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Xyr1 (Xylanase Regulator 1) Regulates both the Hydrolytic Enzyme System and D-Xylose Metabolism in *Hypocrea jecorina*▽†

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Xyr1 (xylanase regulator 1) of the ascomycete *Hypocrea jecorina* (anamorph *Trichoderma reesei*) was recently demonstrated to play an essential role in the transcriptional regulation of the *xyn1* (xylanase 1-encoding) gene expression. Consequently, this study reports on the deletion of the *xyr1* gene from the *H. jecorina* genome. Comparative studies of the growth behavior of the different mutant strains (deleted and retransformed *xyr1*) grown on various carbon sources pointed to the strongly reduced ability of the *xyr1* deletion strain to utilize D-xylose and xylan. Transcriptional analysis of the *xyl1* (D-xylose reductase 1-encoding) gene as well as measurements of corresponding enzymatic activities gave evidence that Xyr1 takes part in the control of the fungal D-xylose pathway, in particular in the regulation of D-xylose reductase. It could be demonstrated that the uptake of D-xylose into the fungal cell is uninfluenced in the Δ*xyr1* strain. Furthermore, transcriptional regulation of the major hydrolytic enzyme-encoding genes *xyn1* and *xyn2* (xylanases 1 and 2), *cbh1* and *cbh2* (cellobiohydrolases 1 and 2), and *egl1* (endoglucanase 1) is strictly dependent on Xyr1. Regulation of the respective genes via Xyr1 is not affected by the substances mediating induction (xylose, xylobiose, and sophorose) and is indispensable for all modes of gene expression (basal, derepressed, and induced). Moreover, Xyr1, it was revealed, activated transcriptional regulation of inducer-providing enzymes such as β-xylosidase BXLI and β-glucosidase BGLI but was not shown to be involved in the regulation of BGLII.

Hypocrea jecorina (anamorph *Trichoderma reesei*) is a fungus of noteworthy industrial importance, mainly because of its employment in both fermentative production of native extracellular enzymes and heterologous protein production. Hydrolases secreted by this fungus have achieved broad areas of applications, e.g., in pulp and paper (4, 35, 50), food and feed (9, 27, 49), and textile (23, 26, 36) industries. The set of hydrolytic enzymes produced by *H. jecorina* comprises two main cellobiohydrolases, CBHI and CBHII (EC 3.2.1.91) (43); endo-β-1,4-glucanases EGI to EGV (EC 3.2.1.4) (37); 1,4-β-glucosidases BGLI and BGLII (EC 3.2.1.21) (8, 40); two major specific endo-β-1,4-xylanases, XYNI and XYNII (EC 3.2.1.8) (45); and one β-xylosidase, BXLI (EC 3.2.1.37) (17), to mention only the best characterized. This set of hydrolases is synergistically working together to attain a complete degradation of biopolymeric substrates, of which cellulose and xylan are predominant. In this particular breakdown process, these enzymes cause hydrolysis to smaller, soluble oligo- and monosaccharides which finally either act directly as low-molecular-weight inducer substances (e.g., xylobiose and xylose) (29, 53) or are converted to their respective inducers (e.g., sophorose) via the transglycosylation activity of some of these enzymes (46).

Whereas in *Aspergillus* spp. the xylanolytic and cellulolytic systems are strictly coregulated via the inducer xylose (10, 15), enzymes participating in the respective *T. reesei* hydrolytic systems are not. Their differential expression levels have been reported in several studies. Briefly summarizing these findings, all discussed hydrolytic genes are inducible by their respective degradation and/or transglycosylation products of xylan and/or cellulose, e.g., the *xyn1* (xylanase 1-encoding) gene is inducible by xylose (30), the *xyn2* (xylanase 2-encoding) gene by xylobiose and sophorose (53), and the *bxl1* (β-xylosidase 1-encoding) gene by xylobiose (33); cellulases such as the *cbh1* (cellobiohydrolase 1-encoding) gene, the *cbh2* (cellobiohydrolase 2-encoding) gene, and the *egl1* (endoglucanase 1-encoding) gene (20); and the *bgl1* (β-glucosidase 1-encoding) gene (8) and the *bgl2* (β-glucosidase 2-encoding) gene (40) by sophorose.

We recently reported that during xylose-mediated induction of *xyn1*, Xyr1 (xylanase regulator 1) plays a main role in *H. jecorina* (38). Xyr1 is a zinc binuclear cluster protein binding to a GGCTAA motif arranged as an inverted repeat in the *xyn1* promoter (38), closely resembling the consensus sequence for binding of the *Aspergillus niger* XlnR transactivator (48). XlnR is not only a central regulator protein responsible for activation of more than 10 genes involved in degradation of xylan and cellulose, it also contributes to the regulation of D-xylose metabolism (10, 15, 47).

Ancillary to Xyr1/XlnR-mediated induction, the carbon catabolite repressor Cre1/A has for both organisms been described as a wide domain repressor of particular hydrolase-encoding genes (6, 7, 21, 30). In *T. reesei*, only some of the major hydrolases, namely *cbh1* and *xyn1*, are under direct Cre1 control (21, 30), whereas other hydrolytic genes

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such as *cbh2*, *xyn2*, and *bgl1* are not Cre1 regulation dependent (31, 33).

In addition, the isolation of the two transcription factors Ace1 and Ace2, potentially involved in the regulation of hydrolase formation in *H. jecorina*, has been reported (2, 39). While the previously described repressor Ace1 (1) was proven to directly antagonize Xyr1 function by competing for one of its binding sites in the *xyn1* promoter (38), deletion of *ace2* was demonstrated to clearly reduce expression levels of the main cellulase genes and of the *xyn2* gene cultivated on cellulose but did not affect induction on sophorose (2). A more detailed study revealed that Ace2 contacts the xylanase-activating element XAE (essential for *xyn2* expression) in the *xyn2* promoter (52) but is not involved in *xyn1* transcription (2, 38). Up until now, no mechanisms involving respective orthologous regulators have been identified in the expression of *Aspergillus* hydrolases.

In this study, we report the deletion of *xyr1* from the *H. jecorina* genome. Strikingly reduced growth on D-xylose and restricted utilization of xylan by the *xyr1* deletion strain could be observed compared to that of the wild type. Consequently, we identified Xyr1 as a general and essential transcriptional activator of not only *xyn1* but also *xyn2*, *cbh1*, *cbh2*, and *egl1* gene transcription. Furthermore, Xyr1 was demonstrated to strictly control xylanolytic as well as cellulolytic enzyme formation under inducing and noninducing conditions in *H. jecorina*. Moreover, Xyr1 could be shown to regulate the gene expression of at least some inducer-providing enzymes, e.g., BGLI and BXLI. Finally, we have proven the involvement of Xyr1 in D-xylose metabolism, namely, its strong impact on the expression of D-xylose reductase activity. Summarizing, we revealed Xyr1 to govern the expression of the xylanolytic and cellulolytic enzyme system as well as D-xylose metabolism in *H. jecorina*.

