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Cloning and DNA Sequence of the Gene Coding for *Bacillus stearothermophilus* T-6 Xylanase

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***Bacillus stearothermophilus* T-6 produces an extracellular thermostable xylanase. Affinity-purified polyclonal serum raised against the enzyme was used to screen a genomic library of *B. stearothermophilus* T-6 constructed in λ-EMBL3. Two positive phages were isolated, both containing similar 13-kb inserts, and their lysates exhibited xylanase activity. A 3,696-bp *SalI-BamHI* fragment containing the xylanase gene was subcloned in *Escherichia coli* and subsequently sequenced. The open reading frame of xylanase T-6 consists of 1,236 bp. On the basis of sequence similarity, two possible −10 and −35 regions, a ribosome-binding site at the 5′ end of the gene and a potential transcriptional termination motif at the 3′ end of the gene, were identified. From the previously known N-terminal amino acid sequence of xylanase T-6 and the possible ribosome-binding site, a putative 28-amino-acid signal peptide was deduced. The mature xylanase T-6 consists of 379 amino acids with a calculated molecular weight and pI of 43,808 and 6.88, respectively. Multiple alignment of β-glycanase amino acid sequences revealed highly conserved regions. Northern (RNA) blot analysis indicated that the xylanase T-6 transcript is about 1.4 kb and that the induction of this enzyme synthesis by xylose is on the transcriptional level.**

Xylanases (1,4-β-D-xylan xylanohydrolase [EC 3.2.1.8]) are hemicellulases that hydrolyze xylan, which accounts for 20 to 30% of the dry weight in different hardwood species. Xylan is composed of β-1,4-linked xylopyranose units with branches containing L-arabinofurannosyl and glucopyranosyl residues. Xylanases are produced by various microorganisms, among which are fungal yeasts and bacterial species; they include, for example, endoxylanases, β-xylosidase, arabinofuranosidase, and acetylxylanesterase (8). Hemicellulases in general and xylanases in particular have several uses and potential applications, including bioconversion of lignocellulose material to fermentative products, clarification of juices, and improvement of animal feed stock digestibility (55). Hemicellulases are also being considered for biopulping and biobleaching in the pulp and paper industry (48). During the process of producing high-quality white paper, the delignified pulp is usually bleached with chlorine-based compounds. Although this bleaching process is highly effective, it results in the release of large quantities of worrisome organochlorine compounds such as chlorinated dioxins and furans (2). Therefore, the paper industry is actively seeking new technologies that will enable it to bleach pulp with less chlorine or even without chlorine. Totally chlorine-free processes usually use oxygen, ozone, and peroxide. Several years ago, Viikari et al. (47, 48) demonstrated that hemicellulases can be used to enhance the delignification and bleaching of unbleached pulp. For economic considerations, if enzymes are to be part of a bleaching process, they have to be produced at low cost and in large volumes and should preferably be active at high temperatures and high pHs. Recently, we have isolated, characterized, and purified an extracellular xylanase from *Bacillus stearothermophilus* T-6 (18, 38). This enzyme was shown to bleach pulp optimally at pH 9 and 65°C and was used in a large-scale biobleaching mill trial (22, 23). The results of that trial were part of a patent application for a totally chlorine-free bleaching process (38). To overproduce the enzyme and further improve its properties via protein engineering, it was essential to clone and sequence the xylanase gene. In this report, we describe the cloning of the xylanase gene from *B. stearothermophilus* T-6, together with the nucleotide sequence and amino acid sequence analysis of the protein.

MATERIALS AND METHODS

Bacterial strains and plasmids. *B. stearothermophilus* T-6 (NCIMB 40221) was isolated by using an enrichment procedure for bacteria capable of producing extracellular thermostable xylanases (39). Strain M-7 is a xylanase-constitutive mutant of strain T-6 (39). *Escherichia coli* strains were KW251 for library construction in λ-EMBL3 (Promega, Madison, Wis.) and XL-1 Blue for cloning in plasmids pBS+/− or pBluescript KS$^+$II (Stratagene, La Jolla, Calif.).

DNA and RNA isolation and manipulation. *B. stearothermophilus* T-6 genomic DNA was isolated by the Marmur method (26) as outlined by Johnson (16). Plasmid DNA was purified with the Qiagen kit (Qiagen Inc., Chatsworth, Calif.). DNA was manipulated by standard procedures (4, 35). Total RNA was isolated essentially as described by Ausubel et al. (4), except that the phenol extraction was carried out with saturated acidic phenol (phenol was brought to pH 4 with HCl and then was saturated with water). Northern (RNA) blot analysis was performed by a procedure described by Moran (29).

Construction of genomic libraries. Genomic DNA was partially digested with *Sau*3A and then separated on a 0.7% agarose gel. DNAs with sizes from 13 to 20 kb were extracted from the gel with activated glass beads (Geneclean II kit; Bio 101, La Jolla, Calif.), ligated into EMBL3 *Bam*HI arms, and packaged into phage particles using the EMBL3 *Bam*HI Arms Cloning system together with the Packgene system (Promega).

Purification of polyclonal antixylanase antibodies and immunological screening of the λ-EMBL3 library. Antixylanase antibodies were purified from antixylanase rabbit antiserum by immunoaffinity chromatography (3, 13). Purified xylanase T-6

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was coupled to cyanogen bromide (CNBr)-activated Sepharose 4B beads (Pharmacia, Uppsala, Sweden), which were then used to build a 5-ml immunoaffinity column. Xylanase T-6 antiserum (25 ml) was passed through the column (5 ml/h), and the column was then washed with 20 volumes of 10 mM Tris-Cl (pH 7.5) and with 20 volumes of the same buffer containing 500 mM NaCl. The bound antixylanase antibodies were eluted at low pH (pH 2.5) with 10 volumes of 100 mM glycine and at high pH (pH 11.5) with 10 volumes of 100 mM triethylamine. Polyclonal antibodies eluted with the basic buffer gave very little cross-reaction with *E. coli* proteins and were therefore used for screening the λ-EMBL3 library for xylanase-positive phages. Detection of positive plaques was performed with alkaline phosphatase conjugated to anti-rabbit immunoglobulin G (BioMakor, Rehovot, Israel) (35).

