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# Cloning, Sequencing, and Regulation of a Xylanase Gene from the Fungus *Aureobasidium pullulans* Y-2311-1

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***Aureobasidium pullulans* Y-2311-1 growing on xylan secretes four major xylanases with different masses and isoelectric points. Two of these enzymes, named APX-I and APX-II, have been purified previously. Their N-terminal amino acid sequences are identical except that APX-I has Asp and APX-II has Asn at position 7. An 83-bp DNA region was amplified by PCR and used as a probe for the xylanase gene cloning. The longest cDNA (*xynA*) obtained by cDNA cloning and PCR amplification consisted of 895 bp. *A. pullulans xynA* had an open reading frame encoding a polypeptide of 221 amino acids with a calculated mass of 23,531 Da and contained a putative 34-amino-acid signal peptide in front of the amino terminus of the mature enzyme. Strong homology was found between the deduced amino acid sequence of XynA and some xylanases from bacterial and fungal sources. It is suggested that *A. pullulans* XynA belongs to the family G glycanases. Northern (RNA blot) analysis revealed that only one transcript of 900 bases was present in cultures grown in medium containing D-xylose or oat spelt xylan. Transcription was completely repressed in the presence of glucose in the medium. Southern blot analysis indicated that *A. pullulans xynA* was present as a single copy in the genome. Comparison between the genomic and cDNA sequences revealed that one intron of 59 bp was present in the coding region. The data presented suggest that the highly active xylanases, APX-I and APX-II, secreted by *A. pullulans* are encoded by the same gene.**

Cellulose, hemicellulose, and lignin are three major components of the plant cell wall. Xylan, a major component of hemicellulose, consists of a backbone of β-1,4-linked xylosyl residues with arabinosyl-, acetyl-, and/or 4-*O*-methylglucurosyl side chains (34). Arabinose is esterified by *p*-coumaric or ferulic acids of lignin (13). Enzymatic conversion of xylan to its monomeric components requires the participation of several enzymes, including xylanase (EC 3.2.1.8), β-xylosidase (EC 3.2.1.37), α-L-arabinofuranosidase (EC 3.2.1.55), α-glucuronidase (EC 3.2.1.1), acetyl xylan esterase (EC 3.1.1.6), and *p*-coumaroyl and feruloyl esterases (3–5, 9). Xylanases are the key enzymes for the breakdown of xylan since they depolymerize the backbone. They have broad potential applications in wood biopulping (8, 10, 27), pulp bleaching (17, 18, 28, 36), pretreatment of animal feed (35), food processing (1), and the conversion of lignocellulosic material into industrial feedstock chemicals and fuels (8, 16).

Considering the industrial potentials of xylanases, an important aspect of xylanase research is to obtain highly active xylanases at low cost. Consequently, several bacteria and fungi have been screened for xylanolytic activity (9, 12, 35). What has become evident is that these microorganisms produce multiple xylanases with various specific activities. The fungus *Aureobasidium pullulans* Y-2311-1 has been reported to produce the highest levels of xylanase among several xylanolytic fungi (20, 24). D-Xylose, xylobiose, xylan, and arabinose all induced production while glucose repressed the production of xylanase activity in this fungus (23). At least four xylanases, differing in isoelectric points (pIs), are secreted into the culture supernatants when *A. pullulans* is grown on oat spelt xylan (OSX) (25). Two of these enzymes, designated APX-I and APX-II (22, 25), have been purified and characterized. The first 45 amino acids at their N termini are almost identical, and both cross-react with polyclonal antiserum against APX-II, but they differ in molecular masses and pIs (21, 25). Here we report the nucleotide and deduced amino acid sequences of the gene (*xynA*) as determined by cDNA cloning and PCR amplification and the regulation of *xynA* in *A. pullulans*.

## MATERIALS AND METHODS

**Strains, vectors, and cultivation conditions.** *A. pullulans* Y-2311-1 is a color variant strain (24). It was grown at 28°C with shaking (200 rpm) in YM medium (24) containing 1.0% (wt/vol) OSX, D-xylose, glucose, or glycerol as the carbon source. Under these conditions, the fungus grew in a yeast-like way, showing the typical cell budding growth pattern. The *Escherichia coli* strains used in this study include INVαF′, SURE, and XL-Blue, and the vectors included pCRII, λZAPII, and pBluescript. They were purchased from either Stratagene Cloning Systems, La Jolla, or Invitrogen Co., San Diego, Calif.

