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The intracellular galactoglycome in *Trichoderma reesei* during growth on lactose

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Abstract Lactose (1,4-0-β-D-galactopyranosyl-D-glucose) is used as a soluble carbon source for the production of cellulases and hemicellulases for—among other purposes—use in biofuel and biorefinery industries. The mechanism how lactose induces cellulase formation in *T. reesei* is enigmatic, however. Previous results from our laboratory raised the hypothesis that intermediates from the two galactose catabolic pathway may give rise to the accumulation of intracellular oligogalactosides that could act as inducer. Here we have therefore used high-performance anion-exchange chromatography–mass spectrometry to study the intracellular galactoglycome of *T. reesei* during growth on lactose, in *T. reesei* mutants impaired in galactose catabolism, and in strains with different cellulase productivities. Lactose, *allo*-lactose, and lactulose were detected in the highest amounts in all strains, and two trisaccharides (Gal-β-1,6-Gal-β-1,4-Glc/Fru and Gal-β-1,4-Gal-β-1,4-Glc/Fru) also accumulated to significant levels. Glucose and galactose, as well as four further oligosaccharides (Gal-β-1,3/1,4/1,6-Gal; Gal-β-1,2-Glc) were only detected in minor amounts. In addition, one unknown disaccharide (Hex-β-1,1-Hex) and four trisaccharides were also detected. The accumulation of the unknown hexose disaccharide was shown to correlate with cellulase formation in the improved mutant strains as well as the galactose pathway mutants, and Gal-β-1,4-Gal-β-1,4-Glc/Fru and two other unknown hexose trisaccharides correlated with cellulase production only in the pathway mutants, suggesting that these compounds could be involved in cellulase induction by lactose. The nature of these oligosaccharides, however, suggests their formation by transglycosylation rather than by glycosyltransferases. Based on our results, the obligate nature of both galactose catabolic pathways for this induction must have another biochemical basis than providing substrates for inducer formation.

Keywords *Trichoderma reesei* · Lactose · Galactoglycome · Cellulase · HPAEC-MS

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Introduction

Lactose (1,4-0-β-D-galactopyranosyl-D-glucose) is worldwide produced in up to 1.2 million tons annually as a by-product from cheese manufacture or from milk processing industries, but utilized only to a low degree and mostly not very profitable (Roelfsema et al. 2010). It can also be used as a soluble carbon source for the filamentous fungus

Trichoderma reesei to produce cellulases and hemicellulases that are needed for food, feed, textile, pulp, and paper industries, as well as the production of biofuels and in biorefineries (Menon and Rao 2012). A drawback of the use of lactose is, however, that the cellulase formation kinetics is slower than on cellulose, thus warranting a deeper understanding of lactose metabolism toward the targeted improvement of enzyme production on this carbon source (Seiboth et al. 2007).

The mechanism how lactose induces cellulase formation in *T. reesei* is still enigmatic: Lactose is catabolized via two pathways (i.e., the canonical Leloir pathway and an oxidoreductive pathway: see Seiboth et al. 2007; Fig. 1), and induction by lactose depends both on phosphorylation of D-galactose by galactokinase in the Leloir pathway, as well as on its catabolism to galactitol in the reductive pathway, whereas subsequent steps in the two pathways have no effect (Hartl et al. 2007; Seiboth et al. 2007). Further, D-galactose must be present in the form of the β-anomer for triggering induction (Fekete et al. 2008). On the other hand, growth on D-galactose or galactitol alone or in combination does not induce cellulases to significant levels (Karaffa et al. 2006). From these data, one may speculate that galactose-1-phosphate and a metabolite from the reducing catabolic pathway may undergo intracellular condensation to an oligosaccharide that could be the inducer of cellulases during growth on lactose.

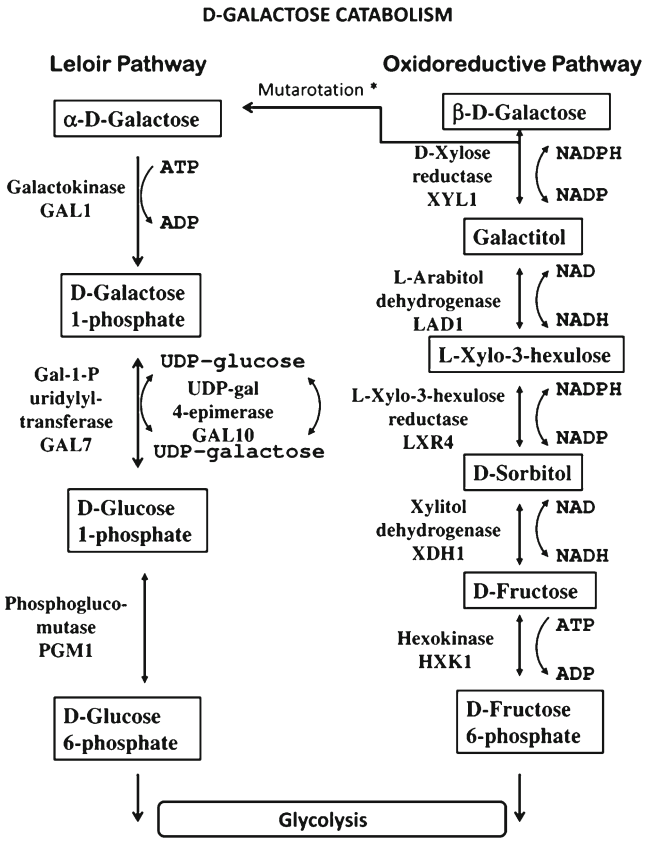

Fig. 1 Metabolic pathways involved in the catabolism of D-galactose by *T. reesei*. *Asterisk* indicates chemical mutarotation (for details, see Fekete et al. 2008)

