Systems Analysis of Lactose Metabolism in *Trichoderma reesei* Identifies a Lactose Permease That Is Essential for Cellulase Induction

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Abstract

*Trichoderma reesei* colonizes predecayed wood in nature and metabolizes cellulose and hemicellulose from the plant biomass. The respective enzymes are industrially produced for application in the biofuel and biorefinery industry. However, these enzymes are also induced in the presence of lactose (1,4-β-D-galactopyranosyl-α-glucose), a waste product from cheese manufacture or whey processing industries. In fact, lactose is the only soluble carbon source that induces these enzymes in *T. reesei* on an industrial level but the reason for this unique phenomenon is not understood. To answer this question, we used systems analysis of the *T. reesei* transcriptome during utilization of lactose. We found that the respective CAZome encoded all glycosyl hydrolases necessary for cellulose degradation and particularly for the attack of monocotyledon xyloglucan, from which β-galactosides could be released that may act as the inducers of *T. reesei*’s cellulases and hemicellulases. In addition, lactose also induces a high number of putative transporters of the major facilitator superfamily. Deletion of fourteen of them identified one gene that is essential for lactose utilization and lactose uptake, and for cellulase induction by lactose (but not sophorose) in pregrown mycelia of *T. reesei*. These data shed new light on the mechanism by which *T. reesei* metabolizes lactose and offers strategies for its improvement. They also illuminate the key role of β-D-galactosides in habitat specificity of this fungus.

Introduction

The β-(1,4)-linked glucose polymer cellulose and hemicellulose polysaccharides of varying composition make up 60–80% of the plant cell wall and arise from the utilization of solar energy and carbon dioxide by plants at an annual production rate of about 7.2 and 6×10¹⁰ tons, respectively [1]. These polymers therefore represent the global reservoir of renewable carbon for the production of biofuels and platform chemicals used in biorefineries.

The fungus *Trichoderma reesei* (the anamorph of the tropical ascomycete *Hypocrea jecorina*) serves as the major producer organism for the enzymes needed to degrade the above mentioned polymers to soluble monosaccharides [2]. Since most of these enzymes are formed only adaptively, the cultivations must be performed in the presence of an inducer which is, in most cases, a cellulose and hemicellulose containing waste material. Interestingly, lactose (1,4-β-D-galactopyranosyl-α-glucose), which is produced to around 1.2 million tons per annum worldwide as a by-product from cheese manufacture or from whey processing industries, also induces cellulase formation in *T. reesei* (but not in other fungi) and is the only economic soluble carbon source for this purpose [3]. On the other side, lactose metabolism is slow and leads to lower cellulase yields than on cellulose, thus warranting a deeper understanding of lactose metabolism towards its targeted improvement [4,5].

In this study, we exploit functional genomics resources to perform a systems analysis of the *T. reesei* transcriptome during utilization of lactose and formation of cellulases and hemicellulases. We have produced strains containing deletions in genes encoding proteins identified from the transcriptome datasets and evaluated them for their ability to use lactose and produce cellulases. From this analysis, we identified a protein whose deletion completely impairs lactose utilization and cellulase gene expression in *T. reesei*. These data shed new light on the mechanism by which lactose induces cellulase formation in *T. reesei*, and illuminate the key role of β-D-galactosides in habitat specificity of this fungus.

Results

The Lactose-regulated Transcriptome of *T. reesei*

In order to identify genes that are induced or repressed by lactose, we determined the transcriptional profiles of *T. reesei* QM 9414 during the initial growth phase (when 25–30% of the carbon source have been consumed) on lactose, glucose and glycerol, and monitored those that were >2-fold different at a p<0.05 between lactose and either of the two other sugars. In order to remove those
functions (which were mostly downregulated). More up- than downregulated members), and genes for ribosomal coupled receptors and transcription factors (which all contained criterion were genes encoding GH-auxiliary proteins, G-protein polysaccharides) were enriched in lactose-grown cultures, and genes acids), and “CAZymes” ( = enzymes acting on extracellular the major facilitator superfamily; “MFS permeases” and of amino the mean variation (p 0.05). Genes involved in solute transport (of the major facilitator superfamily; “MFS permeases” and of amino acids), and “CAZymes” ( = enzymes acting on extracellular polysaccharides) were enriched in lactose-grown cultures, and genes required for proteasome and mitochondrial function were strongly downregulated (Table 2). In addition, genes that only passed the first criterion were genes encoding GH-auxiliary proteins, G-protein coupled receptors and transcription factors (which all contained more up- than downregulated members), and genes for ribosomal functions (which were mostly downregulated).

