D-Xylose Metabolism in *Hypocrea jecorina*: Loss of the Xylitol Dehydrogenase Step Can Be Partially Compensated for by *lad1*-Encoded l-Arabinitol-4-Dehydrogenase

Bernhard Seiboth,* Lukas Hartl, Manuela Pail, and Christian P. Kubicek

*Abteilung für Angewandte Biochemie und Gentechnologie, Institut für Verfahrenstechnik, Umwelttechnik, und Technische Bionwissenschaften, A-1060 Wien, Austria*

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D-Xylose is a major constituent of plant hemicelluloses, where it forms the β-1,4-xylan backbone of hardwood. β-1,4-Xylans are heteropolysaccharides that have a backbone of β-1,4-linked xylopyranosyl residues and contain side groups such as D-glucuronic acid, L-arabinose, p-coumaric acid, and ferulic acid. They constitute 20 to 35% of the approximately 830 gigatons of annually formed renewable plant biomass (23).

Both prokaryotic and eukaryotic microorganisms can use xylan as a carbon source for growth. The bacterial pathway for d-xylose catabolism is well established. It consists of an isomerase and a kinase that sequentially convert d-xylene to d-xylulose and d-xylulose-5-phosphate, which is an intermediate of the pentose phosphate pathway. This pathway is absent from fungi, where d-xylene instead is subjected to NADPH-linked reduction and NADH-linked oxidation reactions before phosphorylation of d-xylulose occurs (Fig. 1). The enzymes catalyzing the first two steps (aldose reductase [EC 1.1.1.21] and xylitol dehydrogenase [EC 1.1.1.9]) have been characterized mainly from yeasts. They are nonspecific and can use other sugars in addition to d-xylene and xylitol, respectively, at approximately the same rates. The genes encoding the enzymes involved in fungal d-xylene catabolism have also been cloned from different yeasts (for a review, see reference 10) and, in part (i.e., aldose reductase and xylulose-5-phosphate kinase), from *Aspergillus niger* (9, 25). Further, cloning of a gene encoding a xylitol dehydrogenase from *Hypocrea jecorina* has been reported, but neither its nucleotide sequence nor its amino acid sequence has been made available (27).

A genetic analysis of the d-xylene-metabolizing pathway in yeasts showed that aldose reductase and xylitol dehydrogenase are indeed essential for d-xylene degradation. In contrast, an aldose reductase knockout mutant of *A. niger* was still able to grow on d-xylene, although at a reduced rate (9), suggesting that multiple enzymes are involved in the first step of the d-xylene catabolic pathway in filamentous fungi.

Xylan breakdown by the ascomycete *H. jecorina* (anamorph: *Trichoderma reesei*) has received the strongest interest because of the application of the corresponding xylanases in the pulp and paper industry and the food industry. The extracellular addition of intermediates of the d-xylene metabolic pathway leads to differential expression of the two major xylanase genes (14), stressing the need to understand this pathway in more detail for the metabolic engineering of xylanase formation in this fungus. As a first step toward the genetic engineering of the d-xylene catabolic pathway in *H. jecorina*, we attempted to identify the genes and proteins involved.

Here we report the cloning of the *xdh1* gene, encoding NAD-xylitol dehydrogenase of *H. jecorina*. We show that deletion of the gene partially affects the growth of *H. jecorina* on d-xylene and that l-arabinitol-4-dehydrogenase (encoded by
The mycelia were then homogenized by sonication of a concentrated mycelial suspension (1 g [wet weight] per 2.5 ml of buffer A [0.1 M Tris-HCl {pH 7.5}, 1 mM EDTA, 5 mM [β-mercaptoethanol]) 10 times for 30 s each time at 2°C. The resulting homogenate was centrifuged at 10,000 × g for 20 min at 4°C. The supernatant, which had a protein content of between 8 and 15 mg/ml, was used as a cell extract.

Enzyme assay. Xyitol dehydrogenase activity was assayed by the procedure of Chakravorty and Horecker (3) with 0.1 M glycine buffer (pH 8.6) and 0.1 M glycylglycine (pH 7.0) for the forward and reverse reactions, respectively. Enzyme (cell extract or purified glutathione S-transferase [GST]-Xdh1 fusion) was added in amounts sufficient to produce a change in the A_{550} of between 0.02 and 0.1/min. The reaction was started by the addition of substrate. Activity was expressed as units, 1 U corresponding to the conversion of 1 μmol of substrate per min, and reported as specific activity (units per milligram of protein). The protein concentration was determined by using a protein assay from Bio-Rad Laboratories, Munich, Germany.