MATERIALS AND METHODS

Strains and growth conditions. *H. jecorina* (anamorph *T. reesei*) QM9414 (ATCC 26921) was used as the parental strain throughout this study and as the recipient strain for the *amdS*-mediated transformation to the *xyr1* deletion strain. It was maintained on malt agar.

Analysis of growth on different carbon sources was performed using plates with Mandels-Andreotti (MA) medium (32) supplemented with 1% (wt/vol) of the corresponding carbon source and 2% (wt/vol) agar-agar at 30°C.

For replacement experiments, mycelia were precultured in 1-liter Erlenmeyer flasks on a rotary shaker (250 rpm) at 30°C for 18 h in 250 ml of MA medium, applying 1% (wt/vol) glycerol as the carbon source. Conidia (10^8 per liter, final concentration) were used as inocula. Pregrown mycelia were washed, and thereafter equal amounts were resuspended in MA medium containing 1% (wt/vol) oat spelt xylan (Sigma, Steinheim, Germany), carboxymethylcellulose (Calbiochem, San Diego, CA), and glucose or xylose as carbon sources. Mycelia were also transferred to MA medium without a carbon source (control) or to medium without a carbon source but supplemented with 2 mM sophorose or xylobiose as the respective inducer molecules. Incubation was continued for 8 and 24 h if mycelia were cultivated on xylan or carboxymethylcellulose; 3, 5, and 8 h if cultivated on xylose or xylobiose or on medium without a carbon source; 5 and 8 h if cultivated on sophorose; and 3 and 5 h if cultivated on glucose.

Direct cultivations were performed in 500-ml Erlenmeyer flasks with 100 ml MA medium containing 1% (wt/vol) xylan or carboxymethylcellulose and inoculated with 10^8 conidia per liter (final concentration). Incubation was performed for 24, 48, and 72 h at 30°C and 250 rpm.

Deletion and retransformation of *xyr1* from the *H. jecorina* genome. The plasmid containing the *xyr1* deletion cassette was constructed as follows. To generate EcoRI and Acc65I terminal sites of a *xyr1* gene fragment, pXR51.1 bearing the 4.3-kb *xyr1* gene (38) served as the template in a four-primer PCR mutagenesis strategy (22) by using overlapping primer pairs Xyr1.1.f and Xyr1.2.r (yielding a 1,021-bp fragment) and Xyr2.1.f. and Xyr2.2.r (yielding a 1,450-bp fragment); a full-length 2.5-kb fragment of *xyr1* was reamplified using the flanking primers Xyr1.1f and Xyr2.2.r. An iCycler (Bio-Rad, Hercules, CA) was used to run 30 cycles of 1 min at 95°C, 1 min at 57°C (59°C for the full-length fragment), and 1 min 30 s (2 min 30 s) at 72°C, applying a Taq2000 polymerase (Stratagene, La Jolla, CA). Primer sequences are given in Table 1. The 2.5-kb fragment was cloned into the pGEM-T vector (Promega, Wisconsin) according to the manufacturer's instructions to create pD0xlr1. In order to remove a SalI restriction site from pD0xlr1, the plasmid was cleaved at NotI/NdeI, treated with Sequenase version 2.0 (Amersham Biosciences, United Kingdom), and religated to obtain pD1xlr1. A SalI/EcoRI *amdS* gene fragment from the plasmid pamdS (pUC19 containing the *amdS* gene) from our department's stock and the SalI/EcoRI fragment of pD1xlr1 were ligated to yield pD2xlr1. For linear transformation, the deletion cassette (6.4 kb) was released from pD2xlr1 by Acc65I digestion. Transformation of *H. jecorina* QM9414 was carried out according to the optimized transformation protocol described in reference 13, using the *amdS* gene of *A. nidulans* (19) as the dominant selection marker. Retransformation of the obtained *xyr1* deletion strain was performed according to an optimized protocol for particle bombardment (16), applying cotransformation of pXR51.1 bearing the 4.3-kb *xyr1* gene (38) and pRLMex$_{30}$ (28) bearing the *hph* (encoding hygromycin B phosphotransferase) gene of *Escherichia coli*.

Southern blot analysis. Fungal genomic DNA was isolated as described previously (12). Southern hybridization was carried out as described in reference 41. Chromosomal DNA of the *xyr1* deletion strain was digested with NdeI, and the obtained blot was hybridized with the [α-^{32}P]dCTP-labeled 6.4-kb deletion cassette, whereas chromosomal DNA of the *xyr1* retransformants was digested with EcoRV, and the obtained blot was hybridized with the [α-^{32}P]dCTP-labeled 1.5-kb SacII fragment of *xyr1*.

Parallel DNA and RNA extraction and reverse transcription real-time PCR. Harvested mycelia were homogenized in 1 ml peqGOLD TriFast DNA/RNA/protein purification system (PEQLAB Biotechnologie, Erlangen, Germany) using a FastPrep FP120 BIO101 model ThermoSavant cell disrupter (Qbiogene, Carlsbad, CA). DNA and RNA were simultaneously isolated in a two-step process according to the manufacturer's instructions.

Synthesis of cDNA from mRNA was carried out by using a RevertAid H Minus First Strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions.

All PCRs were performed in an iCycler iQ real-time detection system (Bio-Rad). The software of the iCycler (iCycler iQ, optical system software, version 3.0a; Bio-Rad) was used to compile PCR protocols and define plate setups. All reactions were accomplished three times in 25-μl mixtures, including 1× iQ Supermix (Bio-Rad), 0.1 μM corresponding TaqMan probe (MWG, Ebersberg, Germany), 0.1 μM forward primer, 0.1 μM reverse primer, 1 mM $MgCl_2$ (used only for *xyn1* and *cbh1* real-time PCR), and as template, DNA or cDNA (100-fold dilution). Primers and probes are given in Table 1. Each run included a blank (sterile bi-distilled water instead of sample) and a no-amplification control (0.01% sodium dodecyl sulfate added to the reaction mixture). The following optimized (to an efficiency of at least 1.9) PCR protocols were followed: 3 min initial denaturation at 95°C, followed by 45 cycles of 15 s at 95°C, 15 s at 60°C (for real-time PCR *of xyr1*) or 59.8°C (for real-time PCR of *cbh1*, *cbh2*, and *egl1*) or 59°C (for real-time PCR of *xyn1* and *xyn2*), and 15 s at 72°C. For all reactions performed, the efficiency was calculated essentially as described in reference 44. Because of the high similarities of the respective PCR efficiencies, no correction factors had to be applied. The threshold level was set automatically to noise-to-signal-ratio conditions by the optical system software. The calculation of relative transcription levels was performed as follows. After choosing a reference sample (in all cases, the sample in which QM9414 mycelium was replaced with MA medium without a carbon source and incubated for 3 h was used), all cycle threshold (C_T) values for DNA samples were referred to the C_T values for the DNA of the reference sample to obtain a correction factor, f_{DNA}, for each DNA sample. Then, C_T values of cDNAs of all samples were corrected by dividing by the f_{DNA} factor. Corrected cDNA C_T values (C_T value$_{corr}$) were subtracted from the C_T values of cDNA of the reference sample to obtain ΔC_T value$_{corr}$. After establishing a relationship between the cDNA C_T values and the DNA C_T values of the reference sample, the differences between the initial amounts of cDNA were calculated. According to the equation $y = 10^{-1/k}$, and with y as the PCR efficiency ($y_{100\%\ efficiency} = 2$) and k as the slope ($k_{100\%\ efficiency} = -3.32$), the ΔC_T value$_{corr}$ was divided by 3.32 to obtain S (calculation factor). Ten was raised to the power of S, because initial target amounts differing in one decimal power should differ in their C_T values by about 3.32. This procedure allows describing differences in gene transcription not in C_T values but in amounts of DNA-corrected cDNA targets. Thus, results of transcription analysis are given in relative amounts of mRNA (cDNA) per gene dose.

