Isolation and characterization of two specific regulatory *Aspergillus niger* mutants shows antagonistic regulation of arabinan and xylan metabolism

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This paper describes two *Aspergillus niger* mutants (*araA* and *araB*) specifically disturbed in the regulation of the arabinanase system in response to the presence of L-arabinose. Expression of the three known L-arabinose-induced arabinanolytic genes, *abfA*, *abfB* and *abnA*, was substantially decreased or absent in the *araA* and *araB* strains compared to the wild-type when incubated in the presence of L-arabinose or L-arabitol. In addition, the intracellular activities of L-arabitol dehydrogenase and L-arabinose reductase, involved in L-arabinose catabolism, were decreased in the *araA* and *araB* strains. Finally, the data show that the gene encoding D-xylulose kinase, *xkiA*, is also under control of the arabinanolytic regulatory system. L-Arabitol, most likely the true inducer of the arabinanolytic and L-arabinose catabolic genes, accumulated to a high intracellular concentration in the *araA* and *araB* mutants. This indicates that the decrease of expression of the arabinanolytic genes was not due to lack of inducer accumulation. Therefore, it is proposed that the *araA* and *araB* mutations are localized in positive-acting components of the regulatory system involved in the expression of the arabinanase-encoding genes and the genes encoding the L-arabinose catabolic pathway.

INTRODUCTION

Degradation of the plant cell wall polysaccharide xylan has been studied in detail in *Aspergillus niger*. Regulation of the xylanolytic system is mediated by the transcriptional activator XLNR (van Peij *et al.*, 1998b). XLNR regulates the expression of a number of genes involved in xylan degradation, such as those encoding β-xylosidase (*xlnD*), arabinoxylan arabinofuranohydrolase (*axhA*), α-glucuronidase (*aguA*) and D-xylose reductase (*xyrA*) (Hasper *et al.*, 2000; van Peij *et al.*, 1998a).

Arabinan is another polysaccharide found in plant cell wall heteropolysaccharides as a side chain of pectin (de Vries & Visser, 2001). *A. niger* is able to degrade arabinan to L-arabinose using its arabinanolytic system. To date three arabinanases have been characterized: two α-L-arabinofuranosidases (ABFA and ABFB) and an arabinan 1,5-α-L-arabinanase (ABNA) (van der Veen *et al.*, 1991). L-Arabinose can be used by the fungus as a carbon source via the L-arabinose catabolic pathway. This pathway converts L-arabinose to D-xylulose 5-phosphate, which is further metabolized via the non-oxidative pentose phosphate pathway. Two *A. niger* genes involved in pentose metabolism have previously been cloned and characterized: x*yrA*, encoding D-xylose reductase (Hasper *et al.*, 2000), and *xkiA*, encoding D-xylulose kinase (vanKuyk *et al.*, 2001).

Little is known about the regulation of the arabinanase system. Arabinanolytic genes are specifically induced when *A. niger* is grown on arabinan-containing substrates or the monomeric compounds L-arabinose and L-arabitol (Flipphi *et al.*, 1994; Gielkens *et al.*, 1997, 1999; Ramon *et al.*, 1993; van der Veen *et al.*, 1991, 1993). One of the intermediates of the L-arabinose catabolic pathway, L-arabitol, is believed to be the low-molecular-mass inducer of the system (van der Veen *et al.*, 1993). In *Aspergillus nidulans* it has been

shown that increased intracellular arabitol accumulation correlates with higher production of the enzymes involved in arabinan breakdown (de Vries *et al.*, 1994), suggesting that L-arabitol is the true inducer of this system. The genes encoding three arabinan-degrading enzymes, *abfA*, *abfB* and *abnA*, have been cloned and characterized (Flipphi *et al.*, 1993a, b, c). Expression analysis of these genes showed that they are co-ordinately expressed. Moreover, insertion of additional copies of one of the arabinanase genes in *A. niger* resulted in lower expression of the other two genes, suggesting a titration effect at the level of a single specific transcriptional activator (Flipphi *et al.*, 1994).

The aim of this study was to find genetic evidence for a positive-acting specific regulatory system involved in the induction of arabinanases in *A. niger*. Furthermore we wanted to assess whether the same system regulates induction of the intracellular enzymes of the L-arabinose catabolic pathway, and investigate the interaction between the regulation of the arabinanase and xylanase systems.

METHODS

Strains and growth conditions. All *A. niger* strains used for this study were derived from *A. niger* N400 (CBS120.49) and are described in Table 1. NW315 was used for mutagenesis and N423 was used as a reference strain. Mycelium cultures were grown at pH 6 in minimal medium (MM) containing, per litre: 6·0 g NaNO$_3$, 1·5 g KH$_2$PO$_4$, 0·5 g KCl, 0·5 g MgSO$_4$, 1 ml trace elements solution (Vishniac & Santer, 1957) and carbon sources as indicated in the text. Culturing was done in a rotary shaker at 250 r.p.m. and 30 °C. For growth of strains with auxotrophic mutations, the necessary supplements were added to the medium. For growth on plates 1·5 % (w/v) agar was added to the medium before autoclaving. In transfer experiments strains were pre-grown in MM containing 1 % (w/v) D-fructose, 0·5 % (w/v) yeast extract and 0·2 % (w/v) Casamino acids. After 16 h mycelium was harvested by suction over a filter, washed with MM without carbon source, and aliquots of 1·5 g (wet weight) were transferred to 50 ml MM containing carbon sources as indicated in the text. The mycelium was harvested after 2, 4 or 8 h, dried between paper and frozen in liquid nitrogen. Mycelium used for the partial purification of intracellular enzymes was washed at 4 °C (10 mM potassium phosphate, pH 7·0) during harvesting. The mycelium samples were stored at −70 °C.

