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Molecular Cloning and Transcriptional Regulation of the *Aspergillus nidulans xlnD* Gene Encoding a β-Xylosidase

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The *xlnD* gene encoding the 85-kDa β-xylosidase was cloned from *Aspergillus nidulans*. The deduced primary structure of the protein exhibits considerable similarity to the primary structures of the *Aspergillus niger* and *Trichoderma reesei* β-xylosidases and some similarity to the primary structures of the class 3 β-glucosidases. *xlnD* is regulated at the transcriptional level; it is induced by xylan and D-xylose and is repressed by D-glucose. Glucose repression is mediated by the product of the *creA* gene. Although several binding sites for the pH regulatory protein PacC were found in the upstream regulatory region, it was not clear from a Northern analysis whether PacC is involved in transcriptional regulation of *xlnD*.

Hydrolysis of xylans is of considerable interest for various biotechnological applications (for reviews see references 7 and 47). Unlike cellulose, xylans are chemically heterogeneous molecules with a characteristic backbone consisting of β-(1,4)-linked D-xylosyl residues replaced with acetyl, L-arabinosyl, and 4-*O*-methyl-glucuronosyl side chains. Natural xylan degradation by microorganisms occurs through the coordinated action of various enzymes, including the endo-(1,4)-β-xylanases (EC 3.2.1.8), which cleave the β-(1,4) glycosidic bonds between D-xylose residues in the main chain to produce xylooligosaccharides, and β-xylosidase (EC 3.2.1.37), which cleaves xylooligosaccharides to produce xylose.

Filamentous fungi are known to be efficient producers of xylanolytic enzymes, and most commercial xylanolytic preparations are obtained from fermentations of *Aspergillus* or *Trichoderma* species. Several genes encoding endo-(1,4)-β-xylanases from these fungal species have been characterized (10, 23, 25, 26, 39, 44), and recently genes encoding β-xylosidases have been cloned from both *Aspergillus niger* (46) and *Trichoderma reesei* (32).

Little is currently known about the molecular mechanisms controlling xylanase gene expression in filamentous fungi. The presence of regulatory elements involved in xylan-specific induction in the promoters of the *Aspergillus tubingensis* and *T. reesei* xylanase-encoding genes (10, 50) and Cre1-mediated carbon catabolite repression of expression of the *T. reesei xln1* gene (31) are the only such data reported so far. The ascomycete *Aspergillus nidulans* is a model organism for studies of gene regulation due to our extensive knowledge of its genetics and the availability of mutants (1, 9). In recent years the molecular basis of glucose repression by the protein product of the regulatory gene *creA* has been investigated; it has been found that this protein is a negatively acting transcription factor which binds to a subset of DNA sequence motifs conforming to the consensus sequence 5′-SYGGRG-3′ (8, 24, 28). In addition, studies of mutants (5) disrupted in their responses to external pH (alkaline growth mimic and acidic growth mimic phenotypes) have revealed a regulatory mechanism comprising a signal transduction pathway, encoded by the *pal* genes, which at alkaline ambient pH results in proteolytic conversion of the PacC transcription factor to its active form. After conversion PacC is able to activate those genes whose expression is appropriate under alkaline conditions and to repress those genes whose expression is suited to acidic ambient pH (3, 12, 33, 34, 43).

When grown in media in which xylan is the only carbon source, *A. nidulans* produces at least three endo-(1,4)-β-xylanases (17, 36) and one predominantly mycelium-bound β-xylosidase (29). These four enzymes have been purified and kinetically characterized (15–18, 29), and the genes encoding the three endo-β-(1,4)-xylanases (*xlnA*, *xlnB*, and *xlnC*) have been cloned and sequenced (30, 35). In this paper we describe the identification, cloning, and nucleotide sequence of an *A. nidulans* gene (*xlnD*) which encodes the previously isolated β-xylosidase (29).