**Isolation of DNA and RNA from *A. pullulans*.** For isolation of genomic DNA, the fungus was grown in medium containing glucose (1.0%, wt/vol) for 3 days. Cells were collected by centrifugation (3,000 × *g*, 15 min) and washed twice with $H_2O$ at 4°C. Protoplast formation, disruption of cells, and recovery of chromosomal DNA were done by the method of Black et al. (2). For isolation of RNA as a template for cDNA library construction, cells were grown for 3 days on 1.0% (wt/vol) OSX as the carbon source and collected as described above. Diethyl pyrocarbonate-treated sterile $H_2O$ was used to wash the cells. Disruption of cells and extraction of RNA were performed with an RNA isolation kit (Stratagene) by following the manufacturer's instructions, except that the cells were broken by shaking (100 rpm) in a water bath at 60°C with glass beads

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TABLE 1. Oligonucleotide primers used in PCR

| Primer | Sequence (5'→3') |
|---|---|
| P0813 | TAT(C)GTT(ACG)CAA(G)AAT(C)TAT(C)AA |
| P3035 | CCA(G)TTA(G)TTCCAA(G)TACAT |
| P200 | GTCGCCATTGACACCGT |
| P3338 | GAAGTCGCCATTGACACCGTTGTT |
| PFW | CGGCACGAGCTCGTGCCGG |
| PRW | GTAGCAAGGTGTCTGACAT |

(212 to 300 μm in size; Sigma Chemical Co., St. Louis, Mo.). Polyadenylated RNA was prepared from total RNA by chromatography on oligo(dT)-cellulose (Boehringer Mannheim Co., Indianapolis, Ind.). For isolation of RNA used in Northern (RNA blot) analysis, cells were first grown on 1.0% (vol/vol) glycerol for 3 days, collected by centrifugation, and washed with diethyl pyrocarbonate-treated sterile $H_2O$ once. The cells were then suspended in medium containing 10 g of glucose or D-xylose or a mixture of 10 g each of glucose and D-xylose or of glucose and OSX per liter and cultured with shaking at 28°C for 20 h before RNA isolation.

**Primer designs and PCR.** The purification of xylanase APX-II and its N-terminal amino acid sequence have been reported (25). Two regions (residues 8 to 13 and 30 to 35) of this sequence were used to design two degenerate nucleotide primers, P0813 and P3035 (Table 1), with redundancies of 128- and 8-fold, respectively. Biotin molecules were attached to the 5' ends of these primers during synthesis by using biotin-ON phosphoramidite (Clontech Laboratories, Inc., Palo Alto, Calif.). With genomic DNA as the template and the Geneamp PCR reagent kit (Perkin Elmer Co., Norwalk, Conn.), DNA fragments were amplified by PCR. Amplification was performed for 30 cycles on a 480 Thermal Cycler (Perkin Elmer Co.), with each cycle including 30 s of melting at 95°C, 30 s of annealing at 50°C, and 45 s of extension at 72°C. PCR was also used to amplify the 5' end of the full-length xylanase cDNA missing in the positive cDNA clones. Lambda DNA was purified from the liquid cDNA library with a λ DNA purification system (Promega Co., Madison, Wis.) and used as the template for the amplification. The T3 promoter sequence and P3338 (Table 1), which was 152 bp from the 5' end of the positive cDNA clones, were used as primers. Amplification was done by 30 cycles of 60 s of melting at 95°C, 60 s of annealing at 55°C, and 90 s of extension at 72°C. PCR products were separated by electrophoresis on agarose gels (2.5 or 4.0%, wt/vol) and visualized by ethidium bromide staining. PCR products were cloned into the pCRII vector (Invitrogen Co.).

**Construction and screening of an *A. pullulans* cDNA library.** Oligo(dT)-cellulose-purified RNA isolated from cells grown on OSX was used as the template for the synthesis of cDNA. cDNA longer than 400 bp as fractionated by a spin column was ligated into λZAPII arms with a ZAP-cDNA synthesis kit and packaged with the Gigapack II packaging extract according to the manufacturer's instructions (Stratagene Cloning Systems). Recombinant phages were screened for hybridization with the 83-bp biotinylated PCR fragments. Briefly, plaques (1 mm in diameter) grown on top agar were transferred to Photogene nylon membranes (Gibco BRL Life Technologies, Inc., Gaithersburg, Md.) by overlaying the membranes on the top agar for 2 min. Positive clones on the membranes were detected with a Photogene nucleic acid detection system according to the manufacturer's instruction (Gibco BRL), except that the membranes were incubated in TBS (100 mM Tris-HCl [pH 7.5], 150 mM NaCl) containing 200 μg of proteinase K per ml at 37°C for 1 h before prehybridization, and washes with 0.1× sterile sodium citrate (SSC) buffer and 1% (wt/vol) sodium dodecyl sulfate (SDS) were done at 44°C instead of 50°C to achieve hybridization stringency. Positive clones were purified by a second round of screening and were then converted into the pBluescriptSK(−) form by in vivo excision as described by Stratagene.

