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L-*xylo*-3-Hexulose Reductase Is the Missing Link in the Oxidoreductive Pathway for D-Galactose Catabolism in Filamentous Fungi*[S]

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Background: There is an oxidoreductive D-galactose pathway in filamentous fungi.
Results: We identified an L-*xylo*-3-hexulose reductase that produces D-sorbitol and that is part of this pathway.
Conclusion: This L-*xylo*-3-hexulose reductase is the missing link in the oxidoreductive D-galactose pathway.
Significance: The alternative pathway for D-galactose catabolism in filamentous fungi is elucidated.

In addition to the well established Leloir pathway for the catabolism of D-galactose in fungi, the oxidoreductive pathway has been recently identified. In this oxidoreductive pathway, D-galactose is converted via a series of NADPH-dependent reductions and NAD^+-dependent oxidations into D-fructose. The pathway intermediates include galactitol, L-*xylo*-3-hexulose, and D-sorbitol. This study identified the missing link in the pathway, the L-*xylo*-3-hexulose reductase that catalyzes the conversion of L-*xylo*-3-hexulose to D-sorbitol. In *Trichoderma reesei* (*Hypocrea jecorina*) and *Aspergillus niger*, we identified the genes *lxr4* and *xhrA*, respectively, that encode the L-*xylo*-3-hexulose reductases. The deletion of these genes resulted in no growth on galactitol and in reduced growth on D-galactose. The LXR4 was heterologously expressed, and the purified protein showed high specificity for L-*xylo*-3-hexulose with a $K_m = 2.0 \pm 0.5$ mM and a $V_{max} = 5.5 \pm 1.0$ units/mg. We also confirmed that the product of the LXR4 reaction is D-sorbitol.

There are several pathways for the catabolism of D-galactose. The most studied is the Leloir pathway, which exists in prokaryotic and eukaryotic microorganisms. In this pathway, α-D-galactose is phosphorylated, and in the subsequent steps, converted to D-glucose-6-phosphate in a redox-neutral way (1). The genes of the Leloir pathway and their regulation have been described in yeast and filamentous fungi (2).

An alternative pathway is the oxidative pathway that is sometimes referred to as the De Ley-Doudoroff pathway and was identified in bacteria (3). In this pathway, D-galactose is first oxidized to D-galactonolactone, which is then hydrolyzed by a lactonase to D-galactonate followed by the removal of a water molecule by a dehydratase to form D-*threo*-3-deoxy-hexulosonate (2-keto-3-deoxy-D-galactonate). D-*threo*-3-Deoxy-hexulosonate is then phosphorylated to D-*threo*-3-deoxy hexulosonate 6-phosphate, which is subsequently split by an aldolase, resulting in pyruvate and D-glyceraldehyde-3-phosphate. In a strain of the mold *Aspergillus niger*, a nonphosphorylated alternative of the De Ley-Doudoroff pathway was described where the D-*threo*-3-deoxy-hexulosonate is split by an aldolase to pyruvate and D-glyceraldehyde instead of being phosphorylated (4).

Another pathway for D-galactose catabolism has also been demonstrated in some filamentous fungi. It was observed that a *Trichoderma reesei (Hypocrea jecorina*) strain with a mutation in the galactokinase gene, *gal1*, is still able to catabolize D-galactose, indicating the existence of an alternative to the Leloir pathway. Because galactitol accumulated in this strain, it was suggested that a pathway exists in *T. reesei* where D-galactose is reduced in the first step (5). In *T. reesei*, an aldose reductase, XYL1, was shown to be responsible for the reduction of D-galactose to galactitol as well as for the reduction of D-xylose and L-arabinose (6). *A. niger* has distinct L-arabinose and D-xylose reductases, LarA and XyrA (7). XyrA had the highest activity with D-galactose and was suggested to be involved in D-galactose catabolism (8).

It was also observed in *T. reesei* that a double mutant with deletions in *gal1* and *lad1*, coding for galactokinase and L-arabitol-4-dehydrogenase, respectively, was not able to catabolize D-galactose. This suggested that the L-arabitol-4-dehydrogenase that is induced on L-arabitol (9) is also part of this alternative reductive pathway. Pail *et al.* (10) showed that purified L-arabitol 4-dehydrogenase, the product of *lad1*, was capable of converting galactitol to L-*xylo*-3-hexulose. In *A. niger*, it was shown that a distinct galactitol dehydrogenase, LadB, exists besides the L-arabitol dehydrogenase, LadA, that produces L-*xylo*-3-hexulose and that it is induced on D-galactose and galactitol (8).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) BK008566 and BK008567.

[S] This article contains supplemental Figs. S1–S3.

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TABLE 1

The transcription of the following genes was tested in *A. niger* using qPCR

The carbon source was D-galactose or galactitol. The genes can be retrieved from the *Aspergillus niger* homepage at the DOE Joint Genome Institute, JGI (genome.jgi-.doe.gov/Aspni5/Aspni5.home.html) using the JGI identifiers or from the *Aspergillus* genome database (AspGD) using the identifiers in parentheses.

JGI: 177738 (An08g01930) *lxrA*
JGI: 184209 (An16g01650) *xhrA*
JGI: 40156 (An07g01830)
JGI: 174212 (An02g00220)
JGI: 177858 (An06g01980)
JGI: 184211 (An16g06440)
JGI: 56312 (An15g02280)
JGI: 212729 (An09g00620)
JGI: 55205 (An04g09990)
JGI: 212936 (An05g01210)
JGI: 52907 (An11g02460)

Seiboth and Metz (11) pointed out the possibility that the L-*xylo*-3-hexulose is reduced to D-sorbitol in a reaction catalyzed by an enzyme related to L-xylulose reductase or by the L-xylulose reductase itself. The complete oxidoreductive D-galactose pathway would then have the intermediates D-galactose, galactitol, L-*xylo*-3-hexulose, D-sorbitol, and D-fructose (see Fig. 1). D-Sorbitol was shown to be an intermediate in the pathway of *A. niger* where the D-fructose-forming D-sorbitol dehydrogenase, *sdhA*, was found to be induced on D-galactose and galactitol and the *sdhA* deletion mutant had reduced growth on galactitol and was unable to grow on D-sorbitol (12). In *T. reesei*, xylitol dehydrogenase, *xdh1*, was proposed to be the enzyme responsible for this reaction.[4] Fekete *et al.* (13) suggested that L-sorbose is produced in *Aspergillus nidulans* from galactitol by L-arabitol dehydrogenase and that it could be further converted to D-sorbitol. The reaction mechanism of the galactitol to L-sorbose conversion, however, has not been proposed and currently remains elusive. Here we set out to identify the enzyme that catalyzes the conversion of L-*xylo*-3-hexulose to D-sorbitol (L-gulitol), which is the missing link in the oxidoreductive D-galactose pathway in the molds *T. reesei* and *A. niger*.