MATERIALS AND METHODS

Strains, plasmids and culture conditions. *Escherichia coli* LE392 [*e14-*(*mcrA*) *hsdR514 supE44 supF58 lacY1 galK2 galT22 metB1 trpR55*] and DH5α [*endA1 hsdR17 gyrA96 thi-1 relA1 supE44 recA1* Δ*lacU169* (ϕ80 *lacZ*Δ*M15*)] were used as hosts for propagation of bacteriophage λ and plasmids, respectively. *A. nidulans biA*1 (= CECT2544) was obtained from the Spanish Type Culture Collection and was used as the wild-type strain. *A. nidulans* G191 (*pabaA*1 *pyrG*89 *fwA*1 *uAY*9) (4) was used as the host in transformation experiments performed with plasmid pGW635, which contains the *A. niger pyrA* gene (20) for selection of transformants. *A. nidulans creA*^{d}30, *biA*1 was a gift from H. N. Arst, Jr., and strains *palA*1, *biA*1, *wA*3 (a strain which mimics growth at acidic pH), and *pacC*^{c}14, *biA*1 (a strain which mimics growth at alkaline pH) were obtained from M. A. Peñalva. *A. nidulans* mycelia were grown from spores in minimal medium (MM) (37) containing various carbon sources (1%, wt/vol) as indicated below; appropriate supplements were included when necessary. In transfer experiments, MM containing D-fructose (1%, wt/vol) and supplemented with 0.5% (wt/vol) Casamino Acids (Difco Laboratories, Detroit, Mich.) was used to generate mycelial biomass. Buffered media were prepared by adding filter-sterilized sodium phosphate after autoclaving from 1 M stock solutions having pH values of 4.1, 6.0, and 8.0 in order to obtain a final phosphate concentration of 100 mM. In all cases the sodium ion concentration was adjusted to 195 mM by adding 5 M NaCl. Induction media were prepared by replacing D-fructose with D-xylose (1%, wt/vol) from a filter-sterilized stock solution (10%, wt/vol).

Cloning and sequencing procedures. An *A. nidulans* genomic library constructed in λ Charon 4A (51) was screened by using hybridization conditions as previously described (35). DNA manipulations were carried out by standard methods as described by Sambrook et al. (40). DNA sequences were determined

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by the dideoxynucleotide chain termination method (41) with a Sequenase sequencing kit from Amersham-USB used according to the supplier's instructions; universal, reverse, and gene-specific oligonucleotides were used as primers. The DNA sequences obtained were analyzed with the Genetics Computer Group sequence analysis software package (13).

DNA isolation and RNA isolation. Fungal high-molecular-weight DNA was isolated as described previously (11). Total RNA was extracted from mycelial tissue by a procedure based on the method of Cathala et al. (6). Mycelia were harvested by filtration and rapidly press dried between sheets of absorbent paper to remove as much liquid as possible. Each mycelial mat was then flash frozen in liquid nitrogen and stored at −70°C. Approximately 100 mg of nitrogen-frozen mycelium was homogenized with a solution containing 600 μl of lysing medium (5 M guanidinium thiocyanate, 10 mM EDTA, 50 mM Tris [pH 7.5]) plus 48 μl of β-mercaptoethanol in a 2-ml screw-cap Eppendorf tube for 45 s at full speed with a Mini-beadbeater (Biospec Products, Bartlesville, Okla.) using five 2-mm-diameter steel balls. The contents reached a temperature of about 50°C. The tube was left at room temperature for 5 min, and the contents were rehomogenized as described above. Mycelial debris was removed by microcentrifugation at 4°C for 15 min. Five hundred microliters of supernatant was removed and added to 1.5 ml of 4 M LiCl, and the preparation was mixed and kept on ice overnight. Precipitated material was collected by microcentrifugation at 4°C for 15 min. The tube was thoroughly drained, and the pelleted material was resolubilized in 500 μl of a solution containing 0.1% (wt/vol) sodium dodecyl sulfate, 1 mM EDTA, and 10 mM Tris (pH 7.5) by homogenization with the Mini-beadbeater for 10 s in the absence of steel balls. The solubilized material was extracted with phenol-chloroform, and the aqueous phase recovered was reextracted with chloroform. Total RNA was precipitated by adding 0.1 volume of 3 M sodium acetate (pH 4.8) and 2.5 volumes of absolute ethanol and then mixing and incubating the preparation at −70°C for 30 to 60 min. RNA was recovered by microcentrifugation at 4°C for 15 min. The tube was drained, and the pellet was dissolved in 100 μl of diethyl pyrocarbonate-treated, MilliQ-filtered water by freezing and thawing.

3-[*N*-Morpholino]propanesulfonic acid (MOPS) (0.6 M)–formaldehyde gels were used to perform a Northern analysis of total RNA. A 1.9-kb DNA fragment containing the *A. niger xlnD* gene was generated with oligonucleotides xylos001 and xylos004 and was used as the *xlnD*-specific probe (46). An 830-bp *Kpn*I-*Nco*I fragment was used as the actin-specific probe (19). Probes were labelled by the random hexanucleotide primer method (14).

***A. nidulans* transformations.** Transformation of *A. nidulans* G191 was carried out as described by Tilburn and coworkers (42) by using plasmids pGW635 (5 μg) and pXDE1 (20 μg); the latter plasmid contained the *A. nidulans xlnD* gene. Transformants were selected for growth on MM in the absence of uridine and were clonally purified. β-Xylosidase activity was extracted and measured as described previously (29).