DNA sequencing. DNA sequencing was performed by the dideoxy chain termination method (36) with a Sequenase version 2.0 kit (U.S. Biochemical Co., Cleveland, Ohio). The sequencing was performed on subclones (pKS$^+$II; Stratagene) using common synthetic KS primers (Stratagene) and custom-made primers. Custom-made oligonucleotide sequences were as follows: p1331, 5′-GGCTTACCTTCCTTGTC-3′ (nucleotides 1348 to 1332); p1910, 5′-CGAGTTCACGATTGGT GCG-3′ (nucleotides 1082 to 1101); p3252, 3′-CGATAAT AGCTGGTGTTTAT-5′ (nucleotides 2124 to 2144).

Computer analysis. Nucleotide and amino acid sequences were analyzed with MacVector (IBI, New Haven, Conn.) and the Sequence Analysis software package of the Genetics Computer Group, version 7.2-UNIX (University of Wisconsin, Madison).

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ nucleotide sequence data bases under the accession number Z29080.

RESULTS AND DISCUSSION

Cloning of the xylanase gene. Several unsuccessful attempts to clone the xylanase gene from *B. stearothermophilus* T-6 were initially made. First, we constructed a genomic library of *B. stearothermophilus* in plasmid pBS following a complete *Eco*RI digest of chromosomal DNA from *B. stearothermophilus* T-6. This library contained plasmids with an average insert size of about 3 kb. More than 4,000 colonies were screened for xylanase and xylosidase activities on Luria-Bertani agar plates containing xylan or *p*-nitrophenyl β-D-xylopyranoside; however, no positive clones were detected. A second genomic library was made in the λ-EMBL3 phage vector. It was constructed following a partial *Sau*3A digest of the chromosomal DNA, ligation into *Bam*HI ends of the phage arms, and in vitro packaging. We obtained more than 100,000 recombinant phages that contained inserts with an average size of 14 kb. This new library was screened in several ways. First, individual phage lysates were tested for hemicellulolytic activities, including xylanase and xylosidase. Of 2,500 phage lysates that were tested, we obtained four independent clones (as shown by their insert sizes) exhibiting xylosidase activity but no clones with xylanase activity. These results indicated that the xylanase gene is probably not well-represented in the λ-EMBL3 library. To screen larger numbers of recombinant phages, we used agar plates containing either xylan (detection was performed with Congo red) or Remazol brilliant blue R-D-xylan. These substrates have been used previously by several groups to clone glycanases genes (9, 24, 25, 42, 54). We failed again to detect positive xylanase phages on these agar plates. Finally, we used immunoaffinity-purified polyclonal

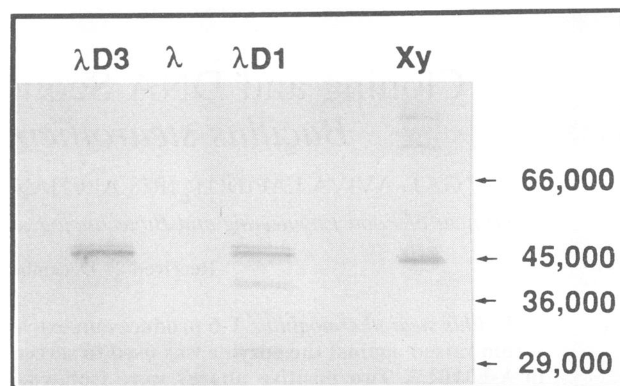

FIG. 1. Western blot analysis of crude lysates from xylanase-positive λ-EMBL3 phages. Crude phage lysates were separated on an SDS-10% PAGE gel, blotted on nitrocellulose paper, reacted with purified antixylanase antibodies, and detected with alkaline phosphatase conjugated to anti-rabbit immunoglobulin G. Lanes: Xy, purified xylanase; λD1 and λD3, xylanase-positive clones; λ, λ-EMBL3.

serum raised against xylanase T-6 to screen the library. Highly purified xylanase T-6 protein was coupled to CNBr-activated Sepharose beads. The resulting xylanase-coupled beads were then used to construct an immunoaffinity column. Xylanase T-6 polyclonal serum was loaded on the column, washed, and then eluted with low and high pH buffers. The basic-pH eluted fraction contained highly specific xylanase antibodies exhibiting low cross-reactivity with *E. coli* proteins. This basic fraction antiserum was therefore used to screen the λ-EMBL3 library. Of about 20,000 phage plaques, two positive clones, λD1 and λD3, were isolated. These two phages formed very small plaques that reacted strongly with antixylanase T-6 antibodies. Moreover, the liquid lysates of these phages contained detectable xylanase and xylosidase activities. Western blot (immunoblot) analysis of the crude λD1 and λD3 lysates indicated that these phages produced a protein with an M_r of 43,000 that was similar in size to xylanase T-6. Above the major protein band, an additional, faint band with a higher M_r could be detected. This band probably represents the prexylanase protein together with its leader peptide (Fig. 1).

Locating the xylanase gene. Both positive phages produced very small plaques and were found to be highly unstable in liquid media. The DNAs of the phages were purified and subjected to restriction enzyme analysis (Fig. 2). Phages λD1 and λD3 contained 13- and 13.4-kb inserts, respectively. The restriction enzyme map indicates that the two inserts are almost identical and, therefore, probably represent a complete fragment of the strain T-6 chromosome. To locate the exact position of the xylanase gene, a synthetic oligonucleotide probe was used. This probe was designed according to the N-terminal amino acid sequence of xylanase T-6 (18), from Tyr-24 to Asn-41, and contained inosine at every third position. Inosine can hybridize with all four bases and neither increases nor decreases hybridization strength (27). The sequence of the 53-mer probe was 5′-TTI TGI A(G/A)I TGI TAI GGI TCI ACI GCI GCI CCI ATI GTI AAI TCI TTI TTI TA-3′. The synthetic probe was labeled and used to probe a Southern blot of a restriction enzyme digest of λD1 and λD3 DNAs. The labeled probe specifically reacted with several of the DNA fragments and was mapped on a 540-bp *Nco*I fragment on the right side of the inserts (results not shown).