MATERIALS AND METHODS

Strains and Chemicals—The *A. niger* strain ATCC 1015 (CBS 113.46) was obtained from the Centraalbureau voor Schimmelcultures (Delft, The Netherlands). The *T. reesei* strain QM9414 (ATCC 26921) was used in this study. For comparison of growth on agar plates, the spores of *A. niger* and *T. reesei* strains were applied to agar plates containing 6.7 g of yeast nitrogen base liter^{-1} (YNB, BD Biosciences), 20 g of agar/liter^{-1}, and 20 g liter^{-1} of carbon source (see "Results" for details). The L-*xylo*-3-hexulose used for the *in vitro* tests was produced as described previously (8).

Transcriptional Analysis—The growth of strains, RNA isolation, and qPCR[5] were performed as described previously (12).

Deletion of the xhrA Gene in the A. niger ΔpyrG Strain—Construction of *A. niger* ATCC 1015 Δ*pyrG* was described previously (7). The cassette for deletion of the *xhrA* gene (Table 1) contained 1644 bp from the *xhrA* promoter region, 1563 bp from the *xhrA* terminator region, and a 1928-bp fragment containing the *pyrG* gene flanked by its native promoter and terminator. These fragments were obtained by PCR from the *A. niger* ATCC 1015 genomic DNA using primers xhrA-5-F, xhrA-5-R, xhrA-3-F, xhrA-3-R, pyrG-del-F_n, and pyrG-del-R_n (Table 2) and the proofreading DNA polymerase Phusion (Finnzymes). The *xhrA* terminator fragment (*xhrA*-3) digested with HindIII (New England Biolabs) was inserted into the plasmid pRSET-A (Invitrogen), which was digested with HindIII and PvuII (both New England Biolabs). This intermediary construct was digested with EcoRV and NheI (both New England Biolabs). The resulting fragment was ligated to the NheI-digested promoter fragment (*xhrA*-5). The resulting vector was digested with EcoRV (New England Biolabs). The *pyrG* DNA fragment, after digestion with SmaI, was inserted between the two *xhrA* flanking regions. The resulting plasmid was verified by restriction analysis and sequencing. The deletion cassette, 5097 bp, containing the *xhrA* flanking regions and the *pyrG* gene, was released by MluI (New England Biolabs) digestion and transformed into the *A. niger* ATCC 1015 Δ*pyrG* strain. Transformants were selected based on their ability to grow in the absence of uracil. Strains with successful deletions were verified by PCR using the primers xhrA_ORF_F and xhrA_ORF_F (Table 2).

Deletion of the lxr4 Gene in T. reesei—A *T. reesei* Δ*tku70* strain was used as the parental strain for transformation (14, 15). The cassette for deletion of the *lxr4* gene (GenBank^{TM} accession number BK008566) contained 937 bp of the promoter region, 1162 bp of the terminator region, and a fragment containing the *pyr4* encoding orotidine-5′-phosphate decarboxylase. The promoter and terminator regions were obtained by PCR from the genomic DNA of the *T. reesei* strain QM9414 using primers Trire22771_XbaI-Ups-fw, Trire22771_XhoI-Ups-rev, Trire22771_XhoI-Dws-fw, and Trire22771_Acc-Dws-rev (Table 2) and the proofreading DNA polymerase Phusion (Finnzymes). Both fragments were ligated into the vector pBluescript SK(+) (Stratagene) after digestion with XbaI and XhoI (Fermentas) for the promoter fragment and with XhoI and Acc65I (Fermentas) for the terminator fragment using ligation mix III (Takara). The SalI fragment of *pyr4* served as the selection marker and was cloned via XhiI restriction between the promoter and terminator regions of *lxr4* in pBluescript SK(+). The resulting plasmid (verified by restriction analysis and sequencing) was linearized using XbaI and Acc65I and transformed into the *T. reesei* Δ*tku70* strain. Transformants were selected based on their ability to grow in the absence of uridine. Strains with successful deletions were verified by PCR using the primers dlxr4_for3 and dlxr4_rev3 (Table 2).

Reintroduction of xhrA into the ΔxhrA Strain—The *xhrA* gene, with its native promoter and terminator (2496-bp genomic fragment), was amplified from the *A. niger* ATCC 1015 genomic DNA using the primers xhrA-genomic_F and xhrA-genomic_R (Table 2). The PCR product was transformed into the Δ*xhrA* strain, and the transformants were selected on medium with galactitol as a sole carbon source. The resulting strains were tested for the presence of the *xhrA* gene by PCR and further analyzed for growth on selected carbon sources.

Expression of the Genes in Saccharomyces cerevisiae—For heterologous expression in *S. cerevisiae*, the open reading

[4] B. Seiboth, unpublished observation.

[5] The abbreviations used are: qPCR, quantitative PCR; TSP, 3-(trimethylsilyl) propionic-2,2,3,3-d4 acid.

TABLE 2
Primers

xhrA-genomic_F	GGCAAGACAACGGACTGAA
xhrA-genomic_R	AATTCCTGGTGATTCGGGTT
xhrA-ORF_F	ATATGAATTCACAATGTCCCTCAAAGGTAAAGTCG
xhrA-ORF_R	ATATGTCGACCTAGATATACAACATCCCACCATT
xhrA-N-HIS_F	ATATGAATTCACAATGCATCACCATCACCATCACGGGTCCCTCAAAGGTAAAGTCG
xhrA-5_F	TATAGCTAGCAGCTGAACGCCTGATACAAA
xhrA-5_R	ATATGATATCGATGGCTTTTGCAGATTGTTTG
xhrA-3_F	ATATGATATCCAGAGCCGTGTTAAATAAGGAATAC
xhrA-3_F	ATTAAAGCTTACGCGTACGAAGCCGCCGAAGATA
act_qPCR_F	CAACATTGTCATGTCTGGTGG
act_qPCR_R	GGAGGAGCAATGATCTTGAC
xhrA_qPCR_F	GATACAGATATGTACCAGGCAG
xhrA_qPCR_R	CTAGATATACAACATCCCACCA
galX_qPCR_F	CTGTGAAATGTTTGGGAAGTC
galX_qPCR_R	GTTTGTTGGTCGTTCGTAGG
pyrG-del-F_n	TATACCCGGGTGATTGAGGTGATTGGCGAT
pyrG-del-R_n	TATACCCGGGTTATCACGCGACGGACAT
lxr4-HIS-N_F	ATATGAATTCACAATGCATCACCATCACCATCACGGGGCCCGTCCGTATGAAGGC
lxr4_ORF_R	TAATGATATCCTACGCAATCGACATGCGCATCC
Trire22771_XbaI-Ups-fw	TCTAGACCATTGTCCCAGCCATCTT
Trire22771_XhoI-Ups-rev	CTCGAGCGACTTGAGCAATCACCAC
Trire22771_XhoI-Dws-fw	CTCGAGGATGTGTACTTGGTGGCTTG
Trire22771_Acc-Dws-rev	GGTACCTCTCTGCTCGTTAAATCCCG
dlxr4_for3	GGCGGAGTTTCTATGGAG
dlxr4_rev3	GGGATTGATATTGTTTGC