Selection and genetic analysis of mutants. Conidiospores were irradiated as described by Witteveen *et al.* (1989). Subsequently, 10^5 spores were spread on solid media plates containing 50 mM L-arabinose and 50 mM sodium D-gluconate. The plates were incubated for 3 days at 30 °C and colonies were purified on the same medium. The mutants were tested by replica plating for growth on D-xylose and combinations of xylitol, D-xylose or L-arabinose with either D-gluconate or L-alanine. Mutants that were still unable to grow on L-arabinose, but showed increased growth on L-arabinose + D-gluconate and L-arabinose + L-alanine, were analysed in more detail.

Genetic localization of the *araA4* and *araB2* mutations was determined by somatic recombination using test strain NW148 as described by Bos *et al.* (1988).

Complementation tests of the *araA4* mutation with other mutations were performed using heterokaryons. Strain NW320 was isolated from somatic recombination experiments using strains NW148//NW316. Heterokaryons were selected from NW320 with some of the other *ara* mutants isolated from NW315. Balanced heterokaryons, recognizable by a good mixture of fawn-coloured and black conidiospores, were tested for growth on MM with nicotinamide and L-arabinose, and MM with nicotinamide and D-glucose. NW320 and NW322 were used as negative controls and N423 as a positive control.

Preparation of cell extracts and protein determination. Mycelia were harvested and disrupted as described previously (Witteveen *et al.*, 1989). Cell extracts were obtained by suspending disrupted frozen mycelium in extraction buffer (10 mM Bistris, 5 mM MgCl$_2$, 1 mM 2-mercaptoethanol, 0·5 mM EDTA pH 6·3) followed by centrifugation at 15 000–20 000 *g* in order to remove cell debris. The entire procedure was performed at 0 to +4 °C. After denaturation with sodium deoxycholate and precipitation of protein with trichloroacetic acid (Bensadoun & Weinstein, 1976) protein concentrations were determined using the bicinchoninic acid method as described by the manufacturer (Sigma).

Fractionation of cell extracts. Cell extracts were fractionated by anion-exchange chromatography. Two millilitres of cell extract was loaded on a ResourceQ column with a bed volume of 1 ml (Pharmacia Biotech). The column was washed with 8 ml extraction buffer. Elution was started with a gradient of 0–0·5 M NaCl over 18 column volumes. The flow rate used was 3 ml min^{-1}. Fractions of 0·5 ml were collected during this gradient and kept on ice until they were used in the enzyme activity measurements.

Enzyme assays. All enzyme assays were performed at 30 °C. L-Arabitol dehydrogenase and xylitol dehydrogenase activities were

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

Strain	Genotype	Reference
N402 (reference)	*cspA1*	Bos *et al.* (1988)
N423 (reference)	*cspA1*; *nicA1*	Bos *et al.* (1988)
NW148 (tester)	*cspA1*; *hisD4*; *bioA1 lysA7*; *leuA1*; *metB10 argB15*; *pabA1*; *cnxC5*; *trpB2*	Bos *et al.* (1988)
NW199 (ΔxlnR)	*cspA1*; *fwnA1*; *goxC17*; *pyrA6 xlnRΔ::pIM240*; *leuA1*	Hasper *et al.* (2000)
NW315 (xkiA1)	*cspA1*; *fwnA1*; *pyrA6*; *xkiA1*; *nicA1*	Witteveen *et al.* (1989)
NW316 (xkiA1, araA4)	*cspA1*; *fwnA1*; *pyrA6*; *xkiA1*; *nicA1 araA4*	This study
NW318 (xkiA1, araB3)	*cspA1*; *fwnA6*; *pyrA6 araB3*; *xkiA1*; *nicA1*	This study
NW319 (xkiA1, araA2)	*cspA1*; *fwnA6*; *pyrA6*; *xkiA1*; *nicA1 araA2*	This study
NW320 (araA4)	*cspA1*; *fwnA6*; *lysA7 bioA1*; *nicA1 araA4*	This study
NW321 (araA4)	*cspA1*; *fwnA6*; *leuA1*; *araA4*	This study
NW322 (araB3)	*cspA1*; *bioA1 lysA7 araB3*; *nicA1*	This study

determined using 100 mM glycine pH 9·6, 0·2 mM NAD$^+$ and 40 mM L-arabitol or xylitol, respectively. L-Arabinose reductase and D-xylose reductase activities were determined using 50 mM sodium phosphate pH 7·8, 0·2 mM NADPH and 40 mM L-arabinose or D-xylose, respectively. Absorbance changes at 340 nm ($\varepsilon = 6\cdot22\ \mathrm{mM}^{-1}\ \mathrm{cm}^{-1}$) were measured using a Cobas Bio autoanalyser (Roche) or a UV-2501PC spectrophotometer (Shimadzu Scientific Instruments).