SDS-PAGE. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed with 10% polyacrylamide gels as described by Ausubel et al. (2). Gels were stained with Coomassie blue.

**Extraction and quantification of mycelial xyitol.** To measure the intracellular xyitol concentration, mycelia of *H. jecorina* were harvested, washed with cold tap water, and resuspended in 1 ml of distilled water. The suspension was then snap-frozen at −75°C for 1 h, thawed, boiled for 10 s, and finally homogenized in a precooled Potter-Elvehjem glass homogenizer. The homogenate was centrifuged (10,000 × g, 4°C, 10 min), and the xyitol concentration in the supernatant was quantified by high-pressure liquid chromatography with an H+ exchange column (Bio-Rad Aminex HPX-87H), with 10 mM H2SO4 at 55°C as the mobile phase, and with isotric elution. Compounds were detected by a refractive index detector.

**Materials and Methods**

*Strains and culture conditions.* The *H. jecorina* parent strains used in this study were QM9414 (ATCC 26921) and the pyr4-negative mutant TU-6 (ATCC MYA-256) (7). All strains were maintained on malt extract agar, and auxotrophic strains were supplemented with uridine (10 mM). Strains were grown in 1-liter Erlenmeyer flasks on a rotary shaker (250 rpm) at 30°C with 280 ml of the medium described by Mandels and Andreotti (16) and containing various carbon sources at final concentrations of 10 g/liter.

For analysis of the effects of different carbon sources on gene expression, the different *H. jecorina* strains were pregrown on 1% (wt/vol) glycerol (20 h), the cultures were harvested after appropriate times, washed extensively with tap water and then distilled water, and dried to a constant weight. To determine hyphal growth on agar plates, 5 g of mycelial dry weight was assumed to be equivalent to a 2.4-ml intracellular volume (22) without consideration of intracellular compartimentalization.

**Cloning of the *H. jecorina* xdh1 gene.** An alignment of different xyitol dehydrogenases from the National Center for Biotechnology Information database revealed the conserved amino acid sequences TGICGSDVH and GHYVQGGM to be potentially suitable for amplifying a corresponding fragment of *H. jecorina*. Consequently, the primers xdhfor1 (5′-ACCGCATCTGCGTCCGATGTCC-3′) and xdhrev2 (5′-CCCGTGTAGTACCGTTCGCCATAC-3′) were deduced directly from the respective nucleotide sequence of a hypothetical protein (NCU00891.1) in the *Neurospora crassa* genome database (http://www.genome.wi.mit.edu/annotation/fungi/neurospora) which shows a high level of similarity to xyitol dehydrogenases. The conditions used for amplification with these primers were as follows: 100 ng of *H. jecorina* QM9414 genomic DNA as a template in a total volume of 50 μl in an automated temperature cycling device (Biotron; Biometra, Göttingen, Germany); a reaction mixture containing 2.5 mM MgCl2, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% (vol/vol) Triton X-100, 0.4 μM each primer, 0.2 mM each deoxynucleoside triphosphate, and 0.5 U of Taq polymerase (Promega); and an amplification program consisting of 1 min of initial denaturation (94°C), 30 cycles of amplification (1 min at 94°C, 1 min at 54°C, and 1 min at 74°C), and 7 min of final extension (74°C). These primers allowed the amplification of a 650-bp fragment, which was isolated and used to screen a genomic λ BlueSTAR library (Novagen) of *H. jecorina* QM9414. The corresponding xdh1 gene was located on a 2.5-kb SacI/SalI fragment, ligated to pBluescript SK+ (Stratagene), resulting in pXDH, and sequenced by means of a Li-cor 4200 automatic sequencer (Li-cor Inc., Lincoln, Nebr.). To confirm the positions of putative introns, a complete cDNA fragment, isolated from a λ HybridZAP cDNA library (Stratagene) of *H. jecorina* QM9414 on d-xylose, was sequenced.

To amplify a 1.2-kb fragment of *H. jecorina* lad1 by PCR, primers lad1fw (5′-ACCAGTCCTCCAGTGGCCCT-3′) and lad1rev (5′-GACCTAATGCAACGACCTC-3′) were derived from the published *H. jecorina* lad1 sequence (18). Standard conditions were used for amplification. The corresponding lad1 gene was located on a cDNA fragment clone H1 and partially sequenced.

**Sequence analysis.** The 2.5-kb fragment was analyzed by using BLAST programs (1), and a multiple sequence alignment was done by using MultiAlin (4). Consensus binding sequences in the xdh1 and lad1 5′ regions were identified manually.

