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The *abfB* gene encoding the major α-L-arabinofuranosidase of *Aspergillus nidulans*: nucleotide sequence, regulation and construction of a disrupted strain

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Using a DNA fragment containing the *Aspergillus niger abfB* gene as a probe, the homologous *Aspergillus nidulans* gene, designated *abfB*, has been cloned from a genomic library containing size-selected *Hin*dIII fragments. The nucleotide sequence of the *A. nidulans abfB* gene shows strong homology with the *A. niger abfB*, *Trichoderma reesei abf-1* and *Trichoderma koningii* α-L-arabinofuranosidase/β-xylosidase genes. Regulation of *abfB* expression has been investigated in cultures induced with L-arabitol. The accumulation of *abfB* mRNA, total α-L-arabinofuranosidase activity and AbfB protein levels have been determined in a wild-type *A. nidulans* strain as well as in different mutant strains. These strains are affected either in their response to ambient pH (*palA1* and *pacC*^{c}*14* mutants), carbon catabolite repression (*creA*^{d}*4* mutant), the ability to utilize L-arabitol as a carbon source (*araA1* mutant) or a combination of both latter genotypes (*araA1 creA*^{d}*4*). The results obtained indicate that the expression of the *A. nidulans abfB* gene was higher at acidic pHs and was superinduced in this double mutant. Furthermore, disruption of the *abfB* gene demonstrated that in *A. nidulans* AbfB is the major *p*-nitrophenyl α-L-arabinofuranoside-hydrolysing activity but at least one minor activity is expressed, which is involved in the release of L-arabinose from polysaccharides.

Keywords: *Aspergillus nidulans*, α-L-arabinofuranosidase, gene disruption, gene regulation

INTRODUCTION

L-Arabinose is a constituent of plant-cell-wall polysaccharides. It is found in a polymeric form in L-arabinan, in which the backbone is formed by 1,5-α-linked L-arabinose residues that can be branched via 1,2-α- and 1,3-α-linked L-arabinofuranose side chains. L-Arabinose is also found as a side chain residue in arabinogalactans (1,3-α or 1,6-α linked), arabinoxylans (1,2-α or 1,3-α linked) and in pectin (1,3-α linked). The enzymic modification of these polysaccharides is technologically relevant in the processing of agricultural products like fruits, vegetables and cereals (Voragen *et al.*, 1982, 1987).

In nature, different micro-organisms secrete endo-arabinanases and α-L-arabinofuranosidases (EC 3.2.1.55) to degrade polysaccharides containing L-arabinose. Most commercial enzyme preparations containing L-arabinanases are obtained from filamentous fungi like *Aspergillus niger*. This fungus secretes two arabinofuranosidases (AbfA and AbfB) and one endo-L-arabinanase; all three have been purified and characterized. AbfA acts only on small linear 1,5-α-linked L-arabinofuranosyl oligosaccharides, whereas AbfB

Abbreviations: ABF, α-L-arabinofuranosidase; MU-ara, 4-methylumbelliferyl α-L-arabinofuranoside; PNP-A, *p*-nitrophenyl α-L-arabinofuranoside.

The EMBL accession number for the sequence reported in this paper is Y13759.

hydrolyses 1,5-α, 1,3-α and 1,2-α linkages in both oligosaccharides and polysaccharides, which contain terminal non-reducing L-arabinofuranoses in side chains (Kaji, 1984; Rombouts *et al.*, 1988; Van der Veen *et al.*, 1991). The encoding genes have been cloned and characterized (Flipphi *et al.*, 1993a, b, c, 1994). In *A. niger*, the synthesis of these enzymes is induced by both L-arabinose, the final product of L-arabinan degradation, and by L-arabitol, an intermediate in the catabolic pathway of L-arabinose, though the latter is a stronger inducer (Van der Veen *et al.*, 1993). Furthermore, the expression of the L-arabinanase system is under carbon-catabolite repression when more preferable carbon sources like D-glucose are present in the medium (Van der Veen *et al.*, 1993; Ruijter *et al.*, 1997).

As in *A. niger*, L-arabinanase biosynthesis in *A. nidulans* is induced by L-arabinose-containing substrates (sugar-beet pulp, for example) by the monomeric sugar L-arabinose and more strongly by L-arabitol (Ramón *et al.*, 1993). When correlating arabinanase biosynthesis and the accumulation of L-arabitol in an L-arabitol dehydrogenase-negative mutant, de Vries *et al.* (1994) obtained evidence that L-arabitol is the actual inducer. Besides an endo-arabinanase activity, only one α-L-arabinofuranosidase (ABF) activity has been found in *A. nidulans*. These two proteins show immunological cross-reactivity with antibodies raised against *A. niger* AbnA and AbfB, respectively, and these enzymes are also similar to the *A. niger* counterparts in their kinetic and physico-chemical properties (Ramón *et al.*, 1993). Van der Veen *et al.* (1994) investigated the regulation of these two extracellular enzyme activities and of the enzymes of the L-arabinose catabolic pathway that generate the inducer, in particular with respect to carbon-catabolite repression.

To extend the previous analysis to the transcriptional level, we have now cloned and characterized the *A. nidulans abfB* gene. Furthermore, disruption of the *abfB* gene will facilitate us to identify possible new minor ABF functions.