Extracellular hydrolytic activities were assayed as follows using *p*-nitrophenyl α-L-arabinofuranoside (*p*NP-A) or *p*-nitrophenyl β-D-xylopyranoside (*p*NP-X) as substrates: 0·01 % *p*NP-substrate, 20–40 µl sample and 25 mM sodium acetate pH 5·0 were used in a total volume of 100 µl, incubated for 1 h at 30 °C (*p*NP-A) or 50 °C (*p*NP-X) and the reaction was subsequently stopped by adding 100 µl 0·25 M Na$_2$CO$_3$. Absorbance was measured at 405 nm in a microtitre platereader (Molecular Devices). The activity was calculated using a standard curve ranging from 0 to 80 nmol *p*-nitrophenol per assay volume.

Polyol extraction and determination. Intracellular polyols were extracted as described previously (Witteveen *et al.*, 1994). Polyol concentrations were measured by HPAEC (Dionex) with a Carbopac MA1 column using isocratic elution with 0·48 M NaOH.

Statistical analysis. Statistical analysis of the polyol and enzyme levels was carried out using the Student's standard *t*-test with a reliability interval of 95 %.

Expression analysis. Total RNA was isolated from powdered mycelium using TRIzol Reagent (Life Technologies), according to the supplier's instructions. For Northern blot analysis 3 µg total RNA was incubated with 3·3 µl 6 M glyoxal, 10 µl DMSO and 2 µl 0·1 M sodium phosphate pH 7 in a total volume of 20 µl for 1 h at 50 °C to denature the RNA. RNA electrophoresis was performed on a 1·5 % agarose gel using 0·01 M sodium phosphate buffer pH 7 and transferred to Hybond-N filters (Amersham) by capillary blotting. Filters were hybridized at 42 °C in a solution of 50 % (v/v) formamide, 10 % (w/v) dextran sulphate, 0·9 M NaCl, 90 mM trisodium citrate, 0·2 % (w/v) Ficoll, 0·2 % (w/v) polyvinylpyrrolidone, 0·2 % (w/v) bovine serum albumin, 0·1 % (w/v) SDS and 100 µg single-stranded herring sperm DNA ml^{-1}. Washing was performed under stringent conditions using 30 mM NaCl, 3 mM trisodium citrate and 0·5 % (w/v) SDS at 68 °C. Probes used were: a 2·0 kb *Pst*I fragment of *abfA* (Flipphi *et al.*, 1994); a 2·8 kb *Pst*I fragment of *abfB* (Flipphi *et al.*, 1994); a 3·1 kb *Hin*dIII fragment of *abnA* (Flipphi *et al.*, 1994); a 0·5 kb *Eco*RV/*Xho*I fragment of *aguA* (de Vries *et al.*, 2001); a 2·8 kb *Pst*I/*Nsi*I fragment of *xlnD* (van Peij *et al.*, 1997); a 0·3 kb *Sal*I/*Xho*I fragment of *xyrA* (Hasper *et al.*, 2000); a 4·0 kb *Sst*I fragment of *xkiA* (vanKuyk *et al.*, 2001) and a 0·7 kb *Eco*RI fragment from the gene encoding the 18S rRNA subunit (Melchers *et al.*, 1994). The 18S probe was used as an RNA loading control.

RESULTS

Isolation of *A. niger* mutants

We developed a selection method to obtain *A. niger* mutants altered in L-arabinose catabolism making use of a parent strain containing the *xkiA1* mutation. The *xkA1*-containing strain lacks D-xylulose kinase activity and is therefore unable to use L-arabinose, L-arabitol, xylitol or D-xylose as a carbon and energy source (Witteveen *et al.*, 1989). Since L-arabinose is able to repress the use of poorer carbon sources such as D-gluconate and L-alanine (Ruijter & Visser, 1997), an *xkiA1* strain fails to grow on a combination of L-arabinose and D-gluconate. By selecting for mutants capable of growth on a combination of L-arabinose and D-gluconate we obtained mutants disturbed in L-arabinose metabolism.

Out of 10^5 spores of the *xkiA1* strain NW315 which were irradiated, 20 colonies were purified. First, we tested these mutants for growth on D-xylose to exclude reversion of the *xkiA1* mutation. Subsequently, derepression of growth on D-gluconate or L-alanine in the presence of L-arabinose, D-xylose or xylitol was tested. Three mutants (NW316, NW318 and NW319) showed increased growth on L-alanine or D-gluconate with L-arabinose and not with D-xylose or xylitol.

Genetic analysis of the *ara* mutations

Two *ara* mutants (NW316 and NW318) were subjected to a somatic recombination experiment with tester strain NW148 for several purposes. Firstly, to gather information on the genetic localization of the *ara* mutations and secondly to remove the *xkiA1* mutation. From the somatic recombination of NW316 (*araA4*) and NW148, 85 recombinants were analysed. Recombination frequencies of the genetic markers on chromosome V with *araA4* were 28 % for *argB13*, 31 % for *nicA1* and 31 % for *metB10*. Recombination frequencies between *araA4* and markers on the other seven chromosomes ranged from 37 % (*trpB2*) to 62 % (*fwnA1*). Recombination frequencies higher than 25 % are not considered to be absolute evidence for linkage, but since none of the markers on the other chromosomes showed a better linkage, we concluded that the *araA4* mutation is located on chromosome V. From the somatic recombination of NW318 (*araB3*) and NW148, 109 recombinants were analysed. No convincing linkage was found with any of the markers used. Best linkage of *araB3* was found with *pyrA6* and *bioA1*, both located on chromosome III, with 38 % recombination. Since recombination percentages with the markers on the other chromosomes ranged from 43 % to 57 %, *araB3* is probably located on chromosome III.