**Xyitol dehydrogenase isoforms and hybridization.** Fungal mycelia were harvested by filtration, washed with tap water, frozen, and ground in liquid nitrogen. Nucleic acids (DNA and total RNA) were extracted as described previously (21). Standard methods (20) were used for electrophoresis, blotting, and hybridization of nucleic acids. Probes used for Northern hybridization were a 1.9-kb Acc65I acc1 fragment (actin encoding), the 1.2-kb lad1 fragment (see above), and a 1.4-kb xdh1 cDNA fragment (without the 5′-untranslated region), and were labeled with [32P]dCTP by random primer labeling. Blots were exposed to x-ray films and autoradiographs were digitized and quantified by densitometric measurements of autoradiographs derived from different exposure times (only values with a linear correlation [r > 0.9] were used).

**FIG. 1.** Fungal d-xylose pathway.
Overexpression of xdh1 in E. coli. To obtain purified H. jecorina xyitol dehydrogenase, the full-length xdh1 cDNA was overexpressed in a GST fusion in E. coli. To this end, the xdh1 coding region was PCR amplified from the cDNA clone with primers GEX-XDHfwd (5' - CGTCCTGGATCCATGGCGGACGCTCAA - 3') and GEX-XDHrev (5' - AGGGCCGCGCCTACCTCTGTTG-3'). PCR amplification was performed with Pfu polymerase (Promega) by using an initial denaturation cycle of 45 s at 94°C, 28 cycles of amplification (45 s at 94°C, 45 s at 50°C, and 3 min at 72°C), and a final extension step of 10 min at 72°C. The amplicon was cut with BamHI and NotI and cloned into pGEX-2T (Amersham Biosciences, Vigo, Spain). After verification by sequencing, the GST-Xdh1 fusion protein was overexpressed in E. coli BL21 (Stratagene) and purified by using glutathione-Sepharose 4B (Amersham Biosciences) according to the manufacturer’s protocol. For storage at −80 or −20°C, 20% (vol/vol) sterile glycerol was added.

Construction of H. jecorina xdh1, lad1, and xdh1 lad1 knockout mutants. To construct an xdh1 knockout vector, a genomic fragment containing 3 kb upstream and 4 kb downstream of the xdh1 coding region was replaced by the hygromycin resistance-conferring expression cassette from pRLMEx30 (15). To this end, a 3-kb ApaI-Clal fragment of the xdh1 upstream region was cloned into pBluescript SK(+), the 2.8-kb XhoI-HindIII hygromycin resistance cassette was cloned into the resulting vector, and finally a 2.8-kb ApaI-SfiI fragment of the xdh1 downstream region was added to this vector to result in the final deletion vector pΔXDH. Transformation of H. jecorina QM9414 was done by the protocol of Gruber et al. (7) with an 8.0-kb Acc65I-NotI fragment of pADXH.

To construct a lad1 knockout strain, the lad1 coding region was replaced by the H. jecorina pyr4 marker (6). To this end, the 2.7-kb Sall-PvuII fragment was cloned between a 3-kb BamHI-Mfl1 upstream fragment and a 2-kb EcoRV-NcoI downstream fragment of lad1 in pBluescript SK(+), resulting in the final deletion vector pΔLAD1. For transformation of H. jecorina TU-6, a 5.7-kb ApaI-XbaI fragment of pΔLAD1 was used.

To obtain strains with deletions of both dehydrogenases, H. jecorina Δlad1 was transformed with the 8.0-kb Acc65I-NotI fragment of pADXH.

Nucleotide sequence accession numbers. The DNA sequences assembled here were deposited in GenBank under the following accession numbers: xdh1, AF428150; and lad1, AJ225444.

RESULTS

Cloning and characterization of H. jecorina xdh1 and its encoded protein. Based on the PCR approach outlined above, a 650-bp H. jecorina xyitol dehydrogenase fragment was amplified and used to isolate a 2.5-kb genomic subclone which included the complete structural xdh1 gene. The results of Southern analysis of chromosomal DNA digested with different restriction nucleases are consistent with the occurrence of a single xdh1 gene in the H. jecorina genome (data not shown).

