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Molecular Cloning and Expression in *Saccharomyces cerevisiae* of Two *Aspergillus nidulans* Xylanase Genes

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Two *Aspergillus nidulans* genes, *xlnA* and *xlnB*, encoding the X_{22} and X_{24} xylanases from this fungus, respectively, have been cloned and sequenced. Their cDNAs have been expressed in a laboratory *Saccharomyces cerevisiae* strain under the control of a constitutive yeast promoter, resulting in the construction of recombinant xylanolytic yeast strains.

The breakdown of xylan, a complex polymer of plant cell walls, requires the action of several enzymes, including xylanases, β-xylosidases, acetyl xylan esterases, and several debranching enzymes (2). Xylanases, which are responsible for depolymerization of the xylan backbone, have potentially broad biotechnological applications (2, 3, 32). At present, most commercial xylanolytic preparations are obtained from filamentous-fungus species. As with other xylanolytic microorganisms, filamentous fungi produce multiple xylanases (33) whose genes have been cloned and sequenced from the following: *Aureobasidium pullulans* (22), *Cochliobolus carbonum* (1), *Penicillium chrysogenum* (17), *Trichoderma reesei* (25, 30), *Aspergillus awamori* (18), *Aspergillus kawachi* (20, 21), and *Aspergillus tubingensis* (4). Little is known about the mechanisms controlling xylanase gene expression. Only in the case of the *A. tubingensis xlnA* gene has a regulatory element involved in xylan-specific induction been clearly identified in the gene promoter (4).

Aspergillus nidulans is genetically well characterized and hence an ideal system for the study of gene regulation. *A. nidulans* produces three major xylanases, X_{22}, X_{24}, and X_{34} (10), so called by virtue of their molecular weights, which have been recently purified and characterized (7–9). This report presents the cloning, sequencing, and yeast heterologous expression of the *xlnA* and *xlnB* genes, which encode the X_{22} and X_{24} xylanases, respectively.

Cloning of the *xlnA* and *xlnB* genes from *A. nidulans*. An *A. nidulans* genomic library constructed in λ Charon 4A (34) was screened by heterologous hybridization using a DNA fragment containing the *A. tubingensis xlnB* gene (5) as a probe; 10 positive plaques were detected and purified. Two different patterns of hybridization signals were observed in Southern analyses of restriction endonuclease digestions of the recombinant phages (results not shown). These patterns correlated well with those seen on blots of *A. nidulans* genomic digests in which at least two specific signals were detected for each restriction-enzyme-treated DNA sample. From the phage DNAs, two fragments hybridizing to the *A. tubingensis xlnB* probe, a 6.2-kb *Bam*HI fragment containing the putative xylanase gene *xlnA* (see below) and a 5-kb *Eco*RI fragment containing the putative xylanase gene *xlnB* (see below), were isolated and subcloned in pUC18.

Nucleotide sequences of the *xlnA* and *xlnB* genes. The DNA fragments hybridizing to the *A. tubingensis xlnB* probe were partially sequenced. In both cases, open reading frames interrupted by introns were identified by homology to the *A. tubingensis xlnA* (4) and *xlnB* (5) genes. The positions of the introns and their processing were confirmed by partial sequencing of the *xlnA* and *xlnB* cDNAs (see below). The intron position is conserved between the *xlnA* genes from both *A. nidulans* and *A. tubingensis*, though the intron is larger in the *A. nidulans* gene (68 versus 49 bp). The N termini of the translated open reading frames of *xlnA* and *xlnB* correspond to the N-terminal amino acid sequences (STPSSTGWSNGYYYSF and STPSSTGTSG) determined for *A. nidulans* X_{22} and X_{24}, respectively.

A signal peptide cleavage site (31) is predicted to be present between residues 19 and 20 after the initiation codon in the *xlnA* product and between residues 18 and 19 in the *xlnB* product (Fig. 1). In addition, 18- and 15-amino-acid putative propeptides preceding the N-terminal amino acids of the X_{22} and X_{24} mature proteins, respectively, would be removed by a protease at the monobasic cleavage sites (26). Such two-step proteolytic processing has been suggested for many other secreted fungal proteins (27, 28, 30) and shown for the *Aspergillus niger* glucoamylase (19). The *xlnA*-encoded mature protein is 187 amino acids long and has a calculated molecular weight of 20,247 and an isoelectric point of 6.33, in good agreement with the values determined for the purified X_{22} enzyme (8). The *xlnB*-encoded mature protein is 182 amino acids long and has a calculated molecular weight of 20,088 and an isoelectric point of 4.22, values which differ from those determined for the purified X_{24} protein (24,000 and <3.6, respectively) (9). The differences between the predicted and determined values may be due to glycosylation or to a particular configuration of this protein. There are no putative N glycosylation sites in the sequence of the *xlnB* product (nor were sites detected in the *xlnA* product sequence), and no sugar modifications were observed for the purified protein (6); hence, the second possibility is more likely.