frames (ORFs) of *xhrA* (Table 1) and *lxr4* (GenBank accession number BK008566) were amplified from the cDNA of *A. niger* or *T. reesei*, respectively, grown in the presence of galactitol with the primers xhrA-H-HIS_F, xhrA-ORF_R, lxr4-HIS-N_F, and lxr4_ORF_R (Table 2). The genes were inserted into the plasmid pYX212 (Ingenius, R&D Systems, Madison, WI) between the EcoRI and SalI sites (in the case of *xhrA*) or the EcoRI and SmaI sites (in the case of *lxr4*), allowing the expression to be controlled by the *TPI1* promoter. All constructs were verified by sequencing.

The *S. cerevisiae* strain CEN.PK2-1D was transformed with the pYX212 plasmids containing the *xhrA* and *lxr4* genes as N-terminal His-tagged variants, and the transformants were selected based on their ability to grow in the absence of uracil. The expression of active reductases was tested in crude cell extracts by enzymatic activity measurements. The *xhrA* was also expressed without a His tag and as a gene that was codon-optimized for expression in *S. cerevisiae*. The gene was also expressed in the *Escherichia coli* strain BL21 (DH3) (Invitrogen) using the pBAT4 expression vector (16) and isopropyl-1-thio-β-D-galactopyranoside induction.

Protein Extraction, Enzyme Activity Measurements, and Analysis of the LXR4 Product—For L-*xylo*-3-hexulose-reductase activity measurements in *A. niger* extracts, the parent strain ATCC 1015 and the Δ*xhrA* strains were cultivated overnight in YPG medium (1% yeast extract, 2% Bacto peptone; 3% gelatin). The mycelia were filtered and transferred to fresh medium containing 1% yeast extract, 2% Bacto peptone, and 2% D-glucose, galactitol or L-arabinose and cultivated for 6 h. After incubation in the inducing conditions, the mycelia were isolated by filtration and washed with water, and ~200 mg of wet mycelia was transferred into 2-ml tubes with 0.6 ml of acid-washed glass beads (Sigma) and 1 ml of lysis buffer containing 50 mM Tris (pH = 7.5) and protease inhibitors (Complete, Roche Applied Science). The cells were disrupted in two 30-s breaking sessions in the Precellys 24 instrument (Bertin Technologies). The cell extracts were clarified by centrifugation, and the supernatants were used in the enzyme assays. The protein concentration was analyzed using the Bio-Rad protein assay kit. In the tests performed with the *A. niger* protein extracts, 10 mM L-*xylo*-3-hexulose, 0.5 mM NADPH, and 50 mM Tris-HCl (pH = 7.5) were used. The enzymatic activity was measured at room temperature by monitoring the NADPH disappearance at 340 nm in microtiter plates (Nunc) using the Varioskan spectrophotometer (Thermo Electron). For the protein extractions from *S. cerevisiae* and the purification of the His-tagged proteins, the same methods were used as described previously (8).

For LXR4 sugar reductase activities, the reactions were set up in 50 mM Tris-HCl (pH = 7.5) with 0.5 mM NADPH and various sugar concentrations (see "Results"). For the LXR4 polyol dehydrogenase activities, the reaction mixtures contained 100 mM Tris-HCl (pH = 8.5), 1 mM $NADP^+$, and various concentrations of polyols (see "Results"). The degradation of NADPH or formation of NADPH was monitored as described above. The K_m and V_{max} were estimated from the Michaelis-Menten equation fitted to the measured data.

The concentration of L-*xylo*-3-hexulose produced by *E. coli* expressing *ladB* was determined by NMR spectroscopy. 480 μl of D_2O containing 0.05% of 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid (TSP, Aldrich) was added to 120 μl of the sample, and a one-dimensional ^1H NMR spectrum was acquired on a 600-MHz Bruker Avance III NMR spectrometer equipped with a QCI CryoProbe (Bruker) using the one-dimensional NOESY pulse sequence for presaturation of the water signal. The concentration of the L-*xylo*-3-hexulose was obtained by comparing the integral over a region of 4.27–4.40 ppm with the integral of TSP.

The *in vitro* reaction with purified LXR4 for the analysis of the reaction product from D-sorbitol was carried out in 1 ml of reaction mix containing 50 μg of the purified LXR4, 100 mM D-sorbitol, and 5 mM $NADP^+$ in 100 mM Tris-HCl (pH = 9.0). The reaction mix was incubated at room temperature for 16 h. The control reaction was terminated immediately after the