The Lactose-induced CAZome

As noted above, and consistent with the hypothesis of this work, genes encoding glycosyl hydrolases and auxiliary enzymes or proteins for them comprised a major group of genes (sixty-three) in the lactose-upregulated transcriptome (Table S1). Among them, we identified both cellobiohydrolases (CEL7A and CEL6A), all endoglucanases (CEL5A, CEL7B, CEL12A, CEL45A), and six β-glucosidases (Table 3). Also four xylanases (XYN2, XYN3, XYN4 and XYN6), two β-xylidoses, six α-galactosidases, two α-fucosidases, two α-arabinofuranosidases, two methyl-α-glucuronidase, as well as one xyl glucanase, one endo-β-1,3/1,4-glucanases, and one pectinase were present. Finally, three acetyl-xylan esterases, two GH61 cellulose monooxygenases [6], one swollenin [a protein containing an expansin and a cellulose binding domain [7] and CIP1 [a protein containing a cellulose-binding domain [8]]; were found, thus completing the above enzymes to a full cellulolytic and hemicellulolytic enzyme spectrum.

Metabolic Characteristics of the Lactose Transcriptome

When the CAZyme encoding genes were not considered, 156 and 120 genes of the up- and downregulated transcriptome, respectively, comprised genes encoding enzymes known to be involved in metabolism. Of these genes, 106 and 92 could be attributed to specific metabolic pathways. Genes encoding enzymes for oxidative reactions and amino acid metabolism accounted for the highest number, but no significant differences were noted between the up- and downregulated genes.

Growth on lactose proceeds slower than on glucose and glycerol, and we therefore tested whether some of the genes specifically expressed on lactose could be subject to regulation by the relationship between growth rate and repression by the carbon catabolite repressor CRE1 as described by Portnoy et al. [9]. We have therefore investigated whether genes found by these authors would overlap with those upregulated on lactose. Indeed, we found 93 genes to be shared between these two conditions (Table S2). However, they were distributed between all nine groups found by Portnoy et al. [9], and also at about equal numbers. Consequently, none of these groups is particularly enriched and we therefore conclude that lactose does not create conditions for carbon catabolite repression or growth rate-dependent derepression.

Most of the transcription factors that were upregulated on lactose comprised members of the fungal-specific Zn(2)Cys(6) cluster family (58 genes), almost all of which have not yet been characterized in fungi. However, the genes encoding the general cellulase regulator XYN1 (Trire2:122208) and the recently described N. crassa cellulase-regulator CLR-2 (Trire2:60289; [10]) were found to be significantly upregulated on lactose (22- and 7-fold vs. glucose and 10 and 5-fold vs. glycerol, respectively).

Interestingly, eight genes that were significantly upregulated during growth on lactose comprised genes involved in sexual differentiation, including the mating type gene mat1-2-1, the α-type pheromone precursor hhp1, two pheromone receptors and genes involved in synthesis and processing of the pheromone precursor (Table 4).

The Lactose-induced MFS Permeases Comprise Members Involved in Transport of Hemicelluloses Monomers

As noted above, MFS permeases comprised one of the largest gene groups upregulated on lactose (Table 2; Table S1). The

### Table 1. Balance of significantly* regulated transcripts during growth of T. reesei on lactose.

| Upregulated | Downregulated |
|-------------|---------------|
| all genes   |               |
| secreted    |               |
| Identified  | Number | %* | Number | %** |
| Identified  | 523     | 58.1 | 88 | 16.8 |
| Unknown     | 298     | 33.1 | 28 | 9.3 |
| Unique      | 78      | 8.8  | 11 | 14.1 |
| Total       | 899     | 127  | 41 | 9.2 |
| Downregulated | Number | %* | Number | %** |
| Identified  | 367     | 57.2 | 25 | 6.8 |
| Unknown     | 247     | 38.5 | 25 | 10.1 |
| Unique      | 27      | 4.3  | 9  | 33.3 |
| Total       | 641     | 59   | 9  | 9.2 |

*either≥or <2-fold vs. glucose and glycerol.

**% values refer to the total number of genes.

*% values refer to the category of genes (e.g. identified, unknown or unique, resp.).

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function of most of them was unknown. However, BLAST analysis of their amino acid sequences resulted in putative mono-or disaccharide transporters of other fungi as next neighbours. One of them (Trire2:104072) has recently been described to enable *S. cerevisiae* to take up D-xylose [11]. To learn more about their function, we prepared deletion strains of the 14 most upregulated MFS permeases, and screened them for their ability to grow on glucose, lactose, D-galactose, and Trire2:104072 also on L-arabinose and D-xylose (Figure 1). The most severe effect was observed with Trire2:3405, whose deletion completely blocked the ability of the strain to grow on lactose containing medium, but not on D-glucose or D-galactose (Figure 2a). A phylogenetic analysis shows that Trire2:3405 is a member of a sister clade of a larger cluster containing the *K. lactis* and *A. nidulans* lactose permeases (Figure 3), and other so far uncharacterized orthologues from other Pezizomycota. All the other deletion mutants did not display any clear phenotype, and their identity therefore remains obscure. Interestingly, none of the mutants exhibited decreased growth on cellobiose, not even in the presence of nojirimycin (to inhibit the extracellular β-glucosidases which could compensate the defect of a putative cellodextrin transporter; Figure S1). The expression of the MFS permeases was verified by qPCR analysis, which confirmed upregulation on lactose after 24 h of growth (Table S3).