METHODS

Strains, media and culture conditions. *Escherichia coli* DH5α was used as a host for cloning experiments. All *Aspergillus* strains used in this paper are described in Table 1. See Clutterbuck (1993) for definition of gene symbols. *Aspergillus* minimal medium was prepared as described by Pontecorvo *et al.* (1953) and contained 0·04 ml trace element solution l^{-1}, as described by Vishniac & Santer (1957). Strains used in the shift experiment were pre-grown for 18 h at 37 °C in 250 ml minimal medium (MM) containing 100 mM D-glucose and 0·05% (w/v) yeast extract. After harvesting, the mycelium was washed with 0·9% (w/v) NaCl and 3 g portions were transferred to 50 ml MM containing 50 mM L-arabitol or 50 mM L-arabitol and 20 mM glycerol. These transfer cultures were grown for additional periods of 6 and 10 h. Where necessary, the media were supplemented with 1·5 mg *p*-aminobenzoate l^{-1} or 4 μg biotin l^{-1}.

DNA isolation and manipulation. Fungal DNA was obtained as described by Ramón *et al.* (1987). Amplification of the *A. niger abfB* gene was done as described previously (Sánchez-Torres *et al.*, 1996) using oligonucleotides Abf2 (5′ GACCTATTTACAAAGCTTTCTCC 3′), which anneals 85 bp downstream of the stop codon, and Abf4 (5′ GAGCCTGCAGTAATGCTCCACAATGTTCTCC 3′), which includes the ATG translation start codon.

Construction and screening of an *A. nidulans* partial genomic library. Southern-blot analyses of *A. nidulans* DNA using a 1·6 kb DNA fragment obtained by PCR, containing the entire *A. niger abfB* gene as a probe, were carried out under various hybridization and washing conditions. This revealed the existence of multiple hybridizing DNA fragments. To avoid isolating false positives upon screening the library, the following scheme was devised. *A. nidulans* DNA was digested with *Hin*dIII and samples were fractionated in triplicate by agarose gel electrophoresis. After transfer and UV fixation of the DNA to a nylon membrane, the membrane was cut into three pieces containing the same samples. Each piece of membrane was hybridized under alternative conditions (last wash step with 4 × SSC and 0·1% SDS at 60 °C) with the complete *A. niger abfB* gene, a 0·9 kb *Kpn*I fragment containing the 5′ region of the *A. niger abfB* gene, or the remaining 0·7 kb fragment which contains the 3′ region of the *A. niger abfB* gene. Comparison of the hybridization patterns showed that a 6 kb *Hin*dIII fragment hybridized with the three probes under the alternative conditions. Subsequently, a partial library of *A. nidulans* was constructed. *A. nidulans* DNA was completely digested with *Hin*dIII and DNA fragments were separated through a 0·7% agarose gel. Fragments between 5 and 7 kb in length were recovered from the gel using a Geneclean kit (Bio 101) and ligated into pBluescript SKII(+) that had previously been digested with *Hin*dIII and dephosphorylated. The ligated plasmids were used to transform competent *E. coli* DH5α cells. The library was screened by colony hybridization under heterologous conditions using the PCR amplified *A. niger abfB* gene as a probe. Approximately 12000 recombinant clones were screened.

Sequence determination and analysis. Double-stranded plasmid DNA was sequenced using the Sequenase 2.0 kit (Amersham). A series of nested deletions were obtained by the exoIII/S1 nuclease method (Henikoff, 1984). In addition, synthetic oligonucleotides were used to determine the sequence on both DNA strands. Computer analysis was done using the PC/GENE program (IntelliGenetics) and version 7 of the Genetics Computer Group package (Madison, WI, USA).

RNA isolation and Northern analysis. Total RNA was isolated using TRIZOL (Life Technologies) according to the manufacturer's instructions. Northern analysis was performed as described by Sambrook *et al.* (1989). Ten micrograms of total RNA was applied per lane. Northern blots were probed with the 0·65 kb *Eco*RI–*Kpn*I *A. nidulans abfB* fragment or with the 0·9 kb *Eco*RI fragment from the *Agaricus bisporus* 28S rRNA gene (EMBL accession no. X91812), which was used as an internal control. The blots were washed down to 0·2 × SSC at 65 °C. RNA levels were quantified by liquid scintillation analysis in a Packard Ultracarb 1500. Samples were corrected for loading differences using the 28S rDNA. All values were normalized to the sample of WG096 transferred to L-arabitol and grown for 10 h.