High-performance anion-exchange chromatography (HPAEC) is an excellent and well-known separation technique for carbohydrates. Hence, HPAEC-mass spectrometry (MS) is a preferred technique for profiling complex mixtures of unknown oligosaccharides and has been successfully applied for this purpose in several studies (Richardson et al. 2001; Rumbold et al. 2002; Okatch et al. 2003; Bruggink et al. 2005; Coulier et al. 2009).

Here we have used HPAEC-MS to study the intracellular galactoglycome of *T. reesei* during growth on lactose, in *T. reesei* mutants impaired in galactose catabolism, and in strains with different cellulase productivity.

Materials and methods

Strains and cultivation conditions

The *T. reesei* strains used in this study are given in Table 1. All cultures were maintained on malt extract (3 %, 1 *w/v*) at 28 °C.

Cultivations were carried out in 2.5 L glass bioreactors (Sartorius Biostat B Plus) with a working volume of 2 L, equipped with one six-blade Rushton disk turbine impeller. Inocula for the fermentations were grown in 500-mL flasks on a rotary shaker (250 rpm) at 28 °C, containing 100 mL of the following medium (initial pH 5.0): 8 gL^{-1} $NH_4H_2PO_4$, 7 gL^{-1} Na_2HPO_4, 4 gL^{-1} KH_2PO_4, 1 gL^{-1} $CaCl_2$, 1 gL^{-1} $MgSO_4$, 0.1 gL^{-1} peptone, 20 mLL^{-1} trace elements (250 mgL^{-1} $FeSO_4$•7 H_2O, 80 mgL^{-1} $MnSO_4$•H_2O, 70 mgL^{-1} $ZnSO_4$•7 H_2O, 85 mgL^{-1} $CoCl_2$) and 10 gL^{-1} of glycerol. Two shake flask cultures grown for 24 h were filtered, the biomass washed with sterile cold tap water and transferred into the fermenter as inocula. Fermentations were run at pH 5.0, 28 °C, blade tip speed 600 rpm, and 0.5 vvm aeration. The fermentation medium was identical to the one above but contained 15 gL^{-1} lactose as a sole carbon source and peptone was omitted. Glass parts of the reactor were treated with the anti-adhesive agent Sigmacot (Sigma) to avoid fungal wall growth, and a few drops of the antifoam agent polypropylene glycol 2000 (Union Carbide Chemicals & Plastics) were injected daily into the reactor through a membrane filter (Millipore).

Sampling of mycelium

Samplings were performed by a method described by Ruijter and Visser (1996). Briefly, this involves direct and rapid transfer of a culture sample containing 0.5 g cellular dry weight from a bioreactor to a buffered solution (culture/

Table 1 *T. reesei* strains used in this study

Strain	Strain description	Source/reference
QM 9414	Parental strain, early cellulase overproducer	ATCC 26921; Le Crom et al. 2009
NG14	Cellulase hyperproducer	ATCC 56767; Le Crom et al. 2009
RUT C-30	Carbon catabolite derepressed cellulase hyperproducer	ATCC 56765; Le Crom et al. 2009
Δ*gal1*	Galactokinase negative (derived from QM 9414)	Seiboth et al. 2004
Δ*xyll1*	D-Xylose (aldose) reductase negative (derived from QM 9414)	Seiboth et al. 2007
Δ*gal1*/Δ*xyl1*	Galactokinase/D-xylose (aldose) reductase negative (derived from QM 9414)	Seiboth et al. 2007

buffer volume ratio 1:4) containing 65 % (*v*/*v*) methanol, 35 % (*v*/*v*) de-mineralized water, 100 mM 1-methyl-imidazole (pH 7.0) at −45 °C, and collecting the mycelium by filtration using Miracloth (Calbiochem, Merck Hungary, Budapest, Hungary) at −35 °C. The mycelium was washed twice at −35 °C with 50 % (*v*/*v*) methanol and 100 mM 1-methyl-imidazole (pH 7.0). The washed pellet is resuspended in 50 % (*v*/*v*) methanol without additions (−40 °C) up to a final volume of 10 mL. This suspension was stored below −40 °C before extraction.

Extraction of the intracellular glycome

The procedure was described in details by Ruijter and Visser (1996). To sum up, mycelial samples obtained were resuspended in a methanol/water solution (1:1 ratio), followed by the addition of chloroform (equal volume as the total methanol/water suspension present) and vigorous shaking at −40 °C. The water–methanol (e.g., the supernatant containing the extracted metabolites) and chloroform phases were separated by centrifugation (12,000 rcf, −20 °C, 15 min), the water–methanol phase was collected, and fresh water–methanol solution (at half original methanol/water volume) was added to the mycelium for repeated extraction. The combined sample from the subsequent extractions was deproteinized using a 10-kDa cut-off filter before chemical analysis.