Both *A. nidulans xlnA* and *xlnB* gene products showed homologies to those low-molecular-weight xylanases of bacterial and fungal (Fig. 1) origins classified in the G family of glycosyl hydrolases (14, 29). However, no clear homology was found with the F family xylanases. The identity values at the nucleotide and amino acid levels between the *xlnA* and *xlnB* genes

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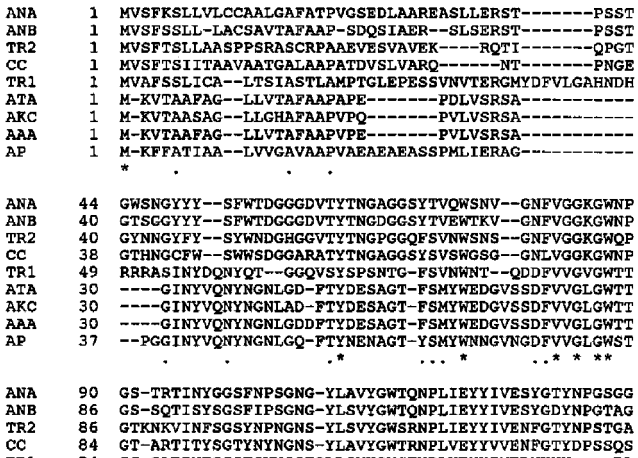

FIG. 1. Multiple protein sequence alignment among G family xylanases of fungal origin and xylanases X_{22} and X_{24}, encoded by the *A. nidulans xlnA* and *xlnB* genes, respectively. Asterisks indicate identical amino acids, and dots indicate similar amino acid residues. ANA, *A. nidulans* X_{22}; ANB, *A. nidulans* X_{24}; TR2, *T. reesei* xylanase II; CC, *C. carbonum* xylanase 1; TR1, *T. reesei* xylanase I; ATA, *A. tubingensis* xylanase A; AKC, *A. kawachi* xylanase C; AAA, *A. awamori* xylanase A; AP, *Aureobasidium pullulans* xylanase.

and their gene products are high (71 and 83%, respectively) and similar to those obtained for the *A. tubingensis xlnB* gene and gene product (about 70 and 75%, respectively) (5); however, the values for the *A. tubingensis xlnA* gene and gene product are much lower (about 58 and 47%, respectively). The *A. nidulans xlnA* and *xlnB* gene products (Fig. 1) and the *A. tubingensis xlnB* gene product are more closely related to *T. reesei* xylanase II (25, 30) and even to *C. carbonum* xylanase 1 (1) than to the *A. tubingensis xlnA* gene product (4), which itself is very similar to *A. kawachi* xylanase C (21), *A. awamori* xylanase A (18), and a xylanase from *Aureobasidium pullulans* (22).

Xylanase overproduction in *A. nidulans* transformants. The *xlnA* and *xlnB* genes, complete with upstream sequences, were introduced into *A. nidulans* G191 (*pabaA1 pyrG89 fwnA1 uAY9*) by cotransformation with plasmid pGW635 (16), which contains the *A. niger pyrA* allele. Uridine prototrophs were selected and analyzed by Southern hybridization. Seven of eight putative *xlnA* transformants were true cotransformants, with different copy numbers of the *xlnA* gene integrated at various locations in the host genome. Similar analyses of 10 putative *xlnB* transformants showed that 8 were also cotransformants. Transformants were tested for xylanase overexpression by growth on *Aspergillus* minimal medium containing 0.5% oat spelt xylan as the carbon source for 40 h. The xylanase activities in culture supernatants were assayed by using azo-birchwood xylan (Megazyme) as the substrate. Reaction mixtures (330 μl) containing 2.5 mg ml of substrate^{-1} and an appropriate volume of sample in 50 mM sodium succinate buffer (pH 5.5) were incubated at 50°C for 20 min. Nondegraded substrate was precipitated with 670 μl of ethanol and removed by centrifugation. A_{590} was measured in supernatants, with 1 U of xylanase activity defined as the amount of enzyme that produces 1 A_{590} U in 1 h. Table 1 summarizes the xylanase activities detected in culture filtrates of several of the *xlnA* and *xlnB* transformants obtained. All transformants showed increased xylanase activity levels compared with that of the host strain; however, whereas all *xlnA* transformants showed similarly high levels, the *xlnB* transformants showed more variable and lower activities. The values determined for the latter transformants seem to correlate with *xlnB* gene copy number, but further studies should be done to confirm this.