Construction of an *abfB*-disruption plasmid. Plasmid pH12S1, containing the 3·6 kb *Sma*I insert of the *abfB* gene, was digested using *Xba*I/*Hin*dIII and ligated into pGEM7,

Table 1. *Aspergillus* strains used in this study

Strain	Genotype	Source/reference
A. niger		
N402	*cspA1*	Derived from CBS 120.49
A. nidulans		
V023	*argB2 biA1 metG1*	M. A. Peñalva*
WG096	*pabaA1 yA2*	FGSC 187
G094	*araA1 biA1 wA2*	Clutterbuck (1981)
creA^{d}4	*biA1 creA^{d}4*	H. N. Arst, Jr†
NW186	*araA1 biA1 creA^{d}4 cnxH4*	This study
pacC^{c}14	*biA1 pacC^{c}14*	H. N. Arst, Jr†
palA1	*pabaA1 palA1*	H. N. Arst, Jr†
NW187	*biA1 creA^{d}4 pyrG90*	This study
NW190	*biA1 creA^{d}4 pyrG90 ΔabfB-pyrA^{+}*	This study

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† Royal Postgraduate Medical School, London.

resulting in pLIG318. *Sal*I-digested pLIG318 was ligated to a 2·4 kb *Xho*I fragment containing the *A. niger pyrA* gene (EMBL accession no. X06626), generating pLIG343. In this plasmid, 400 bp of the *abfB* coding region is replaced by the selection marker *pyrA*. The *Bam*HI site located in the *pyrA* gene was modified to a *Xho*I site using a *Bam*HI–*Xho*I linker (5′ GATCACTCGAGT 3′) that retains the correct reading frame. The resulting plasmid, pIM3005, was digested using *Bam*HI and the 5·6 kb *abfB*-disruption fragment was used for transformation.

Determination of ABF activity and Western blotting. ABF activity was measured at 37 °C as described by Van der Veen *et al.* (1991) using *p*-nitrophenyl α-L-arabinofuranoside (PNP-A) as a substrate. Western blotting was also performed as described by van der Veen *et al.* (1991). Detection of ABF by enzyme staining was conducted as described by Gallego *et al.* (1996) using 4-methylumbelliferyl α-L-arabinofuranoside (MU-ara) in 50 mM sodium acetate buffer, pH 4·0, to visualize ABF activity.

RESULTS AND DISCUSSION

Cloning the *A. nidulans abfB* gene

Southern-blot analyses of *A. nidulans* DNA revealed the existence of multiple hybridizing DNA fragments when probed with a PCR fragment containing the entire *A. niger abfB* gene. A strategy was devised to avoid the isolation of false positives upon screening of a library (see Methods). Twenty-two hybridizing colonies were found when a partial plasmid library was screened. Physical maps of the plasmids isolated from these colonies allowed us to group them into five classes. One member of each class was further analysed by Southern blotting using either the 5′ or the 3′ region of the *A. niger abfB* gene as probes under the alternative conditions. One of these plasmids, designated pH12, showed strong hybridization signals with both probes in an overlapping region, thus being likely to contain a gene homologous to *A. niger abfB*. A 3·6 kb *Sma*I fragment from pH12,

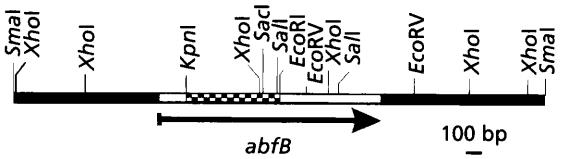

Fig. 1. Restriction map of the *Sma*I fragment containing the *A. nidulans abfB* gene. The *Kpn*I–*Eco*RI DNA fragment that hybridizes with probes derived from both 5′and 3′ regions of the *A. niger abfB* gene is indicated by a boxed pattern.

shown in Fig. 1, was subcloned in both orientations into the *Eco*RV site of pBluescript, yielding pH12S1 and pH12S4. It is interesting to note that of the 22 positive clones initially isolated, six of these, all belonging to the same class, contained the *abfB* gene. The remaining clones were not further analysed since they might represent false positives.

Sequence analysis of the *A. nidulans abfB* gene

The nucleotide sequence of a 3141 bp *Sma*I–*Xho*I fragment from pH12 was determined on both strands. The determined sequence contains an ORF of 1530 bp. The predicted amino acid sequence of the encoded protein contains 510 amino acids and a putative signal peptide of 24 amino acids. The mature protein has a calculated molecular mass of 50·6 kDa and a calculated pI of 3·9.

Similarity searches revealed that *A. nidulans* AbfB is homologous to *A. niger* N400 AbfB (77% identity; Flipphi *et al.*, 1993a), and also to *Trichoderma reesei* Abf1 (Margolles-Clark *et al.*, 1996) and the *Trichoderma koningii* ABF/β-xylosidase (GenBank accession no. U38661) proteins. Alignment of *A. nidulans* AbfB with these other fungal proteins, excluding putative

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ABFBNID  MTMSRSSRSSVLALALATGSLVAAGPCDIYSSGGTPCIAAHSTTRALYSSYNGPLYQVQR
ABFBNIG  MF----SRRNLVALGLAAT--VSAGPCDIYEAGDTPCVAAHSTTRALYSSFSGALYQLQR
ABF1REE  ML---SNARIIAAGCIAAGSLVAAGPCDIYSSGGTPCVAAHSTTRALFSAYTGPLYQVKR
XYL1KON  ML---SNARIIAAGCIAAGSLVAAGPCDIYSSGGTPCVAAHSTTRALFSAYTGPLYQVKR
          *          *   *    * *******  * *** *********  *   * ***  *

