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Regulation of Pentose Catabolic Pathway Genes of *Aspergillus niger*

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Summary

The aim of this study was to obtain a better understanding of the pentose catabolism in *Aspergillus niger* and the regulatory systems that affect it. To this end, we have cloned and characterised the genes encoding *A. niger* L-arabitol dehydrogenase (*ladA*) and xylitol dehydrogenase (*xdhA*), and compared the regulation of these genes to other genes of the pentose catabolic pathway. This demonstrated that activation of the pathway depends on two transcriptional regulators, the xylanolytic activator (XlnR) and an unidentified L-arabinose specific regulator (AraR). These two regulators affect those genes of the pentose catabolic pathway that are related to catabolic conversion of their corresponding inducers (D-xylose and L-arabinose, respectively).

Key words: *Aspergillus niger*, pentose catabolism, regulation, XlnR, AraR, L-arabinose, D-xylose

Introduction

Aspergillus niger is a filamentous ascomycete that predominantly grows on dead or decaying plant material which mostly consists of polysaccharides (cellulose, xylan, pectin, xyloglucan, galactomannan). L-arabinose and/or D-xylose are major components of xylan, pectin and xyloglucan (*1*), reflecting the importance of the catabolic pathway related to these carbon sources for *A. niger*. The pentose catabolic pathway of *A. niger* consists of a number of reversible reduction and oxidation steps leading to D-xylulose, which is irreversibly converted into D-xylulose-5-phosphate (Fig. 1) (*2*), from where it enters the pentose phosphate pathway. L-arabinose reductase and D-xylose reductase from *A. niger* are both active on L-arabinose and D-xylose, but with different affinities (*3*). Conversely, the L-arabitol and xylitol dehydrogenases appeared to be highly specific for their own substrate (*4*). For *A. niger*, so far only the genes encoding D-xylose reductase (*xyrA*) (*5*) and D-xylulokinase (*xkiA*) (*6*) have been described. In *Trichoderma reesei*, genes encoding L-arabitol dehydrogenase (*lad1*) (*7*) and xylitol dehydrogenase (*xdh1*) (*8*) have been identified. Recently, *Magnaporthe grisea* L-arabinose reductase and D-xylose reductase encoding genes and their corresponding proteins have been characterised (de Vries *et al.*, unpublished results).

Induction of the pentose catabolic pathway in *Aspergillus* occurs during growth in the presence of L-arabinose or D-xylose (*2*). The presence of D-xylose results in the activation of the xylanolytic transcriptional activator XlnR (*9*) that regulates the expression of genes encoding extracellular polysaccharide degrading enzymes, as well as the expression of *xyrA*, which was reviewed by de Vries (*10*). XlnR is not responsible for the L-arabinose induction of the pentose catabolic pathway. Analysis of an L-arabitol dehydrogenase mutant of *A. nidulans* demon-

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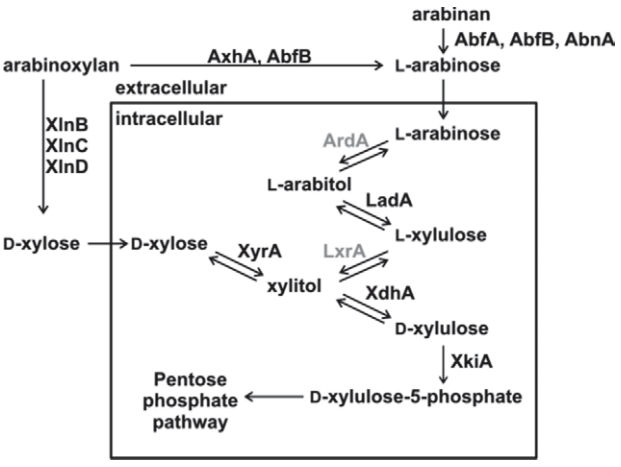