**Sequencing and analysis of an *A. pullulans* xylanase gene.** *E. coli* XL-Blue harboring pBluescript plasmids containing inserted cDNA were grown overnight at 37°C in Luria-Bertani medium containing ampicillin (50 μg/ml). Plasmids were isolated and purified with the Magic Miniprep DNA Purification System (Promega Corp.). The nucleotide sequences of pBluescript plasmids with cDNA inserted and pCRII plasmids with PCR products inserted were determined by PCR sequencing by the *f*mol DNA Sequencing System (Promega) or the Automatic Dyedeoxyl Terminator System (Applied Biosystems Inc., Foster City, Calif.). Plasmids were further purified with Centricon 100 tubes (Amicon, Inc., Beverly, Mass.) before they were subjected to automatic sequencing. Both universal and specific sequencing primers were used to sequence the sense and antisense strands of inserts in the pBluescript and pCRII plasmids. Sequence data were analyzed with the Genetics Computer Group package (University of Wisconsin Biotechnology Center, Madison) on the VAX/VMS system of the BioScience Computing Resource at the University of Georgia.

**Southern and Northern blot analysis.** Genomic DNA was digested to completion with various restriction endonucleases purchased from Boehringer Mannheim Co. or New England Biolabs, Inc. (Beverly, Mass.). Digested DNA fragments were separated on a 1.0% (wt/vol) agarose gel in 1× Tris-acetate-EDTA (TAE) buffer. Biotinylated *Hin*dIII-digested λ DNA fragments (Gibco BRL) were used as size markers. Total RNA samples and molecular weight standards (Gibco BRL) were separated in 2.0% (wt/vol) agarose gels in the presence of formaldehyde as described by Sambrook et al. (31). DNA and RNA fragments in gels were transferred to Photogene nylon membranes by using the vacuum blotter (Bio-Rad Laboratories, Richmond, Calif.) and cross-linked to the surface of the membrane by UV light in a UV 1800 Stratalinker (Stratagene). The biotinylated PCR fragments were denatured by boiling for 5 min and used as a hybridization probe as described for the screening of the cDNA library.

**Analysis of genomic DNA.** Oligonucleotide primers PFW and PRW (Table 1), corresponding to the 5' and 3' ends of the full-length cDNA insert for the *A. pullulans* xylanase, were synthesized. With these oligonucleotides as primers and genomic DNA and DNA purified from the cDNA library as templates, PCR was performed as described above except that each cycle had a denaturation time of 45 s (95°C), an annealing time of 45 s (55°C), and an extension time of 1.5 min (72°C). After 30 cycles, reaction solutions (20 μl) were separated on a 2.5% (wt/vol) agarose gel, and DNA bands were visualized by ethidium bromide staining. The PCR products amplified from genomic DNA were cloned into pCRII and sequenced as described above.

**Nucleotide sequence accession number.** The nucleotide sequence of *xynA* from *A. pullulans* Y-2311-1 has been assigned accession number U10298 in the GenBank, EMBL, and DDBJ libraries.

## RESULTS AND DISCUSSION

**Amplification and cloning of a xylanase-specific DNA probe.** An 83-bp DNA fragment was amplified by the PCR with P0813 and P3035 as primers and *A. pullulans* genomic DNA as the template (Fig. 1). No nonspecific band was observed. The PCR

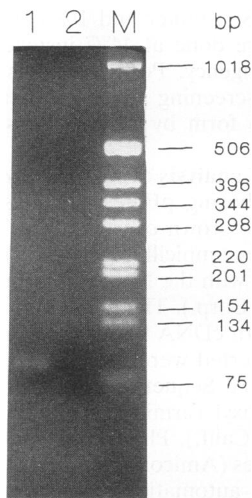


FIG. 1. Amplification of biotinylated xylanase-specific DNA probe by PCR. Design of primers and conditions for amplification and electrophoresis are described in Materials and Methods. Twenty microliters of the reaction solutions (100 μl) with (0.5 ng, lane 1) and without (lane 2) *A. pullulans* genomic DNA were loaded on a 4.0% agarose gel. DNA molecular standards (Gibco BRL) were loaded in lane M. After electrophoresis, DNA bands were visualized by ethidium bromide staining.