ABFBNID  ASDGTTTTITPLSAGGVADASAQDAFCENTTCLITIIYDQSGNGNDLTQAPPGGFNGPDV
ABFBNIG  GSDDTTTTISPLTAGGVADASAQDTFCANTTCLITIIYDQSGNGNHLTQAPPGGFDGPDV
ABF1REE  GSDGATTAISPLSSG-VANAAAQDAFCAGTTCLITIIYDQSGRGNHLTQAPPGGFSGPES
XYL1KON  GSDGATTAISPLSSG-VANAAAQDAFCAGTTCLITIIYDQSGRGNHLREAPPGGFSGPES
          **  ** * **  * ** * *** **  ************* ** *  ****** **

ABFBNID  GGYDNLAGAIGAPVTLNGKKAYGVFVSPGTGYRNNEAIGTATGDEPEGMYAVLDGTHYND
ABFBNIG  DGYDNLASAIGAPVTLNGQKAYGVFMSPGTGYRNNEATGTATGDEPEGMYAVLDGTHYND
ABF1REE  NGYDNLASAIGAPVTLNGQKAYGVFVSPGTGYRNNAASGTAKGDAAEGMYAVLDGTHYNG
XYL1KON  NGYDNLASAIGAPVTLNGQKAYGVFVSPGTGYRNNAASGTAKGDAAEGMYAVLDGTHYNG
          ****** ********** ****** ********* * *** **  *************

ABFBNID  GCCFDYGNAETSSLDTGNGHMEAIYYGTNTAWGYGAGNGPWIMADLENGLFSGQSSDYNA
ABFBNIG  ACCFDYGNAETSSTDTGAGHMEAIYLGNSTTWGYGAGDGPWIMVDMENNLFSGADEGYNS
ABF1REE  ACCFDYGNAETNSRDTGNGHMEAIYFGDSTVWGTGSGKGPWIMADLENGLFSGSSPGNNA
XYL1KON  ACCFDYGNAETNSRDTGNGHMEAIYFGDSTVWGTGSGKGPWIMADLENGLFSGSSPGNNA
          ********** * *** ******* *  * ** * * ***** * ** ****     *

ABFBNID  GDPSISYRFVTAILKGGPNLWALRGGNAASGSLSTYYNGIRPTDASGYNPMSKEGAIILG
ABFBNIG  GDPSISYSFVTAAVKGGADKWAIRGGNAASGSLSTYYSGARP-DYSGYNPMSKEGAIILG
ABF1REE  GDPSISYRFVTAAIKGQPNQWAIRGGNAASGSLSTFYSGARP-QVSGYNPMSKEGAIILG
XYL1KON  GDPSISYRFVTAAIKGQPNQWAIRGGNAASGSLSTFYSGARP-QVSGYNPMSKEGAIILG
          ******* ****  **    ** ************ * * **   **************

ABFBNID  IGGDNSVSAQGTFYEGAMTDGYPDDATENSVQADIVAAKYATTSLISGPALTVGDTVSLK
ABFBNIG  IGGDNSNGAQGTFYEGVMTSGYPSDDVENSVQENIVAAKYVSGSLVSGPSFTSGEVVSLR
ABF1REE  IGGDNSNGAQGTFYEGVMTSGYPSDATENSVQANIVAARYAVAPLTSGPALTVGSSISLR
XYL1KON  IGGDNSNGGQGTFYEGVMTSGYPSDATENSVQANIVAARYAVAPLTSGPALTVGSSISLR
          ******   ******* ** *** *  *****  **** *    * ***  * *   **

ABFBNID  VTTSGYDTRYIAHTGSTINTQVVSSSSSSTLKQQASWTVRTGLASTAAANGCVSFESVDT
ABFBNIG  VTTPGYTTRYIAHTDTTVNTQVVDDDSSTTLKEEASWTVVTGLANSQ----CFSFESVDT
ABF1REE  ATTACCTTRYIAHSGSTVNTQVVSSSSATALKQQASWTVRAGLAN----NACFSFESRDT
XYL1KON  ATTACCTTRYIAHSGSTVNTQVVSSSSATALKQQASWTVRAGLAN----NACFSFESQDT
          **     ******   * *****   *   **  *****  ***        * **** **

ABFBNID  PGSYIRHSNFALLLNANDGTKLFSEDATFCPQDSFNDDGTNSIRSWNYPTRYWRHYENVL
ABFBNIG  PGSYIRHYNFELLLNANDGTKQFHEDATFCPQAPLNGEGT-SLRSWSYPTRYFRHYENVL
ABF1REE  SGSYIRHSNFGLVLNANDGSKLFAEDATFCTQAGINGQGS-SIRSWSYPTRYFRHYNNTL
XYL1KON  SGSYIRHSNFGLVLNANDGSKLFAEDATFCTQAGINGQGS-SIRSWSYPTRYFRHYNNTL
          ****** ** * ****** * * ****** *   *  *  * *** ***** *** * *

ABFBNID  YVASNGGVNTFDAATAFTDDVSWVVADGFA-
ABFBNIG  YAASNGGVQTFDSKTSFNNDVSFEIETAFAS
ABF1REE  YIASNGGVHVFDATAAFNDDVSFVVSGGFA-
XYL1KON  YIASNGGVHVFDATAAFNDDVSFVVSGGFA-
          * ******  **    *  ***       **
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Fig. 2. Amino acid sequence comparison of *A. nidulans* AbfB (ABFBNID), *A. niger* AbfB (ABFBNIG) (Flipphi *et al.*, 1993a), *T. reesei* Abf1 (ABF1REE) (Margolles-Clark *et al.*, 1996) and *T. koningii* Xyl1 (XYL1KON) (GenBank accession no. U38661) sequences. Alignment was done with the CLUSTAL V program. Identical amino acids (*) are shown.