Analysis of the intracellular glycome

Commercially available reference compounds of carbohydrates were purchased from either Sigma-Aldrich Chemie (Zwijndrecht, the Netherlands) or Megazyme International Ltd. (Wicklow, Ireland). All standard solutions were prepared in MilliQ water.

HPAEC-MS was performed on a Thermo Surveyor HPLC system (Thermo Electron Corporation, San Jose, CA, USA) equipped with a Carbopac PA1 column (250×2.0 mm i.d., Dionex, Sunnyvale, CA, USA) operated at 30 °C. Elution was performed with a flow of 215 μL/min, and the injection volume was 5 μL. The following eluents were used: 100 mM NaOH ("A") and 100 mM NaOH + 500 mM NaOAc ("B"). The following gradient was used: 0–5 min, isocratic 100 % "A," 5–78 min, linear gradient from 100 to 74 % "A," followed by a washing step with 100 % "B" for 6 min and re-equilibration for 10 min at 100 % "A." Prior to MS detection, an ASRS300-2 mm suppressor (Dionex) was used as an in-line desalter to convert the eluate into an MS-compatible solution. The membrane was continuously regenerated with acid generated by electrolysis of water. Milli-Q water was fed from an air-pressurized bottle into the regenerant chamber at a flow rate of 3.33 mL/min. A regenerant current of 150 mA was applied. Mass detection was carried out on a Thermo LTQ LT-1000 mass detector using electrospray ionization in the positive ionization modes (ESI spray voltage 3.2 kV, heated capillary temperature 275 °C, sheath gas 25, auxiliary gas 10, full scan range *m*/*z* 125–1,500, number of microscans 3, maximum injection time 200 ms).

HPAEC-MS/MS was performed on the same system and using identical conditions as described above. MS^n-experiments were performed on pre-selected peaks using wide band activation and based on dependent scan settings with a collision energy of 35 % (Xcalibur software V2.0, Thermo Electron Corporation). In addition, samples were also separated using an isocratic mobile phase flow of 15 % "A" and 85 % water to improve separation of co-eluting disaccharides like D-glucose and D-galactose, and lactose, allo-lactose, and lactulose.

Since the analytical method used (*vide supra*) is only semiquantitative, data were expressed as relative abundance, e.g., the percentage (%) of any given compound to the combined amount of mono-, di-, and trisaccharides. Only compounds with a relative abundance over 2 % are presented. In total, these compounds comprised >90 % of the total mono- and oligosaccharides in each sample.

Extraction and analysis of the intracellular adenylate nucleotide pool

To prove that the *in vivo* concentration of metabolites had been preserved during this harvesting procedure, we measured the energy charge $(0.5 \times [(2ATP + ADP)/(ATP + ADP + AMP)])$ by determining the intracellular ATP, ADP, and AMP concentrations (Jörgensen et al. 2010). The nucleotide pool from the mycelium was extracted with ice-cold 1 M perchloric acid, followed by centrifugation and neutralization

of the supernatant with diluted Na_2HPO_4. Samples were analyzed with HPLC (Hewlett-Packard HP-1090, Santa Barbara, CA, USA) with differential UV detection at 258 and 290 nm ($A = A_{258} - A_{290}$). Briefly, 25 μL samples diluted with distilled water were injected onto a Waters SymmetryShield RP18 5 μm (150× 2.1 mm) column coated with 0.1 % dodecyl-trimethyl-ammonium bromide. Elution was performed at 40 °C and a flow rate of 0.5 mL/min, with an aqueous solution composed of 50 mM Na_2HPO_4 and 40 mM $HClO_4$. Only samples with an energy charge >0.85 were considered for further analysis.

Statistical analysis

Pearson's correlation coefficient (Rodgers and Nicewander 1988) was computed to compare the galactoglycome concentrations in different strains. The significance of correlation was tested by calculating the one-way p value (Soper 2012).

Results

The intracellular galactoglycome of *T. reesei*

HPAEC-MS is a useful technique for profiling complex mixtures of (unknown) oligosaccharides in which individual peaks can be characterized or identified based on their retention time, m/z value, and MS/MS fragments. To overcome the problem of quantifying unknown compounds, relative response factors (the peak area corresponding to a certain concentration with respect to a reference compound) were determined for various reference compounds of carbohydrates: Monosaccharides were found to have a relative response factor of 1.0, disaccharides of 1.0–1.3, and trisaccharides of 0.6. These relative response factors were therefore used for unknown carbohydrates based on their degree of polymerization (i.e., 1.15 for unknown disaccharides and 0.6 for unknown trisaccharides).

Figure 2 shows a typical example of carbohydrates that could be detected in *T. reesei* with HPAEC-MS. Although HPAEC has excellent separation power, some carbohydrates still co-elute (e.g., *allo*-lactose, lactose, and lactulose; Coulier et al. 2009). Therefore, an additional isocratic HPAEC-MS was used to separate these three compounds (Fig. 2d).