Fig. 1. Pentose catabolism in *Aspergillus niger.* ArdA=L-arabinose reductase; LadA=L-arabitol dehydrogenase; LxrA=L-xylulose reductase; XdhA=xylitol dehydrogenase; XyrA=D-xylose reductase; XkiA=D-xylulose kinase; AbfA, AbfB=α-L-arabinofuranosidase A and B, respectively; AbnA=endoarabinanase A; AxhA=arabinoxylan arabinofuranohydrolase A; XlnB, XlnC=endoxylanase B and C, respectively; XlnD=β-xylosidase. Abbreviations in grey indicate that the corresponding gene has not yet been identified. ArdA, LxrA and XyrA are NADPH/$NADP^+$-dependent. LadA and XdhA are NADH/NAD^+ dependent

strated that the L-arabinose catabolic pathway and the production of extracellular arabinanolytic enzymes (α-L--arabinofuranosidase and endoarabinanase) are coinduced, and that L-arabitol is most likely the small molecular mass inducer (*11*). Analysis of a D-xylulokinase mutant of *A. niger* and the corresponding gene (*xkiA*) supported the inducer function of L-arabitol (*6*). Two *A. niger* mutants have been described that are specifically disturbed in the regulation of the L-arabinose catabolic pathway and the production of extracellular arabinanolytic enzymes (*12*). These mutants confirmed the coregulation of extracellular arabinanolytic functions and the L-arabinose catabolic enzymes, and showed an antagonistic effect between XlnR and the L-arabinose/L-arabitol responsive regulator.

In this paper we report the characterisation and regulation of the *A. niger* genes encoding L-arabitol dehydrogenase (*ladA*) and xylitol dehydrogenase (*xdhA*).

Material and Methods

Strains, libraries and growth conditions

All *A. niger* strains were derived from *A. niger* N400 (CBS 120.49) and are described in Table 1 (*5,12–14*). *Escherichia coli* DH5αF′, LE392 and M15[pREP4] were used for routine plasmid propagation, as a host for amplification of the cDNA libraries, and for production of LadA and XdhA, respectively. Subcloning was performed using pBluescript SK^+ (*15*), pGEM-T easy (Promega) and pQE32 (Qiagen). A genomic library of *A. niger* was constructed in a similar manner as described previously (*16*). The L-arabinose specific cDNA library had previously been described (*17*). For the D-xylose specific cDNA library, *A. niger* NW176 was grown for 16 h in complete medium (CM) (*18*) with 2 % D-fructose. The mycelium was harvested, washed with minimal medium (MM) (*18*) without carbon source, and aliquots of 3.0 g (wet mass) were transferred to 100 mL of MM containing 50 mM D-xylose. Cultures were harvested at 2, 4, and 8 h after the transfer. Total RNA was isolated as described previously (*19*). Isolation of $PolyA^+$ mRNA and construction of the library were performed in the same manner as had previously been published for the L-arabinose-specific library (*17*).

Table 1. *Aspergillus niger* strains used in this study

Strain no.	Phenotype	Genotype	Reference
N402	Wild type	*cspA1*	(*13*)
NW176	*creA* mutant	*fwnA1, cspA1, hisD4, lysA7, creAd2, nicA1, pabA1*	(*14*)
NW199	*xlnR* disruptant	*cspA1, fwnA1, goxC17, pyrA6, xlnRΔ::pIM240, leuA1*	(*5*)
NW321	*araA* mutant	*cspA1, fwnA6, leuA1, araA4*	(*12*)
NW322	*araB* mutant	*cspA1, bioA1, lysA7, araB3, nicA1*	(*12*)

Liquid cultures were inoculated with 10^6 spores/mL, and incubated at 30 °C in an orbital shaker at 250 rpm. For the growth of strains with auxotrophic mutations, the necessary supplements were added to the medium.

Transfer experiments were performed by pregrowing the strains for 16 h in CM containing 2 % (by mass per volume) D-fructose as carbon source, after which the mycelium was harvested and washed with MM without carbon source. Aliquots (1.5 g) of wet mycelium were then transferred to 50 mL MM containing carbon sources as indicated in the text. After 2 h of incubation in a rotary shaker at 250 rpm and 30 °C, mycelium was harvested, dried between tissue paper, frozen in liquid nitrogen and stored at -70 °C.