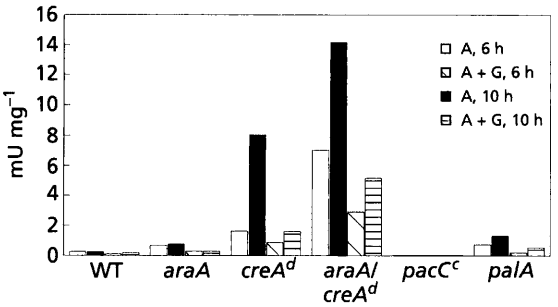

Fig. 3. Determination of PNPase activities in the culture filtrates of various *A. nidulans* strains. Strains were pre-grown on 100 mM D-glucose and shifted to fresh media containing 50 mM L-arabitol with (A+G) or without (A) 20 mM glycerol and grown further for either 6 or 10 h. Activities are expressed in mU mg^{-1} mycelial dry weight.

signal peptides, shows a high overall identity which is calculated to be 64% (Fig. 2).

A. niger AbfB and *T. koningii* ABF/β-xylosidase proteins have recently been included in a new family of glycosyl hydrolases (Family 54; Henrissat & Bairoch, 1996). Family 54 thus includes *A. nidulans* AbfB as well. It is interesting to note that, whereas both *Trichoderma* enzymes also show β-xylosidase activity, this was not found for the *A. niger* equivalents (Flipphi *et al.*, 1993a) or for *A. nidulans* AbfB.

In the promoter region of the *A. nidulans abf*B gene, consensus recognition sequences were found for the wide domain regulators CreA (at −127, −253 and −435) and PacC (at −299, −312, −416, −742, −884, −973 and −985), that mediate carbon-catabolite repression and pH regulation, respectively.

Regulation of *abfB* expression

Van der Veen *et al.* (1994) studied the role of the CreA repressor protein in the expression of several enzymes of the L-arabinose catabolic pathway and in the biosynthesis of AbfB in *A. nidulans*. In the *creA*^{d}*4* and *creA*^{d}*30* strains, very marked, elevated inducibility was noticed for both the extracellular enzyme and for the intracellular enzymes. De Vries *et al.* (1994) characterized an *araA1* mutant, which turned out to lack NAD^+-dependent L-arabitol dehydrogenase activity and is unable to utilize L-arabinose or L-arabitol. This strain featured elevated expression of AbfB, caused by the accumulation of L-arabitol. Here, we have extended these studies by comparing, under inducing conditions only, the expression of *abfB* in the wild-type strain, the *araA1* mutant, a carbon-catabolite derepressed mutant (*creA*^{d}*4*) (Bailey & Arst, 1975) and an *araA1 creA*^{d}*4* double mutant. The strains were cultivated as described in Methods. In this way, any biosynthesis of AbfB can be attributed to induction by L-arabitol, since pre-growth on D-glucose strongly represses AbfB expression and therefore no AbfB is present at the start of the induction period.

We first determined the PNP-A hydrolysing activities in the culture filtrates in the various *A. nidulans* strains, which is shown in Fig. 3. Hydrolysis of PNP-A was significantly increased in the *creA*^{d} mutant compared to that measured in the wild-type. The increase of PNPase activity was less in the *araA* mutant than in the *creA*^{d} mutant. In the *araA creA*^{d}*4* double mutant, however, the level of PNP-A hydrolysing activity was higher than that in the two single-mutant strains. In this double-mutant strain, the combination of the *creA*^{d} mutation and the intracellular accumulation of inducer leads to super-induction.

Previously, ABF induction studies in *A. nidulans* were limited to measurements of enzyme activity, Western blotting and inducer-level effects (van der Veen *et al.*, 1994; de Vries *et al.*, 1994). With the availability of the *abfB* gene, we also studied the *abfB* expression at the transcriptional level. As is shown in Fig. 4, the *abfB* transcript accumulation paralleled the total extra-

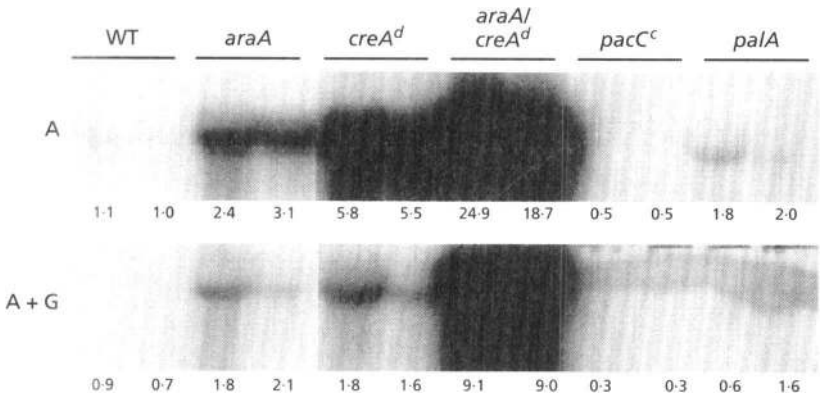

Fig. 4. Northern-blot analysis of *abfB* transcription in various *A. nidulans* strains. Strains were pre-grown on 100 mM D-glucose and shifted to fresh media containing 50 mM L-arabitol with (A+G) or without (A) 20 mM glycerol and grown further for either 6 or 10 h (first and second lane of each strain, respectively). The relative *abfB* mRNA levels, which are expressed in arbitrary units are also shown. The *abfB* transcript levels were corrected for loading differences and all values were normalized for the sample of WG096 transferred to L-arabitol and grown for 10 h.