Nucleotide sequence analysis revealed an open reading frame (ORF) of 1,210 bp, interrupted by a single intron of 118 bp, encoding a 363-amino-acid protein with a calculated mass of 38 kDa. An analysis of the amino acid sequence with PROSITE (http://www.expasy.ch/prosite) identified the protein as a member of the zinc-containing alcohol dehydrogenase family, showing highest sequence identity to the yeast dehydrogenase of Candida sp. strain HA167 (former Galactocandida mustodermitis). When the Xdh1 protein sequence was compared with the Neurospora crassa database, four ORFs showed significant degrees of similarity (E values of 1e−100, 1e−60, 1e−40, and 1e−44). The highest similarity was found with hypothetical protein NCU00891.1, from which the primers were developed. The second highest similarity was obtained with NCU00643.1. However, submitting NCU00643.1 to a BLASTP search revealed that it is highly similar to l-arabinobitol-2-dehydrogenase of H. jecorina (18). The two even less similar proteins (NCU07022.1 and NCU01905.1) could not be identified with reasonable certainty.

A parsimony analysis of the amino acid sequence of H. jecorina Xdh1 and those of several other prokaryotic and eukaryotic proteins to which it showed the highest similarity in a BLAST search revealed that Xdh1 and hypothetical protein NCU00891.1 of N. crassa formed a strongly supported terminal branch within a dichotomous cluster containing all other yeast xyitol and d-sorbitol dehydrogenases and for which two d-sorbitol dehydrogenases of Schizosaccharomyces pombe represented a basal ancestor (Fig. 2). Seven bacterial alcohol and d-sorbitol dehydrogenases formed a sister clade to this cluster. The H. jecorina and N. crassa l-arabinobitol dehydrogenases formed a clearly different cluster. These data provide evidence that the protein encoded by H. jecorina xdh1 clearly is a member of the fungal xyitol and d-sorbitol dehydrogenase family and, in addition, that only one such member is present in the Neurospora genome sequence database.

Figure 3 shows an alignment of the amino acid sequence of H. jecorina xyitol dehydrogenase with those of its closest yeast neighbors: the polyol and coenzyme binding sites as well as the zinc binding sites are well conserved, but there is generally poor conservation outside these areas. It is interesting that H. jecorina xdh1 and N. crassa Xdh1 (NCU00891.1)—in contrast to the yeast xylitol dehydrogenases—have two predicted binding sites for Zn2+ instead of one: one site, consisting of Cys50-H75, and E161, which is typical for d-sorbitol and xyitol dehydrogenases, and a second site, comprising C105-C106-C111, and C119, which is typical for alcohol dehydrogenase and which is not found in the other xyitol dehydrogenases described so far from yeast origins.

Regulation of xdh1 gene expression. The transcriptional regulation of xdh1 was studied by Northern analysis: during growth on D-xylose or xylan, H. jecorina accumulated a single, 1.4-kb xdh1 transcript (Fig. 4). An xdh1 transcript accumulated on l-arabinobitol to an abundance similar to that on D-xylose or xylan, whereas its abundance was found to be higher on l-arabinose but significantly lower on D-galactose, galactitol, lactose, or xyitol. Generally, the xdh1 transcript was most abundant in young cultures, and its abundance was sharply decreased during further cultivation (data not shown). No xdh1 transcript could be detected during growth on D-glucose or glycerol.

Substrate specificity of Xdh1. H. jecorina Xdh1 was overexpressed in E. coli as a fusion to GST, purified to physical homogeneity (Fig. 5), and used to investigate the substrate specificity of the enzyme. The enzyme was active with xyitol and d-sorbitol in the forward reaction and with D-xylulose and D-fructose in the reverse reaction. No activity was observed with l-arabinose or l-arabinobitol as a substrate. NAD or NADH was exclusively required as a coenzyme; NADP or NADPH yielded less than 5% activity (Table 1). We also tested whether Mg2+ was necessary and found full activity in the absence of Mg2+. These data are largely consistent with those reported for xyitol dehydrogenase from the yeast Candida (13) and suggest that the two enzymes—despite having different numbers of predicted zinc binding sites—have similar substrate specificities.

xdh1 is involved in but is not essential for growth on D-xylose. To study whether xyitol dehydrogenase is essential for the metabolism of D-xylose in H. jecorina, a knockout mutant in which the xdh1 coding region was replaced by the E. coli hph
gene (encoding hygromycin B phosphotransferase) under the control of H. jecorina expression signals was constructed (15). Several xdh1 disruptants, verified by Southern analysis (Fig. 6A), were obtained. All of them exhibited considerably slower growth on D-xylose but clearly were still able to grow on this carbon source. Interestingly, growth on xylitol—on which the parent strain already grew rather slowly—was unaffected in the deletion mutant (Fig. 6B and C). Consistent with this finding, cell extracts from the deletion mutant still contained xylitol dehydrogenase activity, albeit at a significantly reduced level (Table 2), thus indicating that the xdh1 gene product does not account for all of the xylitol dehydrogenase activity of H. jecorina XDH1. A microscopic examination of the mutant further showed that during growth on D-xylose, the hyphae of the mutant appeared swollen and contained thicker cell walls (Fig. 6D). This morphology correlates with the accumulation of much higher concentrations of xylitol in the hyphae of strain ΔXDH1 than in those of the parent strain (112 versus 55 mM, respectively). During growth on xylitol, on the other hand, the hyphae of strain ΔXDH1 showed no changes in morphology compared with the morphology of the parent strain, consistent with the similar rates of growth of both strains on xylitol as a carbon source.