Sequencing the xylanase gene. To facilitate the sequencing

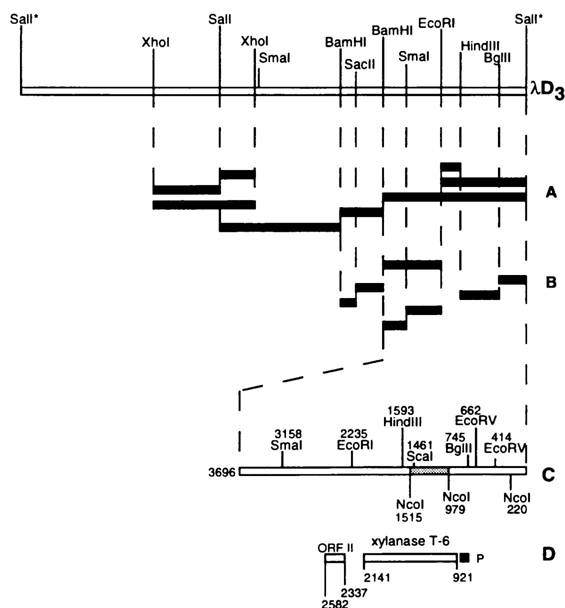

FIG. 2. Restriction map and schematic representation of the λD3 13-kb insert. (A) Fragments cloned directly from λD3 DNA into pKS; (B) fragments cloned from pKS inserts; (C) restriction map of the 3,696-bp *Sal*I-*Bam*HI fragment containing the xylanase gene (the shaded area represents the *Nco*I fragment that hybridized with the synthetic probe); (D) position of the xylanase gene and ORF II. The *Sal*I* sites in λD3 are originated from the cloning site of λ-EMBL3.

of the xylanase gene, we first digested λD3 DNA with various restriction enzymes and subcloned fragments into pBluescript KS$^+$ II vector. The subcloned fragments were as follows: *Sal*I-*Bgl*II, 745 bp; *Bgl*II-*Hin*dIII, 848 bp; *Hin*dIII-*Eco*RI, 642 bp; *Eco*RI-*Sma*I, 923 bp; *Sma*I-*Bam*HI, 538 bp; *Eco*RI-*Bam*HI, 1,461 bp; *Sal*I-*Bam*HI, 3,696 bp; and *Sal*I-*Eco*RI, 2,235 bp. These various subcloned fragments, together with several synthetic primers, were used to sequence the entire 3,696-bp *Sal*I-*Bam*HI fragment (Fig. 3). The sequence revealed one large open reading frame (ORF) (from nucleotides 921 to 2,141), which codes for xylanase T-6, and a smaller ORF (ORF II, from nucleotides 2337 to 2582), which codes for a putative 82-amino-acid peptide.

Sequence analysis of the xylanase T-6 gene. The sequence of the first 45 amino acids from the N terminus of xylanase T-6 was determined previously (18). This sequence appears from amino acid 29 in the large ORF. Ahead of this sequence, there are 28 amino acids leading to the putative start codon. This sequence of 28 amino acids represents a typical gram-positive signal peptide. The first 7-amino-acid end (n region) contains three basic residues and ends with proline (a secondary structure-breaking residue). The center region (h region) contains 12 amino acids, is very hydrophobic, and also ends with proline. The more polar c region is made of 8 amino acids, contains the cleavage recognition site of the signal peptidase, and ends with alanine, which is the most abundant amino acid preceding the cleavage site (49–51, 53). The xylanase gene codes for a 379-amino-acid protein (without the signal peptide) with a calculated molecular weight of 43,808 and a pI of 6.88. These values correspond very closely to those that were obtained for the native protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (M_r, 43,000) and isoelecteric focusing under denaturing conditions (pI 7) (18). Several bases upstream of the ATG start codon there is a deduced ribosome-binding site sequence (5′-AGGGGGA-3′). Identical Shine-Dalgarno sequences were found in other genes, including the α-amylase gene of *B. stearothermophilus* (31), the cellulose gene of *Clostridium cellulovorans* (40), and the *tenA* gene of *Bacillus subtilis* (32). Two putative −10 and −35 regions could be identified upstream of the Shine-Dalgarno sequence. Downstream of the xylanase gene, there is a small ORF encoding a putative 82-amino-acid peptide. The sequence of this peptide was scanned against the EMBL gene bank with the TFASTA program (Genetic Computer Group, Inc.), and no homologous sequences were found. In general, the codon usage of the xylanase gene (Table 1) resembles the codon usage of *B. stearothermophilus* and *B. subtilis* (52), with the following exceptions: the Ser codon AGC is 8 of 13, whereas TCC and AGT are not used; in the Phe codons, the bias is toward the TTC codon (11 of 15). Thirty-two bases downstream of the stop codon, there is a palindromic sequence corresponding to an mRNA hairpin loop with a Δ*G* of −21 kcal (circa −88 kJ)/mol. Following this sequence, there is an AT-rich region. This structure may function as a transcriptional terminator (1, 33).