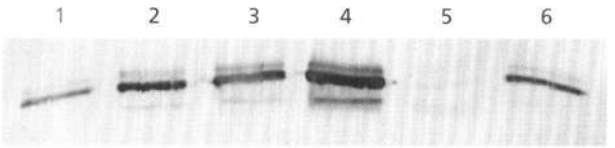

Fig. 5. Western-blot analysis of 15×concentrated culture filtrates using antibodies raised against *A. niger* AbfB. Strains were pre-grown on 100 mM D-glucose and shifted to fresh media containing 50 mM L-arabitol as the sole carbon source and grown further for 10 h. Track order: 1, WG096 (WT); 2, G094 (*araA1*); 3, creA^{d}4 (*creA^{d}4*); 4, NW186 (*araA1 creA^{d}4*); 5, pacC^c (*pacC^{c}14*); 6, palA (*palA1*).

cellular ABF activity and AbfB protein levels in the culture filtrate. This is in agreement with the results obtained for the *abf*B-disruption strain in which it was shown that AbfB is the major component of the PNP-A hydrolysing activities in *A. nidulans* (see below). The *abfB* transcription was also analysed when the strains were transferred to media containing 50 mM L-arabitol and 20 mM glycerol as mixed carbon source. Glycerol was added as a non-repressing carbon source to sustain growth of strains carrying the *araA1* mutation, which are unable to utilize L-arabitol (Figs 3–5). HPLC analysis showed that the concentrations of L-arabitol in the culture filtrates were 30–45 mM after 10 h growth. Those of glycerol, if added to the cultures, were 4–9 mM (data not shown). Although the expression levels were lower than in the L-arabitol cultures, the overall pattern of *abfB* expression is very similar. Under the conditions tested, glycerol repressed the L-arabitol-induced *abfB* expression. This repression by glycerol was also found by de Vries *et al.* (1994) when wild-type *Aspergillus nidulans* was cultured directly on 100 mM L-arabinose and 100 mM glycerol. Transcript levels of the *Agaricus bisporus* genes *cel2* and *cel4*, encoding a cellobiohydrolase I and a β-mannanase, respectively, are also moderately repressed when glycerol was added to cellulose-induced cultures (Yaguë *et al.*, 1997). These findings indicate that glycerol can act as a repressing carbon source in two different organisms, although it can also be a neutral or inducing carbon source on the expression of different enzymes.

As in the case of the *A. nidulans ipnA* (Espeso *et al.*, 1993), *xlnA* and *xlnB* (MacCabe *et al.*, 1998) genes, the presence of putative PacC-binding sites suggest possible regulation in response to external pH. Therefore, we have investigated the potential role of PacC by studying AbfB induction in a constitutive mutant (*pacC^{c}14*), which mimics growth under alkaline conditions, and in a *pal* mutant (*palA1*), which mimics growth under acidic conditions. The level of PNPase activity was higher in the *palA1* strain than the wild-type, whereas in the *pacC^c* strain it was lower than the wild-type (Fig. 3). The repression of AbfB induction by the *pacC^c*14 mutation is shown in Figs 4 and 5. Thus it can be concluded that the expression of the *abf*B gene is pH regulated and is higher at acidic pHs.

Disruption of the *A. nidulans abfB* gene

Ramón *et al.* (1993) found that AbfB was the major ABF activity in *A. nidulans*, since no *abfA* homologue could be detected in *A. nidulans*. We therefore constructed a strain in which the *abfB* gene was disrupted to test this and to explore the possibility that other unknown ABF functions are present in *A. nidulans*. A linear 5·6 kb *Bam*HI fragment containing the *abfB* gene disrupted by a functional clone of the *A. niger pyrA* gene was obtained from pIM3005. This fragment was introduced into a genetic background which displays a high level of ABF activity compared to wild-type, facilitating the possible identification of other minor L-arabinose-releasing activities. For this purpose, we first tried to cross in the *pyrG89* allele in a *creA^{d}4* background. Despite the distance between the *creA* and *pyrG* alleles (3·6 cM), we only obtained recombinant strains that showed a wild-type phenotype for both alleles. Therefore, we introduced a new *pyrG90* mutation, which was obtained by UV mutagenesis and selected using fluoorotic acid, in the carbon-catabolite derepressed strain carrying the *creA^{d}4* allele. The resulting strain, NW187, was used as the recipient strain in the disruption experiment. Disruptant NW187::pIM3005-8, designated NW190, was chosen for further analysis. Strains WG096, NW187 and