Having reliable methods for extraction and quantitation of the galactoglycome from *T. reesei* available, we first followed the accumulation of galactooligosaccharides during cultivation of *T. reesei* QM 9414 strain on lactose (Fig. 3): Lactose, *allo*-lactose (Gal-β-1,6-Glc), and lactulose were detected in the highest amounts and in an average ratio of 7:1:1. Two trisaccharides (Gal-β-1,6-Gal-β-1,4-Glc/Fru and Gal-β-1,4-Gal-β-1,4-Glc/Fru) also accumulated to significant levels. Note that the pairs Gal-β-1,6-Gal-β-1,4-Glc/Gal-β-1,6-Gal-β-1,4-Fru and Gal-β-1,4-Gal-β-1,4-Glc/Gal-β-1,4-Gal-β-1,4-Fru cannot be separated with the method used; thus, we considered these two pairs of oligosaccharides as two single compounds. Glucose and galactose, as well as four further oligosaccharides (Gal-β-1,3/1,4/1,6-Gal and Gal-β-1,2-Glc), were only detected in minor amounts, just like all the other components that could not be identified. A summary of components that could be detected and their tentative identity are given in Table 2. While we cannot rule out that lactose and *allo*-lactose are (in part) contaminants from the medium, the presence of lactulose (D-galactosyl-1,4-β-D-fructoside) argues against an origin of these components as transglycosylation products from the culture filtrate as the medium does not contain D-fructose.

The intracellular galactoglycome in improved cellulase producer strains of *T. reesei*

In an attempt to find out whether any of these components could act as an inducer of cellulase formation, we consequently investigated whether the presence of any of the four oligosaccharides mentioned before would be significantly different in the improved producer strain *T. reesei* NG14 and the hyperproducer *T. reesei* RUT C30 prepared from it (Eveleigh and Montenecourt 1979). To this end, we extracted the glycome also from the *T. reesei* strains NG14 and RUT C30. Since the growth rate of these strains under the cultivation conditions used is different, we chose the time points such that they corresponded to similar stages of carbon source uptake (i.e., rapid growth phase and stationary growth phase, referred to further on as GP and SP, respectively). Relative abundance data of HPAEC-MS chromatograms obtained for samples from *T. reesei* mutant strains NG14 and RUT C30 are shown at Table 3. Most of the carbohydrates data seem profoundly vary with time, particularly in *T. reesei* RUT C30.

In order to test whether the accumulation of any of the detected components would correlate with the increased cellulase expression in the three strains (QM 9414<NG14<RUT C30), we calculated Pearson's correlation coefficient. The data are shown in Fig. 4 a: Correlation was negative for most of the components detected, i.e., they were present in lower abundance in the higher producer strains than in QM 9414. Only the unidentified hexose disaccharide Hex-β-1,1-Hex showed good correlation (0.993; p=0.037), implying that its concentration correlates with cellulase production.

The galactoglycome in *T. reesei* blocked in galactose catabolic pathway mutants

The hypothesis for this work was that growth of *T. reesei* on lactose would lead to the intracellular accumulation of galactooligosaccharides that would induce cellulases. Since this induction is strongly reduced in null mutants in the galactokinase (Δ*gal1*), aldose reductase (Δ*xyl1*), and the