Interestingly, when the xdh1 deletion mutant was tested for growth on other carbon sources, growth on L-arabinitol but not on L-arabinose also was significantly affected: in submerged cultivation, the increase in biomass density was reduced by more than 50%, which is in the same range as the reduction

![Phylogenetic tree](image-url)
observed with D-xylose (Fig. 6B and C), but no apparent morphology changes were observed.

L-Arabinitol-4-dehydrogenase is responsible for residual xylitol dehydrogenase activity in the H. jecorina xdh1 deletion mutant. The results described above demonstrate that at least one more enzyme with xylitol dehydrogenase activity is present in H. jecorina and can partially compensate for the loss of Xdh1 function. A potential candidate for such an enzyme is lad1-encoded L-arabinitol-4-dehydrogenase, because it also catalyzes the oxidation of xylitol to xylulose (18). To study this possibility, the lad1 gene and its flanking regions were cloned.

![Alignment of H. jecorina xylitol dehydrogenase with other xylitol and D-sorbitol dehydrogenases](image1)

FIG. 3. Alignment of H. jecorina xylitol dehydrogenase with other xylitol and D-sorbitol dehydrogenases. Dehydrogenases were as follows: NcXdh1, N. crassa Xdh1 (NCU00891.1); HjXdh1, H. jecorina Xdh1; CaXdh1, Candida sp. strain HA167 Xdh1 (AAC24597.1); ScSor1, S. cerevisiae Sor1 (NP_012693.1); ScXdh1, S. cerevisiae Xdh1 (NP_013171.1); PsXdh1, P. stipitis Xdh1 (P22144); and SpSdh1, S. pombe Sdh1 (P36624). Diamonds indicate the first zinc binding site (C50, H75, and E161), and asterisks indicate the second zinc binding site (C105, C108, C111, and C119). The dashes over the sequence indicate the zinc-containing alcohol dehydrogenase signature, and the plus signs over the sequence indicate an NAD-binding site. Residues in white on a black background are conserved in at least 90% of the proteins; residues in white on a grey background are conserved in 40%.

![Induction of xdh1 transcription in H. jecorina by different carbon sources](image2)

FIG. 4. Induction of xdh1 transcription in H. jecorina by different carbon sources. The results of a Northern analysis of xdh1 transcript levels during growth on various carbon sources are shown. Samples were obtained 6 h after transfer from a glycerol culture to cultures with the carbon sources shown. For the carbon sources lactose and xylan, samples were obtained after 24 h of batch growth (conidial inoculum). Abbreviations: Glc, D-glucose; Gly, glycerol; Gal, D-galactose; Xyl, D-xylose; Ara, L-arabinose; Lac, lactose; Aol, L-arabinitol; Xol, xylitol; and Gol, galactitol.

![SDS-PAGE of purified GST-xylitol dehydrogenase fusion protein](image3)