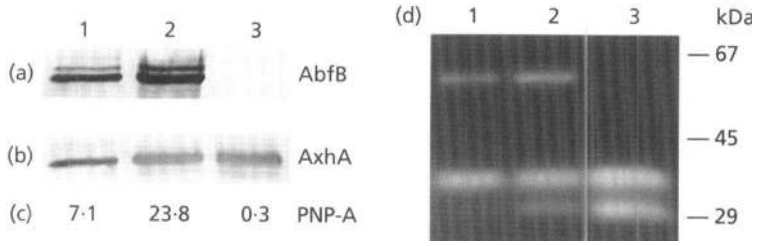

Fig. 6. Western-blot analysis of the 15 × concentrated culture filtrates visualized using antibodies raised against *A. niger* AbfB (a) or *A. tubingensis* AxhA (b). (c) PNP-A activities of 15 × concentrated samples, expressed in mU ml^{-1}. (d) SDS-PAGE followed by zymography of the 15 × concentrated culture filtrates visualized using the chromogenic substrate MU-ara. Strains were pre-grown on 100 mM D-glucose and shifted to fresh media containing 50 mM L-arabitol and grown further for 10 h. Track order: 1, WG096 (WT); 2, NW187 (*creA*^{d}*4*); 3, NW190 (*creA*^{d}*4*, Δ*abfB*).

NW190 were used in transfer cultures as described in Fig. 5. No AbfB protein could be detected in the culture filtrate of the Δ*abfB* strain using *A. niger* AbfB antibodies (Fig. 6a). Furthermore, the PNPase activity was reduced approximately 100-fold to 1·2% of the level seen in control strain NW187 (Fig. 6c). Activities were expressed in mU ml^{-1} since we did not determine the actual dry weights of all samples. This reduction in PNPase activity indicates that AbfB is the major component responsible for the observed PNPase values in *A. nidulans*. No AbfB activity could be detected when SDS-PAGE was conducted followed by a renaturation step and active enzyme staining using MU-ara. However, two other MU-ara-hydrolysing activities having an apparent molecular mass of 30 and 33 kDa, respectively, could be visualized (Fig. 6d). Western-blot analysis demonstrated that the 33 kDa band reacted with antibodies raised against *Aspergillus tubingensis* arabinoxylan arabinofuranohydrolase A (AxhA) (Fig. 6b). This protein is likely to be the *A. nidulans* equivalent of AxhA from *A. tubingensis*. AxhA was found to release L-arabinose residues from arabinoxylans only (Kormelink *et al.*, 1991), and its expression was induced by L-arabitol (Gielkens *et al.*, 1997). The *A. tubingensis* AxhA enzyme has a much higher specific activity towards MU-ara than to PNP-A. The specific activity of *A. tubingensis* AxhA on PNP-A is approximately $1{\cdot}5 \times 10^{-2}$ U mg^{-1} compared to 23·5 U mg^{-1} for AbfB (unpublished results). The AxhA equivalent in *A. nidulans* is therefore a significant component of the residual PNPase activity present in the Δ*abfB* strain. Besides AxhA, another MU-ara-hydrolysing activity corresponding to the 30 kDa band is present in *A. nidulans*, but it is unknown whether this contributes to the PNP-A-hydrolysing activity.

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REFERENCES

Bailey, C. & Arst, H. N., Jr (1975). Carbon catabolite repression in *Apergillus nidulans*. *Eur J Biochem* **51**, 573–577.

Clutterbuck, A. J. (1981). An arabinose non-utilizing mutant *araA1*. *Aspergillus Newsl* **15**, 21.

Clutterbuck, A. J. (1993). *Aspergillus nidulans*. In *Genetic Maps*, vol. 6, pp. 3.71–3.84. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Espeso, E. A., Tilburn, J., Arst, H. N., Jr & Peñalva, M. A. (1993). pH regulation is a major determinant in expression of a fungal penicillin biosynthetic gene. *EMBO J* **12**, 3947–3956.

Flipphi, M. J. A., van Heuvel, M., Van der Veen, P., Visser, J. & de Graaff, L. H. (1993a). Cloning and characterization of the *abfB* gene coding for the major α-L-arabinofuranosidase (ABFB) of *Aspergillus niger*. *Curr Genet* **24**, 525–532.

Flipphi, M. J. A., Panneman, H., Van der Veen, P., Visser, J. & de Graaff, L. H. (1993b). Molecular cloning, expression and structure of the endo-1,5-α-L-arabinase gene of *Aspergillus niger*. *Appl Microbiol Biotechnol* **40**, 318–326.

Flipphi, M. J. A., Visser, J., Van der Veen, P. & de Graaff, L. H. (1993c). Cloning of an *Aspergillus niger* gene encoding α-L-arabinofuranosidase A. *Appl Microbiol Biotechnol* **39**, 335–340.

Flipphi, M. J. A., Visser, J., Van der Veen, P. & de Graaff, L. H. (1994). Arabinase gene expression in *Aspergillus niger*: indications for coordinated regulation. *Microbiology* **140**, 2673–2682.

Gallego, M. V., Pinăga, F., Ramón, D. & Vallés, S. (1996). Production and characterization of an *Aspergillus terreus* α-L-rhamnosidase of oenological interest. *Z Lebensm-Unters-Forsch* **203**, 522–527.