Molecular biology methods

Standard methods were used for DNA manipulations, such as subcloning, DNA digestions and plasmid DNA isolations (*20*). Chromosomal DNA was isolated as previously described (*21*). Sequence analysis was performed using the Big Dye Terminator kit, version 1.1 (Applied Biosystems, Foster City, CA, USA) according to the supplier's instructions. The reactions were analysed with an ABI 310 (Applied Biosystems) or on an ABI 377 (Applied Biosystems), in which case Longranger Single Packs (Cambrex Bio Science, Rockland, Inc., Rockland, ME, USA) were used. Northern blot analysis was performed as described previously (*19*). Per sample, 5 μg of total RNA were loaded on the gel.

The *A. niger ladA* and *xdhA* accession numbers are AJ854040 and AJ854041, respectively.

Sequence analysis

Nucleotide sequences were analysed with computer programs based on those of Devereux *et al.* (*22*). Sequence alignments were performed by using the Blast programs (*23*) at the server of the National Center for

Biotechnology Information, Bethesda, MD, USA (http://www.ncbi. nlm.nih.gov/blast/).

Production of recombinant LadA and XdhA

Based on the genomic sequences of *ladA* and *xdhA*, oligonucleotides were designed to allow cloning of the cDNAs of these genes in *E. coli* expression vector pQE32 (Qiagen). One oligonucleotide was designed just behind the ATG of the genes and contained a *Bam*HI site for *ladA* (5′-GGATCCCTACCGCAACTGTTCTCG-3′) and a *Bgl*II site for *xdhA* (5′-AGATCTGCACCCAGAACACCA ACG-3′). The other was designed based on the sequence behind the STOP codon of the genes and contained a *Kpn*I site for *ladA* (5′-GGTACCACTGTTTAAATCTTCTG ACC-3′) and a *Pst*I site for *xdhA* (5′-CTGCAGAATTCTA TGAATCGACACC-3′). PCRs were performed with these oligonucleotides using the L-arabinose/L-arabitol-specific *A. niger* cDNA library (*17*) and the D-xylose-specific *A. niger* cDNA library as template for *ladA* and *xdhA*, respectively. PCR fragments were cloned in pGEM-T easy (Promega) and confirmed by sequence analysis. Fragments were isolated from these constructs using the restriction enzymes mentioned above and cloned into pQE32. The resulting constructs were transformed to *E. coli* M13 cells as indicated by the supplier (Qiagen). Purification of the recombinant proteins was performed according to the supplier's recommendations.

Enzyme assays

All enzyme assays were performed at 20 °C. Dehydrogenase activities were determined using 100 mM glycine, pH=9.6, 0.4 mM NAD^+ and 100 mM substrate. Reductase activities were determined using 50 mM sodium phosphate, pH=7.6, 0.2 mM NADH and 100 mM substrate. Absorbance changes at 340 nm (ε=6.22 mM^{-1} cm^{-1}) were measured on a Unicam UV-1 spectrophotometer (Spectronic Unicam, Rochester, NY, USA).

Results

Cloning and characterisation of ladA and xdhA

The amino acid sequences of *T. reesei* L-arabitol dehydrogenase and xylitol dehydrogenase were used to identify their paralogues in the *A. niger* genome sequence (DSM, Delft, The Netherlands). The *A. niger* L-arabitol dehydrogenase encoding gene (*ladA*) consists of 1228 bp and contains one intron. The gene encodes a protein of 386 amino acids with a predicted molecular mass of 41 350 Da and a predicted pI of 5.99. The *A. niger* xylitol dehydrogenase encoding gene (*xdhA*) consists of 1262 bp and contains two introns. The gene encodes a protein of 357 amino acids with a predicted molecular mass of 38 836 Da and a predicted pI of 9.05.

Several putative regulatory binding sites were detected in the promoters of *ladA* and *xdhA*. The promoter of *ladA* contains 1 CCAAT box (*24*), 4 putative CreA binding sites (*25*), 2 putative PacC binding sites (*26*), two putative AreA binding sites (*27*), a conserved sequence present in the promoters of pectinolytic genes (CCCTGA) (*28*), and a sequence conserved in the promoters of arabinanolytic genes (YGACRT), modified from the work by Flipphi *et al.* (*29*), based on analysis of the promoters of the arabinanolytic and L-arabinose catabolic genes. The *xdhA* promoter contains 8 CCAAT sites, 2 putative XlnR binding sites (*19*), 2 putative CreA binding sites, 1 putative AreA binding site, and 2 copies of a conserved sequence of arabinanolytic genes (YGACRT).