FIG. 5. SDS-PAGE of purified GST-xylitol dehydrogenase fusion protein. Ten micrograms of protein was loaded on the gel, which was stained with Coomassie blue. MW, molecular weight markers (in thousands).
and used to construct an \textit{xdh1 lad1} double-deletion strain of \textit{H. jecorina}. First, \textit{H. jecorina lad1} deletion mutants were constructed by replacing the \textit{lad1} coding region with the \textit{H. jecorina pyr4} gene (Fig. 7A). The corresponding mutant strains were selected for growth on minimal medium with \textit{D-glucose} as a carbon source and were tested for the disruption of \textit{lad1}. Ten mutants were identified by Southern analysis to have undergone a single integration event in the \textit{lad1} locus. It is noteworthy that, in contrast to previous reports, the percentage of integration at the homologous locus was found to be about 50% for \textit{lad1}. Second, to construct an \textit{xdh1 lad1} double-deletion strain of \textit{H. jecorina}, the \textit{xdh1} coding region was replaced in an \textit{lad1} deletion strain as described above for strain \textit{\Delta XDH1}. Four transformants which showed the expected integration into the \textit{xdh1} locus (Fig. 7B) and in which the \textit{lad1} locus remained disrupted were obtained. The double-deletion mutant and the \textit{lad1} mutant were consequently tested for growth on \textit{D-xylose} and \textit{xylitol} (Fig. 7C). The data clearly show that the double-deletion strain lost the ability to grow on either of these carbon sources. Cell extracts from mycelia of strain \textit{\Delta LADXDH1} that had been pregrown on \textit{glycerol} and then transferred to \textit{D-xylose} for 10 h did not contain any \textit{xylitol} dehydrogenase activity, providing evidence that \textit{lad1} is responsible for the residual \textit{xylitol} dehydrogenase activity still present in the \textit{xdh1} deletion mutant. In contrast, the \textit{lad1} single-deletion strain grew equally well on all of these carbon sources, demonstrating that the \textit{lad1} gene product is not involved in \textit{D-xylose} and \textit{xylitol} degradation in strains of \textit{H. jecorina} when the \textit{xdh1} gene product is still functional.

The compensation of \textit{xdh1} loss of function by the \textit{lad1} gene product is theoretically in conflict with the data of Richard et al. (18) showing that \textit{lad1} is not expressed on \textit{D-xylose}. However, their data were obtained with carbon catabolite-depressed strain RUT C-30. Therefore, we examined \textit{lad1} expression in the QM9414 background and in strain \textit{\Delta XDH1}. Consistent with the findings of Richard et al. (18), we did not find \textit{lad1} expression in strain QM9414, but we did find it in the \textit{xdh1} deletion strain (Fig. 7D). Therefore, \textit{lad1} is transcribed under conditions in which \textit{xdh1} function is impaired.

### DISCUSSION

In the present work, we have characterized a \textit{xylitol dehydrogenase} from the filamentous fungus \textit{H. jecorina} at the molecular and functional levels. According to its primary structure, it belongs to the family of \textit{Zn}\(^{2+}\)-containing long-chain alcohol dehydrogenases, which also includes the \textit{xylitol}, \textit{D-sorbitol}, and \textit{iditol} dehydrogenases of yeasts (8, 11, 17, 19). However, it differs from these enzymes in that it contains two predicted binding sites for \textit{Zn}\(^{2+}\): in addition to a site which has three ligands typical for the binding of an active \textit{Zn}\(^{2+}\) site (\textit{C}_{50p}, \textit{H}_{25a}, and \textit{E}_{161}) and which is conserved in all members of this enzyme family (8, 11, 17, 19), there is a second site (\textit{C}_{105p}, \textit{C}_{105p}, \textit{C}_{1111}, and \textit{C}_{1111}) which is not present in most of the other \textit{xylitol} dehydrogenases (17). Therefore, it was concluded that the ligands of the second \textit{Zn}\(^{2+}\) atom of the long-chain human alcohol dehydrogenase (\textit{C}_{ap}, \textit{C}_{19p}, \textit{C}_{ap}, and \textit{C}_{111}) are not conserved in the yeast \textit{xylitol} dehydrogenase, and it was suggested that this structural zinc atom is missing in all \textit{xylitol} dehydrogenases and that this feature is characteristic of \textit{D-sorbitol} dehydrogenases in general. However, the \textit{D-sorbitol} dehydrogenase of \textit{S. pombe} contains a second zinc binding site which is similar to that in \textit{H. jecorina}, and the putative \textit{N. crassa} \textit{xylitol} dehydrogenase (NCU00891.1) also contains such a site. The phylogenetic analysis of \textit{xylitol} and \textit{D-sorbitol} dehydrogenases in this study showed that both the yeast and the filamentous fungal \textit{xylitol} dehydrogenases arose from an \textit{S. pombe} ancestor, implying that the enzymes in yeasts lost the second zinc binding site during evolution. That \textit{S. pombe} is an evolutionary ancestor of \textit{Saccharomyces} and \textit{Candida} has also been evidenced by 28S gene sequence analysis (12). In addition, and despite containing two zinc binding sites, the \textit{H. jecorina} \textit{xylitol} dehydrogenase has the same substrate specificity as the yeast \textit{xylitol} dehydrogenases (5, 13, 17, 19), namely, oxidation of \textit{xylitol} and \textit{D-sorbitol}, reduction of \textit{D-xylose} and \textit{D-fructose}, and no activity on \textit{L-arabinitol} or \textit{L-arabinose}. Although no extensive kinetic analysis was attempted, we conclude by analogy that this enzyme is a typical \textit{L-iditol-NAD}\(^{2-}\)-oxidoreductase (EC 1.1.1.14).