Complementation of the *araA4* mutation with two other *ara* mutations was tested. Heterokaryons of strains NW318 (*araB3*) and NW319 (*araA2*) with NW320 (*araA4*) were isolated and tested for growth on L-arabinose and D-glucose. NW318 was found to complement the *araA4* defect. NW319 did not complement *araA4*, indicating that the mutations designated *araA2* and *araA4* are allelic. NW315 and the mutants derived from it are pentose non-utilizing due to the *xkiA1* mutation. Growth tests of *xkiA+* recombinants of the original mutants showed that the *araA* and *araB* mutations resulted in strongly reduced growth on L-arabinose and L-arabitol and a slight decrease in growth on xylitol, whereas growth on D-xylose was not affected (Table 2).

Table 2. Growth on plates of the *A. niger* wild-type, *araA2*, *araA4*, *araB3* and *ΔxlnR* strains and *araA*//*araB* heterokaryons, grown for 3 days on solid MM+50 mM carbon source at 30 °C

Growth: +++, good; ++, mediocre; +, poor; −no detectable growth; ND, not determined.

Strain	D-Glucose	D-Xylose	Xylitol	L-Arabinose	L-Arabitol
N423 (reference)	+++	+++	++	+++	++
NW321 (*araA4*)	+++	+++	+	−	−
NW322 (*araB3*)	+++	+++	+	−	−
NW199 (*ΔxlnR*)	+++	++	++	+++	++
NW315//NW148 (*xkiA1*//tester)	+++	ND	ND	+++	ND
NW320//NW318 (*araA4*//*xkiA1, araB3*)	+++	ND	ND	+++	ND
NW320//NW319 (*araA4*//*xkiA1, araA2*)	+++	ND	ND	−	ND

L-Arabitol dehydrogenase and L-arabinose reductase activities are strongly decreased in *araA* and *araB* mutants

Under inducing conditions (D-xylose and L-arabinose) L-arabitol dehydrogenase activity in NW321 (*araA*) and NW322 (*araB*) was fivefold lower on D-xylose and 15-fold lower on L-arabinose compared to the wild-type (N423) and the *ΔxlnR* strain (NW199) (Fig. 1a). L-Arabitol dehydrogenase activities in the wild-type and *ΔxlnR* strains were comparable. L-Arabitol dehydrogenase activity was higher during induction on L-arabinose than on D-xylose.

Xylitol dehydrogenase activity (Fig. 1b) appeared not to be significantly different in the *araA* and *araB* and wild-type strains on either L-arabinose or D-xylose. Xylitol dehydrogenase activity was affected by the disruption of *xlnR*, resulting in a lower activity on D-xylose. Unexpectedly, the xylitol dehydrogenase activity in the *xlnR* disruption strain was higher on L-arabinose than in the wild-type strain.

In order to visualize the relative level of L-arabinose reductase and D-xylose reductase a ratio of the activities was calculated (Fig. 1c). Both these enzymes are able to reduce L-arabinose and D-xylose, and they elute at almost
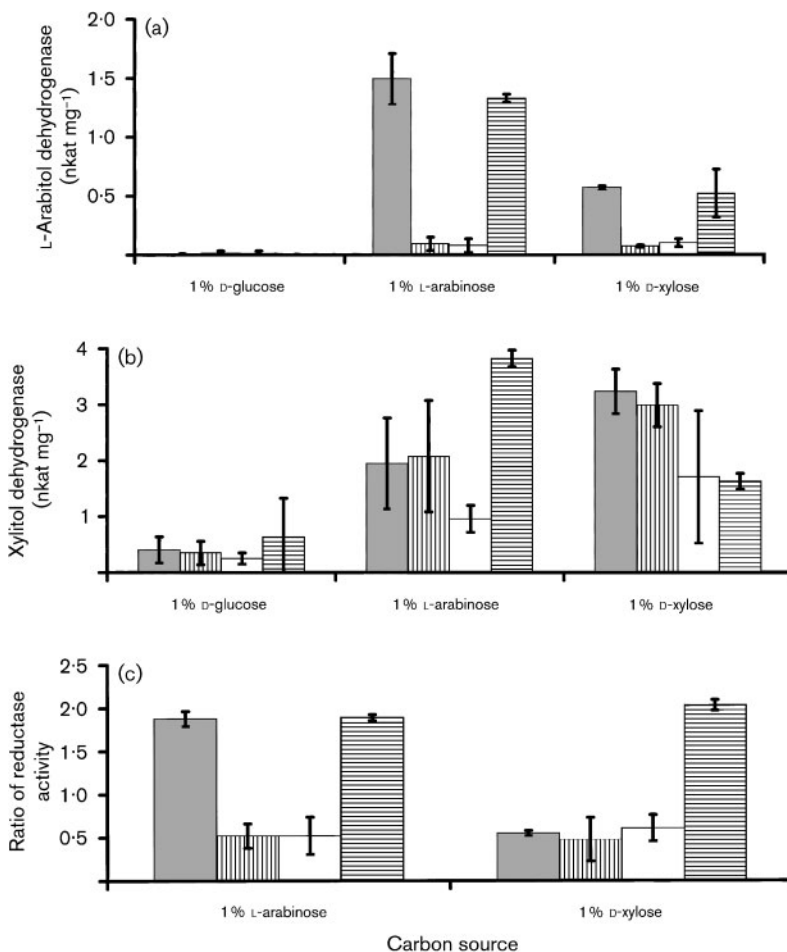

