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

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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 *xyl* (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 *Δxyr1* 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 BXL1 and β-glucosidase BGLI but was not shown to be involved in the regulation of BGLII.

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-xylanase 1-encoding) gene by xylobiose (33); cellulases such as the *cbh1* (cellbiohydrolase 1-encoding) gene, the *cbh2* (cellbiohydrolase 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 hydrolyase-encoding genes (6, 7, 21, 30). In *T. reesei*, only some of the major hydrolyases, namely *cbh1* and *xyn1*, are under direct Cre1 control (21, 30), whereas other hydrolytic genes...
such as cbh2, xyn2, and bgll 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 hydrolyase 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 hydrolyases.

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 cellulytic 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., BGL1 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 cellulytic enzymatic 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-Andreatti (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 Erленмeyer flakes 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 (108 per liter, final concentration) were suspended in 2% (wt/vol) agar-agar at 30°C. It was maintained on malt agar.

Three different strains of T. reesei (Genbank accession numbers D00127, D00126, and D00128) were cultivated on xylose or xylobiose or on medium without a carbon source; 5 and 10 ml samples 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 harvested after equal amounts were resuspended in MA medium containing 1% (wt/vol) glycerol as the carbon source and 2% (wt/vol) agar-agar at 30°C.

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 differences in gene transcription not in amounts of DNA was performed, the efficiency was calculated essentially as described in reference 44.

Several optimizations (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 xyn1 or 59.5°C (for real-time PCR of cbh1, cbh2, and egl1) and 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 containing a carbon source and incubated for 3 h was used), all cycle threshold (Ct) values for DNA samples were referenced to the Ct values for the DNA of the reference sample to obtain a correction factor, . The Ct values of each sample were corrected by the factor. Corrected DNA Ct values (Ct values) were subtracted from the Ct values of DNA of the reference sample to obtain ΔCt values. After establishing a relationship between the DNA Ct values and the DNA Ct values of the reference sample, the differences between the initial amounts of DNA were calculated. According to the equation , where and as the PCR efficiency (100% efficiency = 2) and k as the slope (100% efficiency = 3.32), the ΔCt values were divided by 3.32 to obtain S (calculation factor). Then was raised to the power of S, because initial target amounts differing in one decimal power should differ in their Ct values by about 3.32. This procedure allows describing differences in gene transcription not in Ct values but in amounts of DNA-corrected DNA targets. Thus, results of transcription analysis are given in relative amounts of mRNA (cDNA) per gene dose.
Enzyme assays. α-Xylose reductase activity and t-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 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.