product was cloned directly into pCRII. Eight of 10 white colonies had the 83-bp DNA insert, as revealed by restriction enzyme digestion and nucleotide sequence analysis. The deduced amino acid sequence, YVQNYNGNLGFTYNENAG TYSMYWNNG, matched the corresponding region of the N-terminal amino acid sequence (25). P0813 and P3035 were resynthesized with biotin conjugated to their 5′ ends. The PCR products made with these primers were used as hybridization probes for cDNA library screening and Southern and Northern blot analysis.

**Isolation and sequencing of xylanase-specific cDNA.** A cDNA library was constructed in λZAPII with polyadenylated RNA isolated from a 3-day-old OSX-grown culture. Plaques (approximately 5 × $10^5$) were screened with the biotinylated PCR fragments. During the first round of screening, five plaques contained DNA that hybridized to these probes. A second round of screening yielded pure clones. All five positive plaques were recovered as pBluescriptSK(−) through in vivo excision. All five positive clones had identical restriction patterns. Therefore, only two of these five clones were sequenced. The sequences of both strands of the insert were determined with both universal and specific primers (Fig. 2). The PCR fragment was located 106 bp from the beginning of the insert. The insert had 820 bp, possessing an 18-base poly(A) tail at the 3′ end. Neither xylanase activity nor a polypeptide immunologically cross-reactive with the antiserum against APX-II (25) was found in the supernatant or in the sonicated cell fractions of cultures after induction with isopropylthio-β-D-galactoside (data not shown), suggesting that the cDNA was fused out of frame with the *lacZ* gene.

Based on the N-terminal amino acid sequences of APX-I and APX-II, Genetics Computer Group analysis revealed that this insert encoded a polypeptide of 213 amino acid residues. The N-terminal amino acid Ala of both APX-I and II was located 79 bp from the 5′ end of the insert. The deduced amino acid sequence matched the N-terminal amino acid sequences of APX-I and II except that APX-I had Asp at position 7 (Fig. 3) (21, 25). Upstream of the mature N-terminal sequence was an amino acid sequence rich in hydrophobic residues which could serve as a signal peptide for the secretion of the xylanases. No Met residue was, however, found at the amino side of the signal peptide, suggesting that these cDNA clones might not represent the full-length cDNA of the gene. Using the cDNA library as the template and the T3 promoter sequence and P3339 as primers, the missing 5′ end of the full-length cDNA was amplified by PCR, cloned into pCRII, and sequenced. The full-length cDNA designated *xynA* contained 895 bp (Fig. 2). The nucleotide sequence of *xynA* is given in Fig. 3. The G+C ratio of *xynA* was 52.5%.

A start codon 59 bp downstream of the 5′ end of *xynA* was identified, which began an open reading frame (ORF) of 666 bp. The codon usage in this ORF was significantly biased to C at the third position (Table 2). The percentages of A, T, G, and C at this position were 20.4, 24.8, 24.4, and 29.7%, respectively. These ratios are typical properties of highly expressed genes in fungi (15, 26, 33).