TABLE 1. Primers and probes used throughout the study

Primer name	Sequence (5′–3′)	Employed for
Actf	TGAGAGCGGTGGTATCCACG	Actin real-time PCR
Actr	GGTACCACCAGACATGACAATGTTG	Actin real-time PCR
bgl1f	CAGACAGTCACTCAACATCGGG	*bgl1* real-time PCR
bgl1r	ACCTTATCTTGGAGATTGAGCTTTGCC	*bgl1* real-time PCR
bgl2f	ACGGCTGCCTACCAGATCG	*bgl2* real-time PCR
bgl2r	AGCCGTCGGCGATCTTGC	*bgl2* real-time PCR
bxl1f	GCCAACTTCGCCACCAAGG	*bxl1* real-time PCR
bxl1r	CGGCAATCTGGTGGATCAATGTG	*bxl1* real-time PCR
TaqMan cbh1 FAM	CTGGACTCCACCTACCCGACAAACGAGACC	*cbh1* real-time PCR
chb1f	GATGATGACTACGCCAACATGCTG	*cbh1* real-time PCR
cbh 1Df2	TGTTACAGTACTACGCCAACATGCTG	*cbh1* real-time PCR
cbh 1r	ACGGCACCGGGTGTGG	*cbh1* real-time PCR
TaqMan cbh2 TR	GACTTGCCGGATCGCGATTGCGCTGCCC	*cbh2* real-time PCR
cbh2f	CTATGCCGGACAGTTTGTGGTG	*cbh2* real-time PCR
cbh2Dr	GTCAGGCTCTGGAAGAAGG	*cbh2* real-time PCR
cbh2r	GTCAGGCTCAATAACCAGGAGG	*cbh2* real-time PCR
TaqMan egl1 FAM	CAACTCGAGGGCGAATGCCTTGACCCCTCACTC	*egl1* real-time PCR
egl1f	CTGCAACGAGATGGATATCCTGG	*egl1* real-time PCR
egl1Dr	GAGAGCGCCAGGAAAGGG	*egl1* real-time PCR
egl1r	GTAGTAGCTTTTGTAGCCGCTGC	*egl1* real-time PCR
Xorf	CTGTGACTATGGCAACGAAAAGGAG	*xyl1* real-time PCR
Xorr	CACAGCTTGGACACGATGAAGAG	*xyl1* real-time PCR
TaqMan xyn1 FAM	CGTCCAACCAACGCCCACAACAA	*xyn1* real-time PCR
Taqxyn1f	CAGCTATTCGCCTTCCAACAC	*xyn1* real-time PCR
Taqxyn1Dr	GAGGAGTCCTCCTACGCAGAA	*xyn1* real-time PCR
Taqxyn1r	CCAAAGTTGATGGGAGCAGAA	*xyn1* real-time PCR
TaqMan xyn2 FAM	CTGCCATCCCTTGCCGCC	*xyn2* real-time PCR
Taqxyn2f	GGTCCAACTCGGGCAACTTT	*xyn2* real-time PCR
Taqxyn2Dr	GGTAAGGGTAGGTAGTCTTACTTGTTC	*xyn2* real-time PCR
Taqxyn2r	CCGAGAAGTTGATGACCTTGTTC	*xyn2* real-time PCR
TaqMan xyr1 FAM	CGCGCTTGTGACCAGTGCAACCAGCTTCGTACC	*xyr1* real-time PCR
xyr1f	CCCATTCGGCGGAGGATCAG	*xyr1* real-time PCR
xyr1Dr	GATCAGTACATGTGCTTGAGCGC	*xyr1* real-time PCR
xyr1r	CGAATTCTATACAATGGGCACATGGG	*xyr1* real-time PCR
Xyr1.1.f	GGTACCAATTGTGAGCGCATCAC	Construction of deletion cassette
Xyr1.2.r	CTGTCGACGATGGGAATTCGGGTCAAATGAC	Construction of deletion cassette
Xyr2.1.f	CCGAATTCCCATCGTCGACAGGCACCTGG	Construction of deletion cassette
Xyr2.2.r	GGTACCGAACATAGCCCAACG	Construction of deletion cassette

Enzyme assays. D-Xylose reductase activity and L-arabinose reductase activity were measured as described previously (5). One unit of activity is defined as the amount of enzyme responsible for the consumption of 1 micromole NADPH per minute due to its oxidation in the presence of D-xylose or L-arabinose under the defined assay conditions. Cell extract for the above-mentioned enzyme assay was prepared as described previously (42).

Xylanase activity was measured by applying Xylazyme AX tablets (Megazyme, Wicklow, Ireland) according to the manufacturer's instructions. One unit of activity is defined as the amount of enzyme required to release 1 micromole of xylose reducing sugar equivalents per minute under the defined assay conditions.

Cellulase activity was measured using the dinitrosalicylic acid method according to Miller (34). One unit of activity is defined as the amount of enzyme required to release 1 micromole of glucose reducing sugar equivalents per minute under the defined assay conditions.

The activity of β-glucosidase was assayed as described by Kubicek (25), using *p*-nitrophenyl-β-D-glucoside as substrate. One unit of activity is defined as the amount of enzyme required to release 1 micromole of glucose reducing sugar equivalents per minute under the defined assay conditions.

The activity of β-xylosidase was assayed as described previously (24), using *p*-nitrophenyl-β-D-xylopyranoside as substrate. One unit of activity is defined as the amount of enzyme required to release 1 micromole of xylose reducing sugar equivalents per minute under the defined assay conditions.

Determination of fungal growth on xylan. Harvested mycelia were suspended in 1 ml 0.1 N NaOH in a reaction tube with a screw cap (2 ml; Brand GmbH+Co KG, Wertheim, Germany). Glass beads (0.37-g diameter, 0.1 to 0.01 mm; 0.25-g diameter, 1 mm; glass bead diameter, 3 mm) (Braun Biotech International GmbH, Melsungen, Germany) were added to each mixture. This suspension was solubilized in a Teflon homogenizer (FastPrep 120 BIO101 model, Savant Instruments, Holbrook, NY). After samples were incubated at room temperature for 3 hours, they were centrifuged at 14,000 rpm at 4°C for 10 min. Protein concentration was determined via Bio-Rad protein assay (Bradford) reagent according to the manufacturer's guidelines.