Immunoblot analysis. Mycelia were grown from 2.5×10^8 spores for 14 h at 37°C and 200 rpm in 50 ml of MM containing oat spelt xylan (1%, wt/vol), yeast extract (1%, wt/vol), and Casamino Acids (0.5%, wt/vol) and then extracted with 25 ml of phosphate-buffered saline containing 0.05% Triton X-100 for 24 h at 30°C and 200 rpm. Portions (30 μl) of the mycelial extracts were subjected to sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose membranes. Immunostaining was carried out by the Bio-Rad procedure by using a 1:400 dilution of the *A. nidulans* β-xylosidase antibody (29), followed by incubation with a 1:3,000 dilution of anti-rabbit immunoglobulin G.

Nucleotide sequence accession number. The *A. nidulans xlnD* gene sequence has been deposited in the EMBL nucleotide sequence database under accession no. Y13568.

RESULTS

Cloning of an *A. nidulans* gene coding for a β-xylosidase. An *A. nidulans* genomic library (51) was screened by heterologous hybridization by using a DNA probe corresponding to the *A. niger xlnD* gene, as described above. After two rounds of screening, a positive plaque was detected and purified. The results of a Southern analysis of the phage insert performed with different restriction enzymes correlated well with signals detected previously in Southern blot profiles of restricted *A. nidulans* wild-type genomic DNA (data not shown). A 4.3-kb *Eco*RI DNA fragment that hybridized to the *A. niger xlnD* probe was isolated and subcloned into pUC18, yielding plasmid pXDE1.

Nucleotide sequence of the cloned gene. The sequence of the *A. nidulans* DNA insert (4,243 bp) cloned in plasmid pXDE1 was determined on both strands. A comparison of this sequence with the *A. niger xlnD* gene sequence revealed a 2,406-bp uninterrupted open reading frame (Fig. 1). The deduced amino acid sequence of the *A. nidulans xlnD* gene product exhibited high degrees of similarity to the primary structures of the *A. niger* and *T. reesei* β-xylosidases (64.3 and 61.9% identity, respectively) (Fig. 2) and also exhibited significant levels of similarity to the primary structures of β-glucosidases belonging to glycosidase family 3 (22). There was a predicted signal peptide cleavage site (49) between amino acid residues 17 and 18. Thus, the mature protein is 785 amino acids long and has a predicted molecular mass of 85,320 Da and an isoelectric point of 4.17.

Analysis of the 5′ noncoding sequence of the *A. nidulans xlnD* gene revealed the presence of sequence elements (Fig. 1) that could be involved in transcriptional initiation (21, 45). One TATA box is present 75 bp upstream of the ATG codon, and two CAAT boxes are present 85 and 100 bp upstream of the ATG codon. The start codon is preceded by the sequence TCACC, which resembles the consensus CCPuCC-ATG sequence found in higher eukaryotes (27). In addition, consensus binding target sequences for the *A. nidulans* wide domain regulators CreA (8) and PacC (43) are present. An AATAAA polyadenylation motif is present 120 bp downstream of the proposed stop codon (38).

β-Xylosidase overproduction in *A. nidulans*. Plasmid pXDE1, which contains the *A. nidulans xlnD* gene plus 1,660 bp of upstream sequence, was introduced into *A. nidulans* G191 by cotransformation with plasmid pGW635. Uridine prototrophs were selected and analyzed by Southern hybridization. Five cotransformants were tested for β-xylosidase overexpression by direct growth in MM containing oat spelt xylan as the carbon source. Samples (5 ml) were collected in duplicate after 14, 24, and 36 h of incubation, and the β-xylosidase activities in extracted mycelia were measured (Table 1). In all of the strains analyzed, β-xylosidase activity was lower in the 36-h samples than in the 14-h samples due to the presence of protease activity (data not shown). All of the cotransformants exhibited higher levels of β-xylosidase activity than the nontransformed strain. β-Xylosidase overexpression was greatest in cotransformants TXD1.4 and TXD1.10. These two cotransformants and *A. nidulans* G191 were also grown for 17 h in MM containing D-fructose and Casamino Acids as carbon and nitrogen sources, respectively, and mycelia were then transferred to D-xylose-containing induction medium. Samples (5 ml) were collected in duplicate after 2, 4, and 8 h of posttransfer incubation, and the β-xylosidase activities in extracted mycelia were measured (Table 2). Activity in the nontransformed strain was detected 2 h after transfer and then decreased, and the minimum activity was reached after 8 h. The cotransformants had similar activity profiles but higher absolute levels at all time points after transfer.