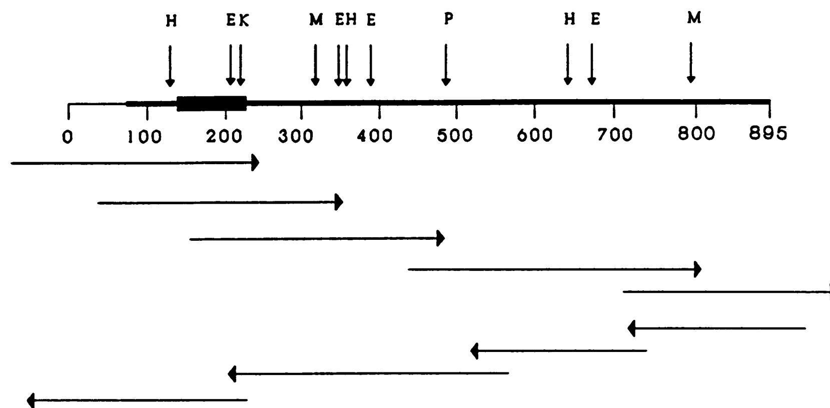


FIG. 2. Restriction map and sequencing strategy of the full-length cDNA for *A. pullulans xynA*. The cDNA insert is shown as a thick line, and the 5′ PCR-amplified cDNA region is shown as a thin line. Solid box, length and location of the 83-bp PCR fragment. Restriction sites for *Eco*RII (E), *Hae*III (H), *Kpn*I (K), *Mbo*I (M), and *Pst*I (P) are shown by vertical arrows. The orientations and lengths of coverage of sequencing primers are shown as horizontal arrows.

```
  1   CGGCACGAGCTCGTGCCGGATCACATCCATTCAAACAATACTTCCAACTCTCTTCAAC

 59  ATGAAGTTCTTCGCCACCATTGCTGCTCTCGTTGTGGGAGCTGTTGCTGCCCCAGTCGCA
      M  K  F  F  A  T  I  A  A  L  V  V  G  A  V  A  A  P  V  A

119  GAGGCTGAGGCTGAGGCCAGCAGCCCCATGCTGgtacgatctcttcgatgaaccattcta
      E  A  E  A  E  A  S  S  P  M  L

179  ttcgagaccatcttgctgatcaaacacaatagATCGAACGTGCCGGTCCCGGTGGCATCA
                                      I  E  R  A  G  P  G  G  I  N

239  ACTACGTCCAGAACTACAACGGCAACCTGGGCCAGTTCACCTACAATGAGAACGCTGGTA
       Y  V  Q  N  Y  N  G  N  L  G  Q  F  T  Y  N  E  N  A  G  T

299  CCTACTCCATGTACTGGAACAACGGTGTCAATGGCGACTTCGTCGTTGGTCTCGGTTGGT
       Y  S  M  Y  W  N  N  G  V  N  G  D  F  V  V  G  L  G  W  S

359  CAACCGGTGCTGCCCGCTCCATCACCTACTCTTCCAACTACCAGGCCAGCGGCGGTTCTT
       T  G  A  A  R  S  I  T  Y  S  S  N  Y  Q  A  S  G  G  S  Y

419  ACCTGTCCGTCTACGGCTGGATCAACAGCCCCCAGGCTGAGTACTACATTGTCGAGTCTT
       L  S  V  Y  G  W  I  N  S  P  Q  A  E  Y  Y  I  V  E  S  Y

479  ACGGCTCGTACAACCCTTGCGGCGCCGGTCAGTCCGGTGTCACTCAGCTCGGCACCGTCT
       G  S  Y  N  P  C  G  A  G  Q  S  G  V  T  Q  L  G  T  V  C

539  GCAGCGATGGCGCTACCTACACCGTCTACACCGACACTCGTACCAACCAGCCCTCCATCA
       S  D  G  A  T  Y  T  V  Y  T  D  T  R  T  N  Q  P  S  I  T

599  CTGGTACTTCTACCTTCAAGCAGTACTGGTCTGTCCGCCAGACTAAGCGTACTTCCGGCA
       G  T  S  T  F  K  Q  Y  W  S  V  R  Q  T  K  R  T  S  G  T

659  CGGTCACCACTGGCAACCACTTTGCTTACTGGGCCAAGTACGGCTTTGGCAACTCTTACA
       V  T  T  G  N  H  F  A  Y  W  A  K  Y  G  F  G  N  S  Y  N

719  ACTTCCAGGTCATGCCTGTCGAGGCTTTCTCTGGCACTGGTAGCGCCAGTGTCACCGTCT
       F  Q  V  M  P  V  E  A  F  S  G  T  G  S  A  S  V  T  V  S

779  CTTAAATGTCGGAACAAGTGGCTGAATTTGGATGTTGGAAAGGAGGTTGTTTGGGATGCG
       *

839  GATGAAACGCTGATGAAGATATGATGTTGATCTGGTTGTGTCCATTTATGCTAGCTTGTC

899  ATTCGTTAGCACAAAGTAAATGTCAGACACCTTGCTACAAAAAAAAAAAAAAAAAA
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FIG. 3. Nucleotide and deduced amino acid sequences of the cDNA and genomic DNA of *A. pullulans xynA*. The N-terminal amino acid sequence of the mature enzyme obtained by protein sequencing is underlined. An intron sequence is shown in lowercase letters. A 34-amino-acid sequence upstream of Ala of the mature enzyme is a putative signal peptide.

The ORF encoded a polypeptide of 221 amino acid residues with a calculated mass of 23,531 Da. The putative signal peptide contained 34 amino acid residues, rich in hydrophobic residues. The mature polypeptide consisted of 187 amino acid residues with a calculated mass of 20,016 Da, which is very close to the value of 20 kDa determined for APX-I (22) but smaller than the 25 kDa determined for APX-II (25). The deduced amino acid composition of this ORF together with that determined for APX-II is shown in Table 3. APX-II has a high percentage