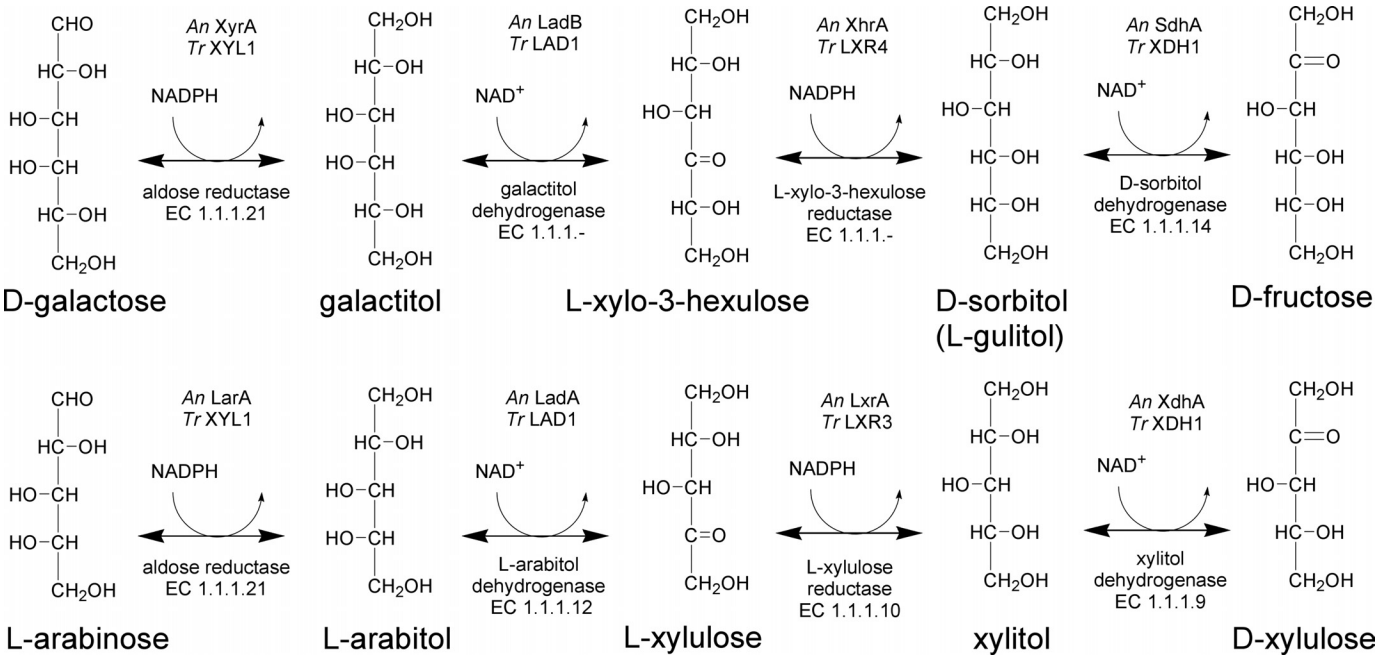

FIGURE 1. **The oxidoreductive D-galactose pathway (*upper part*) and the eukaryotic pathway for L-arabinose catabolism (*lower part*).** The metabolites are in Fischer projection except for the L-*xylo*-3-hexulose and L-xylulose, which are oriented so that the C6 and C5, respectively, are at the *top* to have all molecules in the same orientation. The enzymes for the different reactions in *A. niger* (*An*) and *T. reesei* (*Tr*) are indicated.

components were mixed by incubation at 95 °C for 10 min. The reactions were analyzed by HPLC as described previously (8).

RESULTS

Identification of the L-xylo-3-Hexulose Reductase in A. niger—The suggested oxidoreductive pathway for D-galactose catabolism has similarities with the eukaryotic L-arabinose pathway (Fig. 1). The reductions require NADPH, and the oxidations require NAD^+. The reactions that were described are catalyzed by identical or closely related enzymes. In *T. reesei*, the first and second steps of both pathways are catalyzed by the same enzymes. The *T. reesei* XYL1 is the major enzyme for L-arabinose and D-galactose reduction (6), and LAD1 is the main enzyme for galactitol and L-arabitol oxidation (9, 10). In *A. niger*, close homologues of different enzymes are used; XyrA is used for D-galactose reduction, and LarA is used for L-arabinose reduction (7). Also, the second step uses close homologues of different enzymes. Galactitol is oxidized in *A. niger* by LadB (8), and L-arabitol is oxidized in *A. niger* by LadA (17). This information suggested that a similar phenomenon may also exist for the third step. Thus, the L-*xylo*-3-hexulose reductase may be a homologue of the L-xylulose reductase or identical to the L-xylulose reductase. Because LxrA was identified as the L-xylulose reductase in *A. niger* (18), we examined whether this enzyme could also be the L-*xylo*-3-hexulose reductase.

The LxrA was purified using the histidine tag as described previously (18). The activity was tested with L-xylo-3-hexulose and NADPH and in the reverse direction with D-sorbitol and $NADP^+$. The enzyme had activity with these substrates, suggesting that this enzyme could be the L-xylo-3-hexulose reductase.

We tested the transcription of this gene by qPCR during growth on galactitol or D-galactose. The *lxrA* gene was not up-regulated under these conditions, suggesting that although the LxrA has L-*xylo*-3-hexulose reductase activity, it is not the "true" L-*xylo*-3-hexulose reductase. Also, the Δ*lxrA* strain does not confer any growth defect in the presence of galactitol when compared with the wild type strain (data not shown). We then tested the transcription of several close homologues to *lxrA* on galactitol or D-galactose, which are listed in Table 1. The closest *lxrA* homologue (E-value = 1.45×10^{-31}) JGI: 184209 (An16g01650) was found to be up-regulated on galactitol and D-galactose (Fig. 2*A*). We called this gene *xhrA* for L-*xylo*-3-hexulose reductase.

The *xhrA* gene was also up-regulated on galactitol and D-galactose in a strain where *ladB* was deleted with the up-regulation being significantly higher (Fig. 2*B*). The LadB converts galactitol to L-*xylo*-3-hexulose, and the *ladB* mutant cannot grow on galactitol (8). This suggests that galactitol, which probably accumulates in the Δ*ladB* strain and not L-*xylo*-3-hexulose, is required for the induction of *xhrA*.

A homologue of *galX* is located next to *xhrA* on the chromosome. The *galX* gene was identified in *A. nidulans* as the gene encoding a regulator that controls the D-galactose utilization, and it was shown to be up-regulated in the presence of galactitol and D-galactose (19). Likewise in *A. niger*, the transcription of *galX* was up-regulated in the presence of galactitol and D-galactose. In addition, *galX* transcription was further enhanced in the Δ*ladB* strain in a similar manner as observed in the case of *xhrA* expression (Fig. 2, *C* and *D*).

Identification of the L-xylo-3-Hexulose Reductase in T. reesei—In *T. reesei*, several homologues of L-xylulose reductase exist. The product of the *lxr1* gene tre74194 (www.jgi.doe.gov) that was described to be active with L-xylulose (20) turned out to be a D-mannitol dehydrogenase (21). Another candidate LXR3 (GenBank accession number BK008567), which was identified