Comparison of the deduced amino acid sequence of xylanase T-6. The deduced amino acid sequence of xylanase T-6 was scanned against the EMBL gene bank with the TFASTA program (Genetic Computer Group, Inc.). Xylanase T-6 showed high homology to several β-glycanases which belong to group F, according to Gilkes et al. (11). The relationship among these various enzymes is illustrated in a dendrogram (Fig. 4) prepared by PILEUP, a computer-generated multiple sequence alignment (Genetic Computer Group, Inc.). *B. stearothermophilus* T-6 xylanase has a 58.7% identity (375-amino-acid overlap) with *Bacillus* sp. strain C-125 xylanase (12, 14), a 44.2% identity (312-amino-acid overlap) with xylanase A from *Clostridium saccharolyticum* (37), and a 41.1% identity (365-amino-acid overlap) with endoxylanase from *Thermoanaerobacterium saccharolyticum* B6A-R1 (21). Amino acid sequence alignment of enzymes related to xylanase T-6 revealed several highly conserved regions (Fig. 5). In the N-terminal region of the enzyme, there are three highly conserved regions, EN-MK, RGHTLVWH, and WDVVNE. At least five more conserved regions can be identified further along the gene. The enzymatic hydrolysis of xylan is likely to occur via an acid catalysis mechanism similar to that known for lysozyme. Some sequence homology has been found between the active site of lysozyme and the sequences of fungal cellulases (19, 56). Several groups have shown that glutamic and aspartic acid residues are involved in the catalytic domains of β-1,4-glycanases (5, 34, 41, 45). Recently, Lee et al. (21) have demonstrated by site-specific mutagenesis that in *T. saccharolyticum* xylanase, Asp-537, Asp-602, and Glu-600 are essential for activity. These amino acids are found in the conserved regions, and, on the basis of homology, their correspondents in xylanase T-6 are Asp-202, Asp-267, and Glu-265. Tryptophan was also shown to be involved in the active sites of different xylanases (6, 17, 18). We have previously shown that *N*-bromosuccinimide (a tryptophan modifier) completely inhibits the activity of xylanase T-6. However, xylan protects the enzyme from this reagent (18). The amino acid sequence alignment indicates that there are six conserved tryptophan residues. By replacing these conserved tryptophan residues via site-directed mutagenesis, we will be able to determine which of these residues are essential for activity.

Induction of xylanase T-6, transcript length, and putative regulatory sites. Northern blot analysis of the xylanase gene was performed on RNAs isolated from strain T-6 cultures grown with or without xylose and from strain M-7 by using a

FIG. 3. Nucleotide and deduced amino acid sequences of the *B. stearothermophilus* T-6 xylanase gene. The putative ribosome-binding sites (RBS; Shine-Dalgarno), the −10 and −35 regions, potential Hpr (*hpr*) and catabolite repression (c.r.) binding sites, and the 3′ end palindrome (potential transcriptional terminator) sequences are overlined. The 28-amino-acid putative signal peptide is underlined, and the upward arrow indicates the cleavage site (based on the N-terminal amino acid sequence of the mature protein).

*Bgl*II-*Eco*RI fragment (Fig. 2) as a probe. The analysis indicated that the xylanase T-6 transcript is about 1.4 kb long (Fig. 6). This size agrees with the sequence analysis of the xylanase gene. The xylanase transcript was detected only in the xylanase-constitutive strain, M-7, or in wild-type cultures grown in the presence of xylose. Thus, in *B. stearothermophilus* T-6, the induction of xylanase synthesis by xylose is controlled on the transcriptional level. The regulation is probably mediated by a repressor protein, since all of the xylanase-constitutive mutants that we have previously obtained were also constitutive to β-xylosidase (39). We have recently cloned the β-xylosidase gene of strain T-6 (results not shown), and it appears to be located about 10 kb downstream of the xylanase gene and is not part of the same operon. The *xyl* operon of *B. subtilis* W23 and 168 is also negatively regulated at the level of transcription (10, 20). The production of xylanase T-6 occurs toward and during the stationary phase. In addition, the synthesis of the enzyme is repressed when readily metabolized carbon sources are present in the growth medium (39). These results suggest that the synthesis of the enzyme is controlled by transition state regulators and catabolite repression. From sequence analysis of the xylanase gene, several putative regulatory sites could be identified. The region preceding the upstream promoter is very AT rich (76%). AT-rich regions were found upstream of other genes; among them are the *degR* gene of *Bacillus natto* (30) and the *aprE* gene of *B. subtilis* (46). The AT-rich region preceding the *aprE* gene results in a DNA with a static curvature that affects the migration properties of this DNA in a polyacrylamide gel (46). It is possible that these AT-rich regions interact or facilitate the interaction with regulators, such as is the case with the transition state regulator AbrB (43). Upstream of the xylanase gene, there are several sequences that resemble the

TABLE 1. Codon usage in the xylanase gene of *B. stearothermophilus* T-6

Codon	Amino acid	Count	Percent
TTT	Phe	4	1.0
TTC	Phe	11	2.7
TTA	Leu	5	1.2
TTG	Leu	7	1.7
TCT	Ser	2	0.5
TCC	Ser	2	0.5
TCA	Ser	1	0.2
TCG	Ser	0	0.0
TAT	Tyr	10	2.5
TAC	Tyr	11	2.7
TGT	Cys	0	0.0
TGC	Cys	0	0.0
TGG	Trp	9	2.2
CTT	Leu	5	1.2
CTC	Leu	4	1.0
CTA	Leu	3	0.7
CTG	Leu	5	1.2
CCT	Pro	5	1.2
CCC	Pro	2	0.5
CCA	Pro	6	1.5
CCG	Pro	10	2.5
CAT	His	4	1.0
CAC	His	5	1.2
CAA	Gln	18	4.4
CAG	Gln	1	0.2
CGT	Arg	4	1.0
CGC	Arg	6	1.5
CGA	Arg	3	0.7
CGG	Arg	2	0.5
ATT	Ile	13	3.2
ATC	Ile	14	3.4
ATA	Ile	0	0.0
ATG	Met	10	2.5
ACT	Thr	2	0.5
ACC	Thr	2	0.5
ACA	Thr	6	1.5
ACG	Thr	6	1.5
AAT	Asn	13	3.2
AAC	Asn	11	2.7
AAA	Lys	28	6.9
AAG	Lys	11	2.7
AGT	Ser	0	0.0
AGC	Ser	8	2.0
AGA	Arg	1	0.2
AGG	Arg	0	0.0
GTT	Val	9	2.2
GTC	Val	6	1.5
GTA	Val	5	1.2
GTG	Val	9	2.2
GCT	Ala	8	2.0
GCC	Ala	7	1.7
GCA	Ala	11	2.7
GCG	Ala	7	1.7
GAT	Asp	15	3.7
GAC	Asp	15	3.7
GAA	Glu	15	3.7
GAG	Glu	8	2.0
GGT	Gly	5	1.2
GGC	Gly	7	1.7
GGA	Gly	7	1.7
GGG	Gly	3	0.7