Determination of D-xylose uptake. QM9414 and Δ*xyr1* mycelia were pregrown as described above and transferred to 5 ml phosphate buffer (20 mM, pH 5) containing 2 mM D-xylose pulsed with $2 \cdot 10^{-6}$ mM [^{14}C]D-xylose (1.85 MBq/250 μl) (Amersham Biosciences, United Kingdom). After an incubation time of 20 min at 30°C, 0.1 M D-xylose was added to a final concentration of 50 mM. Mycelia were harvested, washed thoroughly, and measured in an LKB Wallac model 1219 Rack-beta (PerkinElmer, Boston, MA) scintillation counter.

RESULTS

Deletion of the *xyr1* gene of *H. jecorina*. To delete *xyr1* (GenBank accession no. AF479644) from the *H. jecorina* genome, transformation was performed with a deletion cassette containing the *xyr1* up- and downstream regulatory regions interrupted by the *amdS* gene of *A. nidulans* (GenBank accession no. M16371), thereby replacing the *xyr1* encoding region and yielding two mitotically stable transformants. Retransformation of *xyr1* was carried out via cotransformation using the full-length *xyr1* gene (38) and the pRLMex$_{30}$ vector, conferring

TABLE 2. Growth of the parental strain and strains Δ*xyr1* and Re*xyr1* on plates containing MA medium supplemented with different carbon sources[a]

Carbon source	Avg mycelial growth zone diam (cm)[b]					
	After 3 days			After 6 days		
	QM9414	Δ*xyr1*	Re*xyr1*A	QM9414	Δ*xyr1*	Re*xyr1*A
Glucose	3.7 ± 0.29	3.9 ± 0.17	3.5 ± 0.19	8.0 ± 0.00	8.0 ± 0.00	8.1 ± 0.04
Glycerol	3.0 ± 0.15	3.4 ± 0.00	3.2 ± 0.12	7.2 ± 0.35	7.1 ± 0.10	7.2 ± 0.12
L-(+)-Arabinose	3.3 ± 0.10	3.5 ± 0.21	3.5 ± 0.17	7.4 ± 0.12	7.5 ± 0.40	7.3 ± 0.15
L-(−)-Arabitol	3.1 ± 0.17	3.7 ± 0.06	3.3 ± 0.08	7.7 ± 0.15	7.7 ± 0.12	7.6 ± 0.22
D-(+)-Xylose	4.1 ± 0.23	1.1 ± 0.10	4.2 ± 0.21	8.0 ± 0.00	2.4 ± 0.06	8.2 ± 0.16
Xylitol	3.3 ± 0.12	3.5 ± 0.06	3.5 ± 0.12	7.5 ± 0.06	7.5 ± 0.00	7.6 ± 0.17
Xylan	2.0 ± 0.17	2.5 ± 0.12	2.2 ± 0.19	5.5 ± 0.38	4.6 ± 0.12	5.6 ± 0.16
Cellulose	2.8 ± 0.17	3.3 ± 0.17	3.0 ± 0.14	5.7 ± 0.10	6.0 ± 0.00	5.9 ± 0.11

[a] MA medium (1% wt/vol) cultured at 30°C.
[b] Values are the means ± SD of results from three independent experiments.

hygromycin B resistance, and yielding seven strains. Southern blot analysis revealed the deletion of *xyr1* and the insertion of one to three copies of *xyr1* on ectopic loci (data not shown). Transcriptional analysis via real-time PCR indicated the absence or regain of *xyr1* transcript in all deletion or retransformation strains, respectively. It should be noted that transcript levels of the *xyr1* gene did not vary importantly in the retransformant strains compared to those in the wild type (data not shown). The Δ*xyr1* strain showed similar growth rates on malt extract and synthetic medium supplemented with different low-molecular-weight carbon sources, with the exception of D-xylose (Table 2), compared to those of the QM9414 parental strain. In all cases tested, neither conidiospore formation on agar plates nor germination times differed between the mutant and the parental strain.

Xyr1 controls D-xylose reductase activity. To examine the influence of Xyr1 on the utilization of different carbon sources, we conducted a series of growth experiments on agar plates. In detail, growth of the parental strain and of respective deletion and retransformation strains on plates containing glucose, glycerol, L-arabinose, L-arabitol, D-xylose, xylitol, xylan, and cellulose were observed for 6 days (Table 2). While the parental strain and the *xyr1* retransformation strain (data are shown for one strain, Re*xyr1*A) were able to utilize all carbon sources investigated, the Δ*xyr1* strain showed strongly reduced growth on D-xylose as the sole carbon source (Fig. 1A). As is known from the D-xylose pathway, in the first step of D-xylose metabolism, D-xylose reductase converts D-xylose into xylitol. Xylitol is reduced to D-xylulose and then goes into the pentose phosphate pathway (Fig. 1B). In contrast to D-xylose, when applying xylitol (next intermediate of the D-xylose utilization pathway) as the sole C source, no growth differences between the parental and the Δ*xyr1* strains could be observed (Fig. 1A). As fungi are not able to reduce D-xylose by means of a xylose isomerase (51), D-xylose is converted into xylitol via D-xylose reductase in *H. jecorina*. Consequently, the reg-

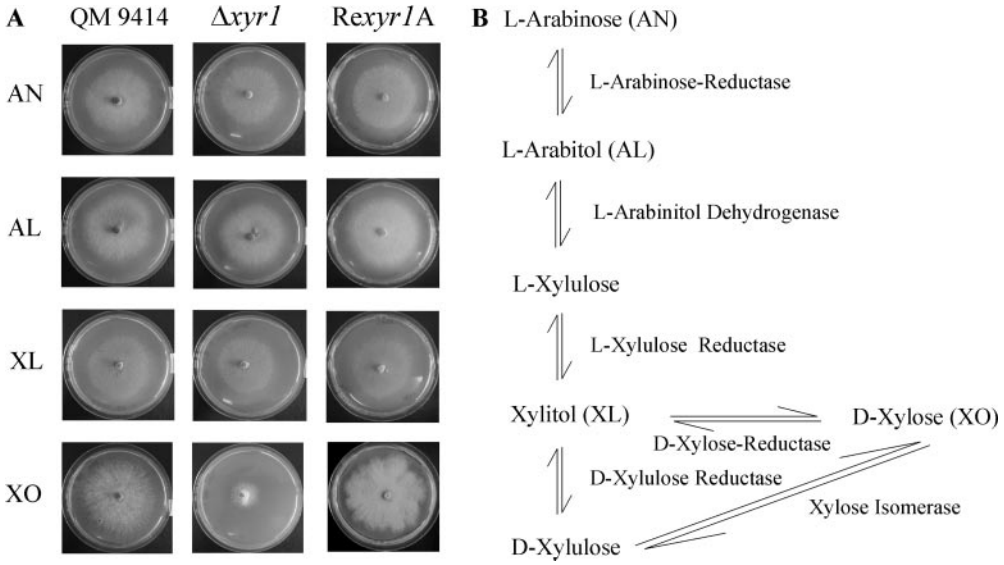

FIG. 1. (A) Growth of parental strain QM9414, the *xyr1* retransformation strain (Re*xyr1*A), and the Δ*xyr1* strain on plates containing MA medium supplemented with 1% (wt/vol) L-arabinose (AN), L-arabitol (AL), xylitol (XL), and D-xylose (XO) and 2% (wt/vol) agar-agar at 30°C after 4 days. (B) Schematic presentation of the D-xylose catabolic pathway. In *H. jecorina*, D-xylose cannot be directly converted into D-xylulose because fungi do not have xylulose isomerase at their disposal (51).