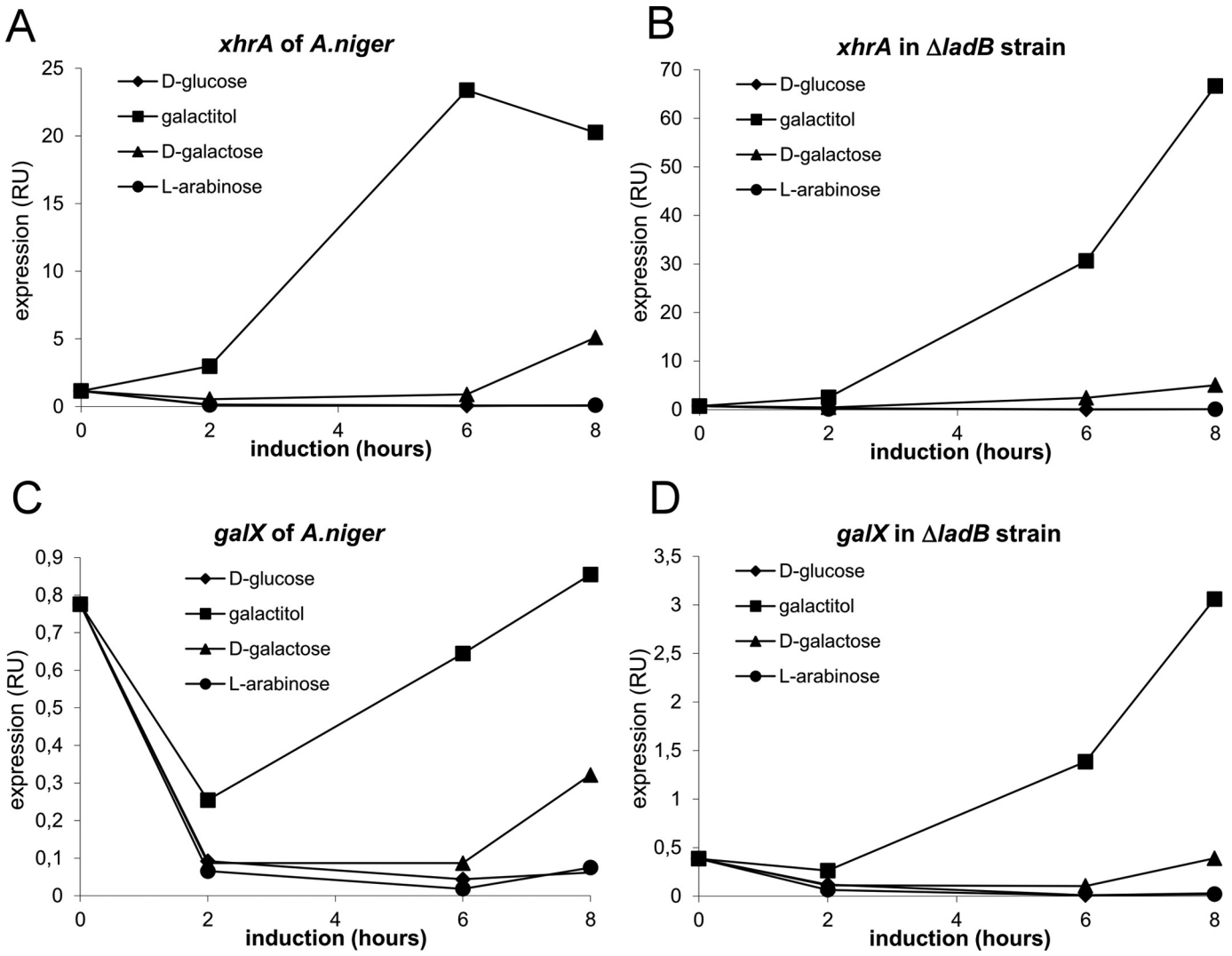

FIGURE 2. **Transcription profiles of the *xhrA* and *galX* genes in *A. niger*.** *A*, the *xhrA* gene is up-regulated in the presence of galactitol and to a lesser extent and with a delay on D-galactose. The expression on L-arabinose and D-glucose is not affected. *RU*, response units. *B*, the transcriptional activation of *xhrA* is further increased in the Δ*ladB* strain on galactitol. *C* and *D*, the *galX* encoding the transcription factor responsible for control of the D-galactose catabolic genes is up-regulated on galactitol (*C*), and its transcription is increased in the Δ*ladB* strain (*D*).

and characterized as a gene encoding the true L-xylulose reductase of *T. reesei*, showed only activity with L-xylulose but not with L-*xylo*-3-hexulose *in vitro*.[6] Moreover, none of these genes are close homologues of *xhrA*. By searching the *T. reesei* genome, we identified a gene that we called *lxr4* (GenBank accession number BK008566) as the closest orthologue for *lxrA*.

Deletion of the L-xylo-3-Hexulose Reductase Genes in A. niger and T. reesei—To test whether the *xhrA* is essential for the pathway, we deleted the gene in *A. niger*. The resulting strain was then tested for growth on different carbon sources and compared with the ATCC 1015 parent strain (Fig. 3). The mutant showed no growth on galactitol, demonstrating that the *xhrA* is an essential gene for its utilization. To prove that this phenotype is only related to the deletion of the *xhrA*, we retransformed the *xhrA* to the Δ*xhrA* strain. In the strain expressing the *xhrA* in the mutant background, growth on galactitol is fully restored (data not shown). The mutant and parent strain do not grow on D-galactose. Only when small amounts of D-xylose (0.025%) are supplemented does the parent strain grow, and the Δ*xhrA* strain showed reduced growth. 0.025% D-xylose alone does not result in significant growth as described previously (8). The growth on D-glucose and L-arabinose is not affected because the *xhrA* gene is not required for the metabolism of these sugars.

The deletion of *lxr4* in *T. reesei* resulted in no growth on galactitol. The phenotype of this mutant is similar to the *xhrA* deletion in *A. niger* (Fig. 3). Growth on galactitol was abolished, but growth on D-glucose and L-arabinose was not affected. Growth on D-galactose is significantly slower but not absent. This is expected because it is established that *T. reesei* has a functional Leloir pathway for D-galactose catabolism and that the oxidoreductive pathway only partially contributes to the D-galactose catabolism. The oxidoreductive pathway only becomes essential if the Leloir pathway is disrupted, for example, when the gene encoding the galactokinase is deleted (5).

[6] B. Metz, D. Mojzita, S. Herold, C. P. Kubicek, P. Richard, and B. Seiboth, manuscript in preparation.

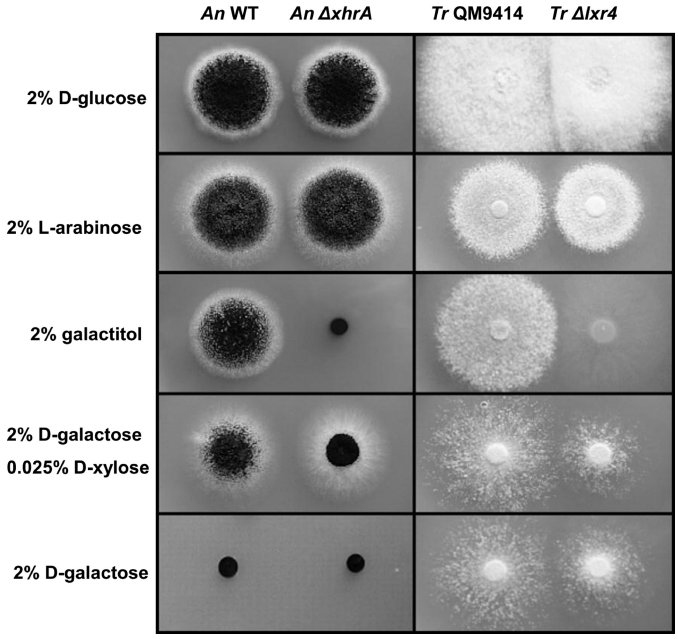

FIGURE 3. **Growth of the *A. niger* (*An*) and *T. reesei* (*Tr*) strains with and without a deletion of the gene encoding L-*xylo*-3-hexulose reductase on solidified medium containing different carbon sources.** Equal amounts of spores were applied and grown for 4 days at 28 °C.