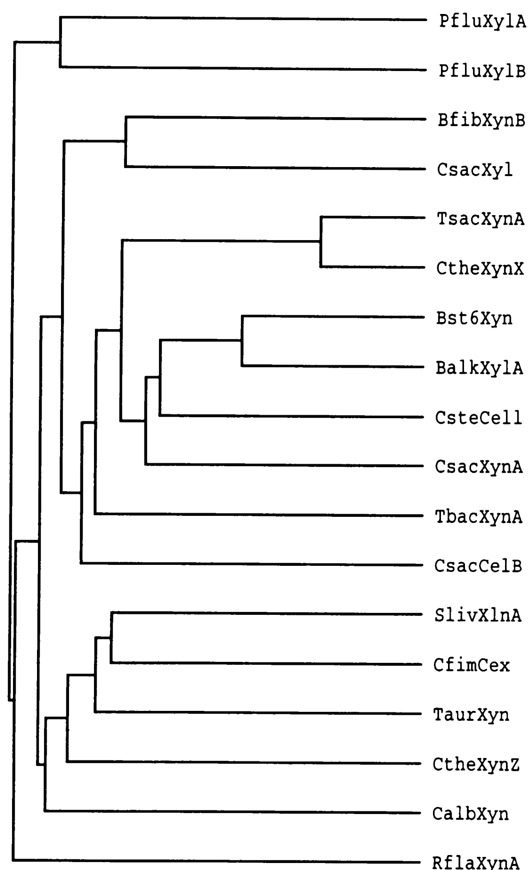

FIG. 4. Dendrogram illustrating the relationships among 18 β-glycanases from family F. The alignment was made with PILEUP, a computer-generated multiple-sequence alignment (Genetic Computer Group, Inc.). The distance along the branch point connecting two sequences is proportional to the difference between the sequences. The glycanases are as follows (data base accession numbers in parentheses): PfluXylA, *Pseudomonas fluorescens* xylanase A (P14768); PfluXylB, *P. fluorescens* xylanase B (P23030); BfibXynB, *Butyrivibrio fibrisolvens* xylanase (P26223); CsacXyl, *Caldocellum saccharolyticum* xylanase (P23557); TsacXynA, *Thermoanaerobacter saccharolyticum* B6A-RI xylanase (M97882); CtheXynX, *Clostridium thermocellum* xylanase (M67438); Bst6Xyn, *B. stearothermophilus* T-6 (Z29080); BalkXylA, *Bacillus* sp. C-125 xylanase (P07528); CsteCell, *Clostridium stercorarium* F-9 celloxylanase (D12504); CsacXynA, *C. saccharolyticum* xylanase (P23556); TbacXynA, thermophilic bacterial sp. Rt8.B4 xylanase (L18965); CsacCelB, *Caldocellum saccharolyticum* cellulase (P10474); SlivXlnA, *Streptomyces lividans* xylanase (P26514); CfimCex, *Cellulomonas fimi* exoglucanase (M15824); TaurXyn, *Thermoascus aurantiacus* xylanase (42a); CtheXynZ, *Clostridium thermocellum* xylanase Z (P10478); CalbXyn, *Cryptococcus albidus* xylanase (P07529); RflaXynA, *Ruminococcus flavefaciens* bifunctional xylanase (Z11127).

consensus binding sequence (AATANTATT) of the transition state regulator *hpr* (44) (Fig. 3). A putative catabolite repression sequence can be found 230 bp inside the xylanase structural gene (Fig. 2). The catabolite repression consensus sequence is T G T/A A A N C | G N T N A/T C A, where underlined letters represent the most critical bases, N is any base, and the vertical line denotes an axis of symmetry (7). In *B. subtilis*, this consensus sequence was found inside the structural genes in several operons, including the *xyl* operon (7, 15, 28). We are now trying to determine the exact role of these putative regulatory sites.

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PfluXylA  pvssssssls sssvvssirs ssssssssva tgnglaslad fpigvavaas ggnadiftss arqnivraeF NqitaENiMK msymysgsn. ..........
PfluXylB  vragncgsvs ss...ssvqs ssssssssaa sakkfi.... .......... ...gnittsg avrsdftryW NqitpENesK wgsvegtrnv ..........
BfibXynB  .......... .......... .......mn Lktayepy.. ....fkigaa isrwnl.htp ahtkllaeqF NsftcENdMK pmyyldrean kkdpekynls
CsacXyl   .......... .......... .......... .......... .......... .......... .......... ........MK qqylldyeat kaskngmpvc
TsacXynA  nptlefyidd fsvigeisnn qitiqndipd Lysvfkdy.. ....fpigva vdpsrlndad phaqltakhF NmlvaENaMK peslqpteg. ...n......
CtheXynX  .......... ....geisnn qitiqndipd Lssvfkdy.. ....fpigva vdpsrlndtd phaqltakhF NmlvaENaMK peslqpteg. ...n......
Bst6Xyn   sakn...... ...adsyakk phisalnapq Ldqrykne.. ....ftigaa vepyql.qne kdvqmlkrhF NsivaENvMK pisiqpeeg. ...k......
BalkXylA  aaaqggppks gvfgenekrn dqpfawqvas Lseryqeq.. ....fdigaa vepyql.e.g rqaqilkhhY NslvaENaMK peslqpreg. ...e......
CsteCell  .......... .......... ........ps Laeafrdy.. ....fpigaa iep.gy.ttg qiaelykkhv NmlvaENaMK paslqpteg. ...n......
CsacXynA  .......... ....mrcliv cenlemlnls Laktykdy.. ....fkigaa vtakdl.egv hrdillk.hF NsltpENaMK fenihpeeq. ...r......
TbacXynA  aaaisyyldl ysisdenwqq pvp.dynlps Lcekykny.. ....fkigva v.pyraltnp vdvevikrhF NsitpENeMK peslqpyeg. ...g......
CsacCelB  iafiasislv gamsyfpvet qaapdwsips Lcesykdd.. ....fmigva i.parclsnd tdkrmvlkhF NsitaENeMK pesllagqts tgls......
SlivXlnA  gtataliapp gahaaestlg aaaaqsg.ry Fgtaiasg.. .......... ....rl.sds tytsiagreF NmvtaENeMK idatepqrgq ..........
CfimCex   ..atvvlpaq aa....ttlk eaadgag.rd Fgfaldpn.. .......... ....rl.sea qykaiadseF NlvvaENaMK wdatepsqns ..........
TaurXyn   .......... .......... .......... .......... .......... .......... .......... ....pENrMK wdatepsqgn ..........
CtheXynZ  gqgdvqtpnp svtptqtpip tisgnal.rd Yaeargik.. ....igtcvn ypfynn.sdp tynsilqreF smvvcENeMK fdalqprqnv ..........
CalbXyn   lsalaltsvq aapadknsld ylankagkry Lgtavqsp.. ....ql.... .....v.pgs qyvqilesqF daitpENeMK wevveptegn ..........
RflaXynA  gqqqnndwnn qnnwnqgqqn nnnsagssds Lkgafsky.. ....fkigts vsphelnsg. ..adflkkhY NsitpENeLK pesildqgac qqkgn..nvn
Consensus ---------- ---------- ---------- L--------- ---------- ---------- ---------F N----EN-MK ---------- ----------