TABLE 1. Xylanase activities detected in culture filtrates of several fungal transformants

Strain[a]	Xylanase activity (U/ml)
A. nidulans G191	79.5
A. nidulans::pBA7.1 #3	516.0
A. nidulans::pBA7.1 #6	510.0
A. nidulans::pBA7.1 #8	523.0
A. nidulans::pBA7.1 #10	504.0
A. nidulans::pEA8.1 #1	516.6
A. nidulans::pEA8.1 #5	459.0
A. nidulans::pEA8.1 #8	204.3
A. nidulans::pEA8.1 #11	487.0

[a] Plasmids pBA7.1 and pEA8.1 contain the *xlnA* and *xlnB* genes, respectively. The numbers after number signs denote individual transformants.

Expression of *xlnA* and *xlnB* cDNAs in *Saccharomyces cerevisiae*. A simplified method of rapid amplification of cDNA ends (11) was used to synthesize the *A. nidulans xlnA* and *xlnB* cDNAs essentially as described previously (15). Oligonucleotides 5′-ACAGGATCTAGACAATGG-3′ and 5′-CCTCTA GACGTCAACAACCGGCAACATGG-3′, similar to the 5′ termini around the ATG start codons of *xlnA* and *xlnB*, respectively, but modified in order to create an artificial *Xba*I recognition site, were used for gene-specific amplification of double-stranded cDNAs. The amplified fragments were cloned in pUC18. An *S. cerevisiae* DNA fragment containing the actin gene promoter (23), obtained by PCR amplification as described previously (24), was placed as a *Sma*I-*Xba*I DNA fragment upstream of the *xlnA* and *xlnB* cDNA inserts. The actin gene promoter-*xlnA* and -*xlnB* fusions were moved to Yeplac181 (13), and the resulting plasmids, pYLA1 and pYLB1, were transformed into *S. cerevisiae* OL1 (α *leu2-3 leu2-112 his3-11 his 3-15 ura3-251 ura3-337*) by the lithium acetate method, as modified by Gietz et al. (12). Recombinant clones growing on uracil-deficient plates were tested for the production of xylanase activity by plate assay (15) using oat spelt xylan as the substrate. *xlnA*- and *xlnB*-transformed *S. cerevisiae* clones produced clear halos, indicating substrate hydrolysis by a secreted xylanase activity (results not shown). The halos produced by *xlnA*-transformed strains were larger than those produced by *xlnB*-transformed strains. The production of X_{22} and X_{24} xy-

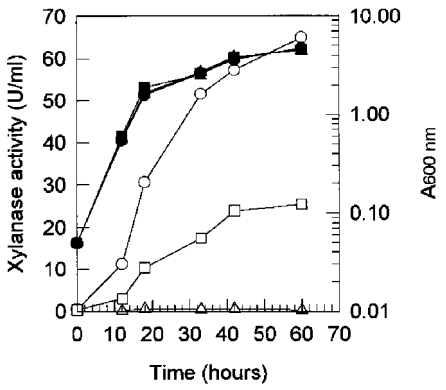

FIG. 2. Growth (filled symbols) and xylanase activities (open symbols) in culture media inoculated with *S. cerevisiae* OL1::Yeplac181 (triangles), *S. cerevisiae* OL1::pYLA1 (circles), and *S. cerevisiae* OL1::pYLB1 (squares).