The expression of \textit{xdh1} is adaptive, as no \textit{xdh1} transcript could be detected in mycelia grown on \textit{D-glucose} or \textit{glycerol}, whereas it accumulated during growth on \textit{D-xylose}, \textit{xylitol}, \textit{L-arabinose}, and \textit{L-arabinobiose}. The effect of the latter two components may be direct or indirect, since the catabolism of \textit{L-arabinobiose} forms \textit{xylitol}. The lack of \textit{xdh1} expression on \textit{D-glucose} and \textit{glycerol} would be indicative of regulation by carbon catabolite repression, but since the \textit{xdh1} transcript also does not accumulate during growth on \textit{glycerol} in the \textit{cre1} truncated mutant \textit{H. jecorina} RUT C-30 (unpublished data), this is clearly not the case. Therefore, the presence of the \textit{xdh1} transcript during growth on \textit{D-xylose} is most likely due to induction, which would be consistent with the mode of regulation of the other two genes of the \textit{xylose} catabolic pathway in \textit{A. niger} (those for \textit{xylose} reductase and \textit{xylulose}-5-phosphate kinase) (9, 25). In addition, the \textit{Aspergillus} \textit{xylulose}-5-phosphate kinase was shown to be induced by \textit{L-arabinose} and \textit{L-arabinobiose} but not by \textit{xylitol}. As for the transactivator protein mediating this response, the \textit{A. niger} \textit{xylose} reductase was shown to be under the control of \textit{XlnR}, the \textit{A. niger} transcriptional activator of \textit{xylanase} and cellulase biosynthesis (24, 26), whereas its \textit{xylanokinase} was still inducible by \textit{D-xylose} in an \textit{XlnR}-negative mutant. Whether an \textit{XlnR} homologue would also be involved in the regulation of \textit{xdh1} by \textit{D-xylose} in \textit{H. jecorina} is unclear, however, as we did not detect any nucleo-

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**TABLE 1. Substrate specificity of recombinant Xdh1 of \textit{H. jecorina}**

| Substrate | Cosubstrate (mM) | Mean \(\pm SD\) |
|-----------|-----------------|------------------|
| Xyliot    | NAD (0.3)       | 25 ± 7 0.06 ± 0.02 |
| D-Sorbitol| NAD (0.3)       | 24 ± 7 0.04 ± 0.01 |
| Galactitol| NAD (0.3)       | ND <0.002 ± 0.001 |
| L-Arabinitol| NAD (0.3)   | ND <0.002 ± 0.001 |
| D-Xyliot  | NADH (0.15)     | 4.5 ± 0.8 0.10 ± 0.03 |
| D-Fructose| NADH (0.15)     | 400 ± 120 0.15 ± 0.04 |
| D-Galactitol| NADH (0.15) | ND <0.005 ± 0.002 |
| NAD\(^+\)  | Xyliot (50)     | 0.025 ± 0.010 0.06 ± 0.002 |
| NADH\(^+\) | d-Xyliot (3)    | 0.35 ± 0.014 0.10 ± 0.03 |
| NADP\(^+\) | Xyliot (50)     | ND <0.005 ± 0.001 |

\(^{a}\) ND, not determined.
tide sequences matching the consensus sequence for the binding of XlnR (GGCTAA) in the *xdh1* 5′ upstream region. In *H. jecorina*, the genes encoding the two xylanases are differently expressed, and only *xyn1* and not *xyn2* is regulated by the XlnR homologue Xyr1 (14, 28). It is therefore possible that this coordinated regulation of genes for xylan and D-xylose metabolism in *A. niger* does not exist in *H. jecorina*, a possibility which may reflect the different natural habitats of the two fungi.