### Table 2. Major changes in the transcriptome during cultivation of *T. reesei* on lactose.

| FunCat       | function                          | metabolic function | regulation | T      | G      | genome % | fold (up/down) |
|--------------|-----------------------------------|--------------------|------------|--------|--------|----------|----------------|
|              |                                   |                    | up         | down   | 1540   | 9143     | 16.8           | 1.49           |
| 98           | Oxidation                         |                    | up         | down   | 15     | 27       | 13.3           | 1.3            |
| 98           | Short chain dehydrogenases/reductases |                    | up         | down   | 15     | 12       |                |                |
| 01_05        | Carbohydrate metabolism           |                    | up         | down   | 14     | 12       |                |                |
| 98           | Metal ion metabolism              |                    | up         | down   | 7      | 26       |                |                |
| 01_01        | Amino acid metabolism             |                    | up         | down   | 7      | 6        |                |                |
| 01_06        | Lipid metabolism                  |                    | up         | down   | 12     | 6        |                |                |
| 01_20        | Secondary metabolism              |                    | up         | down   | 2      | 5        |                |                |
| ND           | Others                            |                    | up         | down   | 50     | 28       |                |                |
| 01,05,01     | Glycosyl hydrolases               |                    | up         | down   | 59     | 10       | 69             | 201            | 34.3 | 5.9  |
| 01,25        | GH auxiliary proteins             |                    | up         | down   | 8      | 1        | 9              | 35             | 25.7 | 8    |
| 01,25,03     | Proteases                         |                    | up         | down   | 17     | 7        | 24             | 82             | 29.2 | 2.42 |
| 4,16         | Mitochondrial function            |                    | up         | down   | 3      | 34       | 37             | 74             | 50   | 0.088|
| 11,02,03,04  | Transcription factors             |                    | up         | down   | 42     | 10       | 52             | 351            | 14.8 | 4.2  |
| 12,4         | Translation                       |                    | up         | down   | 1      | 12       | 13             | 88             | 14.7 | 0.083|
| 14,13        | Proteosomal function              |                    | up         | down   | 0      | 12       | 12             | 27             | 44.4 | N*   |
| 30,05,02,24  | GPCR                              |                    | up         | down   | 5      | 1        | 6              | 21             | 28.5 | 5    |
| 20,03        | Major facilitator superfamily     |                    | up         | down   | 111    | 36       | 147            | 260            | 56.5 | 3.1  |
| 99           | Orphan protein                    |                    | up         | down   | 78     | 32       | 110            | 506            | 21.7 | 2.4  |
| 99           | Unknown proteins                  |                    | up         | down   | 298    | 245      | 543            | 3530           | 15.4 | 1.21 |
| **Total**    |                                   |                    | up         | down   | 622    | 400      |                |                |

Data on grey background were statistically significant (p < 0.05). *N*, not calculated because of zero in one condition. doi:10.1371/journal.pone.0062631.t002

The Putative Lactose Permease is Essential for Cellulase Induction by Lactose

Having identified a putative lactose transporter of *T. reesei*, we were also interested whether this transporter would be relevant for cellulase production on lactose. To this end, we cultivated the deletion strain in Trire2:3405 on glycerol and then transferred it to lactose and recorded the formation of cellulases and the accumulation of cellulase transcripts (Figure 2 b–e). The D3405 strain did not grow after transfer to lactose, and also did not take it up; cellulase activities were reduced to zero and the levels of the *cel6A* and *cel7A* transcripts eliminated, as determined with qPCR. Placing Trire2:3405 under the strong and constitutive expression signals of the *tef1* promoter [12] resulted in an earlier appearance of the *cel6A* and *cel7A* transcripts (after 16 h of growth on 1% lactose) compared to the parental strain, and this finding was also reflected in the appearance of these two enzymes at 16 h in the culture filtrate but not in the parental strain, as shown by SDS-PAGE analysis (Figure 2e).

We also tested whether induction by sophorose, a strong inducer of cellulase formation in *T. reesei* (8), would also be affected by the knock out in the lactose transporter. However, pregrowth on glycerol and subsequent transfer to 1 mM sophorose revealed the same abundance of *cel7A* and *cel6A* transcripts in QM 9414 and Δ3405 after 4 and 6 hrs of incubation (data not shown). We therefore conclude that the lactose permease Trire2:3405 is specifically involved in cellulase induction by lactose.
### Table 3. The lactose-induced CAZome of *T. reesei*.