**TABLE 1. Primers and probes used throughout the study**

| Primer name | Sequence (5’-3’) | Employed for |
|-------------|------------------|--------------|
| Actf | TGAGAAGGGGTTGATCCACG | Actin real-time PCR |
| Actr | GGTACCCACAGACATACTGAA | Actin real-time PCR |
| bgf1 | CAGCAGTCTACAACACCTCG | bgf1 real-time PCR |
| bgf1r | ACCCTAATCCGAGATGACATTCGG | bgf1 real-time PCR |
| bgf2 | ACGGCTGCTACCAGATCG | bgf2 real-time PCR |
| bgf2r | AGCCTGCGGACGACTC | bgf2 real-time PCR |
| bx1 | GCGAATCTTGGACGATGATGG | bx1 real-time PCR |
| bx1r | CGGACCATTGCTGATATGGTG | bx1 real-time PCR |
| TaqMan cbb1 FAM | CTGTACCCATACCCGACACAGACC | cbb1 real-time PCR |
| TaqMan cbb1 Tr | GATGGACTACTCCCAGACATCTCG | cbb1 real-time PCR |
| TaqMan cbb2 TR | CTATGCGGACAGTTCGTTG | cbb2 real-time PCR |
| TaqMan eg1 FAM | CAACTCAGGGGCGATCGTCGTACCTGTCG | eg1 real-time PCR |
| TaqMan eg1 Tr | CTGCAAAGGATGATGATCTG | eg1 real-time PCR |
| TaqMan xyn1 FAM | CGCTGACTATGGCAACGAAAACG | xyn1 real-time PCR |
| TaqMan xyn1 Tr | CAGCTATTCGCTATGGG | xyn1 real-time PCR |
| TaqMan xyn2 FAM | GGTCCAGCTCGGGCAACTT | xyn2 real-time PCR |
| TaqMan xyn2 Tr | CTGCCATCCCTTGCCG | xyn2 real-time PCR |
| TaqMan egl1 FAM | CGAATTCTATACAATGGGCACATGGG | egl1 real-time PCR |
| TaqMan egl1 Tr | GCAGCTATTCGCTATGGG | egl1 real-time PCR |
| TaqMan cbh1 FAM | CGAATTCTATACAATGGGCACATGGG | cbh1 real-time PCR |
| TaqMan cbh1 Tr | GCAGCTATTCGCTATGGG | cbh1 real-time PCR |
| TaqMan xyl1 FAM | Xorr CACAGCTTGGACACGATGAAGAG | xyl1 real-time PCR |
| TaqMan xyl1 Tr | Xorr CACAGCTTGGACACGATGAAGAG | xyl1 real-time PCR |
| TaqMan cbh2 Dr | GATCAGTACATGTGCTTGAGCGC | cbh2 real-time PCR |
| TaqMan cbh2 Tr | GATCAGTACATGTGCTTGAGCGC | cbh2 real-time PCR |
| TaqMan egl1 Dr | GAGGAGTCCTCCTACGCAGAA | egl1 real-time PCR |
| TaqMan eg1 Dr | GAGGAGTCCTCCTACGCAGAA | eg1 real-time PCR |
| TaqMan egl1 Fr | CAACTCGAGGGCGAATGCCTTGACCCCTC | egl1 real-time PCR |
| TaqMan egl1 Tr | CAACTCGAGGGCGAATGCCTTGACCCCTC | egl1 real-time PCR |
| TaqMan cbh1 Dr | GATCAGTACATGTGCTTGAGCGC | cbh1 real-time PCR |
| TaqMan cbh1 Tr | GATCAGTACATGTGCTTGAGCGC | cbh1 real-time PCR |
| TaqMan xyl1 Dr | GATCAGTACATGTGCTTGAGCGC | xyl1 real-time PCR |
| TaqMan xyl1 Tr | GATCAGTACATGTGCTTGAGCGC | xyl1 real-time PCR |
| TaqMan egl1 Fr | CAACTCGAGGGCGAATGCCTTGACCCCTC | egl1 real-time PCR |
| TaqMan egl1 Tr | CAACTCGAGGGCGAATGCCTTGACCCCTC | egl1 real-time PCR |
| TaqMan cbh2 Fr | GATCAGTACATGTGCTTGAGCGC | cbh2 real-time PCR |
| TaqMan cbh2 Tr | GATCAGTACATGTGCTTGAGCGC | cbh2 real-time PCR |
| TaqMan egl1 Fr | CAACTCGAGGGCGAATGCCTTGACCCCTC | egl1 real-time PCR |
| TaqMan egl1 Tr | CAACTCGAGGGCGAATGCCTTGACCCCTC | egl1 real-time PCR |
| TaqMan cbh2 Dr | GATCAGTACATGTGCTTGAGCGC | cbh2 real-time PCR |
| TaqMan cbh2 Tr | GATCAGTACATGTGCTTGAGCGC | cbh2 real-time PCR |
| TaqMan egl1 Dr | GAGGAGTCCTCCTACGCAGAA | eg1 real-time PCR |
| TaqMan eg1 Dr | GAGGAGTCCTCCTACGCAGAA | eg1 real-time PCR |

**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 pRLMex30 vector, conferring 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 α-xylose uptake.** OM9414 and A. nidulans mycelia were pregrown as described above and transferred to 5 ml phosphate buffer (20 mM, pH 5) containing 2 mM α-xylose pulsed with 2·10^[14] M (14C)D-xylose (1.85 MBq/250 μl) (Amersham Biosciences, United Kingdom). After an incubation time of 20 min at 30°C, 0.1 M α-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.
we conducted a series of growth experiments on agar plates. In order to examine the influence of Xyr1 on the utilization of different carbon sources, we investigated the Δxyr1 strain and the insertion of Xyr1 on plates containing glucose, glycerol, L-arabinose, L-arabitol, D-xylose, xylitol, xylose, and cellulose. The Δxyr1 strain showed strongly reduced growth compared to the parental strain. In all cases tested, neither conidiospore formation on agar plates nor germination times differed between the mutant strain 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, xylobiose, and 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, Rexyr1A) were able to utilize all carbon sources investigated, the Δxyr1 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 Δxyr1 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-

**TABLE 2. Growth of the parental strain and strains Δxyr1 and Rexyr1 on plates containing MA medium supplemented with different carbon sources**

| Carbon source | After 3 days | After 6 days |
|---------------|-------------|-------------|
|               | QM9414      | Δxyr1       | Rexyr1A     |
| 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 Δxyr1 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.

**FIG. 1.** (A) Growth of parental strain QM9414, the xyr1 retransformation strain (Rexyr1A), and the Δxyr1 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).
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 preincubated 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 Δxyn1 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 Δxyn1 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 Δxyn1 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

![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 H9004 (QM) and the Δxyn1 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 means of results from three independent experiments. Error bars indicate standard deviations.](image)
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 CBHII, CBHIII, 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 system 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., xylene, 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 BXL1 (11, 17, 40, 46), are also affected by Xyr1. By examination of the transcriptional levels of *bgl1, bgl2,*
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 *H9004* *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 *H9004* *xyr1* strain (Fig. 2B). Therefore, we presume that the weak residual growth on D-xylose plates of the *H9004* *xyr1* strain is the result of a more general aldose reductase activity of