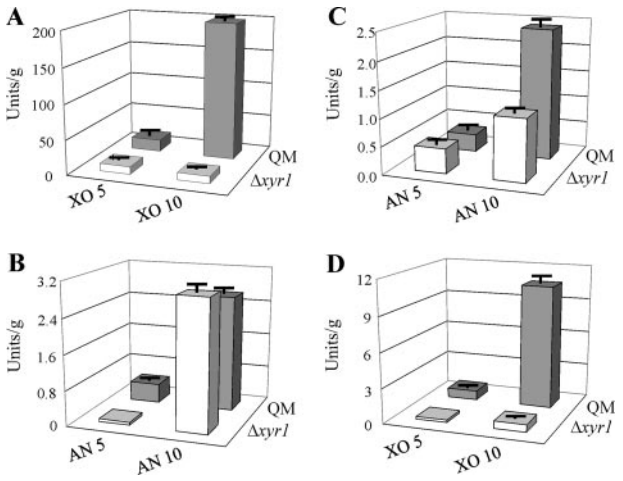

FIG. 2. Enzyme activities of D-xylose reductase (A and C) and L-arabinose reductase (B and D) given in U/g, measured in cell extracts of QM9414 (QM) and the Δ*xyr1* strain. All strains were precultured in MA medium containing 1% (wt/vol) glycerol. For analysis of both reductase activities, mycelia were transferred to MA medium containing 1% (wt/vol) D-xylose (XO) (A and D) and L-arabinose (AN) (B and C) and grown for 5 and 10 h. One unit of activity is defined as the amount of enzyme responsible for the consumption of 1 micromole NADPH per minute due to its oxidation in the presence of D-xylose or L-arabinose under the defined assay conditions (5). Values are the means of results from three independent experiments. Error bars indicate standard deviations.

ulatory influence of Xyr1 on this enzyme system was examined further.

Transcriptional analysis via real-time PCR of the parental and Δ*xyr1* and *xyr1* retransformation strains replaced on D-xylose as the carbon source showed the *xyl1* (D-xylose reductase-encoding) transcript to be absent in the Δ*xyr1* strain under all conditions tested. A regain of D-xylose-dependent *xyl1* transcript formation was observed for the retransformation strains for all time points (5 or 10 h of cultivation) (data not shown). Measuring D-xylose reductase activities in cell extracts also provided evidence for a regulatory impact of Xyr1 on D-xylose reductase, since strongly reduced activities could be detected from the deletion strain (Fig. 2A).

To investigate whether D-xylose reductase could at least partially be replaced by L-arabinose reductase, both reductase activities were analyzed using cell extracts of mycelia replaced with either D-xylose or L-arabinose. Measurements of L-arabinose reductase activity in cell extracts of mycelia of both strains grown on L-arabinose showed similar activities (Fig. 2B). We therefore conclude that L-arabinose reductase is not or only weakly affected by Xyr1. D-Xylose reductase activities could also be detected in the parental strain and to a lesser extent in the Δ*xyr1* strain when replaced with L-arabinose (Fig. 2C) and vice versa (Fig. 2D). These findings indicate that those two enzymes are at least partially able to substitute for one another in *H. jecorina* in their activities, thereby explaining the weak residual growth of the deletion strain on D-xylose plates (Table 2).

Transport of D-xylose into the cell is not influenced by Xyr1. Results described above demonstrate that Xyr1 has a major regulatory influence on D-xylose reductase, whereas only negligible to no effects on the downstream enzymes of the D-xylose pathway can be deduced. Summarizing this set of data, it still remains unclear whether the deletion of *xyr1* additionally causes an inhibition of the transport of D-xylose into the cell. Therefore, the parental and the deletion strains were precultivated on glycerol and thereafter transferred into a D-xylose-containing medium spiked with [^{14}C]D-xylose. Measuring the mycelia after a 20-min incubation in a liquid scintillation counter gave 13,400 cpm/g dry weight for the Δ*xyr1* strain and 12,700 cpm/g dry weight (both values are means from the results of three independent experiments) for the wild type, indicating that the uptake of D-xylose into the cell is uninfluenced by Xyr1.

Xyr1 regulates the transcription of *xyn1*, *xyn2*, *cbh1*, *cbh2*, and *egl1*. The fact that a GGCTAA palindrome within the *xyn1* promoter is bound by Xyr1 under inducing and noninducing conditions, as recently shown (38), prompted us to examine *xyn1* transcript formation in the respective mutant strains. After precultivation, the parental and the Δ*xyr1* strain were transferred to medium without a carbon source or medium containing either glucose, D-xylose, or xylan as the sole carbon source and incubated 3 and 5 h or 3, 5, and 8 h or 8 and 24 h, respectively. After parallel DNA and RNA extraction, followed by cDNA synthesis, transcription levels were analyzed via real-time PCR. Whereas the parental strain showed transcript formation on xylan and on D-xylose, both carbon sources already known to activate *xyn1* expression (30), no *xyn1* transcript could be detected from the Δ*xyr1* strain under those conditions (Table 3). No transcript formation occurred from any strain on glucose, a carbon source known to completely repress *xyn1* transcription (30).

Consequently, *xyn2* transcription in both strains replaced with medium either without a carbon source or with xylan or

TABLE 3. Relative *xyn1* transcription levels of the wild-type and the Δ*xyr1* strains

Carbon source (cultivation time, h)	Amt of mRNA (cDNA) per gene dose[a]	
	QM9414	Δ*xyr1*
No carbon source (3)	1.0 ± 0.5	ND[b]
No carbon source (5)	0.5 ± 0.1	ND
No carbon source (8)	0.4 ± 0.2	ND
Glucose (3)	ND	ND
Glucose (5)	ND	ND
Xylose (3)	1.9 ± 0.4	ND
Xylose (5)	169.3 ± 16.5	ND
Xylose (8)	0.0 ± 0.0	ND
Xylan (8)	16.1 ± 2.6	ND
Xylan (24)	9.8 ± 0.0	ND
No carbon source (3)	1.0 ± 0.1	ND
No carbon source (5)	3.8 ± 0.5	ND
No carbon source (8)	ND	ND
Sophorose (5)	97.9 ± 9.6	ND
Sophorose (8)	6,701.7 ± 531.1	ND
Xylobiose (3)	6,085.9 ± 637.1	ND
Xylobiose (5)	265.6 ± 11.2	ND
Xylobiose (8)	37.5 ± 3.2	ND
Xylan (8)	4,305.9 ± 415.3	ND
Xylan (24)	3,819.4 ± 277.6	ND

[a] Values are the means ± SD of results from three independent experiments.
[b] ND, no detection.