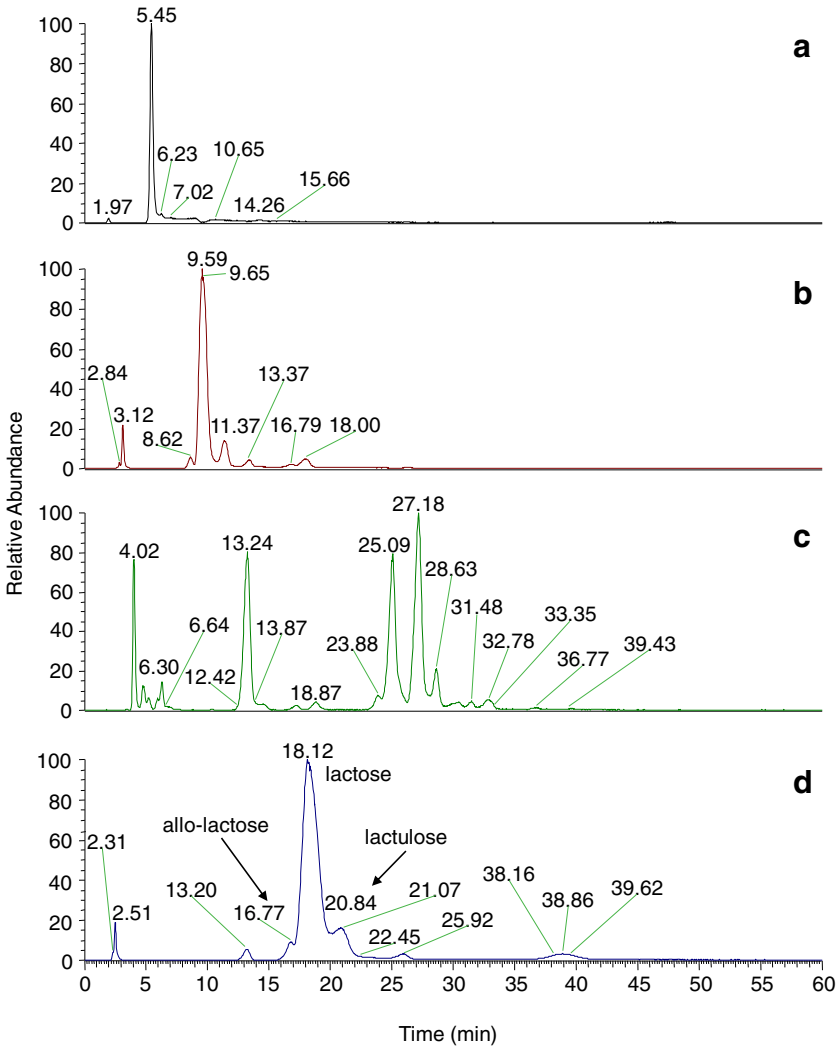

Fig. 2 Example of extracted ion HPAEC-MS chromatograms (*T. reesei* NG14 strain at cultivation time of 16.5 h, corresponding to the rapid growth phase): **a** *m/z* 203.1 corresponding to monohexoses, **b** *m/z* 365.2 corresponding to dihexoses, **c** *m/z* 527.2 corresponding to trihexoses, and **d** *m/z* 365.2 corresponding to dihexoses with isocratic flow to improve separation

respective double knockout strain (Δ*gal1*/Δ*xyl1*), our hypothesis implies that the putative inducer should be present in these strains only in reduced concentrations or be absent. We therefore analyzed the galactoglycome of these strains in the same way as that of the higher producer strains described above. Because single knock out mutants have a reduced growth rate on lactose compared to the producer strains, samples were taken at the time of the (relatively) most intense carbon uptake only. The double -knockout *T. reesei* strain does not grow on lactose at all. We therefore pregrew it on glucose and then pulsed it with a final concentration of 10 gL^{-1} lactose. This led to the formation of cellulase activity and transcription of the two major cellulase genes *cel7A* and *cel6A* in a pattern consistent with previous data (Seiboth et al. 2004, 2007; data not shown).

Analysis of the corresponding galactoglycome showed (Table 3) that the two mutants in which cellulase gene expression was the strongest affected (Δ*gal1* and Δ*gal1*/Δ*xyl1*) accumulated even higher amounts of some of the di- and trigalactooligosaccharides detected in QM 9414. Correlation analysis again identified the unknown hexose disaccharide Hex-β-1,1-Hex (*vide supra*) as one of the positively correlating oligosaccharides (Pearson's coefficient 0.836), yet with a lower probability (p=0.082). In addition, three trisaccharides (two of unknown structure and Gal-β-1,6-Gal-β-1,4-Glc) showed good correlation (0.945, 0.843, and 0.873; p values 0.027, 0.078, and 0.063, respectively) with the cellulase production by QM 9414 and the three pathway mutants (Fig. 4b).

Discussion

In this paper, we tested the hypothesis that metabolites from the galactose catabolic pathway would form oligosaccharides that could act as inducers of cellulase formation during growth on lactose. To this end, we adapted a method for rapid metabolite extraction and the subsequent analysis of potentially occurring oligosaccharides. Our data show that the method indeed led to a extraction of rapidly turning over metabolites such as ATP,