PfluXylA  ..FsFtnsDr lvsWAaqngq tvhGHaLVWH psyQlPnWa. .......... ......sd.s nanfrqdFar hIdtvaahF. .....agqVk sWDVVNEalf
PfluXylB  ..YnWaplDr iyaYArqnni pvkaHTFVW. .gaQsPsWl. .......... ......nnls gpevaveieq wIrdycarY. .....pdtam i.DVVNEav.
BfibXynB  paLtFenaip yleFAkdnki amRGHTLVWH n..QtPkWff cerynenf.. ......pmad retilarLes yIhgvldfvq tny..pgiIy aWDVVNE.i.
CsacXyl   ...kFdscip alqFckengi kmRGHvLVWH n..QtPeWff hkdydvsk.. ......plvd aatmerrLes yIkqviefcq kny..pgvVy cWDVVNEai.
TsacXynA  ..FtFdnaDk ivdYAiahnm kmRGHTLlWH n..QvPdWff qdpsd..... ....psksas rdlllqrLkt hIttvldhFk tkygsqnpIi gWDVVNEvl.
CtheXynX  ..FtFdnaDr ivdYAiahnm kmRGHTLlWH n..QvPdWff qdpsd..... ....ptkpas rdlllqrLkt hIttvldhFk tkygaqnpIi gWDVVNEvl.
Bst6Xyn   ..FnFeqaDr ivkFAkangm diRfHTLVWH s..QvPqWff ldkegkpmvn etdpvkreqn kqlllkrLet hIktiverYk ......ddIk yWDVVNEvv.
BalkXylA  ..WnWegaDk iveFArkhnm elRfHTLVWH s..QvPeWff idedgnrmvd etdpdkrean kqlllerMen hIktvverYk ......ddVt sWDVVNEvi.
CsteCell  ..FqWadaDr ivqFAkengm elRfHTLVWH n..QtPtgfs ldkegkpmve etdpqkreen rklllqrLen yIravvlrYk ......ddIk sWDVVNEvi.
CsacXynA  ..YnFeevar ikeFAikndm klRGHTLVWH n..QtPgWvf ldknge.... .......eas kelvierLre hIktlcerYk ......dvVy aWDVVNEav.
TbacXynA  ..FsFsiaDe yvdFckkdni slRGHTLVWH q..QtPsWff tnpetgekl. ...tnsekd keilldrLkk hIqtvvgrYk ......gkVy aWDVVNEai.
CsacCelB  ..YrFstaDa fvdFAstnki giRGHTLVWH n..QtPdWff kdsngqrl.. .........s kdallarLkq yIydvvgrYk ......gkVy aWDVVNEai.
SlivXlnA  ..FnFssaDr vynWAvqngk qvRGHTLaWH s..QqPgWm. .qsl...... .........s grplrqaMid hIngvmahYk ......gkIv qWDVVNEaf.
CfimCex   ..FsFgagDr vasYAadtgk elyGHTLVWH s..QlPdWa. .knl...... .........n gsafesaMvn hVtkvadhFe ......gkVa sWDVVNEaf.
TaurXyn   ..FnFagaDy lvnWAqqngk liyGHTLVWW s..QlPpWv. .vs....... .......... .......... .ItdkmknYi ......gkaf aWDVVNEaf.
CtheXynZ  ..FdFskgDq llaFAerngm qmRGHTLIWH n..QnPsWlt ngnw...... .........n rdsllavMkn hIttvmthYk ......gkIv eWDVaNECm.
CalbXyn   ..FdFtgtDk ivaeAkktgs llRGHnicWd s..Q...... .......... .......... .......... .......... .......... ..........
RflaXynA  tqisLsraaq tlkFceqngi alRGHTFVWy s..QtPdWff renfsqn... .....gayvs kdimnqrLes mIkntfaaLk sqypnld.Vy sYDVcNElf.
Consensus --F-F---D- ---FA----- --RGHTLVWH ---Q-P-W-- ---------- ---------- -------L-- -I------Y- --------V- -WDVVNE---