Heterologous Expression of the L-xylo-3-Hexulose Reductase—The *xhrA* of *A. niger* and the *lxr4* of *T. reesei* were expressed in *S. cerevisiae* from a multicopy plasmid with a strong constitutive promoter. In the crude *S. cerevisiae* extracts, we could detect L-*xylo*-3-hexulose reductase activity when the *lxr4* was expressed, but not when the *xhrA* was expressed. We also tested the expression of a yeast codon-optimized version of the *xhrA* gene in yeast as well as the expression of cDNA in *E. coli* using the *lac* promoter and isopropyl-1-thio-β-D-galactopyranoside induction, but we were not able to detect L-*xylo*-3-hexulose reductase activity (data not shown).

Therefore, we used the *T. reesei* enzyme for the *in vitro* characterization of L-*xylo*-3-hexulose reductase. LXR4 was produced in *S. cerevisiae* as a recombinant N-terminally His_6-tagged protein and subsequently purified. The purified enzyme was NADP(H)-specific and conferred high and specific activity with L-*xylo*-3-hexulose with a $K_m = 2.0 \pm 0.5$ mM and a $V_{max} = 5.5 \pm 1.0$ units/mg (Fig. 4*A*). The enzyme was also active with D-ribulose and L-xylulose, $K_m = 47 \pm 3$ mM and $V_{max} = 14 \pm 2$ units/mg and $K_m = 22 \pm 3$ mM and $V_{max} = 4.2 \pm 1$ units/mg, respectively. The enzyme also showed some activity with D-xylulose and very low activity with D-fructose and L- and D-sorbose (Fig. 4, *B* and *D*). In the reverse reaction, LXR4 showed activity with D-sorbitol and D-mannitol, low activity with xylitol, and no activity with galactitol, ribitol, and L- and D-arabitol (Fig. 4, *C* and *D*).

L-xylo-3-Hexulose Reductase Activity in Crude Extracts of A. niger—Because we were not able to produce an active XhrA in a heterologous host, we tested whether this activity could be detected in *A. niger*. We made a crude cell extract from mycelia that were shifted to different carbon sources. Extracts from mycelia on D-glucose showed the lowest L-*xylo*-3-hexulose reductase activity. The activity was significantly higher on galactitol and L-arabinose, which is expected because *xhrA* and *lxrA*, respectively, are up-regulated under these conditions. In the *xhrA* deletion mutant, the activity is not increased on galactitol, indicating that the XhrA is mainly contributing to the L-*xylo*-3-hexulose reductase activity under these conditions. On L-arabinose, the activity is not decreased by the *xhrA* deletion. This activity is likely due to *lxrA*, which is up-regulated on L-arabinose and also has high L-*xylo*-3-hexulose reductase activity (Table 3).

L-xylo-3-Hexulose Is Produced from D-Sorbitol in the Reverse Reaction—To demonstrate that the L-*xylo*-3-hexulose reductase is catalyzing the conversion of L-*xylo*-3-hexulose to D-sorbitol, we tested the reverse reaction with D-sorbitol as the substrate and analyzed the reaction product by HPLC. The product of the reverse reaction of LXR4 with D-sorbitol and $NADP^+$ was identified as L-*xylo*-3-hexulose. It had the same retention time as the L-*xylo*-3-hexulose produced from galactitol using LadB (8) (Fig. 5) or LAD1 (not shown). D-Fructose has a different retention time and was not produced.

The L-*xylo*-3-hexulose that we had produced from galactitol (8) still contained large amounts of galactitol, which overlaps the D-sorbitol signal in the HPLC. This made it problematic to test whether in the forward reaction D-sorbitol was indeed produced from L-*xylo*-3-hexulose.

DISCUSSION

In this study, we identified an enzyme with activity for the conversion of L-*xylo*-3-hexulose to D-sorbitol using NADPH as a cofactor. Such an enzyme activity has not, to the best of our knowledge, been described previously. The enzyme is encoded by *xhrA* in *A. niger* and by *lxr4* in *T. reesei*, and deletion mutants are unable to grow on galactitol. This enzyme activity is the missing link in the oxidoreductive pathway for D-galactose catabolism that had been demonstrated to exist in filamentous fungi.

The oxidoreductive D-galactose pathway and the fungal L-arabinose pathway are very similar (Fig. 1). In both cases, the sequence of reactions is reduction, oxidation, reduction and oxidation. In both cases, the reductions are NADPH-linked, and the oxidations are NAD^+-linked. In *A. niger*, D-galactose is reduced by XyrA, and L-arabinose is reduced by LarA. In *T. reesei*, the same enzyme, XYL1, is used for the reduction of D-galactose and L-arabinose. In *A. niger*, the second step consisting of the oxidation of galactitol and L-arabitol is carried out by two different enzymes, LadB and LadA, respectively. Again, the same enzyme in *T. reesei*, LAD1, oxidizes both galactitol and L-arabitol. So far, it seems that in *A. niger*, two different pathways exist for D-galactose and L-arabinose, whereas in *T. reesei*, the enzymes of the L-arabinose pathway are also the enzymes of the D-galactose pathway. However, this pattern is different in the third step.