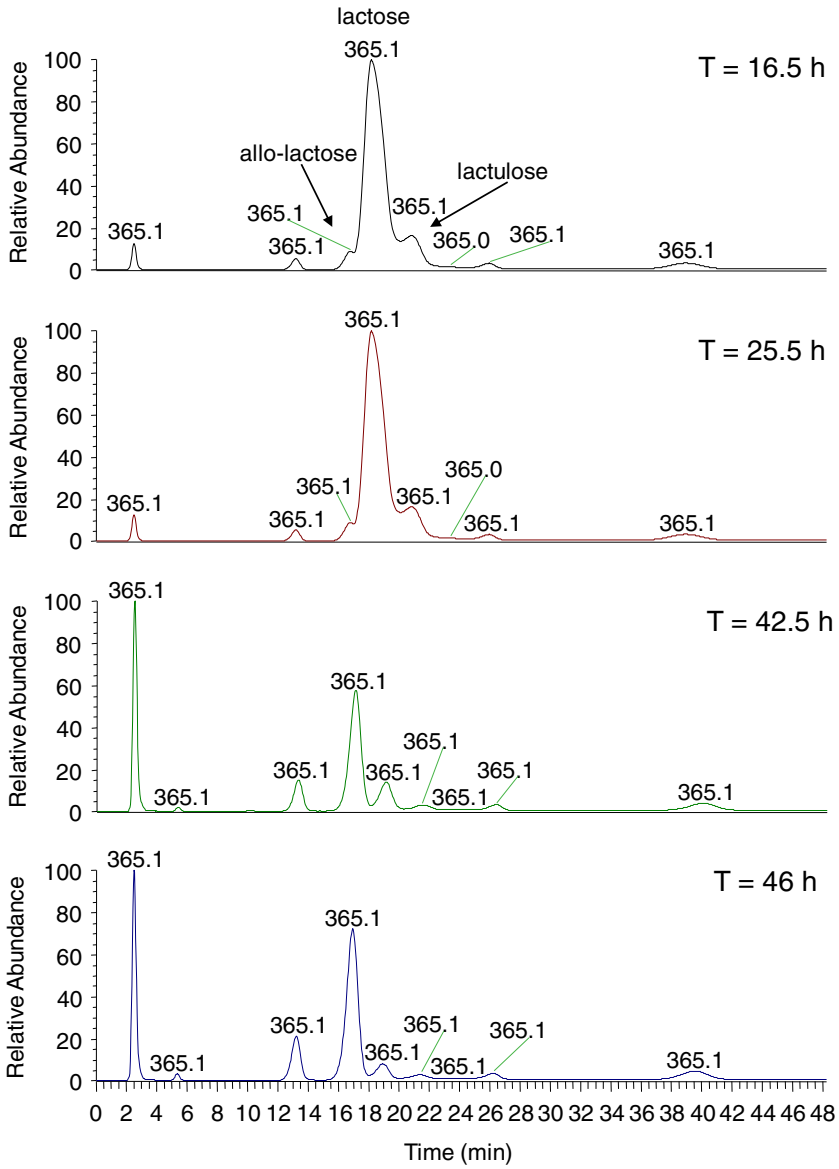

Fig. 3 Separation of galactooligosaccharides of *T. reesei* QM 9414 growing on lactose for different time-points by isocratic mobile phase flow

ADP, and AMP (evidenced by the calculation of an energy charge from their concentrations that resembles that of intact cells), and we can hence assume that the probably slower turning-over oligosaccharides also are present in the extracts at proportions that resemble the in vivo situation.

A drawback of the rapid harvesting and extraction procedure, however, is that washing of the mycelia must occur within a very short time, which may not be sufficient to remove all the lactose bound to the cell walls. The binding of mono- and oligosaccharides to cell wall polysaccharides is a well-known phenomenon in cellular interactions (for review, see Bucior and Burger 2004), and their removal from fungal cell walls requires extensive washing at higher salt concentration (Röhr et al. 1981). Therefore, we do not believe that lactose, which was detected as the most abundant compound by HPAEC-MS, is actually most abundant in the cytosol of *T. reesei*. One may argue that also the other oligosaccharides detected are simply transglycosylation products formed by the extracellular β-galactosidase and that (partially) bound to the cell walls. However, we have several arguments against this: One is the occurrence of lactulose (D-galactosyl-1,4-β-D-fructoside) in the oligogalactoglycome pool of *T. reesei*. Since fructose does not occur in the medium of *T. reesei*, lactulose must be formed from fructose present in the intracellular pool of *T. reesei*. The second argument comes from the relative percentage of the other oligosaccharides to lactose: If we assume that they all would bind with approximately the same strength to the cell walls, then some of them (e.g., Gal-β-1,4-Gal-β-1,4-Glc/Fru) would have to be present in the medium up to 25 % of that of lactose. HPLC analysis of culture filtrates of *T. reesei* growing on lactose, however, never detected such components (unpublished data). Finally, since β-galactosidase activity is higher in strain RUT C30, we would have to observe

Table 2 Hexoses detected and tentatively identified by HPAEC-MS

t_r (min)	Component
3.2	Unknown Hex-β-(1→1)-Hex
4.1	Unknown Hex-β-(1→4)-Hex-β-(1→1)-Hex
4.8	Unknown Hex-β-(1→6)-Hex-β-(1→1)-Hex
5.4	Galactose/glucose[a]
6.3	Unknown Hex-Hex-Hex containing β-(1→1) linkage
8.5	Gal-β-(1→6)-Gal
9.7	Gal-β-(1→6)-Glc/Gal-β-(1→4)-Glc/Gal-β-(1→4)Fru[a]
11.2	Gal-β-(1→3)-Gal
13.2	Gal-β-(1→6)-Gal-β-(1→4)-Glc/Gal-β-(1→6)-Gal-β-(1→4)-Fru
13.4	Gal-β-(1→4)-Gal
16.7	Gal-β-(1→3)-Glc
17.6	Gal-β-(1→2)-Glc
25.0	Gal-β-(1→4)-Gal-β-(1→4)-Glc/Gal-β-(1→4)-Gal-β-(1→4)-Fru
27.2	Gal-β-(1→6)-Gal-β-(1→X)-Glc
28.6	Unknown Hex-Hex-Hex (no β-(1→1) or β-(1→2) linkages)
30.4	Gal-β-(1→4)-Gal-β-(1→2)-Glc/Gal-β-(1→4)-Gal-β-(1→3)-Glc

[a] Separated by the isocratic mobile phase flow

these oligosaccharides at higher proportions in this strain. However, in this study, most oligosaccharides negatively correlated with the increased production by the three strains, thus rejecting this assumption. In summary, we therefore believe that the oligosaccharide pool that we extracted indeed reflects the composition of the intracellular pool.

The second prerequisite for this work was the adaptation of the HPAEC-MS analysis for separating the intracellular glycome of *T. reesei*. The method indeed detected 13 oligosaccharides, besides lactose, and its hydrolysis products glucose and galactose. Eight of them could be reliably identified based on standard components, but the identity of five of them (one disaccharide and four trisaccharides) remained elusive.