Western blot analysis of the overproduced β-xylosidase. In previous work in our laboratory Kumar and Ramón isolated and characterized an 85-kDa β-xylosidase from *A. nidulans* (29). Antibodies raised against this enzyme (29) were used to probe a Western blot of mycelial extracts prepared from G191 (the untransformed strain) and the overexpressing transformants TXD1.4 and TXD1.10. Figure 3 shows that in the extracts from the overproducing transformants there were increased levels of a specific band detected by the *A. nidulans* β-xylosidase antibody whose mobility was identical to the mobility of a band found in the untransformed strain extract. The intensities of staining of the bands corresponded to the levels of β-xylosidase activity measured in the mycelial extracts.

Transcriptional regulation of *xlnD*. The levels of expression of *xlnD* were investigated by performing a Northern blot analysis of transfer cultures and in all cases were compared to the

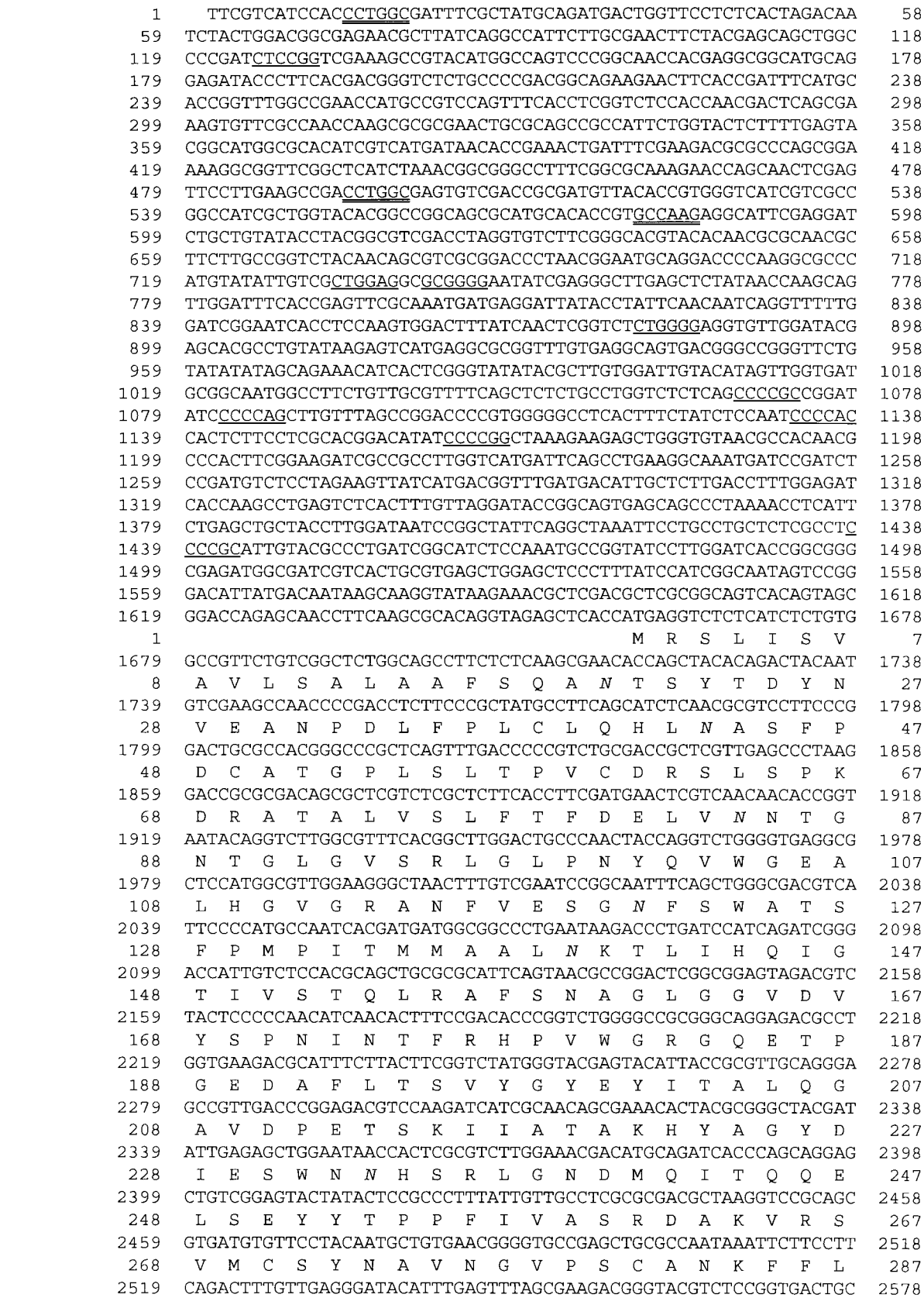

FIG. 1. Nucleotide sequence of the *A. nidulans xlnD* gene and the deduced amino acid sequence of the gene product. Putative CreA and PacC binding sites are underlined and double underlined, respectively. Potential N-glycosylation sites are in italics.