![FIG. 4. Xylanase activities in culture supernatants of the parental strain QM9414 and the Δxyr1 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.](image)

**TABLE 4. Xylanase and cellulase activities in supernatants from direct cultivations of the parental strain and the Δxyr1 strains with xylan or cellulose as respective carbon sources**

| Cultivation time (h) | Xylanase activity (U/μg protein) in xylan supernatants | Cellulase activity (U/μg protein) in cellulose supernatants |
|----------------------|---------------------------------------------|-------------------------------------------------|
|                      | QM9414 | Δxyr1 | QM9414 | Δxyr1 |
| 24                   | ND     | 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 Δxyr1 strains**

| Analyzed gene, carbon source, and incubation time (h) | Transcript ratio |
|--------------------------------------------------------|------------------|
|                                                        | QM9414 | Δxyr1 |
| bgl1, sophorose (8)                                     | 1.0±0.1 | ND   |
| 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.
\textbf{FIG. 5.} (A) Total (extracellular and mycelial-bound) \(\beta\)-glucosidase activity (+) and activity in the culture supernatant (−) of the parental and the \(\Delta\text{xyr1}\) strains after replacement of glycerol pregrown mycelia into medium containing 2 mM sophorose (SO) and incubation for 5 and 8 h. (B) Activity of \(\beta\)-xylosidase in supernatants of the parental and the \(\Delta\text{xyr1}\) 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.

\textsuperscript{t}-arabinose reductase. It should be noted that a similar mechanism has previously been proposed for \textit{A. niger} (15).

It is a well established fact that \(\text{D-xylose is an inducer of xyn1}\) gene expression in \textit{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 \textit{xyn1}. Recently, we demonstrated the basic necessity for Xyr1 binding to the \textit{xyn1} promoter for both derepressed (release from Cre1-mediated glucose repression [30]) and induced \textit{xyn1} gene expression (38). In this study we could demonstrate that the transcript formation of \textit{xyn1} is completely abolished on \(\text{D-xylose and xylan}\) in the \(\Delta\text{xyr1}\) strain (Table 3). While in the parental strain both a basal \textit{xyn2} transcription level as well as clear induction on sophorose, xylobiose, and xylan could be detected, the \(\Delta\text{xyr1}\) 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 \textit{xyn2} transcription. As transcription analysis of the \textit{cbh1}, \textit{cbh2}, and \textit{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 \(\Delta\text{xyr1}\) strain to form xylanolytic enzymes (Fig. 4) and thus asserts the findings of analysis of the transcriptional level. Deletion of \textit{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 \textit{xyn1} expression regulated in a Cre1-dependent manner (30). Such an assumption is also in accordance with the fact that \textit{xyn1} expression is strictly dependent on a Xyr1 binding motif even in the background of inactivated Cre1 sites in the \textit{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 \textit{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 \(\Delta\text{xyr1}\) strain (Table 4), thereby strongly pointing to Xyr1 as the general regulator of the \textit{H. jecorina} xylanolytic and cellulolytic enzyme systems. A similar regulatory function has previously been described for XlnR, the Xyr1 orthologue of \textit{A. niger} (10, 15).

In addition, Xyr1 was shown to regulate the inducer-providing enzymes BGLI and BXL1 (Fig. 5A and B; Table 5). Transcriptional levels of the \textit{bgl2} gene (40) are uninfluenced, in contrast to those of \textit{bgl1} that are absent in the \(\Delta\text{xyr1}\) strain (Table 5). Measuring \(\beta\)-glucosidase activity in the supernatants confirmed regulation via Xyr1, since we could not detect any activity in the \(\Delta\text{xyr1}\) strain (Fig. 5A). Previously, it has been published that the \textit{bgl1} gene product is secreted mainly into the medium (29), whereas antibodies raised against BGLII showed the presence of this enzyme in \textit{H. jecorina} cell lysates but not in the culture supernatant (40). The fact that we measured reduced total \(\beta\)-glucosidase activities (mycelia plus supernatant) from the \(\Delta\text{xyr1}\) strain together with the findings that the transcription of \textit{bgl1} but not \textit{bgl2} is activated in a Xyr1-dependent manner (Table 5, Fig. 5A) leads us to the conclusion that only the \(\beta\)-glucosidase activity present in the supernatant is regulated by Xyr1 and is exclusively due to the \textit{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 (\textit{bxl1} and \textit{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 \textit{H. jecorina} relates it to \textit{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 hydrolyase-encoding genes as \textit{cbhA}, \textit{cbhB}, \textit{xlnB}, and \textit{xlnD} in \textit{A. niger} require D-xylose (10, 15), the induction pattern of the best characterized hydrolytic enzymes in \textit{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 xylooligosaccharide, respectively (53), or the inducibility of xyn2 by xylooligosaccharide 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-specific 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 accordan 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 tetramer, respectively, thereby providing the opportunity for the xylanolytic transcriptosome and pose a powerful tool for strain design and protein production improvement.

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