Fig. 1. (a, b) Intracellular enzyme activities of (a) L-arabitol dehydrogenase (EC 1.1.1.12) and (b) xylitol dehydrogenase (NAD$^+$-dependent) (EC 1.1.1.9) with the different carbon sources shown. (c) Ratio of L-arabinose reductase/D-xylose reductase (EC 1.1.1.21) activity. Strains: ■, N423 (wild-type); ▦, NW321 (*araA*); □, NW322 (*araB*); ▤, NW199 (*ΔxlnR*). Activity is given in nkat (mg protein)$^{-1}$. The error bars represent the reliability interval of 95 %.

the same salt concentration during the fractionation of cell extracts. As D-xylose reductase activity is not induced in the ΔxlnR strain on D-xylose (Hasper et al., 2000) we concluded that a ratio of L-arabinose reductase/D-xylose reductase activity of 0·5 to 0·6 represents a situation in which D-xylose reductase is mostly present, and as one would expect to find only L-arabinose reductase when inducing the wild-type on L-arabinose a ratio of 1·9 to 2·2 represents a situation in which L-arabinose reductase is predominantly present. In the wild-type strain L-arabinose reductase is induced on L-arabinose and D-xylose reductase on D-xylose. In the ΔxlnR strain the L-arabinose reductase/ D-xylose reductase ratio was 2 on both L-arabinose and D-xylose, which is consistent with the absence of expression of xyrA encoding D-xylose reductase (Hasper et al., 2000). The L-arabinose reductase/D-xylose reductase ratio in the araA and araB strains after incubation on L-arabinose and D-xylose resembled that of the wild-type grown on D-xylose. In other words only D-xylose reductase can be detected and L-arabinose reductase was not present in the ara mutant strains.

α-L-Arabinofuranosidase is absent in the araA and araB strains

Extracellular α-L-arabinofuranosidase activity levels (Fig. 2a) were strongly decreased in the araA and araB strains compared to the wild-type and ΔxlnR strains when grown on L-arabinose. There also appeared to be some production of α-L-arabinofuranosidase by the wild-type when grown on D-xylose, although not to the same level as when grown on L-arabinose. In NW321 (araA), NW322 (araB) and NW199 (ΔxlnR) there was no production of α-L-arabinofuranosidase on D-xylose apart from the basal level observed on D-glucose.

β-Xylosidase activity (Fig. 2b) was induced in the wild-type strain on D-xylose, but not on L-arabinose. There was no production of this activity in the ΔxlnR strain on either carbon source. With the araA strain there was a normal level of β-xylosidase activity on D-xylose and an elevated production on L-arabinose compared to the wild-type strain. In the araB strain the production of β-xylosidase activity was low on D-xylose and approximately twofold on L-arabinose compared to the basal level in the wild-type strain.

The araA and araB strains accumulate arabitol

Intracellular arabitol levels (Fig. 3a) were at least fivefold higher in the araA and araB strains grown on L-arabinose than in the wild-type and ΔxlnR strains. Arabitol was present at a low level in all four strains on D-xylose and D-glucose. The arabitol produced during growth on D-glucose is most likely D-arabitol produced from an intermediate of the pentose phosphate pathway. Xylitol levels (Fig. 3b) were lower in the araA and araB mutants compared to the wild-type strain on L-arabinose, but comparable to the level in the wild-type on D-xylose. Xylitol was not detected in mycelium grown on D-glucose in any of the four strains.

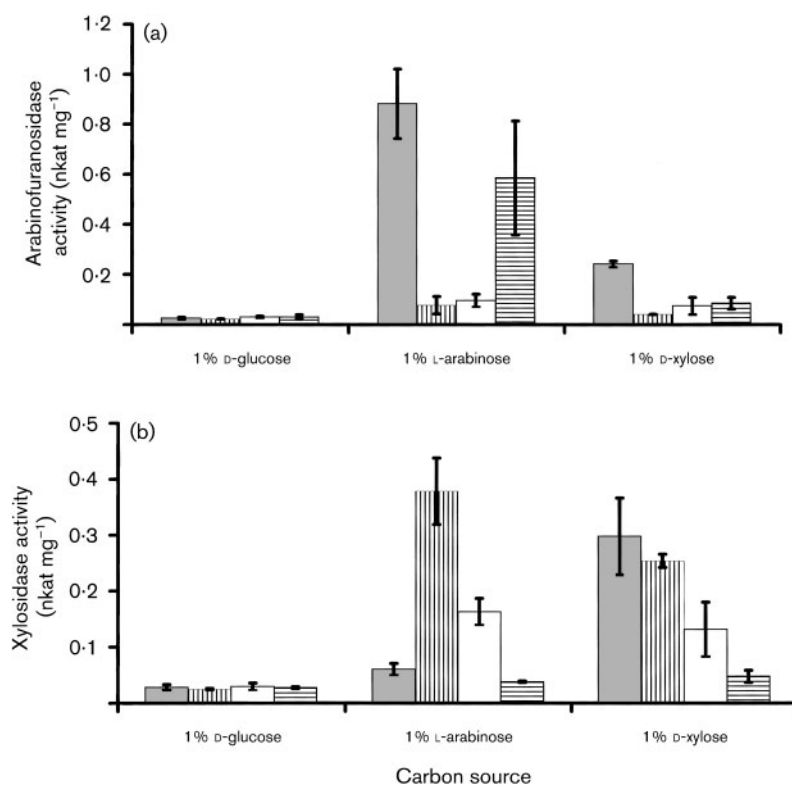Levels of other polyols, such as glycerol and erythritol,