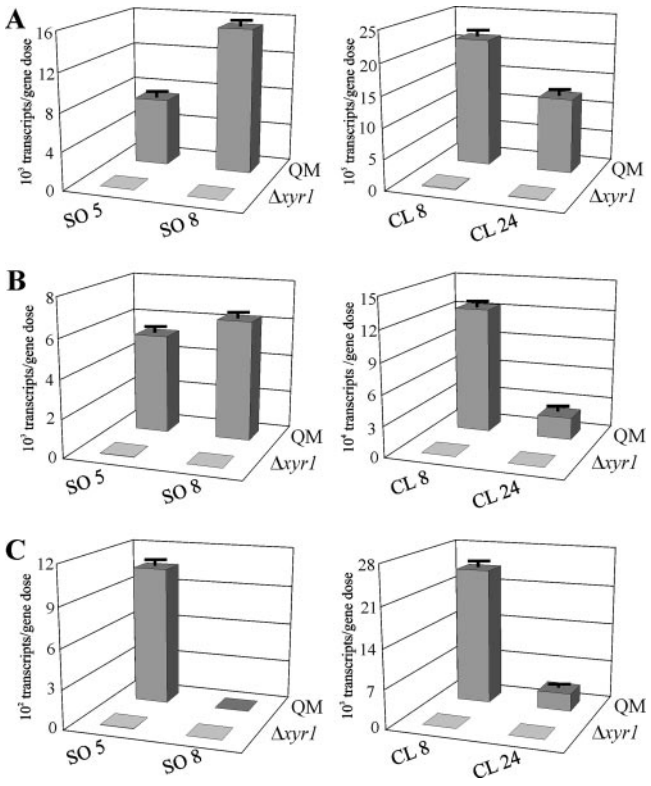

FIG. 3. Relative transcription levels of *cbh1* (A), *cbh2* (B), and *egl1* (C). The parental strain QM9414 (QM) and the Δ*xyr1* strain were precultured with glycerol and thereafter transferred to MA medium containing 2 mM sophorose (SO) as inducer or 1% (wt/vol) cellulose (CL) as the sole carbon source and incubated 5 and 8 h or 8 and 24 h, respectively. After parallel extraction of RNA and DNA and reverse transcription of RNA, real-time PCR analysis of the respective genes was performed. Values are the means of results from three independent experiments. Error bars indicate standard deviations.

with a medium containing 2 mM sophorose or xylobiose as inducers was examined, and similar results were found. On the activating carbon source xylan and the inducing compounds sophorose and xylobiose, *xyn2* transcription in the parental strain QM9414 was strongly induced, as previously published (53), but no *xyn2* transcript arose from the Δ*xyr1* strain (Table 3). In contrast to the parental strain, from which small amounts of transcript could also be detected when replaced with medium without a carbon source (Table 3), no *xyn2* transcript was formed by the Δ*xyr1* strain (Table 3), strongly indicating that Xyr1 is also involved in the regulation of the previously described carbon source independent (basal) transcriptional level of *xyn2* (52, 53).

To ascertain whether Xyr1 also influences transcription of cellulase-encoding genes, we accordingly determined *cbh1* (Fig. 3A), *cbh2* (Fig. 3B), and *egl1* (Fig. 3C) transcription levels, all of which gave similar results. Under activating conditions applying either 2 mM sophorose as the inducer or cellulose as the carbon source, no transcripts of any of those three genes were observed for the Δ*xyr1* strain, although the parental strain showed high amounts of transcript formation, which is in strict accordance with previous publications (20) (Fig. 3).

Role of Xyr1 in the regulation of the expression of xylanolytic and cellulolytic enzyme system. To investigate whether the transcriptional regulation of the above-mentioned genes via Xyr1 can be extended to a general influence on xylanolytic and cellulolytic enzyme activity formation in *H. jecorina*, xylanase activity was measured in supernatants of cultivations after transferring equal amounts of mycelia to media with corresponding carbon sources. Xylanase activity from the parental strain could be detected either when the low-molecular-weight inducers sophorose or xylobiose were applied or during growth on D-xylose. Very high activities were formed if the strain was grown on xylan or cellulose, which is in accordance with former results (18) (Fig. 4). Only marginal but still detectable activity could be obtained from the parental strain grown on medium without a carbon source after 8 h, due most probably to derepression of *xyn1* expression (30). In contrast, no xylanase activity was yielded from the Δ*xyr1* strain under any conditions tested (Fig. 4). This finding perfectly fits the observation that the cultivation of the Δ*xyr1* strain on xylan plates led to no distinct clearing zone formation due to xylanolytic activity (data not shown), indicating that the strain is no longer able to degrade the D-xylose backbone of xylan. The remaining residual growth on xylan plates (Table 2) is most likely due to the utilization of the carbon sources comprising the side groups linked to the D-xylose backbone of xylan. The oat spelt xylan used during this study (Sigma, Steinheim, Germany) contains approximately 10% arabinose and 15% glucose residues, according to the manufacturer.

Obtained results strongly point to Xyr1 as the general regulator of the xylanolytic and cellulolytic enzyme system of *H. jecorina*. This assumption is further affirmed by measuring enzyme activities in supernatants of direct cultivations of longer time periods. To this end, both strains were directly cultivated on xylan and cellulose in a time course experiment (24, 48, and 72 h). As expected, the parental strain showed formation of xylanase as well as cellulase activity after 48 and 72 h on the respective carbon sources, whereas the Δ*xyr1* strain never formed those enzyme activities (Table 4).

In order to exclude the possibility that the deletion of *xyr1* leads to the formation of inactive enzymes, Western blot analysis of supernatants of the replacement experiment and the direct cultivation of both strains were performed, applying monoclonal antibodies against CBHI, CBHII, XYNI, and XYNII. Distinct bands from supernatants of strain QM9414, applying 2 mM sophorose or xylobiose as the respective inducers for 8 h and from cellulose or xylan cultures grown for 48 h and 72 h, could be obtained, but no bands appeared from supernatants of the Δ*xyr1* strain (data not shown).

In summary, we conclude that Xyr1 is the general regulator of the xylanolytic and cellulolytic enzyme activity formation in *H. jecorina*, independent of whatever inducer or inducing carbon source is used.