Gielkens, M. M. C., Visser, J. & de Graaff, L. H. (1997). Arabinoxylan degradation by fungi: characterization of the arabinoxylan-arabinofuranohydrolase encoding genes from *Aspergillus niger* and *Aspergillus tubingensis*. *Curr Genet* **31**, 22–29.

Henikoff, S. (1984). Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene* **28**, 351–359.

Henrissat, B. & Bairoch, A. (1996). Updating the sequence-based classification of glycosyl hydrolases. *Biochem J* **316**, 695–696.

Kaji, A. (1984). L-Arabinosidases. *Adv Carbohydr Chem Biochem* **42**, 383–394.

Kormelink, F. J. M., Searle-van Leeuwen, M. J. F., Wood, T. M. & Voragen, A. G. J. (1991). Purification and characterization of a (1,4)-β-D-arabinoxylan arabinofuranohydrolase from *Aspergillus awamori*. *Appl Microbiol Biotechnol* **35**, 753–758.

MacCabe, A. P., Orejas, M., Pérez-González, J. A. & Ramón, D. (1998). Opposite patterns of expression of two *Aspergillus nidulans* xylanase genes with respect to ambient pH. *J Bacteriol* **180**, 1331–1333.

Margolles-Clark, E., Tenkanen, M., Nakari-Setala, T. & Pentilla, M. (1996). Cloning of genes encoding α-L-arabinofuranosidase and β-xylosidase from *Trichoderma reesei* by expression in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* **62**, 3840–3846.

Pontecorvo, G., Roper, J. A., Hemmons, L. J., MacDonald, K. D. & Bufton, A. W. J. (1953). The genetics of *Aspergillus nidulans*. *Adv Genet* **5**, 141–238.

Ramón, D., Carramolino, L., Patiño, C., Sánchez, F. & Peñalva, M. A. (1987). Cloning and characterisation of the isopenicillin N synthetase gene mediating the formation of the β-lactam ring in *Aspergillus nidulans*. *Gene* **57**, 171–181.

Ramón, D., Van der Veen, P. & Visser, J. (1993). Arabinan degrading enzymes from *Aspergillus nidulans*: induction and purification. *FEMS Microbiol Lett* **113**, 15–22.

Rombouts, F. M., Voragen, A. G. J., Searle-van Leeuwen, M. J. F., Geraeds, C. C. J. M., Schols, H. A. & Pilnik, W. (1988). The arabinases of *Aspergillus niger*: purification and characterisation of two α-L-arabinofuranosidases and an endo-1,5-α-L-arabinase. *Carbohydr Polym* **9**, 25–47.

Ruijter, G. J. G., Vanhanen, S. A., Gielkens, M. M. C., Van de Vondervoort, P. J. I. & Visser, J. (1997). Isolation of *Aspergillus niger creA* mutants and effects of the mutations on expression of arabinases and L-arabinose catabolic enzymes. *Microbiology* **143**, 2991–2998.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sánchez-Torres, P., González-Candelas, L. & Ramón, D. (1996). Expression in a wine yeast strain of the *Aspergillus niger abfB* gene. *FEMS Microbiol Lett* **145**, 189–194.

Van der Veen, P., Flipphi, M. J. A., Voragen, A. G. J. & Visser, J. (1991). Induction, purification and characterisation of arabinases produced by *Aspergillus niger*. *Arch Microbiol* **157**, 23–28.

Van der Veen, P., Flipphi, M. J. A., Voragen, A. G. J. & Visser, J. (1993). Induction of extracellular arabinases on monomeric substrates in *Aspergillus niger*. *Arch Microbiol* **159**, 66–71.

Van der Veen, P., Arst, H. N., Jr, Flipphi, M. J. A. & Visser, J. (1994). Extracellular arabinases in *Aspergillus nidulans*: the effect of different *cre* mutations on enzyme levels. *Arch Microbiol* **162**, 433–440.

Vishniac, W. & Santer, M. (1957). The thiobacilli. *Bacteriol Rev* **21**, 195–213.

Voragen, A. G. J., Geerst, F. & Pilnik, W. (1982). Hemicellulase in enzymic fruit processing. In *Use of Enzymes in Food Technology*, pp. 497–502. Edited by P. Dupuy. Paris: Technique et Documentation Lavoisier.

Voragen, A. G. J., Rombouts, F. M., Searle-van Leeuwen, M. J. F., Schols, H. A. & Pilnik, W. (1987). The degradation of arabinans by endo-arabinase and arabinofuranosidases purified from *Aspergillus niger*. *Food Hydrocolloids* **1**, 423–437.

de Vries, R. P., Flipphi, M. J. A., Witteveen, C. F. B. & Visser, J. (1994). Characterization of an *Aspergillus nidulans* L-arabitol dehydrogenase mutant. *FEMS Microbiol Lett* **123**, 83–90.

Yaguë, E., Mehak-Zunic, M., Morgan, L., Wood, D. A. & Thurston, C. F. (1997). Expression of CEL2 and CEL4, two proteins from *Agaricus bisporus* with similarity to fungal cellobiohydrolase I and β-mannanase, respectively, is regulated by the carbon source. *Microbiology* **143**, 239–244.

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