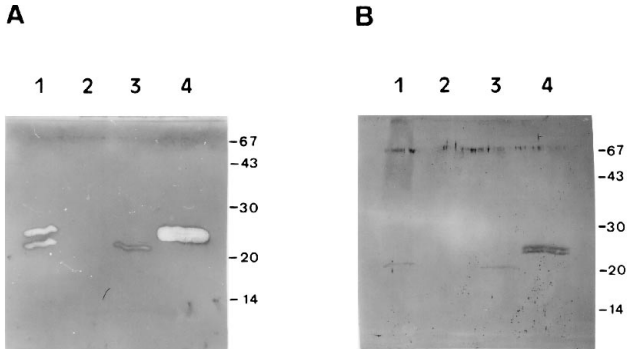

FIG. 3. SDS-PAGE followed by zymogram analysis (A) or immunostaining (B) of extracellular xylanases produced by *A. nidulans* and *S. cerevisiae*. Culture filtrates (16 μl) were applied from *A. nidulans* grown for 40 h in *Aspergillus* minimal medium containing 0.5% oat spelt xylan (lanes 1), *S. cerevisiae* OL1::Yeplac181 grown for 42 h in YPD (lanes 2), *S. cerevisiae* OL1::pYLA1 grown for 42 h in YPD (lanes 3), and *S. cerevisiae* OL1::pYLB1 grown for 42 h in YPD (lanes 4). The positions of molecular weight markers (in thousands) are indicated on the right.

lanase activities was also tested in liquid cultures. Very low xylanase activities, measured as described above, were detected in uracil-deficient minimal medium culture filtrates for both recombinant yeast strains. However, activity was easily detectable in supernatants of yeast extract-peptone-dextrose (YPD)-rich medium cultures (Fig. 2). Although this is not a selective medium, the stabilities of both 2μm-based plasmids were found to be high by analysis of Ura^+ and Ura^- phenotypes (80% of cells retained the Ura^+ phenotype after 60-h culture). Xylanase activity was observed from the beginning of growth for both recombinant strains (Fig. 2), which is consistent with constitutive expression directed by the actin gene promoter. The xylanase activity of the *xlnA*-transformed strain was higher than that of the *xlnB*-transformed strain, but neither of the recombinant strains reached the level of activity detected in *A. nidulans* transformants or even in the *A. nidulans* G191 host strain (Table 1).

Zymograms of culture filtrates were made in order to detect xylanase activities after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Equal amounts of each sample (16 μl from original culture filtrates) were loaded onto gels. As can be seen in Fig. 3A, two clear bands corresponding to X_{22} and X_{24} xylanase activities were detected in the *A. nidulans* sample. A band detected in the *S. cerevisiae* OL1::pYLA1 culture filtrate corresponded to that produced by X_{22} in *A. nidulans*. A second band, corresponding to that produced by X_{24} in *A. nidulans* but more intense, was detected in the culture filtrate from *S. cerevisiae* OL1::pYLB1. No bands were detected in the *S. cerevisiae* OL1::Yeplac181 control sample. For immunoassay, 16-μl samples of culture filtrates were run on an SDS-PAGE gel, blotted to nitrocellulose, and exposed to antibody prepared against *A. tubingensis* xylanase A (Fig. 3B). A faint band corresponding to X_{22} was detected in the *A. nidulans* sample. As expected, no band corresponding to X_{24} xylanase was detected, as it is a very minor protein in *A. nidulans* culture filtrates (the zymogram was more sensitive, as it detected this activity). A faint protein band with the same mobility as that of X_{22} was detected in the culture filtrate from *S. cerevisiae* OL1::pYLA1. Two closely spaced and more intense protein bands were observed in *S. cerevisiae* OL1::pYLB1. From their mobilities, these proteins could correspond to X_{24}, with the larger one possibly being a glycosylated form of this protein. No bands were detected in the *S. cerevisiae*::Yeplac181 control. The results from the zymogram and immunoassay analyses confirm that both the *xlnA* and *xlnB* genes are expressed in the corresponding *S. cerevisiae* recombinant strains.

The previous activity assays performed with liquid (Fig. 2) and solid media showed that *S. cerevisiae* OL1::pYLB1 produced less xylanase activity than did *S. cerevisiae* OL1::pYLA1. However, the activity detected on the zymogram and the amount of protein detected in the immunoassay were much higher in the former than they were in the latter. The lower level of X_{22} activity detected on the zymogram could be explained by our observation that X_{22} protein renaturation after SDS-PAGE is more difficult than X_{24} protein renaturation (23a). The specific activity of X_{22} on birchwood xylan and oat spelt xylan is three times that of X_{24} (6) and may explain why *S. cerevisiae* OL1::pYLA1 showed more activity in liquid and solid media than did *S. cerevisiae* OL1::pYLB1, which secretes more protein, as detected by immunoassay. In addition, it is also possible that some modification of X_{24} by yeast cells inactivates the enzyme.

Nucleotide sequence accession numbers. The EMBL accession numbers of the *A. nidulans xlnA* and *xlnB* gene sequences are Z49892 and Z49893, respectively.

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