Fig. 2. Extracellular enzyme activities of (a) α-L-arabinofuranosidase (EC 3.2.1.55) and (b) 1,4-β-xylosidase (EC 3.2.1.37). Strains: ■, N423 (wild-type); ▥, NW321 (araA); □, NW322 (araB); ▤, NW199 (ΔxlnR). Activity is given in nkat (mg dry weight)$^{-1}$. The error bars represent the reliability interval of 95 %.
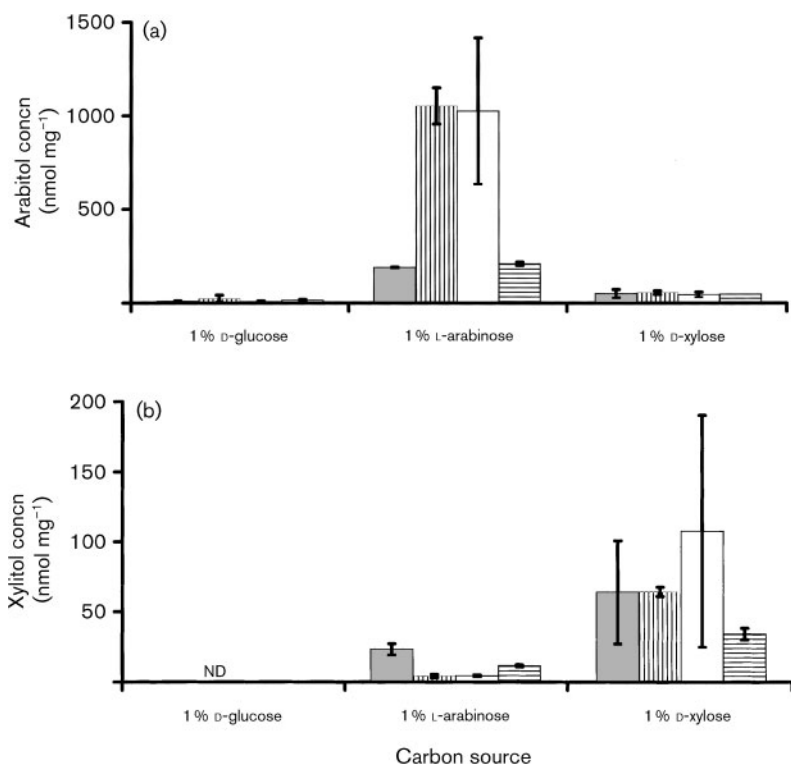
Fig. 3. Intracellular (a) arabitol and (b) xylitol accumulation. Strains: ■, N423 (wild-type); ▥, NW321 (*araA*); □, NW322 (*araB*); ▤, NW199 (Δ*xlnR*). The error bars represent the reliability interval of 95 %. ND, Not detected.

appeared not to be affected by disruption of *xlnR* or the *araA* and *araB* mutations under the conditions tested (data not shown).

Expression of genes of the arabinanase system is decreased in the *ara* mutants

Expression of the known L-arabinose-induced genes, *abfA* and *abfB*, was severely decreased or completely absent in the *araA* and *araB* strains on L-arabinose or L-arabitol compared to the expression in the wild-type (Fig. 4). The data show that *xkiA* is regulated by both L-arabinose and D-xylose induction. Expression of *xkiA* is normal in the *araA* and *araB* strains on D-xylose. In the Δ*xlnR* strain a higher expression level of the arabinanase-encoding genes and *xkiA* was detected. Conversely, the expression of the XLNR-regulated genes, *xlnD*, *aguA* and *xyrA*, was decreased or absent in the Δ*xlnR* strain and elevated in the two *ara* mutants. Expression of *xyrA* in the Δ*xlnR* strain is less than in the wild-type strain but not zero.
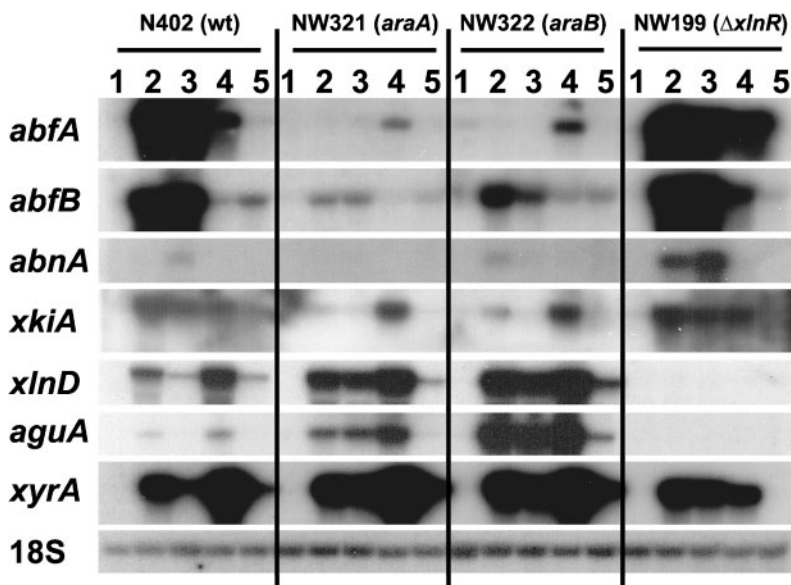

Fig. 4. Northern blot analysis showing the expression of *abfA*, *abfB*, *abnA*, *xkiA*, *xlnD*, *aguA*, *xyrA* and 18S (loading control) in the different strains used: N423 (wild-type), NW321 (*araA*), NW322 (*araB*) and NW199 (Δ*xlnR*). The numbers represent the following carbon sources at a concentration of 15 mM: 1, D-fructose; 2, L-arabinose; 3, L-arabitol; 4, D-xylose; 5, xylitol.