of Ala, Gly, Ser, and Thr. When the mature polypeptide was used for comparison, the overall amino acid composition was in fairly good agreement with that for APX-II (Table 3) (25) but very different from that published for APX-I (21), which, after careful consideration of the data, appears to be erroneous. We suggest that APX-I and APX-II are both encoded by *xynA*. This suggestion is based on their almost identical N-terminal amino acid sequences, immunological and regulatory relatedness, and the presence of a single copy of the gene and the transcript (see below). Purified APX-I and APX-II differ in mass. Posttranslational modifications such as glycosylation, proteolysis, or both could contribute to this phenomenon. The sequence Asn-X-Ser/Thr required for N-linked glycosylation was not found in this polypeptide (29); therefore, it is suggested that APX-II may be more glycosylated by O-linked oligosaccharides than APX-I. The xylanases had high percentages of Ser (11.6%) and Thr (10.0%) residues. Thus, the extent of glycosylation of these residues may contribute to the differences in mass of APX-I and APX-II. More glycosylation was detected on APX-II than on APX-I by carbohydrate staining of protein bands on SDS-polyacrylamide gel electrophoresis gels (data not shown).

TABLE 2. Codon usage of *A. pullulans xynA*

| Amino acid | Codon | Usage | Amino acid | Codon | Usage |
|---|---|---|---|---|---|
| Gly | GGG | 0 | Thr | ACG | 1 |
| | GGA | 1 | | ACA | 0 |
| | GGT | 12 | | ACT | 8 |
| | GGC | 15 | | ACC | 13 |
| Glu | GAG | 7 | Trp | TGG | 5 |
| | GAA | 1 | | | |
| | | | Met | ATG | 4 |
| Gln | CAG | 10 | | | |
| | CAA | 0 | Cys | TGT | 0 |
| | | | | TGC | 2 |
| Asp | GAT | 1 | | | |
| | GAC | 2 | Tyr | TAT | 0 |
| | | | | TAC | 19 |
| Asn | AAT | 2 | | | |
| | AAC | 14 | Leu | TTG | 0 |
| | | | | TTA | 0 |
| Val | GTG | 1 | | CTG | 3 |
| | GTA | 0 | | CTA | 0 |
| | GTT | 3 | | CTT | 0 |
| | GTC | 15 | | CTC | 3 |
| Ala | GCG | 0 | Phe | TTT | 2 |
| | GCA | 1 | | TTC | 7 |
| | GCT | 12 | | | |
| | GCC | 9 | Ser | AGT | 1 |
| | | | | AGC | 6 |
| Lys | AAG | 4 | | TCG | 1 |
| | AAA | 0 | | TCA | 1 |
| | | | | TCT | 8 |
| Ile | ATA | 0 | | TCC | 7 |
| | ATT | 2 | | | |
| | ATC | 5 | His | CAT | 0 |
| | | | | CAC | 1 |
| Arg | AGG | 0 | | | |
| | AGA | 0 | Pro | CCG | 0 |
| | CGG | 0 | | CCA | 1 |
| | CGA | 0 | | CCT | 2 |
| | CGT | 3 | | CCC | 4 |
| | CGC | 2 | | | |

**Homology of the sequence with other xylanases.** The deduced amino acid sequence of XynA was used to search for the homologous sequences in the SWISS-PROT database. This sequence showed significant homology only to some xylanases. The alignments of these homologous sequences are given in Fig. 4. Sequence identity was 45.6% for *Bacillus pumilus*; 33.8% for *Ruminococcus flaveficiens*; 41.4 and 38.7% for *Streptomyces lividans* B and C, respectively; 41.7% for *Bacillus circulans*; and 38.8% for *Neocallimastix patriciarum* xylanases (11, 32). Certain regions, including YGW at positions 97 to 99, EYY at positions 106 to 108, and TFKQYWSVRQ at positions 157 to 166, were highly conserved among these enzymes. These residues might play critical roles in substrate binding and catalysis. A complete elucidation of the importance of these residues must await further studies on the expression of this gene and the site-directed mutagenesis of these residues. X-ray

TABLE 3. Amino acid composition of *A. pullulans* xylanase