| Protein ID | Lact vs Glc | Lact vs Gly | p-value | annotation |
|------------|-------------|-------------|---------|------------|
| 22197      | 4.052       | 9.923       | 0.00338 | GH1 β-glucosidase |
| 120749     | 21.174      | 116.676     | 0.00635 | GH1 β-glucosidase |
| 120229     | 8.373       | 8.239       | 0.00323 | GH10 xylanase   |
| 123818     | 28.587      | 25.835      | 0.00273 | GH11 xylanase XYN2 |
| 123232     | 72.563      | 73.564      | 0.000887| GH12 endo-β-1,4-glucanase |
| 108477     | 5.864       | 3.143       | 0.0104  | GH13 α-glucosidase |
| 55886      | 4.553       | 3.907       | 0.0369  | GH16 endo-β-1,3/1,4-glucanase |
| 65162      | 7.393       | 2.217       | 0.00458 | GH18 chitinase |
| 77299      | 6.826       | 4.682       | 0.0121  | GH2 exo-β-D-glucosaminidase |
| 57857      | 5.956       | 4.869       | 0.0039  | GH2 β-mannosidase |
| 69245      | 101.158     | 38.535      | 0.00396 | GH2 β-mannosidase |
| 103458     | 2.346       | 2.738       | 0.00439 | GH25 lysozyme |
| 72632      | 76.354      | 48.322      | 0.00442 | GH27 α-galactosidase |
| 72704      | 7.091       | 2.103       | 0.00501 | GH27 α-galactosidase |
| 65986      | 35.526      | 21.134      | 0.00119 | GH27 α-galactosidase |
| 27259      | 3.285       | 3.374       | 0.0194  | GH27 α-galactosidase |
| 59391      | 23.278      | 25.785      | 0.00291 | GH27 α-galactosidase |
| 55999      | 5.679       | 4.295       | 0.0069  | GH27 α-galactosidase |
| 82227      | 8.106       | 8.403       | 0.0014  | GH3 β-glucosidase |
| 46816      | 5.255       | 6.969       | 0.0106  | GH3 β-glucosidase |
| 108671     | 2.69        | 2.545       | 0.0221  | GH3 β-glucosidase |
| 76672      | 2.108       | 2.121       | 0.0421  | GH3 β-glucosidase |
| 121127     | 107.07      | 91.618      | 0.0011  | GH3 β-xylosidase |
| 111849     | 16.879      | 12.353      | 0.0035  | GH30 glucuronoyl-xylanase |
| 69276      | 4.691       | 3.5         | 0.00897 | GH30 glucuroyl xylanase XYN6 |
| 60085      | 3.819       | 11.31       | 0.00989 | GH31 α-glucosidase |
| 69944      | 3.73        | 4.186       | 0.0167  | GH31 α-xylanase |
| 64827      | 2.979       | 2.612       | 0.00495 | GH36 α-galactosidase |
| 124016     | 12.7        | 5.756       | 0.00915 | GH36 α-galactosidase |
| 123226     | 35.271      | 14.146      | 0.00183 | GH37 trehalase |
| 3739       | 233.319     | 63.254      | 0.00112 | GH43 β-xylosidase/α-L-arabinofuranosidase |
| 49976      | 12.862      | 24.602      | 0.00443 | GH45 endoglucanase V; C-terminal CBM1 module |
| 45717      | 11.069      | 8.48        | 0.00302 | GH47 α-1,2-mannosidase |
| 120312     | 163.479     | 152.15      | 0.00132 | GH5 endoglucanase Cel5A |
| 81087      | 62.912      | 76.828      | 0.00108 | GH5 glycoside hydrolase |
| 123283     | 4.169       | 5.102       | 0.00817 | GH54 α-L-arabinofuranosidase |
| 55319      | 3.309       | 5.048       | 0.00178 | GH54 α-L-arabinofuranosidase; C-terminal CBM42 module |
| 72567      | 273.081     | 274.233     | 0.00225 | GH6 cellobiohydrodase II (Cel6A) |
| 120961     | 62.728      | 62.882      | 0.00128 | GH61 polysaccharide monooxygenase |
| 73643      | 21.043      | 25.74       | 0.000785| GH61 polysaccharide monooxygenase |
| 76210      | 10.795      | 11.667      | 0.00228 | GH62 α-L-arabinofuranosidase |
| 65137      | 3.654       | 6.227       | 0.0134  | GH64 β-1,3-glucanase |
| 25224      | 5.701       | 8.362       | 0.00653 | GH65 α,α-trehalase |
| 123456     | 11.275      | 2.234       | 0.00412 | GH65 α,α-trehalase |
| 72526      | 275.106     | 218.78      | 0.00163 | GH67 α-glucuronidase |
| 123989     | 246.472     | 91.566      | 0.00151 | GH7 cellobiohydrodase I |
| 122081     | 3.3         | 3.913       | 0.0095  | GH7 endoglucanase Cel7B |
| 71532      | 2.649       | 7.183       | 0.00359 | GH71 α-1,3-glucanase |
| 49081      | 5.419       | 3.008       | 0.0137  | GH74 xylglucanase; C-terminal CBM1 module |
Consistent with recent findings that induction of the cellulase regulator XYR1 does not require metabolism of D-galactose (14), expression of \textit{xyr1} remained unaffected in the \textit{D3405} strain.

Similarly, formation of the extracellular \(\beta\)-galactosidase BGA1 was unaffected in strain 3405 (Figure 2f).