DISCUSSION

In this report we describe the *A. niger araA* and *araB* mutations which give rise to a decrease in the expression and production of the arabinanases. In addition, these mutations affect intracellular catabolism, since L-arabinose reductase, L-arabitol dehydrogenase activities and D-xylulose kinase expression are strongly decreased.

L-Arabitol, the most potent inducer of arabinanase biosynthesis and L-arabinose catabolism, accumulates to much higher levels in the *araA* and *araB* strains than in the wild-type, indicating that the lack of expression and production of the arabinanase system is not due to lack of inducer accumulation. Therefore, we propose that *araA* and *araB* are mutations in components of the arabinanolytic regulatory system. These mutations are either at the level of the transcriptional activator itself, or at the level of a second element which modulates the transcriptional activator activity. The intracellular accumulation of arabitol in the *araA* and *araB* mutants in the presence of L-arabinose can be explained by the ability of D-xylose reductase to reduce L-arabinose to L-arabitol and the low L-arabitol dehydrogenase activity.

The accumulation of arabitol also indicates that L-arabinose is still taken up in the *araA* and *araB* mutants. This indicates that L-arabinose uptake is not strictly regulated by *araA* or *araB*. If *araA* and *araB* affect not only L-arabinose catabolism, but also an L-arabinose transport system, this would indicate the presence of a second transporter for L-arabinose that is not under control of *araA* or *araB*. Alternatively, L-arabinose uptake might be completely independent of *araA* and *araB*.

The expression of *xkiA*, encoding D-xylulose kinase, was previously shown not to be controlled by XLNR (vanKuyk *et al.*, 2001), even though expression was observed on both L-arabinose and D-xylose. This suggests that expression of *xkiA* on L-arabinose is induced by the regulatory system that also activates the expression of the arabinanolytic genes.

Genes of the xylanolytic system, such as *xlnD* and *aguA*, were not expressed in the *xlnR* disruptant (Fig. 3 *xlnD*, *aguA*), which is in agreement with previous results (van Peij *et al.*, 1998a). The expression of *xyrA*, encoding D-xylose reductase, was previously reported to be controlled by XLNR (Hasper *et al.*, 2000) and absence of expression of *xyrA* in a *xlnR* disruption strain was reported in that study. In our investigation, however, expression of *xyrA* in the same *xlnR* disruption strain was reduced compared to wild-type but not absent. XLNR control of *xyrA* expression is therefore not absolute. The differences in expression level between the two studies in the *xlnR* disruption strain can be explained by the different D-xylose concentrations used. It was demonstrated previously (de Vries *et al.*, 1999) that apart from being an inducer of xylanolytic gene expression mediated by XLNR, D-xylose also represses the expression of these genes. This repression is stronger at higher xylose concentrations. In our study we used 15 mM D-xylose whereas Hasper *et al.* (2000) used 50 mM, resulting in lower expression levels for *xyrA*.

Expression of *xlnD* and *aguA* was increased in the *araA* and *araB* mutants, especially on L-arabinose, compared to the expression in the wild-type. Conversely, expression of *abfA*, *abfB*, *abnA* and *xkiA* was higher in the *xlnR* disruptant compared to the wild-type. This suggests an interaction between the *xlnR* and the *ara* system and may be explained by a relief of repression when either regulatory system is not functional or by a change in intracellular inducer concentration. The latter was observed for L-arabitol; however, the L-arabitol concentration was much higher in the *araA* and *araB* mutants compared to the wild-type, which suggests a high level of induction if the regulatory system was present (de Vries *et al.*, 1994; van der Veen *et al.*, 1993). The intracellular concentration of D-xylose, which is believed to be the inducer of the XLNR system, cannot be measured accurately. This supports a model (Fig. 5) in which the repression caused by the presence of D-xylose or L-arabinose is relieved and that XLNR and the arabinose regulatory system are involved in the signalling of this repression. In the presence of L-arabinose, *araA* and *araB* play a role in the activation of carbon catabolite repression of other catabolic systems, e.g. xylanolytic functions. Mutations in *araA* or *araB* relieve this repression. This model explains how the mutants have been selected, i.e. selecting for a relief of repression of L-arabinose towards D-gluconate or L-alanine. This catabolite repression could be mediated either directly by the *ara* or XLNR system, or indirectly by the changes in metabolite levels.

It is well known that D-glucose in aspergilli is the most repressing carbon source (Ruijter & Visser, 1997). We found a clear order of repression using combinations of pentoses with other carbon sources. The order in degree of repression of the carbon sources tested is D-xylose, L-arabinose, xylitol, L-arabitol, D-gluconate and L-alanine. The *araA* and *araB* mutants show decreased carbon catabolite repression specifically for L-arabinose on the repressible carbon sources D-gluconate and L-alanine. This implies that the *araA* and *araB* mutations are located in genes that are not only involved in a pathway leading to arabinanase induction, but also have an impact on carbon catabolite repression by L-arabinose. It was demonstrated quite clearly that the simultaneous induction and repression of hemicellulases by D-xylose is regulated by XLNR and CREA (de Vries *et al.*, 1999), but the role of XLNR in signalling the presence of D-xylose, and resulting in carbon catabolite repression by CREA in the presence of D-xylose, was not investigated. Such a role, however, could at least partially explain the elevated induction of the L-arabinose system in the *xlnR* disruptant.