| Amino acid | No. of molecules/mol of enzyme[a] | | |
|---|---|---|---|
| | Xylanase | | APX-II |
| | Whole ORF | Mature enzyme | |
| Ala | 22 | 12 | 14 |
| Arg | 5 | 4 | 5 |
| Asx | 19 | 19 | 23 |
| Cys | 2 | 2 | 0 |
| Glx | 18 | 14 | 15 |
| Gly | 28 | 27 | 29 |
| His | 1 | 1 | 5 |
| Ile | 7 | 5 | 4 |
| Leu | 6 | 5 | 5 |
| Lys | 4 | 3 | 4 |
| Met | 4 | 2 | 1 |
| Phe | 9 | 7 | 6 |
| Pro | 7 | 5 | 5 |
| Ser | 24 | 22 | 20 |
| Thr | 22 | 21 | 22 |
| Trp | 5 | 5 | ND[b] |
| Tyr | 19 | 19 | 13 |
| Val | 19 | 15 | 11 |
| Total | 221 | 187 | 182 |

[a] Data for the whole ORF and mature enzyme are from this study. Values for APX-II were calculated from the data published in reference 25.

[b] ND, not determined.

crystallographic and site-directed mutagenesis studies suggested that Glu-93 and Glu-182 in a *B. pumilus* xylanase are essential for its active site, whereas Asp-21 was not as critical (19). The sequence alignment (Fig. 4) strongly supported this statement, since Glu-73 and Glu-157 in *A. pullulans* XynA were conserved in all xylanases, whereas Gly-14 of *A. pullulans* XynA was in the position of Asp-21 of *B. pumilus* xylanase. *A. pullulans* XynA was not significantly homologous to the cellobiohydrolase of *Cellulomonas fimi* or xylanase Z of *Clostridium thermocellum*, which were the two representative enzymes classified as family F β-glycanases by hydrophobic cluster analysis (14). In contrast, the *A. pullulans* XynA deduced amino acid sequence was significantly homologous to those of XlnB and XlnC of *S. lividans* and XlnB of *R. flaveficiens*, which were recently classified as family G β-glycanases (12). Thus, *A. pullulans* XynA may be grouped in this family.

**Regulation of gene expression of xylanase in *A. pullulans* by growth substrates.** The regulation of *xynA* expression by growth substrates was investigated by Northern blot analysis with the biotinylated PCR fragments as hybridization probes. An RNA band of about 900 bases was detected in cells grown in medium containing 1% (wt/vol) D-xylose or OSX but not in medium containing 1% glucose, 1% D-xylose plus 1% glucose, or 1% OSX plus 1% glucose (all wt/vol) (Fig. 5). The intensities of the RNA bands from the samples of different cultures detected by ethidium bromide staining were similar (data not shown). Three times as much cross-hybridization was obtained from cells grown in medium containing OSX as from cells grown in medium containing D-xylose. This correlated with the levels of xylanase activity in the supernatants of OSX- and

FIG. 4. Alignments of homologous xylanase sequences with the *A. pullulans* XynA sequence. Sequences listed include those of xylanases from *A. pullulans* in this study (Xyna-Aurpu), *B. pumilus* (Xyna-Bacpu), *R. flavefaciens* (Xyna-Rumfl), *S. lividans* B (Xynb-Strli) and C (Xync-Strli), *B. circulans* (Xyna-Bacci), and *N. patriciarum* (Xyna-Neopa).

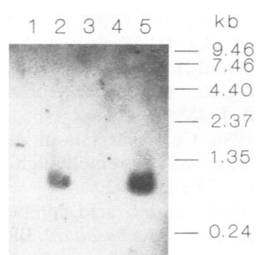


FIG. 5. Northern blot analysis of *A. pullulans xynA*. Total RNA samples (10 μg each) were isolated from cells grown in medium containing glucose (lane 1), D-xylose (lane 2), glucose plus D-xylose (lane 3), glucose plus OSX (lane 4), or OSX (lane 5). The positions of migration of RNA molecular standards are shown on the right.