Characterisation of recombinant LadA and XdhA

Recombinant LadA and XdhA were purified from *E. coli* using the HIS tag present in the expression plasmid (pQE30, Quiagen) and substrate specificities were determined for both enzymes. L-arabitol dehydrogenase (LadA) could only use NAD^+ as a cofactor and had the highest dehydrogenase activity on L-arabitol, while the activity on xylitol, D-sorbitol and D-galactitol was 2.4-, 17-, and 25-fold lower, respectively (Table 2). The reductase activity of LadA on L-xylulose, D-xylulose, and D-fructose was 2.8- and 1.4-fold higher, and 33-fold lower, respectively, than the dehydrogenase activity on L-arabitol. Xylitol dehydrogenase (XdhA) was also specific for NAD^+ and had the highest dehydrogenase activity on xylitol, while the activity on D-sorbitol and L-arabitol was 1.8- and 33-fold lower, respectively, and no activity was detected on D-galactitol. XdhA had reductase activity on D-xylulose and D-fructose, and it was 1.7-fold higher and 14-fold lower, respectively, than the dehydrogenase activity on xylitol. No activity for either enzyme was found using D-arabitol, D-mannitol, glycerol, ethanol, L- or D-arabinose, L- or D-xylose, D-glucose, D-galactose, D-ribose, D-mannose, L-rhamnose, L- or D-sorbose, L- or D-fucose, or D-tagatose as substrates.

Table 2. Specific activity of recombinant L-arabitol dehydrogenase and xylitol dehydrogenase

Enzyme substrate	LadA	XdhA
	Specific activity/(U/mg)	
L-arabitol	211±25	12±3
xylitol	86±6	380±8
D-sorbitol	12.3±0.3	218±3
D-galactitol	9±3	n.d.
L-xylulose	597±9	n.d.
D-xylulose	302±2	650±6
D-fructose	3.1±0.1	28±6

n.d.=not detected

Regulation of the expression of ladA and xdhA

The expression of *ladA* and *xdhA* was studied in a transfer experiment (see Materials and Methods) using a wild type, two arabinanolytic mutants (*12*) and an *xlnR* disruptant strain (*5*), and compared to the previously determined expression of *xkiA*, *xyrA*, and *abfB* (encoding α-L-arabinofuranosidase B) (*12*). High expression of *ladA* was observed in the wild type and the *xlnR* disruptant on L-arabinose and L-arabitol, while the expression on D-xylose was very low in all strains except for the *xlnR* disruptant. In the arabinanolytic mutants, very low *ladA* expression on these three carbon sources was detected.

For *xdhA*, expression was highest on L-arabinose and L-arabitol in the wild type, but significant expression levels were also detected on D-xylose (Fig. 2). The expression on L-arabitol and L-arabinose in the arabinanolytic

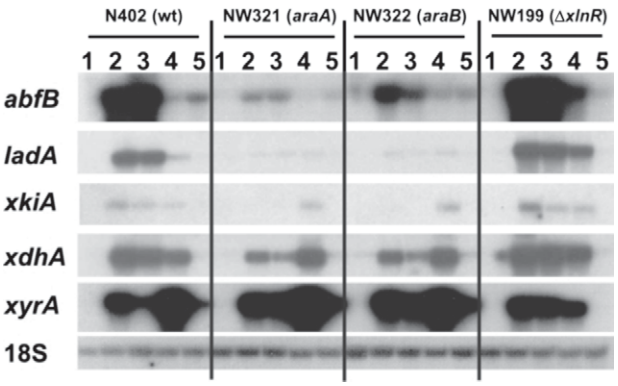

Fig. 2. Comparison of the expression of *ladA* and *xdhA* to other pentose catabolic genes and *abfB*
1=D-fructose, 2=L-arabinose, 3=L-arabitol, 4=D-xylose, 5=xylitol

mutants was decreased, while a small increase was observed on D-xylose. In the *xlnR* disruptant, increased expression on L-arabinose and L-arabitol was observed. No expression was detected for any of the genes tested in the presence of xylitol.