In *A. niger*, the oxidation of L-*xylo*-3-hexulose or L-xylulose is performed by two different but highly homologous enzymes, XhrA and LxrA, respectively. However, in this case, *T. reesei* also uses two different enzymes, LXR4 for the L-*xylo*-3-hexulose reduction and LXR3 for the L-xylulose reduction.[6] The LXR4 has the closest sequence similarity of the *A. niger* enzymes LxrA and XhrA in *T. reesei*. The LXR3, however, is

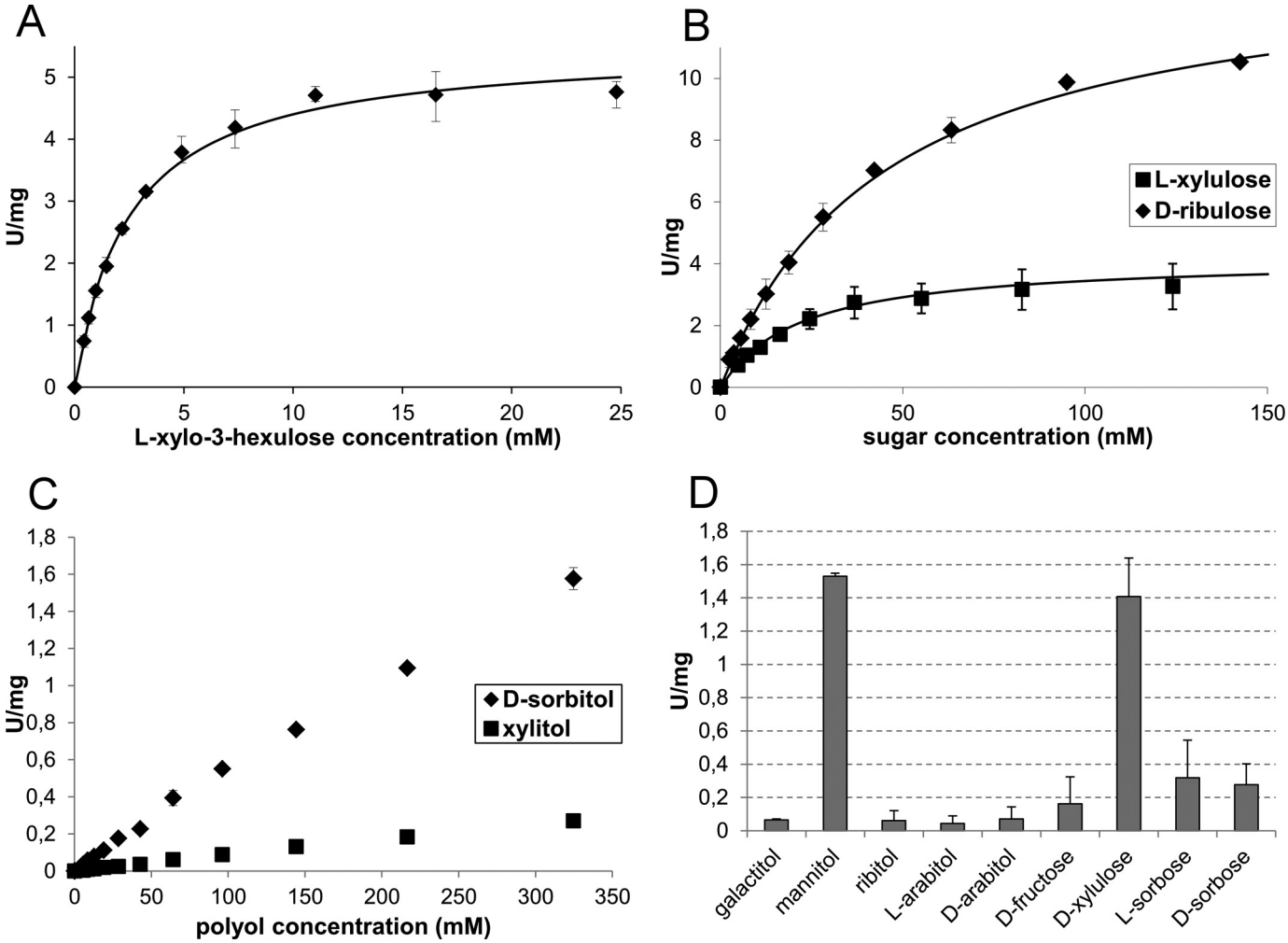

FIGURE 4. ***In vitro* activity of purified LXR4.** *A*, initial reaction rate at different L-*xylo*-3-hexulose concentrations to obtain the Michaelis-Menten-constants: $K_m = 2.0 \pm 0.5$ mM and $V_{max} = 5.5 \pm 1$ units/mg. *B*, initial reaction rates of L-xylulose and D-ribulose. The Michaelis-Menten constants are: $K_m = 22 \pm 3$ mM and $V_{max} = 4.2 \pm 1$ units/mg for L-xylulose and $K_m = 47 \pm 3$ mM and $V_{max} = 14 \pm 2$ units/mg for D-ribulose. *C*, the reverse reaction with the polyols D-sorbitol and xylitol. In the concentration range tested, the rate increased linearly with the substrate concentration, so the Michaelis-Menten-constants could not be determined. *D*, activity with other substrates. The reactions were carried out at room temperature at pH = 7.5 with 0.5 mM NADPH and 50 mM sugars unless otherwise specified. In the reverse direction, 300 mM polyols, 1 mM $NADP^+$, and pH = 8.5 were used. *Error bars* in *panels A–D* indicate S.D.

TABLE 3

L-*xylo*-3-hexulose activity in crude extracts of *A. niger*

The activities of the crude extract are given in milliunits/mg of extracted protein. The mycelia were pre-grown in YPG medium and then shifted for 6 h to YP medium supplemented with the 2% carbon sources indicated.

	A. niger (WT)	*A. niger* Δ*xhrA*
D-Glucose	7 ± 0.2	7 ± 0.8
Galactitol	25 ± 0.8	9 ± 1.0
L-Arabinose	42 ± 3.4	60 ± 2.3

more distant in terms of sequence similarity (supplemental Fig. S1), and it also confers considerably different substrate specificity. For example, it shows no activity with L-*xylo*-3-hexulose, unlike LxrA, whereas it is active with L-sorbose and D-fructose.

In the natural habitats of *T. reesei* and *A. niger*, D-galactose is often accompanied by pentose sugars, which indicates that both L-arabinose and D-galactose pathways are active simultaneously in such conditions. In *T. reesei*, only LXR4 is responsible for the conversion of L-*xylo*-3-hexulose, whereas in *A. niger*, XhrA and LxrA both contribute to the reaction.

Surprisingly, we could not obtain an active XhrA after heterologous expression, although the activity was detected in the *A. niger* crude extracts, and it was reduced in the *xhrA* deletion mutant. That we could not express the active XhrA in a heterologous host could be due to the protein instability, folding problems, or other issues that we did not pursue as we were able to analyze the *T. reesei* homologue (LXR4) *in vitro*.