PfluXylA  dsaddpdgrg sangyrqsvF yrqfGgpeYI deAFrrApr. adptaeLYyN DFNtEeng.a KttalvnlVq rLlnnGvPId GVGfqmhvm. ..........
PfluXylB  ......pghq pa.gyaqraF .....gnnWI qrvFqlArq. ycpnsiLilN DYNnirwq.h n.....efIa lakaqGnyId aVGlqahel. ..........
BfibXynB  .....vdega f..r..ksiW tetvGed.Ff ikAFefArky aapevsLFyN DYet.aqp.w KrdfiLekVl gplidkklId GmGmqshl.. ..........
CsacXyl   .....lddgs w..reinnnW ytimkek.YV ekAFyyArky akkdvaLFyN DYNv.flp.a KreaiYn.la qklkekglId GlGlqptv.. ..........
TsacXynA  .....d..dn g..nlrnskW lqiiGpd.YI ekAFeyAhe. adpsmkLFiN DYNiEnng.v KtqamYdlVk kLkseGvPId GIGmqmhi.. ..........
CtheXynX  .....d..dn g..slrnskW lqiiGpd.YI ekAFeyAhe. adpsmkLFiN DYNiEnng.v KtqamYdlVk kLkseGvPIs GIGmqmhi.. ..........
Bst6Xyn   .....g..dd g..klrnspW yqiaGid.YI kvAFqaArky ggdnikLYmN DYNtE.ve.p KrtalYnlVk qLkeeGvPId GIGhqshi.. ..........
BalkXylA  .....d..dg g..glresew yqitGtd.YI kvAFetArky ggeeakLYiN DYNtE.vp.s KrddlYnlVk dLleqGvPId GVGhqshi.. ..........
CsteCell  .....epndp g..gmrnspW yqitGte.YI evAFratrea ggsdikLYiN DYNtD.dp.v KrdilYelVk nLlekGvPId GVGhqthi.. ..........
CsacXynA  .....edkte k..llresnW rkiiGdd.YI kiAFeiArey ag.dakLFyN DYNnE.mp.y KlektYkvlk eLlerGtPId GIGiqahw.. ..........
TbacXynA  .....denqp d..gyrrsdW ynilGpe.YI ekAFiwAhe. adpkakLFyN DYstE.dp.y KrefiYklIk nLkakGvPVh GVGlqchi.. ..........
CsacCelB  .....denqp d..syrrstW yeicGpe.YI ekAFiwAhe. adpnakLFyN DYNtE.is.k KrdfiYnmVk nLkskGiPIh GIGmqchi.. ..........
SlivXlnA  .....ad.gs s..garrdsn lqrsGnd.WI evAFrtAra. adpsakLCyN DYNvEnwtwa KtqamYnmVr dFkqrGvPId cVGf...... ..........
CfimCex   .....ad.gd g..ppqdsaF qqklGng.YI etAFraAra. adptakLCiN DYNvEg.ina KsnslYdlVk dFkarGvPld cVGf...... ..........
TaurXyn   .....ni.t. .......... ......g.Rl evA..aAsr. tdpnakLYiN DYNlDsaryp KtqaivnrVk qwcaaGvPIi GIGn...... ..........
CtheXynZ  .....dd.sg n..glrssiW rnviGqd.Yl dyAFryAre. adpdalLFyN DYNiEdlg.p KsnavFnmIk sMkerGvPId GVGf...... ..........
CalbXyn   .......... .......... .......... ...LryAhe. vapkmkLCiN DYNiEt.vna Ksqamakvaa gLlakGaPlh cIGmfknakr rssgllirta
RflaXynA  .....lnngg gmrgadnsnW vkiyGddsFV inAFkyArqy apagckLYlN DYN..eyipa KtndiYnmam kLkqlGy.Id GIGmqshl.. ..........
Consensus ---------- ---------W ----G---YI --AF--A--- ------LF-N DYN-E----- K----Y--V- -L---G-PI- G-G------- ----------

PfluXylA  ..ndy..... .psianirqa mqkivalspt lkIkiTELDv rlnnpydgns sndytnrndc avscagldrq karykeivqa ylevvppgrr ggItvWGiaD
PfluXylB  ..kgmtaaqv ktaidniwnq vgkpiyis.e ydIgdTndqv qlqn...... .......... .......... .......fqa hfpvfynhph vhgitsGicg
BfibXynB  ..lmdhp... ..diseyrta lemYgst..G lqIhiTELDm ....hna... .....dpsee smhala.try qeffqtYl.. dakksgkani tsVtfWnllD
CsacXyl   ..glnypeld sddidsfktt letFakl..G lqIhiTELnf eikgdes... .....nrtpe nlkkqa.dry yemmklLlke dtdnggpcni tcVtvFGicD
TsacXynA  ..ni...... nsnidnikas iekLasl..G veIqvTELDm nmng...... .....nisne allkqa.rly kqlfdlF... ...kaekqyi taVvfWGvsD
CtheXynX  ..ni...... nsnidnikas iekLasl..G veIqvTELDm nmng...... .....nvsne allkqa.rly kqlfdlF... ...kaekqyi taVvfWGvsD
Bst6Xyn   ..qi.....g wpseaeiekt inmFaal..G ldnqiTELDv smygwppr.a yptydaipkq kfldqa.ary drlfklY... ...eklsdki snVtfWGiaD
BalkXylA  ..qi.....g wpsiedtras fekFtsl..G ldnqvTELDm slygwpptga ytsyddipae llqaqa.dry dqlfelY... ...eelaadi ssVtfWGiaD
CsteCell  ..di.....y nppveriies ikkFagl..G ldniiTELDm siyswndrsd yg..dsipdy iltlqa.kry qelfdaL... ...kenkdiv saVvfWGisD
CsacXynA  ..ni....wd knlvsnlkka ievYasl..G leIhiTELDi svfefedkrt .dlfeptp.e mlelqa.kvy edvfavF... ...reykdvi tsVtlWGisD
TbacXynA  ..sl.....d wpdvseieet vklFsri.pG leIhfTEiDi si.....akn mtdddaynry llvqqa.qkl kaifdvL... ...kkyrnvv tsVtfWGlkD
CsacCelB  ..nv.....n wpsvseiens iklFssi.pG ieIhiTELDm slynygssen ys...tppqd llqkqs.qky keiftmL... ...kkyknvv ksVtfWGlkD
SlivXlnA  .qs..hfnsg spynsnfrtt lqnFaal..G vdVaiTELDi q......... .......... .....g.apa ....stYanv tndclavsrc lgItvWGvrD
CfimCex   .qs..hlivg .qvpgdfrqn lqrFadl..G vdVriTELDi rm........ .....rtpsd .atkla.tqa ....adYkkv vqacmqvtrc qgVtvWGitD
TaurXyn   .qtaraaitv wgvadpdrww lrevigi..c wggvpnqFD. .......... .......... ..asag.tpe ....afLnvi gedyipiafk ntlt......
CtheXynZ  .qchfingms peylasidqn ikrYaei..G viVsfTEiDi ri........ .....pqsen patafq.vqa ....nnYkel mkiclanpnc ntfvmWGftD
CalbXyn   ssgleshfig gstpkdipaa mnlFsdq..G leVpmTELDv ri........ .....pvngn dmpana.tva keqvddYyts vsaclgndlc pgVsiWqfaD
RflaXynA  .......atn ypdantyeta lkkFlst..G leVqiTELDi tctns..... .......... ..aeqa.dly ekifkl.... ..amqnsaqi paVtiWGtqD
Consensus ---------- ---------- ---F-----G --I--TELD- ---------- ---------- ---------- ------Y--- ---------- --V--WG--D