**Discussion**

In their natural habitat, fungi are unlikely to encounter the disaccharide lactose frequently as a carbon source. However, \textit{T. reesei} is able to grow on it and simultaneously secretes cellulases. The present data offer some explanations for this enigma: besides a complete cellulase system, the upregulated CAZome also includes the cellulase accessory proteins CIP1, swollenin and two of the newly detected GH61 cellulose monooxygenases. Further, it comprises about half of the hemicellulases (xylanases, \(\alpha\)- and \(\beta\)-D-galactosidases, \(\alpha\)-L-arabinofuranosidases and cellulose/hemicellulose deacetylases), three of four \(\beta\)-L-fucosidases and all enzymes acting on D-glucuronosyl-side chains (GH30 glucuronoyl xylanases, and GH67/GH79 \(\alpha\)-D-glucuronidases). An indirect stimulation of expression of these genes (e.g. by a lower growth rate and/or relieve from carbon catabolite repression) appears unlikely when the respective transcriptomes displayed under these conditions are compared \(\text{(vide supra)}\). A plausible explanation therefore is that lactose mimics substance(s) present in the natural habitat of \textit{T. reesei} (i.e. predegraded and decaying wood) that signal the availability of plant biomass to the fungus. This physiological signal is likely another \(\beta\)-D-galactoside, as some of them have been shown to be able to induce cellulases [13]. Such \(\beta\)-D-galactooligosaccharides would typically occur in xyloglucans of the XXGG type [14], in which xylose residues can be substituted with \(\alpha\)-1,2-L-fucopyranose-\(\beta\)-1,2-D-galactopyranose and \(\alpha\)-1,2-L-galactopyranose-\(\beta\)-1,2-D-galactopyranose disaccharides and O-linked acetyl groups, side chains for which the respective hydrolytic enzymes are all induced in \textit{T. reesei} by lactose. Further, the xyloglucans of dicotyledons are partially replaced by glucuronoarabinoxylan, which has a linear \(\beta\)-1,4-linked D-xylopyranosyl backbone with glucuronosyl or 4-O-methyl glucuronosyl side chains, which are again structures for which \textit{T. reesei} enzymes are induced. Xyloglucans are the hemicelluloses in the primary cell wall of dicotyledons and are strongly associated with cellulose by crosslinking cellulose microfibrils [15]. Our data leads to speculate that the xyloglucans are the initial target for \textit{T. reesei} when feeding on plant biomass.

The Zn(2)Cys(6) transcriptional regulator XYR1 has been demonstrated to be responsible for the induction of cellulases, xylanases and also some of the \(\alpha\)-L-arabinofuranosidases in \textit{T. reesei}.

**Table 3. Cont.**

| Protein ID | Lact vs Glc | Lact vs Gly | p-value | annotation          |
|------------|-------------|-------------|---------|---------------------|
| 42152      | 2.83        | 6.149       | 0.0115  | GH75 chitosanase     |
| 27395      | 3.292       | 2.494       | 0.0144  | GH76 \(\alpha\)-1,6-mannanase |
| 55802      | 2.158       | 3.146       | 0.0206  | GH76 \(\alpha\)-1,6-mannanase |
| 122495     | 19.358      | 20.326      | 0.00113 | GH76 \(\alpha\)-1,6-mannanase |
| 106575     | 5.015       | 3.634       | 0.0091  | GH79 methyl-\(\beta\)-glucuronidase |
| 79921      | 7.183       | 5.67        | 0.00323 | GH92 \(\alpha\)-1,2-mannosidase |
| 74198      | 40.21       | 20.923      | 0.00652 | GH92 \(\alpha\)-1,2-mannosidase |
| 5807       | 33.976      | 37.228      | 0.00346 | GH95 \(\alpha\)-L-fucosidase |
| 58802      | 15.405      | 21.841      | 0.00663 | GH95 \(\alpha\)-L-fucosidase |
| 123992     | 15.986      | 4.71        | 0.00527 | swollenin           |
| 65215      | 12.104      | 2.749       | 0.00615 | CE4 polysacharide deacetylase |
| 54219      | 5.676       | 5.861       | 0.00149 | CES acetyl xylan esterase |
| 73632      | 5.339       | 7.325       | 0.00556 | CES acetyl xylan esterase AXE1 |
| 73638      | 29.011      | 30.747      | 0.00204 | CIP1                |

**Table 4.** Genes involved in sexual development that are upregulated in \textit{T. reesei} during growth on lactose.

| Protein ID | lact vs gluc* | lact vs glyc | p-value | annotation |
|------------|---------------|--------------|---------|------------|
| 62693      | 11.378        | 7.257        | 0.001   | ABC-transporter Ste6p |
| 34493      | 7.451         | 5.858        | 0.002   | \(\alpha\)-type peptide pheromone precursor \text{hpp1} |
| 124222     | 2.898         | 4.37         | 0.010   | CaX-protease, related to \textit{E. nidulans rce1}, involved in signal transduction |
| 57526      | 15.982        | 16.307       | 0.003   | GPCR, mating type pheromone G-protein coupled receptor |
| 64018      | 12.801        | 6.654        | 0.003   | GPCR, mating type pheromone G-protein coupled receptor |
| 31134      | 7.111         | 5.816        | 0.002   | isoprenylcysteine carboxyl methyltransferase |
| 124341     | 18.462        | 4.564        | 0.003   | mating protein MAT1-2-1 |
| 22093      | 3.354         | 4.094        | 0.004   | Protein farnesyltransferase, alpha subunit |

*fold change.