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 288   Q  T  L  L  R  D  T  F  E  F  S  E  D  G  Y  V  S  G  D  C    307
2579  GGTGCTGTCTACAACGTGTGGAACCCTCATGGGTATGCGAGCAACGAGGCGGCTGCGTCC  2638
 308   G  A  V  Y  N  V  W  N  P  H  G  Y  A  S  N  E  A  A  A  S    327
2639  GCGGATTCGATTCTTGCCGGCACAGACATCGACTGTGGAACTTCATACCAGTGGCACTCT  2698
 328   A  D  S  I  L  A  G  T  D  I  D  C  G  T  S  Y  Q  W  H  S    347
2699  GAGGATGCATTCGAGGACAGTCTCGTCTCGCGATCGGACATTGAGCGCGGCGTTATCCGC  2758
 348   E  D  A  F  E  D  S  L  V  S  R  S  D  I  E  R  G  V  I  R    367
2759  CTCTACAGCAACCTCGTGCAAGCTGGGTACTTTGACGGCGAAGACGCGCCATACCGCGAT  2818
 368   L  Y  S  N  L  V  Q  A  G  Y  F  D  G  E  D  A  P  Y  R  D    387
2819  ATTACATGGGACGACGTCCTCAGCACCGACGCCTGGAACATCGCGTACGAGGCAGCCGTC  2878
 388   I  T  W  D  D  V  L  S  T  D  A  W  N  I  A  Y  E  A  A  V    407
2879  GAGGGAATCGTCCTGCTTAAGAATGATGAGACGCTCCCCCTTTCCAAGGACATCAAGAGC  2938
 408   E  G  I  V  L  L  K  N  D  E  T  L  P  L  S  K  D  I  K  S    427
2939  GTCGCGGTGATCGGTCCTTGGGCGAACGTCACCGAGGAACTCCAGGGCAACTATTTTGGC  2998
 428   V  A  V  I  G  P  W  A  N  V  T  E  E  L  Q  G  N  Y  F  G    447
2999  CCAGCGCCCTACCTCATTAGCCCACTGACTGGCTTCCGCGACTCTGGTCTGGACGTCCAC  3058
 448   P  A  P  Y  L  I  S  P  L  T  G  F  R  D  S  G  L  D  V  H    467
3059  TACGCGCTCGGCACCAACCTGACCTCGCACTCGACATCCGGTTTCGAAGAGGCGCTCACC  3118
 468   Y  A  L  G  T  N  L  T  S  H  S  T  S  G  F  E  E  A  L  T    487
3119  GCAGCTAAGCAAGCCGACGCAATCATCTTTGCTGGCGGAATTGACAACACAATCGAAGCC  3178
 488   A  A  K  Q  A  D  A  I  I  F  A  G  G  I  D  N  T  I  E  A    507
3179  GAAGCCATGGACCGCGAGAATATCACTTGGCCTGGGAACCAGCTCGACCTAATCAGCAAG  3238
 508   E  A  M  D  R  E  N  I  T  W  P  G  N  Q  L  D  L  I  S  K    527
3239  CTCAGCGAGCTCGGCAAGCCGCTTGTCGTCCTGCAAATGGGCGGTGGTCAGGTTGATTCA  3298
 528   L  S  E  L  G  K  P  L  V  V  L  Q  M  G  G  G  Q  V  D  S    547
3299  TCCTCCCTAAAAGACAACGACAACGTCAACGCACTCATCTGGGGCGGATACCCCGGCCAG  3358
 548   S  S  L  K  D  N  D  N  V  N  A  L  I  W  G  G  Y  P  G  Q    567
3359  TCGGGTGGGCATGCACTCGCCGATATCATTACCGGCAAGCGCGCGCCTGCTGGCCGCCTC  3418
 568   S  G  G  H  A  L  A  D  I  I  T  G  K  R  A  P  A  G  R  L    587
3419  GTGACAACGCAATATCCTGCTGAATATGCAGAAGTGTTCCCGGCCATCGATATGAACCTG  3478
 588   V  T  T  Q  Y  P  A  E  Y  A  E  V  F  P  A  I  D  M  N  L    607
3479  AGGCCGAATGAGACTAGCGGGAACCCTGGGCAGACGTACATGTGGTACACTGGGACTCCA  3538
 608   R  P  N  E  T  S  G  N  P  G  Q  T  Y  M  W  Y  T  G  T  P    627
3539  GTCTATGAGTTCGGACACGGCCTTTTCTATACAACTTTCGAGGAGTCGACGGAGACAACA  3598
 628   V  Y  E  F  G  H  G  L  F  Y  T  T  F  E  E  S  T  E  T  T    647
3599  GATGCGGGATCTTTCAACATTCAGACAGTCCTTACGACGCCGCACTCGGGATACGAGCAC  3658
 648   D  A  G  S  F  N  I  Q  T  V  L  T  T  P  H  S  G  Y  E  H    667
3659  GCTCAGCAGAAGACACTCCTAAACTTCACCGCGACGGTCAAGAACACTGGCGAGCGTGAG  3718
 668   A  Q  Q  K  T  L  L  N  F  T  A  T  V  K  N  T  G  E  R  E    687
3719  TCCGACTACACGGCACTAGTCTACGTTAACACCACCGCTGGTCCGGCGCCGTATCCCAAG  3778
 688   S  D  Y  T  A  L  V  Y  V  N  T  T  A  G  P  A  P  Y  P  K    707
3779  AAGTGGGTTGTTGGGTTCGACCGGCTCGGAGGATTGGAACCGGGTGACTCGCAGACCCTG  3838
 708   K  W  V  V  G  F  D  R  L  G  G  L  E  P  G  D  S  Q  T  L    727
3839  ACGGTCCCTGTGACGGTTGAGAGCGTCGCCAGGACAGACGAGCAGGGAAACAGGGTGTTG  3898
 728   T  V  P  V  T  V  E  S  V  A  R  T  D  E  Q  G  N  R  V  L    747
3899  TATCCTGGCTCTTATGACGTGGCGCTGAACAACGAGCGCTCTGTTGTTGTGAAGTTTGAG  3958
 748   Y  P  G  S  Y  D  V  A  L  N  N  E  R  S  V  V  V  K  F  E    767
3959  CTGAAAGGCGAGGAGGCGGTTATTCTGAGTTGGCCGGAGGATACGACCTCGGACTTTGTT  4018
 768   L  K  G  E  E  A  V  I  L  S  W  P  E  D  T  T  S  D  F  V    787
4019  TCTAGCATTGACGGAGGCCTTGACAGGAAGCAGGATGTTATTGCTTAGTGTTTCTATCTG  4078
 788   S  S  I  D  G  G  L  D  R  K  Q  D  V  I  A  *                803
4079  GATATGCCTAGCATTAATATTGGAGCTGCAATATCATAGACAGACTGCCTGTCTTCACTT  4138
4139  CGCCTCTTCAGAACTCTACGAGTAGTCTTCATAGTGGCCAGAATAAATCGCAAGTGTCCA  4198
4199  ACTATAACCGTAAGCACTGAATCATAGAATCTCTCAACTGAATTC                 4243
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FIG. 1—*Continued.*