D-xylose-grown cells (25). Only one transcript is synthesized from this gene under induction. These results confirmed the previous suggestion (25) that expression is controlled at the transcriptional level rather than at the translational or post-translational level. Glucose behaved as a repressor. Xylan and D-xylose, the end product of xylan degradation, are both able to induce transcription only when glucose is absent or reaches certain low levels in the culture. Thus, the regulation of xylanase gene expression in *A. pullulans* does not parallel cellulase gene expression in cellulolytic fungi such as *Trichoderma reesei*, in which glucose, the end product of cellulose degradation, completely repressed the transcription of cellulose genes (7). The establishment of the exact regulatory mechanism for xylanase gene expression in this fungus needs further investigation.

**Characterization of genomic DNA.** Genomic DNA was digested with various restriction enzymes, and Southern blot analysis was performed with the biotinylated PCR fragment as the hybridization probe (Fig. 6). Only one band was obtained for *Eco*RI (8.5 kbp), *Hin*dIII (9.4 kbp), *Eco*RI plus *Hin*dIII (6.8 kbp), *Hae*III (0.5 kbp), *Hae*III plus *Eco*RI (0.5 kbp), *Hae*III plus *Hin*dIII (0.5 kbp), and *Xho*I (1.0 kbp). The minor bands from *Hae*III plus *Eco*RI (3.0 kbp) and *Hae*III plus *Hin*dIII (6.5 kbp) digestions might be caused by incomplete digestion. These data indicated that the *A. pullulans* genome possesses only a single copy of this gene. Two 19-base oligonucleotide primers (PFW and PRW, Table 1) corresponding to the 5′ and 3′ ends of *xynA* were synthesized. With these primers and genomic DNA and cDNA as templates, both genomic DNA and cDNA gave a band of approximately 900 bp after amplification by PCR (Fig. 7). It appeared that the band amplified from genomic DNA was slightly larger than the one amplified from cDNA, indicating that genomic DNA in this region might possess short introns. Sequencing of this region after cloning into pCRII and comparing the sequences revealed that it indeed had an intron of 59 bp (Fig. 3). This intron sequence started with GTA and ended with TAG, which matched the general consensus sequences for intron ends (6, 30).

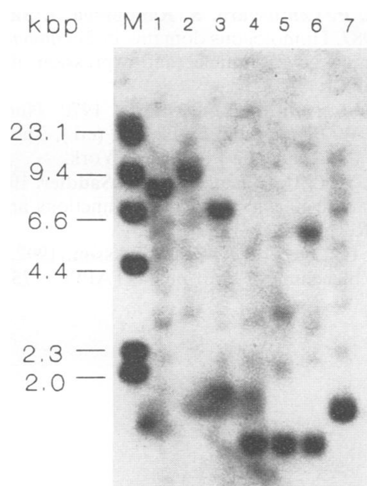


FIG. 6. Southern blot analysis of *A. pullulans* genomic DNA. Genomic DNA (40 μg each) was digested by *Eco*RI (lane 1), *Hin*dIII (lane 2), *Eco*RI plus *Hin*dIII (lane 3), *Hae*III (lane 4), *Eco*RI plus *Hae*III (lane 5), *Hin*dIII plus *Hae*III (lane 6), and *Xho*I (lane 7) and analyzed as described in Materials and Methods. The migration of biotinylated *Hin*dIII-digested λ DNA fragments (lane M) is indicated on the left.

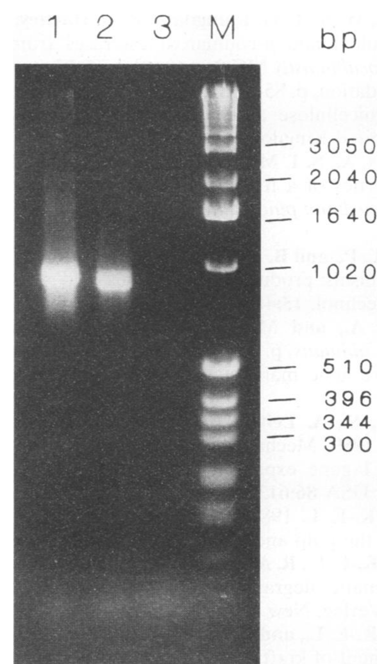


FIG. 7. Amplification of genomic DNA of *A. pullulans* xylanase gene by PCR. Reaction solutions (20 μl) with genomic DNA (lane 1) and cDNA (lane 2) as the templates and without template (lane 3) were loaded on a 2.5% agarose gel. DNA molecular standards were loaded in lane M. After electrophoresis, DNA bands were visualized by ethidium bromide staining.


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