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proteasome are also altered. At this time, the physiological metal ion uptake), mitochondrial functions and components of the areas of metabolism (such as amino acid and lipid metabolism or metabolism of lactose, our data show that other shift in illustrating that growth on lactose involves a major physiological studies on carbon catabolite repression or conidiation [9,22], during growth on lactose is higher than that recorded in previous superiority of its secretome in cellulose hydrolysis.

Superiority of its secretome in cellulose hydrolysis. would be an important step in further understanding the operation machinery of CAZymes by Fenton-type like reactions. Although the operation of these enzymes is considered to be involved in lignin degradation by white rot fungi [19], and aid in the attack of brown rot fungi on cellulose by Fenton chemistry [20]. We consider it possible that lactose, as a trigger for formation of the plant biomass decomposing machinery of T. reesei, also induces enzymes that can aid the action of the CAZymes by Fenton-type like reactions. Although the operation of Fenton-type chemistry in cellulose degradation has so far only been shown in brown-rot fungi, recent data in N. crassa also pointed in this direction [21]. The demonstration of Fenton-type chemistry in T. reesei would be an important step in further understanding the superiority of its secretome in cellulose hydrolysis.

The number of genes -1540 - that are significantly regulated [12,16], but it is not known whether this extends to all of the CAZyme genes upregulated on lactose. In this context, it is interesting that the orthologue of the recently described N. crassa cellulase regulator CLR-2 [10] was found to be upregulated by lactose. Since in vivo and in vitro studies have shown that only the binding sites for Xyr1 and the HAP2/3/5 complex are in the cell64 promoter are occupied and are thus necessary for transcription of the cellulases in T. reesei [17], and a xyr1 knock out is unable to induce cellulase gene transcription [16], it is unclear how CLR-2 could contribute to cellulase induction. It will be interesting to investigate whether it is responsible for the expression of some of the hemicellulase genes for which the regulator is as yet unknown.

Lactose also induces the genes for a number of oxidative enzymes such as a GMC oxidoreductase, multicopper oxidase, tyrosinase, and FAD-dependent oxidases and monoxygenases. The majority of these proteins were predicted to possess a signal peptide (by SignalP; p<0.05; [18]) and are thus putative secreted proteins. Their induction by lactose is enigmatic, because no oxidation products of lactose or D-galactose are present in the medium. On the other hand, these enzymes are considered to be involved in lignin degradation by white rot fungi [19], and aid in the attack of brown rot fungi on cellulose by Fenton chemistry [20]. We consider it possible that lactose, as a trigger for formation of the plant biomass decomposing machinery of T. reesei, also induces enzymes that can aid the action of the CAZymes by Fenton-type like reactions. Although the operation of Fenton-type chemistry in cellulose degradation has so far only been shown in brown-rot fungi, recent data in N. crassa also pointed in this direction [21]. The demonstration of Fenton-type chemistry in T. reesei would be an important step in further understanding the superiority of its secretome in cellulose hydrolysis.

The number of genes -1540 - that are significantly regulated during growth on lactose is higher than that recorded in previous studies on carbon catabolite repression or conidiation [9,22], illustrating that growth on lactose involves a major physiological shift in T. reesei. While one would expect changes in genes associated with metabolism of lactose, our data show that other areas of metabolism (such as amino acid and lipid metabolism or metal ion uptake), mitochondrial functions and components of the proteasome are also altered. At this time, the physiological consequences of these changes are unknown; however they do not seem to be related to the different growth rate on lactose in comparison to glucose and glycerol, as only a few genes were shared between the present study and that by Portnoy et al. who used chemostat cultures at different growth rates [9]. Interestingly, secondary metabolism - which was reported to correlate with cellulase and hemicellulase gene upregulation [23] - was almost not affected by lactose: only two nonribosomal peptide synthases (Trire2:60751 and Trire2:67189) and none of the polyketide synthases at all were upregulated.

The lactose upregulated transcriptome also included a vast number of putative transporters of the major facilitator superfamily. The fact that gene deletion could only identify the function of one of them is likely due to a redundancy of their function. However, one of them turned out to be essential for lactose utilization. Interestingly, this transporter is not a member of the major fungal lactose permease clade identified recently ([24]; cf. Figure 3). The fact that its knock-out impairs triggering of cellulase gene expression by lactose also in resting cells, which do not require lactose for growth, illustrates that either the uptake or the intracellular presence and/or metabolism of lactose is essential for cellulase induction. These findings reject our previous assumption that lactose metabolism in T. reesei proceeds only via extracellular hydrolysis and subsequent uptake and metabolism of the monomers D-glucose and D-galactose [25,26]. This theory was based on the findings that the genome inventory of T. reesei appears not to contain a gene encoding an intracellular β-galactosidase. Intracellular β-galactosidases belong to GH family 2. While the genome of T. reesei contains seven members of this group [6], and four have also been found in this study to be induced on lactose, a Blastp search identified all of them as β-mannosidases (five), exo-β-D-glucosaminidase (one) and β-glucuronidase (one) and none as a β-galactosidase. While we cannot rule out that one of them (e.g. the β-glucuronidase) has also a β-galactosidase activity, it is also possible that T. reesei has an intracellular β-galactosidase that does not belong to family 2. Identification of the respective enzyme would provide essential information for genetic manipulation of lactose metabolism and thus cellulase formation, and for the understanding of the utilization of this intriguing disaccharide in T. reesei.