The detection of a considerable number of oligogalactosides in the intracellular metabolite pool of *T. reesei* then raises two questions: Which enzymes are responsible for their formation? And what is the purpose of their accumulation? As for the first question, most of their chemical structure suggests an origin by a typical transglycosylation reaction catalyzed by β-galactosidases (Rodriguez-Fernandez et al. 2011; Lu et al. 2012). The presence of such an intracellular β-galactosidase is also warranted by our recent findings of a lactose permease in *T. reesei*, whose function is essential for growth on lactose (Ivanova et al., submitted for publication). However, the genome of *T. reesei* has not been reported to contain a gene encoding an intracellular β-galactosidase: Intracellular β-galactosidases identified until now belong to glycosyl hydrolase family 2 (GH2), of which *T. reesei* has seven members (Martinez et al. 2008). A Blastp search identifies five of them as β-mannosidases, one as exo-β-D-glucosaminidase, and one as unknown GH2 glycoside hydrolase (Table 4). The latter (Trire2:76852) has been annotated as β-glucuronidase (Martinez et al. 2008). However, a blastp search against the NCBI database (http://blast.ncbi.nlm.nih.gov) reveals most

Table 3 Relative abundance of intracellular carbohydrates in *T. reesei* QM 9414 and various mutant strains

Component	QM9414	GP NG14	SP NG14	GP Rut C30	SP Rut C30	GP Δ-*gal1*	GP Δ*xyl1*	GP Δ*xyl1*/*gal-1*
Gal	0.01	0.01	0.00	0.00	0.00	0.13	0.03	0.10
Glc	0.01	0.01	0.02	0.01	0.01	0.01	0.02	0.01
Unknown Hex-β-1,1-Hex[a]	0.03	0.04	0.15	0.08	0.06	0.02	0.02	0.01
Gal-β-1,6-Gal	0.01	0.03	0.06	0.00	0.00	0.06	0.01	0.04
Gal-β-1,3-Gal	0.02	0.02	0.01	0.00	0.00	0.10	0.05	0.09
allo-Lactose (Gal-β-1,6-Glc)	0.04	0.08	0.26	0.00	0.00	0.05	0.06	0.02
Lactose (Gal-β-1,4-Glc)	0.47	0.40	0.04	0.69	0.74	0.17	0.26	0.32
Lactulose (Gal-β-1,4-Fru)	0.05	0.04	0.01	0.06	0.02	0.15	0.09	0.16
Gal-β-1,4-Gal	0.01	0.03	0.00	0.00	0.00	0.06	0.02	0.04
Gal-β-1,2-Glc	0.01	0.04	0.04	0.01	0.01	0.08	0.03	0.05
Unknown Hex-β-1,6-Hex-β-1,1-Hex	0.02	0.01	0.01	0.01	0.01	0.00	0.01	0.00
Unknown Hex-β-1,1-Hex-β-1,1-Hex	0.01	0.02	0.08	0.00	0.00	0.00	0.01	0.00
Gal-β-1,6-Gal-β-1,4-Glc/Fru	0.07	0.09	0.16	0.01	0.03	0.03	0.06	0.02
Gal-β-1,4-Gal-β-1,4-Glc/Fru	0.11	0.07	0.04	0.11	0.10	0.04	0.12	0.02
Unknown Hex-β-1,4-Hex-β-1,1-Hex	0.11	0.08	0.08	0.01	0.01	0.03	0.12	0.03
Unknown Hex-Hex-Hex[b]	0.01	0.01	0.01	0.00	0.00	0.02	0.03	0.03

Only carbohydrates >2 % were taken

[a] Hex means any hexose

[b] Glycosidic linkage could not be determined

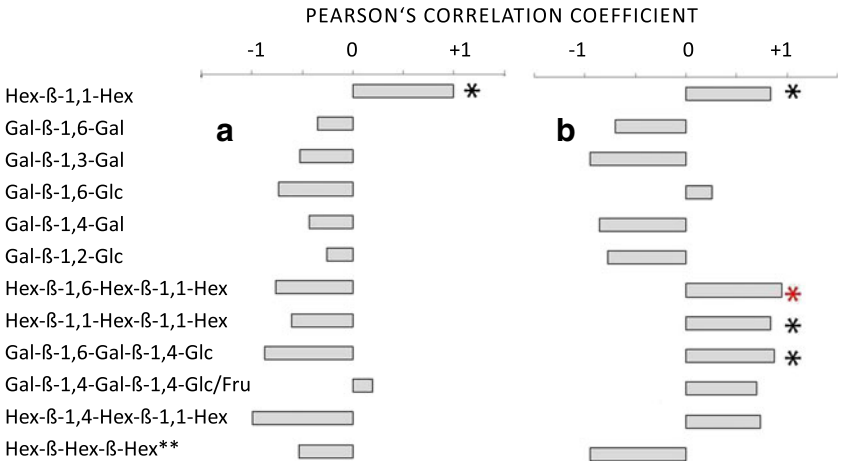

Fig. 4 Correlation between cellulase production and accumulation of individual oligosaccharides in *T. reesei* improved producer strains (**a**) and galactose pathway mutants (**b**). *Asterisk* indicates Pearson coefficients that were characterized by $p<0.1$ or $p<0.05$ (*asterisk* in *red*). *Double asterisk*s glycosidic linkage could not be determined with certainty