levels of actin transcripts as an internal control. Mycelial biomass was grown from spores for 17 h in D-fructose-containing MM supplemented with Casamino Acids and then transferred to D-xylose-containing induction medium. Mycelial samples taken 1, 2, 4, and 6 h after the transfer were used to prepare total RNA. No *xlnD* expression was detected after growth of the *A. nidulans* wild-type strain in D-fructose-containing medium. However, within 1 h of transfer to inducing conditions a

FIG. 2. Alignment of the β-xylosidase amino acid sequences of *A. nidulans*, *A. niger* (46), and *T. reesei* (34a). Asterisks indicate identical amino acids, and dots indicate conservative changes.

strong *xlnD* transcript signal was detected, the level of which remained high throughout the time course analyzed (Fig. 4A, lanes X).

The carbon catabolite repressibility of *xlnD* expression was investigated with the severe carbon catabolite repression mutant *creA*^{d}30 (2). Mycelial biomasses from the wild type and a *creA*^{d}30 mutant were each divided into two halves and transferred to induction medium and induction medium supplemented with D-glucose. In the wild-type mycelial samples, *xlnD* transcript levels were considerably reduced at the early time points in the presence of D-glucose (Fig. 4A, lanes XG). In the case of *creA*^{d}30, elevated levels of the *xlnD* transcript were observed in the presence of glucose at all of the time points examined, although the transcript levels never attained the levels seen in the samples that were induced and derepressed (samples containing D-xylose but not D-glucose) (Fig. 4B).