Figure 1. Growth of several MFS-knock out strains on D-glucose, lactose, D-galactose, and where appropriate, D-xylose, and L-arabinose. Pictures were taken 96 h after of incubation on minimal medium supplemented with the appropriate carbon source (1% w/v).

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Materials and Methods

Strains, Cultivations and Measurement of Growth, Lactose Consumption and Cellulase Formation

*T. reesei* QM9414 (ATCC 26921), an early cellulase producing mutant was used throughout this work and kept on potato dextrose agar (Sigma, St. Louis, MO) at 28°C. *T. reesei*Δtku70 strain [27], an uridine auxotrophic strain lacking the *tku70* gene required for non-homologous end joining DNA-repair, was maintained on malt extract medium supplemented with uridine (10 mM).

Growth on different carbon sources was assessed on minimal medium containing (1 g/L MgSO₄, 10 g/L KH₂PO₄, 6 g/L (NH₄)₂SO₄, 3 g/L Na₃citrate·2H₂O, 1% glucose, 20 ml/L trace elements (5 mg/L FeSO₄·7H₂O, 1.6 mg/L MnSO₄·H₂O, 1.4 mg/L ZnSO₄·H₂O and 2 mg/L CoCl₂·2H₂O) and 15 g/L agar) 1% (w/v) of the respective carbon source [28].

**Figure 2.** (a) Growth comparison of QM 9414 to the Δ3405 strain on minimal media supplemented with D-glucose, lactose and D-galactose. Pictures were taken on day 4. (b-g) Comparison of strains QM 9414 (grey), Δ3405 (white) and ptef::3405 (black) pregrown in minimal medium supplemented with 1% glycerol and, after washing of the mycelium extensively with sterile water, transferred to minimal medium containing 1% lactose. (b) Accumulation of *cel6a*, *cel7a* and *xyr1* transcripts measured by qPCR. Samples were taken 5, 8, 16 and 24 h after replacement to 1% lactose. The expression is given in relation to *tef1* gene expression, where the *tef1* expression value equals one. Mean values ± SD of three independent experiments are shown. (c) Lactose concentration in the medium during growth of QM 9414 (filled diamonds), Δ3405 (empty circles) and ptef::3405 (empty squares) on 1% lactose. (d) Biomass formation of strains QM 9414 (filled diamonds), Δ3405 (empty circles) and ptef::3405 (empty squares) on 1% lactose. Mean values ± SD of three independent experiments are given. (e) SDS-PAGE analysis of culture filtrates from QM 9414, Δ3405 and ptef::3405. Samples taken at 16 and 24 h after replacement to lactose. (f) β-galactosidase activity determined with o-nitrophenyl-β-D-galactopyranoside as the substrate. Error bars indicate the standard deviation of three independent experiments.

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cultivation in submerged cultures (shake flasks), strains were pregrown in 1% (w/v) glycerol for 24 h, mycelia were harvested by filtration and washed with sterile water, and equal amounts of mycelia then transferred to flasks containing the appropriate carbon source (1% w/v), and cultivated for up to 96 h. Growth on solid medium was tested by inoculating agar plates with a small agar piece (5 mm diameter). The biomass in submerged culture was determined by filtering portions of culture onto Whatman no. 1 preweighed filter papers. The harvested biomass was dried at 80°C for 3 days, and then weighted. Cellulase and β-galactosidase activities were assayed as described previously [29] with ρ-nitrophenyl-β-D-galactopyranoside and ρ-nitrophenyl-β-D-galactopyranoside as the respective substrates. Lactose concentration in the medium was determined with the 3,5-dinitrosalicylic acid [30].

SDS-PAGE was performed as described by Ausubel et al. [31] using 10% polyacrylamide gels. To this end, the proteins from the culture supernatant were precipitated by the addition of 2 vol EtOH and then purified. cDNA synthesis, labelling and hybridization was performed by Roche NimbleGen (Roche-NimbleGen, Inc., Madison, WI, USA) with a high density oligonucleotide specific for the upstream region outside of the deletion cassette (primer abbreviated with “ch” in Table S4) and the second specific for the ppy4 marker gene (ch_ppy4 neu).