PfluXylA  pdsWlythqn l......... .......... .....pdwPL lFndnlqpKp AYqgVveals gr........ .......... .......... ..........
PfluXylB  gqdl...... .......... .......... ......drrL rFdpgqwhta pgndVvd... .......... .......... .......... ..........
BfibXynB  ensWlsgfrr .......... .......... ....etsyPL vFkgkceaKe AYyaVlkaav sddsidk... .......... .......wvp dyseedyklq
CsacXyl   dyplyknfkq .......... .......... .......cmY lWdkncnpKp cFysflqagl ..dwkas... .......... .......lls k.........
TsacXynA  dvtWl..... .......... .......... ...skpnaPL lFdsklqaKp AFwaVvdpsk aipdiqsaka legsptigan vdsswklvkp lyvntyvegt
CtheXynX  dvtWl..... .......... .......... ...skpnaPL lFdsklqaKp AYwaIadpsk aipdiqsaka legsptigan vdsswklvkp lyantyvegt
Bst6Xyn   nhtWldsrad vyydangnvv vdpnapyakv ekgkgkdaPF vFgpdykvKp AYwaIidhk. .......... .......... .......... ..........
BalkXylA  nhtWldgrar ey........ .......... nngvgidaPF vFdhnyrvKp AYwrIid... .......... .......... .......... ..........
CsteCell  kysWlngfp. .......... .......... ..vkrtnaPL lFdrnfmpKp AFwaIvdpsr lre....... .......... .......... ..........
CsacXynA  rhtWkdnfpv k......... .......... ...grkdwPL lFdvngkpKe ALyrIlrf.. .......... .......... .......... ..........
TbacXynA  dysWl..... .......... .......... ....rgdmPL lsdkdyqpKf AFwslidpsv vpke...... .......... .......... ..........
CsacCelB  dysWlrsf.. .......... .......... ..ygkndwPL lFfedysaKp AYwaVieasg vtts...... ...sptptpt ptvtvtptpt ptptptvtat
SlivXlnA  .....sd... ..swrse... .......... ......qtPL lFnndgskKa AYtaVldaln ggdssep... .padggqikg vgsgrc.... .ldvpdasts
CfimCex   kysWvpd... ..vfpge... .......... ......gaaL vWdasyakKp AYaaVmeafg asptptpttp tptpttptpt ptsgpagcqv lwgvnqwntg
TaurXyn   ..nvmkn... ..siras... .......... ......ttPL lFdgnfnpKp AYnaIvqglq .......... .......... .......... ..........
CtheXynZ  kytWipg... ..tfpgy... .......... ......gnPL iYdsnynpKp AYnaIkealm gy........ .......... .......... ..........
CalbXyn   ptsWipg... ..vfkgklia vsctfsgcll qycvgygaaL lYdaqyqpKs tYyvVqqalk dgknsgskfh gikl...... .......... ..........
RflaXynA  tvsWrss... .......... .......... ......qnPL lFsagyqpKp AYdrVmalak .......... .......... .......... ..........
Consensus ---W------ ---------- ---------- --------PL -F------K- AY--V----- ---------- ---------- ---------- ----------
```

FIG. 5. Amino acid sequence alignment of β-glycanases from family F with *B. stearothermophilus* T-6 xylanase. The alignment was made with the programs PILEUP and PRETTY (Genetic Computer Group, Inc.) by using a plurality of 14 for the consensus sequence. Amino acids agreeing with the consensus are in uppercase letters. For definitions of acronyms, see the legend to Fig. 4.

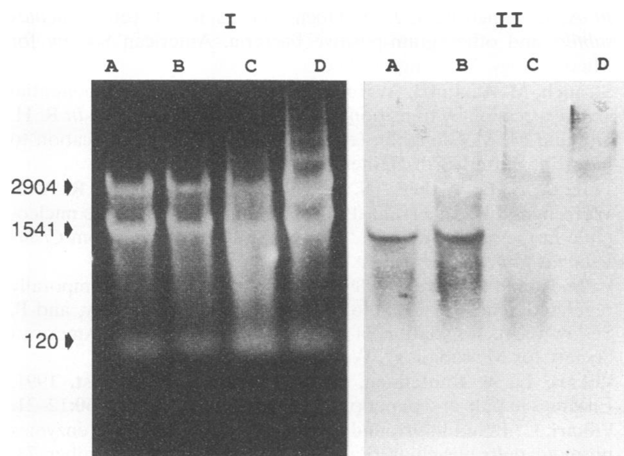

FIG. 6. Northern blot analysis of the xylanase gene transcript from *B. stearothermophilus* T-6. Total RNA was isolated from *B. stearothermophilus* and probed with a 1,481-bp *Bgl*II-*Eco*RI fragment containing the xylanase gene. (I) Total RNA separated on a denaturing agarose gel and stained with ethidium bromide; (II) Northern blot of total RNA. Lanes: A, RNA from strain M-7, a xylanase-constitutive mutant; B, RNA from strain T-6 grown on Luria-Bertani broth plus xylose; C and D, RNA from strain T-6 grown on Luria-Bertani agar. The analysis was performed with the enhanced chemiluminescence random prime labeling and detection system (Amersham, Buckinghamshire, England).

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