, respectively. The PCR amplification protocol consisted of an initial denaturation step (2 min, 95°C) followed by 30 cycles with a denaturation step (1 min, 95°C), an annealing step (1 min, 50°C) and an elongation step (1.5 min, 72°C). Reaction was completed with a final 10-min elongation step at 72°C. The resulting 1,043 bp $GAL10^{1068-2100}$ fragment was ligated into the XbaI and NsiI sites of the vector pRLM$_{ex}$30 (29). As a genetic marker the *H. jecorina* 2.7-kb SalI *pyr4* fragment (27) was introduced in

the XhoI site of this vector, and the resulting plasmid was designated pGAL10$_{Kicsi}$. Sequence of the $GAL10^{1068-2100}$ fragment was confirmed by sequencing (MWG).

Construction of a Mutarotase-Expressing *H. jecorina* Strain. The expression cassette of $GAL10^{1068-2100}$ (Fig. 2*A*) was amplified by M13 primers (Promega), and the ≈5.5-kb PCR amplicon was subsequently used for transformation of *H. jecorina* TU-6 as described (27). Approximately 10 μg of DNA were used for transformation. Twenty-four primary colonies were obtained, of which eight grew repeatedly on uridine-free MM1 medium (30) that was used to select for transformants.

Southern analysis was used to verify the integration of the construct and determine the copy number. Chromosomal DNA was digested with SalI, which has a single restriction site in $GAL10^{1068-2100}$ only (Fig. 2*A*). pGAL10$_{Kicsi}$ linearized with XhoI was used as a control. Primers MRfw and MRrev (used also for the amplification of $GAL10^{1068-2100}$) were used for probe construction. Copy number (n) of $GAL10^{1068-2100}$ was hence determined by the number of $n + 1$ SalI fragments, respectively.

Analytical Methods. Mycelial dry weight was determined by withdrawing 2 × 5-ml aliquots from the culture, doing suction filtration through a preweighed glass wool filter, and drying in an oven at 80°C to constant weight. Data were averaged and deviated by not >14%.

The concentration of D-glucose, D-galactose, and lactose was determined by HPLC analysis, using an H⁺ exchange column (Bio-Rad Aminex HPX-H⁺), with 10 mM H_2SO_4 at 55°C as mobile phase with isocratic elution and a refractive index detection.

The specific activity of the mutarotase was assayed by using the NAD⁺ and β-D-glucose dehydrogenase coupled assay (13). Specific enzyme activities are related to mg protein, which was determined by a modified Lowry method (31), using BSA for calibration.

The specific activity of the extracellular β-galactosidase was determined with *o*-nitrophenyl-β-D-galactopyranoside as substrate (21). Specific enzyme activities are related to mg dry cell mass.

Reproducibility. Analytical data are means of three to five independent experiments. The data were analyzed by Sigmaplot (SPSS), and standard deviations were determined for each procedure. The SD values were always <14% of the means.

Chemicals. All chemicals were of analytical grade, and, except where noted otherwise, were purchased from Sigma-Aldrich.

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APPLIED BIOLOGICAL SCIENCES

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