Vector Construction and Generation of Gene Deletion Mutants

Deletion cassettes consisting of 1.4 to 1.5 kb fragments of the gene-specific flanking regions interrupted by the T. reesei ppy4 marker gene (encoding orotidine-5′-monophosphate decarboxylase) were assembled by yeast recombinational cloning [32]. Oligonucleotides (10 μM) 5′F +5R and 3′F +3R were used for amplification of the individual flanking regions from genomic T. reesei DNA using Taq Polymerase (Promega). By PCR approximately 19 bp were introduced at each flanking end that overlap with the pRS426 (URA +) yeast shuttle vector or the ppy4 gene to allow homologous recombination. A 3.2 kb fragment of the T. reesei ppy4 marker gene was amplified with oligonucleotides Pyr4 5′F and Pyr4 rev. A PCR touchdown program ranging from 62°C to 58°C for annealing was used for amplification. Oligonucleotide sequences are shown in Table S4. Yeast transformation was performed as described [33] using the lithium chloride/polyethylene glycol procedure. Yeast strain WW-YH10 (ATCC 208405) was transformed with both flanking regions, the ppy4 marker gene and an EcoRI/XbaI digested plasmid pRS426 [34]. Transformants were selected on synthetic complete dropout media (SC-URA with uracil dropped out). Following total DNA isolation from liquid SC-URA media [35], plasmids were introduced into chemically competent JM109 E. coli cells (Promega). The outside primers 5′F +3R were used to synthesize the complete deletion cassette from S. cerevisiae.

For construction of a strain overexpressing Trire2:3405 under the constitutive tef1 promoter, the coding region of Trire2:3405 plus approximately 500 bp downstream of it was amplified using primer st_infusion_1 and st_infusion_2 (Table S4) and inserted into the SalI/HindIII linearized plasmid pLHhph1-tef1 [12] using the In-Fusion™ HD Cloning Kit (Clontech). Transformation of T. reesei protoplasts was performed as described by Gruber et al. [36]. Integration of the ppy4::3405 fragment was verified using primer Ptf1 F-ch and St R-ch (Table S4).

Transformation of T. reesei and Analysis of Transformants

All deletion strains were generated in the Atka70 strain [27] and the QM 9414 strain (ATCC 26921) was used as a control in all experiments. Protoplast preparation and transformation were performed as previously described [36]. The deletion cassettes were purified from agarose gels (QiAquick Gel Extraction kit, QiAGEN) and concentrations were determined (Nanodrop Spectrophotometer, Peqlab). After transformation protoplasts were stabilized and regen-

ated on minimal mediansupplemented with 1 M D-Sorbitol. In the case of the QM9414 tef1::3405 strain the selection media additionally contained 100 μg/mL hygromycin B (Roth). For sporulation, transformants were transferred to small plates and purified by plating conidiospores onto plates with 0.1% Triton X-100 as colony restricter. Single colonies were transferred to selective media and screened for correct integration of the deletion cassettes or of the tef1::3405 fragment, respectively. Genomic DNA of the transformants was extracted [37] and transformants were screened for the presence of the deletion cassettes by amplifying a fragment by PCR with one oligonucleotide specific for the upstream region outside of the deletion cassette (primer abbreviated with “ch” in Table S4) and the second specific for the ppy4 marker gene (ch_ppy4 neu).

Transcriptome Analysis

Mycelia were harvested from cultures growing on lactose, glucose and glyceral, respectively, for 24 hrs. Total RNAs were extracted using TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer’s instructions, and then purified. cDNA synthesis, labelling and hybridization was performed by Roche NimbleGen (Roche-NimbleGen, Inc., Madison, WI, USA) with a high density oligonucleotide specific for the upstream region outside of the deletion cassette (primer abbreviated with “ch” in Table S4) and the second specific for the ppy4 marker gene (ch_ppy4 neu).

Real Time PCR

DNase treated (DNase I, RNase free; Fermentas) RNA (5 μg) was reverse transcribed with the ReverTaq™ First Strand cDNA Kit (Fermentas) according to the manufacturer’s protocol with a combination (1:1) of the provided oligo-dT and random hexamer primers. All assays were carried out in 96-well plates which were covered with optical tape, as described by Metz et al. [22]. Primers, amplification efficiency and R-square values are given in Table S5. Determination of the PCR efficiency was performed using triplicate reactions from a dilution series of cDNA, and the amplification efficiency was then calculated from the given slopes in the IQ5 Software. All samples were analyzed in two independent experiments with three replicates in each run.

Phylogenetic Analysis

Phylogenomic relationship between Trire2:3405 and its closest neighbors in BLAST (blastP) was studied by performing a randomized bootstrap maximum-likelihood analysis using RAxML software [39], setting the bootstrap analysis to 1000 runs and the bootstrap random seed value to 12,311. The Dayhoff mutation data matrix was used for the analysis of the alignment.
Figure 3. Phylogenetic analysis of the putative lactose permease Tr ire2:3405.
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Supporting Information

Figure S1  Growth of the MFS-knock out strains on glucose and cellobiose (1%, w/v, each) in the presence of 20 and 50 μg/mL nojirimycin.

(DOCX)

Table S1  Genes that are significantly (>2-fold) and consistently up- or downregulated in T. reesei on lactose when compared to glucose and glycerol.

(DOCX)

Table S2  Genes significantly regulated on lactose and by CRE1 and/or growth rate.

(DOCX)

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