TABLE 1. Time course of β-xylosidase production during growth of *A. nidulans* G191 and five *xlnD* cotransformants on MM containing 1% oat spelt xylan as the sole carbon source

Strain	β-Xylosidase activity (U/g of mycelial dry wt) after growth for:		
	14 h	24 h	36 h
G191	6.28	2.13	0.46
TXD1.1	158.57	5.42	2.90
TXD1.3	131.42	7.85	23.33
TXD1.4	617.14	62.85	20.33
TXD1.6	70.00	13.57	2.90
TXD1.10	442.85	100.71	28.33

TABLE 2. Time course of β-xylosidase production by *A. nidulans* G191 and two *xlnD* cotransformants during incubation of washed, D-fructose-grown mycelia in D-xylose-containing induction media

Strain	β-Xylosidase activity (U/g of mycelial dry wt) after growth for:			
	0 h	2 h	4 h	8 h
G191	1.60	27.71	12.33	4.91
TXD1.4	3.25	83.14	80.11	63.75
TXD1.10	3.60	183.57	156.22	141.08

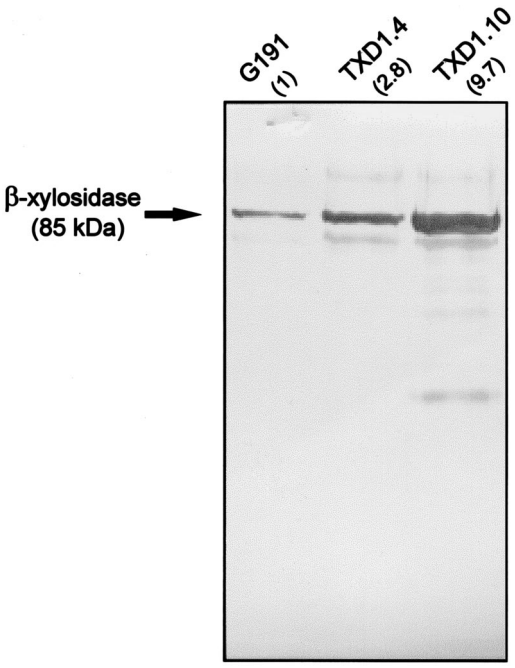

FIG. 3. Western blot. Mycelial extracts of G191 (an untransformed strain) and transformed β-xylosidase overproducers TXD1.4 and TXD1.10 were probed with an antibody raised against the *A. nidulans* 85-kDa β-xylosidase (29). The numbers in parentheses are the relative (compared to nontransformed extract) β-xylosidase activities in the mycelial extracts.

The influence of pH on *xlnD* expression was investigated by the following two techniques: (i) by examining the consequences of transferring wild-type mycelial biomass to acidic (pH 4), neutral (pH 6), and alkaline (pH 8) buffered induction media, and (ii) by analyzing the expression of *xlnD* in *A. nidulans* pH regulatory mutants. Transfer of D-fructose-grown wild-type mycelium to sodium phosphate-buffered induction media revealed no apparent influence of pH at any of the time points analyzed (Fig. 5A). The pH of each medium was measured at the time of harvest to ensure that the buffering capacity was adequate throughout the experiment, and no significant pH shifts were detected (data not shown). *xlnD* expression in both the acid mimic mutant *palA*1 and the alkaline mimic mutant *pacC*^{c}14 exhibited induction patterns similar to the pattern observed for the wild-type strain after mycelial biomass was transferred to nonbuffered induction media, although induction in the *pacC*^{c}14 mutant seemed to be partially reduced (Fig. 5B). The transcript levels appeared to decrease at later time points in the mutants, whereas the transcript level remained essentially the same in the wild type.

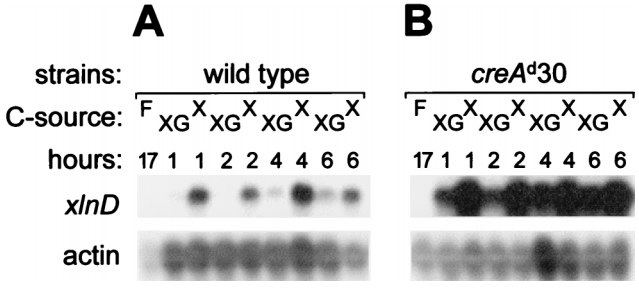

FIG. 4. Northern blots of total RNAs extracted from wild-type mycelia (A) and the *creA*^{d}30 mutant (B) after growth in the presence of D-fructose (lanes F) for 17 h and transfer to inducing conditions (1% D-xylose) (lanes X) and inducing-repressing conditions (1% D-xylose plus 1% D-glucose) (lanes XG).