Involvement of Xyr1 in the regulation of *bgl1*, *bgl2*, and *bxl1*. As already mentioned, hydrolytic enzyme-encoding genes are inducible by their respective degradation/transglycosylation products, e.g., xylose, xylobiose, and sophorose. Since the activation of expression of those genes is dependent on Xyr1, we wondered if enzymes partaking in providing those inducers, such as BGLI, BGLII, and BXLI (11, 17, 40, 46), are also affected by Xyr1. By examination of the transcriptional levels of *bgl1*, *bgl2*,

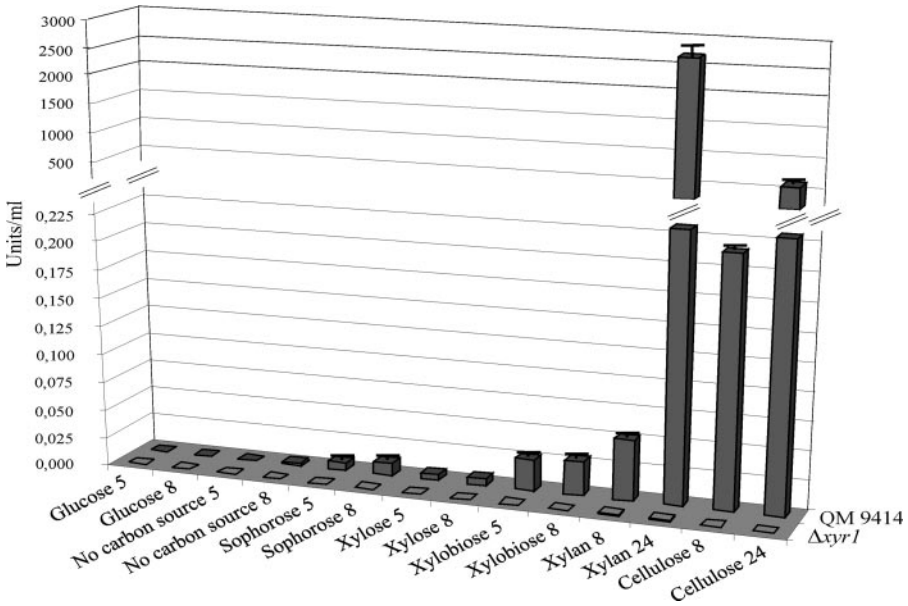

FIG. 4. Xylanase activities in culture supernatants of the parental strain QM9414 and the Δ*xyr1* strain. Both strains were precultured in MA medium containing 1% (wt/vol) glycerol. Detection of xylanase activity formation was accomplished after transfer of equal amounts of wet weight of mycelia to MA medium without a carbon source or to medium containing 2 mM sophorose or xylobiose as inducer and incubated for 5 and 8 h, or to medium containing 1% (wt/vol) D-xylose or glucose grown for 5 and 8 h, or to MA medium containing 1% (wt/vol) oat spelt xylan or carboxymethylcellulose and grown for 8 and 24 h. One unit of activity is defined as the amount of enzyme required to release 1 micromole of xylose reducing sugar equivalents per minute at 40°C. Data are the means of results from three independent experiments. Error bars indicate standard deviations.

and *bxl1* from glycerol pregrown mycelia replaced with medium containing 2 mM sophorose or xylobiose as inducers and incubated for 8 or 5 h, respectively (Table 5), and by determination of corresponding enzyme activities (Fig. 5A and B), we found that Xyr1 strictly regulates *bgl1* and *bxl1* expression (Table 5; Fig. 5A and B) but is not involved in the activation of *bgl2* expression (Table 5).

DISCUSSION

In the absence of easily utilizable carbon sources, saprophytes such as *H. jecorina* are able to metabolize heterogeneous polysaccharides. The naturally high secretory capacity of this fungus was further improved and recently led to *H. jecorina* strains secreting up to 100 g/liter of extracellular protein (M. Ward, personal communication). A set of its native enzymes allows degradation of xylan to smaller saccharides and finally to monomeric D-xylose (3), which can enter the fungal cells and acts as an inducer (30). In this study we report the effects of the deletion of the *xyr1* gene from the *H. jecorina* genome with D-xylose utilization. The observation that a Δ*xyr1* strain was almost unable to grow on D-xylose plates (Table 2; Fig. 1A) prompted us to examine transcription levels of *xyl1* as well as activities of D-xylose reductase and L-arabinose reductase. As can be inferred from Fig. 2A, D-xylose reductase is tightly regulated by Xyr1, in contrast to L-arabinose reductase, which is not affected in the Δ*xyr1* strain (Fig. 2B). Therefore, we presume that the weak residual growth on D-xylose plates of the Δ*xyr1* strain is the result of a more general aldose reductase activity of

TABLE 4. Xylanase and cellulase activities in supernatants from direct cultivations of the parental strain and the Δ*xyr1* strains with xylan or cellulose as respective carbon sources

Cultivation time (h)	Xylanase activity (U/μg protein) in xylan supernatants[a,c]		Cellulase activity (U/μg protein) in cellulose supernatants[b,c]	
	QM9414	Δ*xyr1*	QM9414	Δ*xyr1*
24	ND[d]	ND	0.003 ± 0.000	ND
48	0.206 ± 0.015	ND	0.655 ± 0.012	ND
72	0.142 ± 0.009	ND	0.885 ± 0.022	ND

[a] One unit of activity is defined as the amount of enzyme required to release 1 micromole of xylose reducing sugar equivalents per min under the defined assay conditions.
[b] One unit of activity is defined as the amount of enzyme required to release 1 micromole of glucose reducing sugar equivalents per min under the defined assay conditions.
[c] Values are the means ± SD of results from three independent experiments.
[d] ND, no detection.

TABLE 5. Relative transcript levels of *bgl1*, *bgl2*, and *bxl1* in the wild-type and the Δ*xyr1* strains

Analyzed gene, carbon source, and incubation time (h)	Transcript ratio[a,b]	
	QM9414	Δ*xyr1*
bgl1, sophorose (8)	1.0 ± 0.1	ND[c]
bgl2, sophorose (8)	1.3 ± 0.2	1.1 ± 0.0
bxl1, xylobiose (5)	58.2 ± 0.4	ND

[a] Values were calculated with reference to analyses of QM9414, *bgl1*, sophorose cultivated for 8 h.
[b] Values are means ± SD from results of three independent experiments.
[c] ND, no detection.

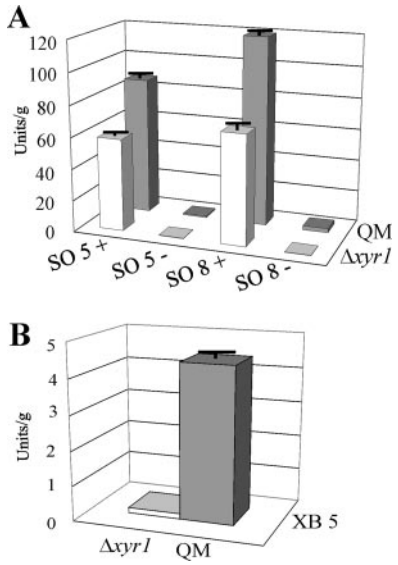

FIG. 5. (A) Total (extracellular and mycelial-bound) β-glucosidase activity (+) and activity in the culture supernatant (−) of the parental and the Δ*xyr1* strains after replacement of glycerol pregrown mycelia into medium containing 2 mM sophorose (SO) and incubation for 5 and 8 h. (B) Activity of β-xylosidase in supernatants of the parental and the Δ*xyr1* strains after replacement of glycerol pregrown mycelia into medium containing 2 mM xylobiose (XB) and incubation for 5 h. All data are the means of results from three independent experiments. Error bars indicate standard deviations.

L-arabinose reductase. It should be noted that a similar mechanism has previously been proposed for *A. niger* (15).