Discussion

It has been shown that two regulatory systems are involved in the pentose catabolic pathway, responding to either D-xylose (XlnR) or L-arabinose/L-arabitol (unidentified arabinanolytic regulator), and that they act in an antagonistic fashion (*12*). XlnR appeared to regulate D-xylose reductase (*xyrA*) (*5*), but not D-xylulokinase (*xkiA*) (*6*), while the putative arabinanolytic regulator activates *xkiA*, but not *xyrA* (*12*). The effect of these regulators on the other members of the catabolic pathway could only be studied using enzyme activities, as the genes have not yet been identified. This was complicated due to the fact that L-arabinose reductase and D-xylose reductase are active on both L-arabinose and D-xylose. The analysis of the expression of *ladA* and *xdhA*, and reanalysis of the expression of *abfB*, *xkiA* and *xyrA* performed in this study clearly shows the influence of the two regulatory systems on the pentose catabolic pathway in *A. niger*. Expression of *ladA* and *abfB* on L-arabinose and L-arabitol is strongly reduced in the arabinanolytic mutants, while significant expression on D-xylose is only detected in the *xlnR* disruptant. Taking into account the antagonistic action of the two regulatory systems (*12*), this indicates that *ladA* and *abfB* are predominantly regulated by the arabinanolytic system, while XlnR has no or very little influence on their expression. The expression of *xkiA* and *xdhA* is more complex. They have reduced expression on L-arabinose and L-arabitol in the arabinanolytic mutants and increased expression on D-xylose in the *xlnR* disruptant, indicating that they are under the control of the arabinanolytic regulator. However, they also have increased expression on D-xylose in the arabinanolytic mutants and on L-arabinose and L-arabitol in the *xlnR* disruptant, indicating regulation by XlnR. Based on the changes in expression levels, the effect of XlnR on the expression of these genes appears to be smaller than the effect of the arabinanolytic regulator. Expression of *xyrA* is increased on D-xylose in the arabinanolytic mutants and reduced in the *xlnR* disruptant, demonstrating that XlnR regulates this gene. Contrary to what was demonstrated for a number of other XlnR-regulated genes (*30*) and to what was suggested previously (*5*), the expression of *xyrA* on D-xylose in the *xlnR* disruptant is not completely absent. This indicates that XlnR is not the only factor affecting *xyrA* expression. The expression of *xyrA* on L-arabinose does not decrease in the arabinanolytic mutants, suggesting that the arabinanolytic regulator is not involved in *xyrA* expression. In fact, the *xyrA* expression on L-arabinose could be the result of the small amount of D-xylose present in the L-arabinose preparation used in these studies (*31*).

These data are supported by promoter analysis of the genes. In the promoters of *xdhA* and *xkiA*, putative binding sites for XlnR have been detected as well as a sequence commonly found in the promoter of genes that are influenced by the arabinanolytic regulator. However, the promoter of *ladA* only contains this conserved arabinanolytic sequence, while the promoter of *xyrA* only contains putative XlnR binding sites. Such a system can be explained from a fungal perspective as it ensures expression of all the relevant genes in the presence of either L-arabinose or D-xylose. The effects observed are not due to starvation or other stress factors, as all strains can grow on the carbon sources used for the study (*12*).

Conclusions

Cloning of the genes encoding L-arabitol dehydrogenase and xylitol dehydrogenase, which are part of the pentose catabolic pathway, has been performed. Analysis of the regulation of these genes as well as other genes of the pathway demonstrated that regulation of the pathway depends on two specific regulators. The previously characterised transcriptional activator XlnR regulates the conversion of D-xylose into xylitol and to a lesser extent the two-step conversion of xylitol into D-xylulose-5-phosphate. A second regulator, named AraR, which has not yet been identified, regulates the expression of all genes involved in the conversion of L-arabinose into D-xylulose--5-phosphates. This demonstrates that the combined steps of D-xylose and L-arabinose catabolism are controlled by both regulators, while the steps specific for either L-arabinose or D-xylose are controlled by a single regulator.

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