An intriguing finding of this study was that a loss-of-function mutant of *xdh1* was still capable of growing on xylitol and—although at lower growth rates—on D-xylose, implying the presence of at least one more enzyme oxidizing xylitol or a less effective new pathway for D-xylose catabolism. The results obtained with the *xdh1 lad1* double-deletion mutant conclusively showed that the enzyme responsible for this residual activity is L-arabinitol-4-dehydrogenase, Lad1. The lack of both Xdh1 and Lad1 leads to a complete loss of the ability to grow on D-xylose and xylitol. Lad1 and Xdh1 belong to the same family.
of zinc-containing alcohol dehydrogenases, and a phylogenetic analysis of Xdh1 and Lad1 from various sources showed that the clade containing Lad1 is basal to that of Xdh1; these data imply that the more specific Xdh1 protein may have evolved from the rather broadly specific Lad1 protein. Such an assumption is also supported by the finding that \textit{H. jecorina} xdh1 was not able to enable a loss-of-function mutant of lad1 to grow on \textit{L}-arabinose, consistent with the inability of Xdh1 to oxidize \textit{L}-arabinitol. Compensation of loss-of-function mutants in one pentose catabolic pathway by enzymes from another pathway may also explain the results obtained with \textit{A. niger}, i.e., that a xylose reductase mutant was still able to grow at a reduced rate on d-xylose (9). The pathways for d-xylose and \textit{L}-arabinose catabolism are both initiated by an aldose reductase, but at present it is not known whether the same enzyme, two isoenzymes with the same substrate specificity, or two enzymes with different substrate specificities catalyze the steps in the two pathways.

Despite of the lack of \textit{L}-arabinitol-oxidizing activity of Xdh1, the xdh1 mutant showed a significantly decreased growth rate on \textit{L}-arabinitol, an intriguing result in view of the lack of effect on growth on xylitol in this mutant. Although we cannot rule

| Substrate (mM) | Mean ± SD U/mg of protein in: |
|---------------|-------------------------------|
| Xylitol (150) | 0.33 ± 0.05 0.11 ± 0.03 |
| d-Sorbitol (150) | 0.27 ± 0.04 0.08 ± 0.02 |
| L-Arabinitol (150) | 0.08 ± 0.02 0.06 ± 0.02 |
| Galactitol (150) | <0.01 ± 0.005 |
| d-Fructose (200) | 0.02 ± 0.01 <0.01 ± 0.005 |
| d-Xylulose (10) | 0.08 ± 0.02 0.01 ± 0.005 |

*Strains were grown for 24 h on d-xylose as a carbon source, and activities were determined as described in Materials and Methods.

![FIG. 7. Characterization of \textit{H. jecorina} lad1 and xdh1 deletion mutants. (A) Southern analysis of lad1 deletion strains. Genomic DNAs of strain QM9414 and lad1 deletion strains were digested with BamHI and probed with a 1-kb BamHI-MluI fragment of lad1. In the lad1 deletion strains, replacement of the lad1 coding region by the \textit{H. jecorina} pyr4 marker leads to a 1.7-kb increase in the size of the hybridizing fragment. This change leads to an increase in the size of the 3-kb hybridizing fragment in strain QM9414 to an ~4.7-kb fragment in the lad1 deletion strains. (B) Southern analysis of xdh1 deletion strains, performed as described in the legend to Fig. 6A. (C) Comparison of the growth behaviors of parent strain QM9414 and deletion strains on plates containing d-glucose (Glc), d-xylose (Xyl), and xylitol (Xol) as carbon sources. (D) Northern analysis of lad1 and xdh1 transcript levels in strain QM9414 and deletion strains. Samples were obtained 6 h after transfer from a glycerol culture (Gly) to \textit{L}-arabinose (Ara) and d-xylose (Xyl) cultures.|
out the possibility that the xdh1 gene product also has a regulatory function, we interpret the findings in terms of an imbalance in substrates, products, and/or coenzymes such that flux like that in the wild type cannot be maintained. The fact that these findings are observed only when the first (NADPH-specific) step (L-arabinose reductase) is omitted suggests that the NADPH/NADP ratio may be the critical variable: filamentous fungi possess a cytosolic pyridine nucleotide transhydrogenase (e.g., *N. crassa* genome database entry NCU01140.1; http://www-genome.wi.mit.edu/annotation/fungi/neurospora) which is responsible for maintaining a balance between NADH/NAD and NADPH/NADP ratios. The catabolism of L-arabinose to L-xylulose-5-phosphate requires two NADPH and two NADP+ molecules, and it is likely that the transhydrogenase is at least partially involved in the regeneration of NAD+ for the L-arabinitol-4-dehydrogenase and xylitol dehydrogenase reactions through reduction of the NADP+ formed in the aldose reductase reactions. Thus, under conditions where only L-arabinitol-4-dehydrogenase accounts for pentitol reductions, the regeneration of NAD+ on behalf of only one NADPH molecule generated by the D-xylulose reductase reaction located downstream may not produce sufficient activity to catalyze L-arabinitol oxidation with the same velocity as in the wild type. This interpretation is also supported by the generally lower growth rate of the wild type on both L-arabinitol and xylitol. However, the proposed role of the pyridine nucleotide transhydrogenase needs to be verified by reverse genetics.

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