hits as "unknown GH2 glycosyl hydrolase" except for *Verticilium dahliae* (EGY21219.1) and *Zymoseptoria tritici* (XP_003848790.1) where the respective ortholog (*e* value in both cases zero, 99 and 96 % coverage, 59 % identity for *V. dahliae*, and 46 % for *Z. tritici*) was annotated as β-galactosidase. The respective protein has not been characterized from any fungus, however. While this does of course not prove that Trire2:76852 is indeed the missing intracellular β-galactosidase, it makes an investigation of this protein worthwhile. Such enzyme source may also have practical applications for the synthesis of oligogalactosides which are desired components for prebiotic food (Sangwan et al. 2011; Lamsal 2012). Arguments against Trire2:76852, however, are that it contains a signal peptide, which is atypical for GH2 glycoside hydrolases (http://www.cazy.org/GH2_characterized), but—if functional—would drive its secreted protein out of the cell and that it is induced about 6-fold on both cellulose and lactose (unpublished data) implying that its function is not restricted to growth on lactose.

The oligosaccharides that contain β-1,1-linkages, however, can probably not be formed by transglycosylation. At least, we are not aware of any β-galactosidase which can form this glycosidic bond. Alternatively, these linkages could be formed by a glycosyltransferase, but one that transfers galactose (or another hexose) in a β-1,1-linkage to another hexose has so far not been described. Likewise, β-1,1-linkages seem not be present in natural oligo- or polysaccharides. Their detection in the intracellular pool of *T. reesei* is therefore enigmatic.

The purpose of accumulation of the observed oligogalactosides is even more difficult to answer. Most of the galactooligosaccharides show increased accumulation in the galactose pathway mutants, which argues against their origin by glycosyl transferases because they would require the presence of UDP-sugar moieties (Lairson et al. 2008) which were not detected. Apparently, blockage in D-galactose catabolism stimulates their accumulation. Therefore, their accumulation could represent a mechanism by which the fungal cell sequesters an excess of sugars under conditions where it cannot further degrade it efficiently. Accumulation of oligosaccharides has also been reported in plants, algae, and bacteria (Nagai et al. 2012). Alternatively, they could function in the stabilization of some enzymes (Prasad and Roy 2010). It will be interesting to learn whether a similar accumulation of oligosaccharides also occurs on other carbon sources and in other fungi.

Despite the analysis of the intracellular galactoglycome in *T. reesei*, the induction of its cellulase formation by lactose

Table 4 GH2 members of *T. reesei*

Trire2	Best hit	Annotated as	*e*-value	Maximal identity
5836	*Aspergillus oryzae* gi|83766925	Candidate β-mannosidase	*e*0	60 %
59689	*Aspergillus niger* gi|134079322	Candidate β-mannosidase	*e*0	45
69245	*Thielavia terrestris* gi|367044434	Candidate β-mannosidase	*e*0	55
62166	*Cordyceps militaris* gi|346325910	Candidate β-mannosidase	*e*0	65
57857	*Beauveria bassiana* gi|400597449	Candidate β-mannosidase	*e*0	64
77299	*Trichoderma reesei* gi|67625669	Exo-β-D-glucosaminidase GLS93	*e*0	100
76852	*Verticillium dahliae* gi|346977767	Candidate β-galactosidase	*e*0	59

Protein models were taken from the *T. reesei* genome website (http://genome.jgi-psf.org/Trire2/Trire2.home.html) and subjected to blastp at the NCBI server by querying the non-redundant protein sequence database and an expect threshold of 10. Data shown were obtained on December 14, 2012

remains enigmatic: A comparison of the improved producer mutant line identified the unknown hexose disaccharide to correlate with cellulase formation in the improved mutant strains as well as the galactose pathway mutants, and Gal-β-1,4-Gal-β-1,4-Glc and two other unknown hexose trisaccharides to correlate with cellulase production only in the pathway mutants. These compounds could therefore be the components mediating lactose induction. The fact that the latter trisaccharides did not significantly accumulated in strains NG14 and RUT C30 does not necessarily argue against this because it is not known whether inducer formation in these two strains has indeed been elevated. Analysis of the mutations in the genome that had occurred during their mutagenesis has revealed a number of targets that could be responsible for their enhanced cellulase productivity even if the response to the inducer would have remained unaffected (Le Crom et al. 2009). Further work on the identification of these unknown oligosaccharides and their role as inducer of cellulase formation will thus be worthwhile. However, the hypothesis that oligosaccharides formed from both galactose catabolic pathways (i.e., galactose and galactitol) would be the inducers of cellulase gene expression was not supported by the present data because no galactitol-containing oligosaccharides were detected among the components that accumulated to detectable amounts. The obligate nature of both galactose catabolic pathways for this induction must therefore have another biochemical basis. Having said that, the correlation of some intracellular oligosaccharides with cellulase formation in the galactose pathway mutants does not yet rule out that one of them could be an inducer. Identifying the enzymes that are synthesizing these oligogalactosides will not only enable the direct testing of this hypothesis but also provide new β-galactosidases with eventually new properties for biotechnology.

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