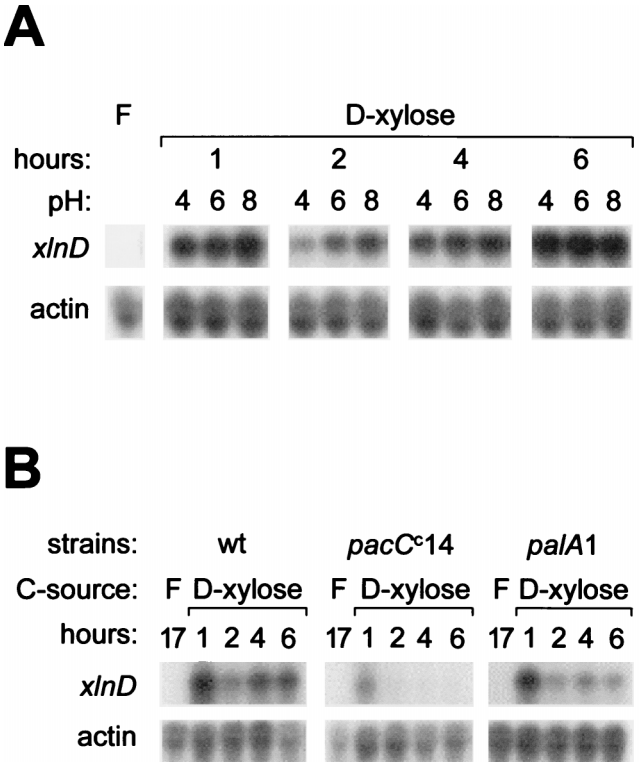

FIG. 5. (A) Northern blots of total RNA extracted from wild-type mycelia after growth in the presence of D-fructose (lane F) for 17 h and transfer to inducing conditions in media buffered with sodium phosphate to pH 4, 6, and 8. (B) Similar experiment performed with mycelia from the wild type (wt) and the *pacC*^{c}14 and *palA*1 mutants after fructose-grown biomass was transferred to nonbuffered inducing conditions (1% D-xylose) for 1, 2, 4, and 6 h.

DISCUSSION

An *A. nidulans* 4.3-kb *Eco*RI genomic DNA fragment subcloned in pXDE1 harbors a functional β-xylosidase gene (designated *xlnD*) since *A. nidulans* multicopy transformants exhibit significant β-xylosidase overexpression (10- to 100-fold greater expression) compared to the wild type. Western blot analysis performed with a polyclonal antibody raised against the *A. nidulans* 85-kDa β-xylosidase revealed elevated levels of production of this enzyme in overexpressing transformants. Taken together, these data show that the cloned sequences present in pXDE1 encode the previously characterized (29) 85-kDa β-xylosidase.

The nucleotide sequence of *xlnD* has been determined. The coding region of the gene consists of an uninterrupted 2,406-bp open reading frame which encodes an 802-amino-acid protein. The deduced molecular mass of the mature protein (85.3 kDa) corresponds closely to the molecular mass previously determined for the purified *A. nidulans* β-xylosidase (29). This implies that although a number of potential N-glycosylation sites occur within the primary structure, the protein is either not glycosylated to a high degree or not glycosylated at all. The vast majority of enzymic activity (>90%) appears to be cell wall associated (data not shown). The basis of this association might be a consequence of the enzyme's molecular size since the purified activity has been found to be a dimer (29) and this might result in its capture within the cell wall, a situation

analogous to the situation which has been described for the *A. niger* glucose oxidase (48). The deduced primary structure of the cloned β-xylosidase exhibits a high degree of similarity to the primary structures of other previously characterized fungal β-xylosidases (Fig. 2), confirming that the cloned gene encodes the *A. nidulans* 85-kDa β-xylosidase.

As in *A. niger*, expression of *xlnD* is specifically induced by oat spelt xylan, as well as by D-xylose (46). Transcription of *xlnD* does not appear to be influenced by the external pH. No significant differences were found in *xlnD* transcript levels after D-xylose induction at pH 4, 6, and 8. This finding is consistent with the expression data obtained with pH regulatory mutants, although the level of transcription in the *pacC*^{c}14 alkaline mimic mutant seems to be somewhat lower than the level of transcription in the wild type. In contrast, *xlnD* expression is subject to carbon catabolite repression by D-glucose, indicating that the glucose repression of β-xylosidase activity observed previously (29) occurs at the level of mRNA transcription; a transcript is observed upon induction by D-xylose but not in the presence of D-glucose. *xlnD* is, however, transcribed in the presence of D-glucose in the *creA*^{d}30 mutant, from which it can be concluded that carbon catabolite repression of the gene is, at least in part, controlled by CreA. Carbon catabolite repression mediated by CreA has also been observed in other fungal genes encoding xylanolytic enzymes (10, 31, 36). Nine putative CreA binding sites are located in the *xlnD* upstream sequences. Deletion analysis of these sites is in progress in order to determine their in vivo function.

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