The last step of the oxidoreductive D-galactose pathway is catalyzed by the D-sorbitol dehydrogenase, SdhA, which was identified in *A. niger*. This enzyme was shown to be part of the pathway because it is up-regulated on D-galactose and galactitol, and the Δ*sdhA* strain showed reduced growth on galactitol (12). In *A. niger*, the corresponding enzyme in the L-arabinose pathway is the xylitol dehydrogenase XdhA (17). In *T. reesei*, it is apparently a single enzyme, XDH1, that carries out the sorbitol dehydrogenase reaction and the xylitol dehydrogenase reaction. The enzyme is active with D-sorbitol and xylitol, and the *xdh1* is up-regulated on the carbon sources L-arabinose and D-galactose (22). Moreover, the Δ*xdh1* strain fails to grow on galactitol.[5]

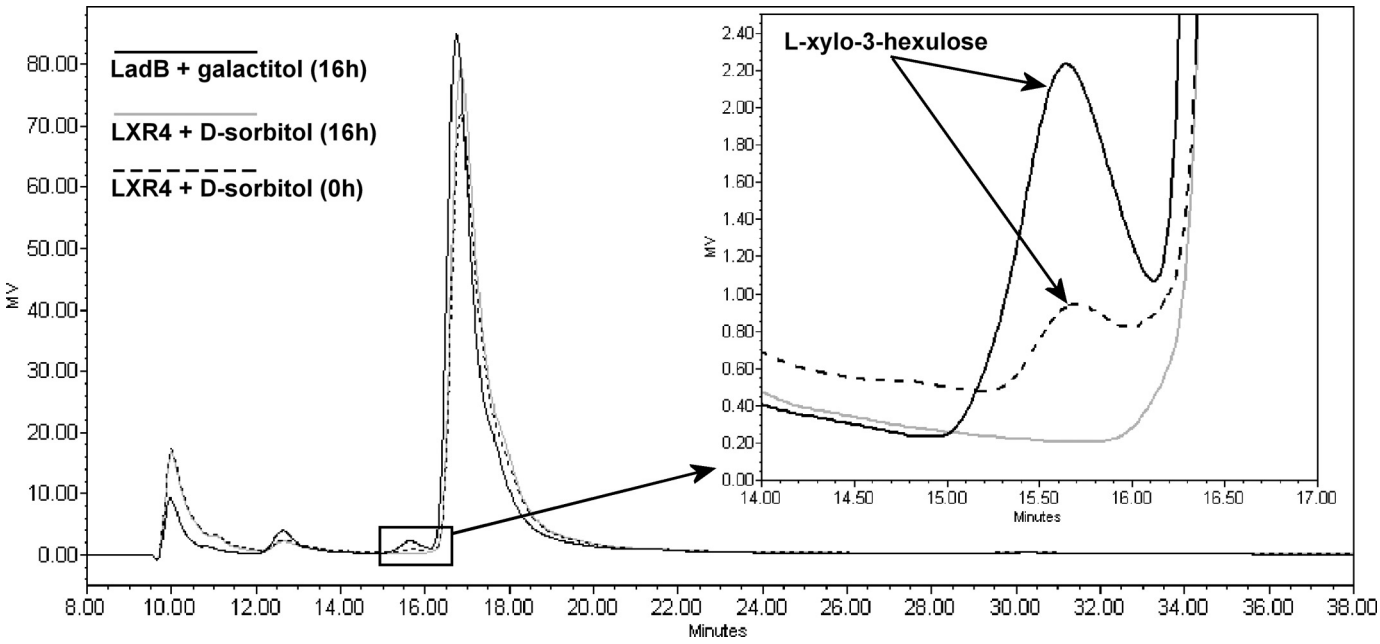

FIGURE 5. **HPLC elution profile of the reaction products of LadB and LXR4.** The reaction product formed from galactitol by LadB is L-*xylo*-3-hexulose. The reaction product that is formed from D-sorbitol by LXR4 has the same retention time, which is different from the retention time of D-fructose.

Although the regulation of the Leloir pathway has been studied and the regulation factors have been identified, not much is known about the regulation of the oxidoreductive pathway. We have suggested previously that galactitol might be the inducing compound for the genes of this pathway (8). There are four observations that support this. 1)The expression of *ladB* is up-regulated sooner and more strongly in the presence of galactitol than in the presence of D-galactose (8). 2) The up-regulation of the *sdhA* is still observed on galactitol in the Δ*ladB* strain when the pathway is blocked and the production of D-sorbitol, which is the main inducer of *sdhA,* is reduced or even absent (12). 3) The expression of the *xhrA* gene is significantly enhanced on galactitol in the Δ*ladB* strain when compared with the wild type strain (Fig. 2*B*). 4) The expression of the *galX* gene, which encodes for the transcription factor involved in the regulation of the Leloir pathways genes, and of the *ladB* gene (19) is significantly more expressed on galactitol in the Δ*ladB* strain (Fig. 2*C*).

In *A. nidulans*, the D-galactose catabolism and its regulation seem to be different from other filamentous fungi. In this fungus, the use of different D-galactose pathways is pH-dependent. The Leloir pathway is used between pH 4.0 and 6.5 and via some other route at pH = 7.5 (23). This other route showed oxidation to galactitol, but the subsequent steps were unidentified. It was suggested that the pathway proceeds via L-sorbose and D-sorbitol or even via sorbose 6-phosphate and D-tagatose 1,6-bisphosphate (24). *A. nidulans* has, in addition to GalX, an additional transcriptional regulator, GalR, which is unique among ascomycetes (19). When comparing the different *Aspergillus* species using the comparative analysis tool on the JGI *A. niger* v3.0 database (supplemental Fig. S2), *A. nidulans* does not have a close homologue of xhrA in the same location as the other *Aspergillus* species. However, it has a close homologue in a different place. The closest homologue to the xhrA is the gene with the identifier ANID_03400.1 (E-value = 2.93×10^{-41}).

Until recently, *A. niger* was considered unable to use D-galactose as a carbon source (25, 26). In the latest demonstrations of D-galactose utilization in *A. niger*, the oxidoreductive, but not the Leloir pathway, was shown to be employed, and the D-galactose utilization was enabled with the addition of a small amount of D-xylose (8, 12). Fekete *et al.* (27) recently suggested that the reason for the inability of *A. niger* to germinate on D-galactose is due to nonfunctional D-galactose uptake in the conidiospores, whereas the uptake is active in the mycelium. The authors showed that once the spores germinated on a different carbon source, the mycelium continued to grow on D-galactose; however, it was unable to sporulate. In addition, evidence of a functional Leloir pathway was presented. In the work of Fekete *et al.* (27), the strain N402 (28) was used. The strain ATCC 1015 that we used in our current and previous studies, nonetheless, behaved differently. The pregrown mycelium was not able to continue growth on D-galactose, but the strain can sporulate in its presence. In addition, the Leloir pathway is not active, and the growth on D-galactose is facilitated by the addition of a small amount of D-xylose but not D-glucose (8) (supplemental Fig. S3).

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