It is a well established fact that D-xylose is an inducer of *xyn1* gene expression in *H. jecorina* (30, 38, 53). Since Xyr1 affects the metabolism of that inducer, it stood to reason whether and how Xyr1 influences the expression of *xyn1*. Recently, we demonstrated the basic necessity for Xyr1 binding to the *xyn1* promoter for both derepressed (release from Cre1-mediated glucose repression [30]) and induced *xyn1* gene expression (38). In this study we could demonstrate that the transcript formation of *xyn1* is completely abolished on D-xylose and xylan in the Δ*xyr1* strain (Table 3). While in the parental strain both a basal *xyn2* transcription level as well as clear induction on sophorose, xylobiose, and xylan could be detected, the Δ*xyr1* strain did not form any corresponding transcript (Table 3). Hence, we conclude that there is an indispensable Xyr1 dependence on both induction and on low basal *xyn2* transcription. As transcription analysis of the *cbh1*, *cbh2*, and *egl1* genes of both strains portrayed the same picture (Fig. 3A, B, and C), consequently, the formation of expression products of the above-mentioned genes was checked by the determination of activities of hydrolytic enzymes.

Measuring xylanase activities in supernatants of mycelia replaced with various carbon sources confirms the inability of the Δ*xyr1* strain to form xylanolytic enzymes (Fig. 4) and thus asserts the findings of analysis of the transcriptional level. Deletion of *xyr1* leads not only to the loss of induction of xylanase formation but also to the absence of low activity on medium without any carbon source that could be detected in the parental strain (Fig. 4). That low activity could, on the one hand, result from derepression of *xyn1* expression regulated in a Cre1-dependent manner (30). Such an assumption is also in accordance with the fact that *xyn1* expression is strictly dependent on a Xyr1 binding motif even in the background of inactivated Cre1 sites in the *xyn1* promoter (38), hence strongly indicating the involvement of Xyr1 in the derepression mechanism. On the other hand, the low xylanase activity on medium without a carbon source could be due to a basal level of *xyn2* transcription (52, 53). However, the induction of xylanase enzyme activity formation is strictly dependent on Xyr1, and it suggests that Xyr1 is additionally involved in both derepression and basal expression mechanisms.

Determination of cellulase activities again revealed a complete loss of enzyme formation in the Δ*xyr1* strain (Table 4), thereby strongly pointing to Xyr1 as the general regulator of the *H. jecorina* xylanolytic and cellulolytic enzyme systems. A similar regulatory function has previously been described for XlnR, the Xyr1 orthologue of *A. niger* (10, 15).

In addition, Xyr1 was shown to regulate the inducer-providing enzymes BGLI and BXLI (Fig. 5A and B; Table 5). Transcriptional levels of the *bgl2* gene (40) are uninfluenced, in contrast to those of *bgl1* that are absent in the Δ*xyr1* strain (Table 5). Measuring β-glucosidase activity in the supernatants confirmed regulation via Xyr1, since we could not detect any activity in the Δ*xyr1* strain (Fig. 5A). Previously, it has been published that the *bgl1* gene product is secreted mainly into the medium (29), whereas antibodies raised against BGLII showed the presence of this enzyme in *H. jecorina* cell lysates but not in the culture supernatant (40). The fact that we measured reduced total β-glucosidase activities (mycelia plus supernatant) from the Δ*xyr1* strain together with the findings that the transcription of *bgl1* but not *bgl2* is activated in a Xyr1-dependent manner (Table 5, Fig. 5A) leads us to the conclusion that only the β-glucosidase activity present in the supernatant is regulated by Xyr1 and is exclusively due to the *bgl1* gene product.

Thus, Xyr1 regulates, on the one hand, the expression of the main xylanolytic and cellulolytic genes regardless of which inducer substances they respond to and, on the other hand, some genes whose products are responsible for making these inducers available (*bxl1* and *bgl1*). A respective model outlining the extensive impact and involvement of Xyr1 on mechanisms leading to xylan and cellulose degradation can be found in Fig. S1 in the supplemental material.

The role of Xyr1 as the central activator of the expression of main xylanolytic and cellulolytic enzymes in *H. jecorina* relates it to *A. niger* XlnR, which is known to be the main transcriptional activator of cellobiohydrolase- and xylanase-encoding genes (10, 47). Although both are regarded as the main regulatory factors in the corresponding hydrolytic enzyme systems and D-xylose metabolism of their respective fungi, the molecular mechanisms of transcriptional activation involving Xyr1 must significantly differ from those concerning XlnR. Whereas XlnR-mediated induction of such important hydrolase-encoding genes as *cbhA*, *cbhB*, *xlnB*, and *xlnD* in *A. niger* require D-xylose (10, 15), the induction pattern of the best characterized hydrolytic enzymes in *H. jecorina* is more differentiated.

In recent years, such low-molecular-weight inducer substances as xylobiose and D-xylose as well as cellobiose and sophorose were shown to act as inducers of the formation of

the main hydrolases or transcription of the encoding genes of XYNI, XYNII, CBHI, and CBHII (18, 20, 30, 33, 53).

Activation of the corresponding genes was previously proven to be caused by different inducers, e.g., the different inducibility of *xyn1* and *xyn2* by D-xylose and xylobiose, respectively (53), or the inducibility of *xyn2* by xylobiose and sophorose in contrast to that of *cbh1* only by sophorose (33). Nevertheless, in the transcriptional regulation of all of them, one common regulator plays the essential role. Therefore our current working hypothesis includes the existence of a general, substrate-unspecific activator, Xyr1, as well as inducer-specific transcriptional regulators, that is responsible for the fine tuning of the regulation of the corresponding gene, such as that recently shown by the example of the antagonistic relationship of Xyr1 and Ace1 concerning *xyn1* regulation (38). This working model concurs with the indication that Xyr1 binds to an inverted repeat within the *xyn1* promoter, as either a homo- or a heterodimer, respectively, thereby providing the opportunity for specific regulatory proteins to interact with the accordant promoter and/or Xyr1. Moreover, preliminary studies suggest that Ace2 poses a *xyn2* gene-specific transcriptional factor modulating activation caused by Xyr1 (A. R. Stricker, P. Trefflinger, and R. L. Mach, unpublished data). For the *A. niger* XlnR protein, the possible participation of Ace1 in transcriptional regulation of hydrolase-encoding genes has not yet been shown, and an Ace2 homologue could not even be found in the genome. In addition, it was suggested that XlnR binds as a monomer since it was shown to bind to a nonpalindromic consensus in the promoter (14, 48). In contrast to Xyr1, XlnR seems to act as an unspecific transactivator of a wide variety of genes encoding hydrolytic enzymes responding mainly to D-xylose induction, largely independent of other substrate-specific regulatory proteins.

It remains to be resolved if and how Ace2 interacts with Xyr1 and which role further proteins involved in the regulation of *xyn2* under repressing conditions are playing (e.g., the postulated AGAA-binding repressor protein of the *xyn2* promoter [52]). Above all, a fundamental understanding of the regulation of Xyr1 itself would reveal important mechanisms within the xylanolytic transcriptosome and pose a powerful tool for strain design and protein production improvement.

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