The function of the two specific inducers (L-arabitol and D-xylose), of the *ara* and XLNR systems respectively, both as
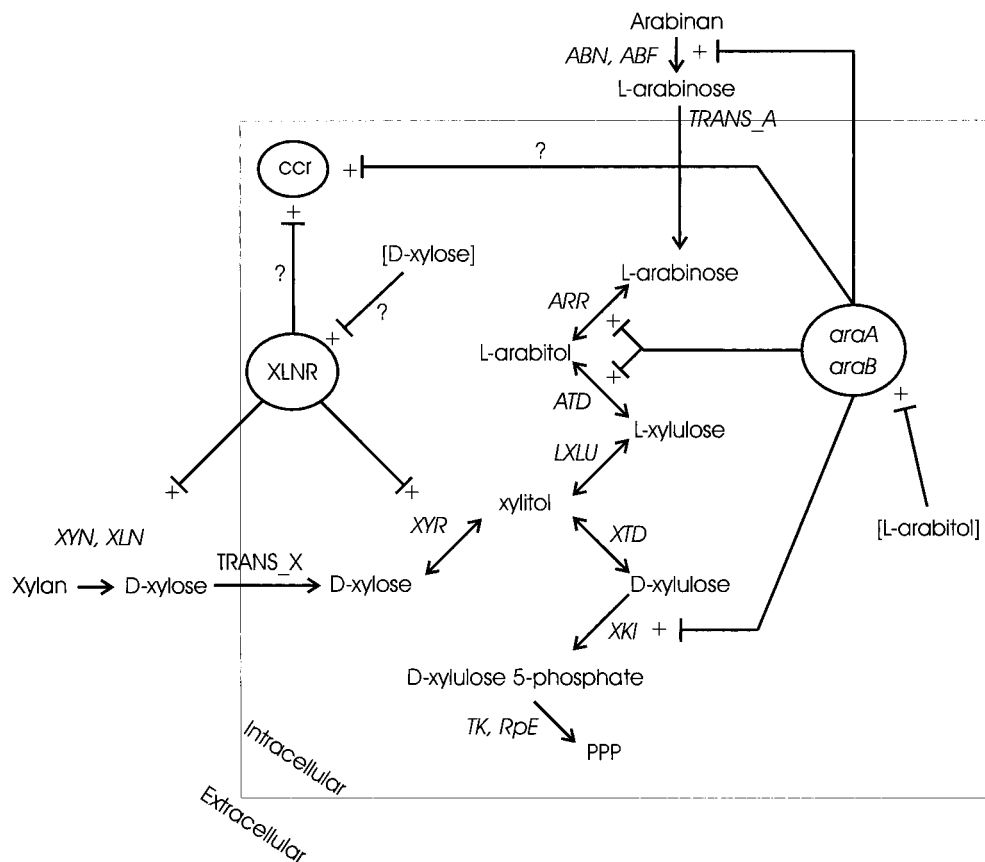
Fig. 5. Model showing the regulation, by XLNR and the proteins encoded by *araA* and *araB*, of the extracellular and intracellular components of the arabinan- and xylan-degrading systems. The italic abbreviations represent the following enzymes/steps: *ABN*, *ABF*, α-L-arabinofuranosidases (EC 3.2.1.55) (ABFA, ABFB, AXHA) and arabinan endo-1,5-α-L-arabinosidase (EC 3.2.1.99) (ABNA); *TRANS_A*, transport of L-arabinose; *ARR*, L-arabinose reductase (EC 1.1.1.21); *ATD*, L-arabitol dehydrogenase (EC 1.1.1.12); *LXLU*, L-xylulose reductase (EC 1.1.1.10); *XTD*, xylitol dehydrogenase (D-xylulose reductase) (EC 1.1.1.9); *XKI*, D-xylulokinase (EC 2.7.1.17) (XKIA); *XYR*, D-xylose reductase (EC 1.1.1.21) (XYRA); *TRANS_X*, transport of D-xylose; *XYN*, *XLN*, endo-1,4-β-xylanase (EC 3.2.1.8) (XYN1) and xylan 1,4-β-xylosidase (XLND); *TK*, *RpE*, transketolase (EC 2.2.1.1) and ribulose-phosphate 3-epimerase (EC 5.1.3.1). PPP, non-oxidative pentose phosphate pathway; ccr, carbon catabolite repression. +, Positive regulation effect. The question marks represent effects of which the mechanism is unknown; such effect is found if one of the regulatory factors (XLNR, *araA* and *araB*) is dysfunctional. This could be mediated either directly by the regulatory system, or indirectly by changes in the metabolite level.

an inducer for their own targets, and as carbon catabolite repressors mediated by CREA or by a more specific system, enable *A. niger* to respond specifically to the presence of different pentoses and hemicelluloses. Since the occurrence of these two pentoses in hemicellulose is never completely separate, such a system would function to fine-tune the regulation of the arabinanase- and xylanase-encoding genes. The metabolism of L-arabitol and D-xylose is linked, both by enzymic equilibrium and by the broad substrate specificity of the enzymes involved, and always leads to induction of both the arabinan- and xylan-degrading systems. The bias created by the induction–repression system proposed reduces the production of unnecessary enzymes and is therefore beneficial to the organism.

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