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Sequence Analysis and Expression of Xylanase Gene (*xynY*) from Alkalophilic *Bacillus* sp. YC-335

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The nucleotide sequence of the xylanase gene (*xynY*) from alkalophilic *Bacillus* sp. YC-335 was determined and analyzed. An open reading frame of 1,062 base pairs for *xynY* gene was observed and encoded for a protein of 354 amino acids with a molecular weight of 38,915. S1 nuclease mapping showed that the transcription initiation sites of the *xynY* gene were different in *Bacillus* sp. YC-335 and *Escherichia coli* HB101 (pYS55). S1 mapping also showed that −10 region of the *xynY* gene recognized by RNA polymerases of *E. coli* and *Bacillus sp.* YC-335 were 'TACAGT' and 'TATGAT', respectively. A ribosome binding site sequence with the free energy of −17.0 Kcal/mol was observed 9 base pairs upstream from the unusual initiation codon, TTG. The proposed signal sequence consisted of 27 amino acids, 2 of which were basic amino acid residues and 21 were hydrophobic amino acid residues. When the amino acid sequences of xylanases were compared, *Bacillus* sp. YC-335 xylanase showed more than 50% homology with xylanases from *B. pumilus, B. subtilis,* and *B. circulans.*

Xylan, a β 1-4 linked polymer of xylose, is the principal constituent of plant hemicelluloses and is regarded as the most important biologically renewable resource for bioconversion. The enzymatic hydrolysis of xylan is accomplished by the action of endo-β-1- 4 -xylanase (EC 3.2.1.8) and β-D-xylosidase (EC 3.2.1.38). Studies on xylanase have been done to elucidate the relationship between its protein structure and function. And several xylanase genes have been cloned and sequenced (2, 8, 10, 11, 15, 22, 28, 40). However, no data regarding the regulation of expression of the xylanase genes are available as yet.

In our laboratory, several alkalophilic *Bacillus* strains were isolated from soil under alkaline conditions (13, 34, 35). These strains produced various useful enzymes (4, 5, 32, 33) and the properties and expression of genes coding for these enzymes were investigated (14, 21, 23, 30, 31, 36, 37, 38). Among these strains, *Bacillus* sp. YC-335 (4), which produced a large amount of xylanase in the culture broth, was selected and its xylanase gene was cloned (39).

In this paper, we determine the nucleotide sequence of the *xynY* gene, and the transcription initiation sites recognized in *Bacillus* and *Escherichia coli*. In addition, the complete primary structure of the xylanase was compared with those of other xylanases.

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Key words: xylanase gene, transcription initiation site, amino acid homology, alkalophilic Bacillus sp.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Alkalophilic *Bacillus* sp. YC-335 (34) was the donor strain of the xylanase gene, and *E. coli* HB101 [*supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*] (3) was used as the host for the expression of the cloned gene. *E. coli* JM109 (r_k^-, m_k^-, *rec*A1, *end*A 1, *gyr*A96, *thi*, *hsd*R17, *sup*E44, *rel*A1, λ^-, Δ(*lac-pro*AB) /F', *tra*D36, *pro*AB, *lacI*^{q} ZΔM15) (29) was used as a recipient for bacteriophage M13mp vectors. Plasmid pYS 55 (39) was recombinant plasmid harboring xylanase gene.

Media

E. coli cells were cultured on 2YT broth (16 g Bacto-peptone [Difco Laboratories, Detroit, Mich.], 10 g yeast extract [Difco], 5 g NaCl, in 1 liter of distilled water, pH 7.5) for the purpose of producing single strand and replicative form (RF) DNA. *E. coli* transformants were selected on the YT agar broth (8 g Bacto-peptone [Di-

fco], 5 g yeast extract [Difco], 5 g NaCl, in 1 liter distilled water, pH 7.5, 15 g Bacto-agar [Difco]) and 3 ml of YT top agar containing 10 μl of 100 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 50 μl of 2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). To isolate RNA, *Bacillus* sp. YC-335 and *E. coli* cells were cultured in LB medium (10 g Bacto-tryptone [Difco], 5 g yeast extract [Difco], 10 g NaCl, in 1 liter of distilled water, pH 7.5) (16).

Enzymes and Reagents

All restriction endonucleases and T4 DNA ligase were purchased from Promega (Madison, Wis.) and New England Biolabs (Beverly, Mass.) and were used as recommended by the suppliers. Cyclone I Biosystem was obtained from International Biotechnologies, Inc. (New Haven, CT) Isopropyl-β-D-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and ribonucleoside-vanadyl complex were from Sigma Chemical Co. (St. Louis, Mo.).

DNA Isolation

Rapid isolation of plasmid DNA from *E. coli* was done by the miniscreen method (24). Bacteriophage RF DNA was isolated from *E. coli* by the method of Messing *et al.* (17). Large-scale plasmid purification was obtained by banding in CsCl-ethidium bromide density gradients (12). DNAs were resolved by doing horizontal gel electrophoresis in Tris-acetate or Tris-borate buffer (16).

Construction of Overlapping Deletion Subclones

Overlapping deletions were generated with Cyclone I Biosystem as described by Dale *et al.* (6). To subclone into M13mp19 DNA, plasmid pYS55 (39) was digested with *Hin*dIII, and the 1.5 kb DNA fragment was ligated with *Hin*dIII-digested M13mp19. The 1.5 kb insert DNA fragments were deleted with exonuclease activity of T4 DNA polymerase from the 3'-end of the inserted DNA, tailed with poly A, ligated , and transformed into *E. coli* JM109. Single strand DNAs were isolated from the transformed white plaque, electrophoresed and fractionated.

Nucleotide Sequence Analysis

Fragments of appropriate size in the M13 deletion subclones were sequenced by the dideoxy chain termination method of Sanger *et al.* (25). DNA chain termination sequencing with sequenase enzyme (United States Biochemicals Corp., Cleveland, Ohio) and [α-^{35}S]dATP (Amersham Corp., Arlington Heights, Ill.) were performed according to the protocols given by U.S. Biochemicals. Electrophoresis was carried out on a 6% polyacrylamide/8 M urea gel. The sequences were read from both strands, and each sequence was read an average of 4 times in each directions. Sequence data were analyzed by the Pustell DNA Analysis Program (International Biotechnologies, Inc., New Haven, CT). The theoretical free energy (ΔG) of secondary structure was calculated by the method of Tinoco *et al.*(26).

Isolation of RNA

Total cellular RNAs were extracted from *Bacillus* sp. YC-335 cells and *E. coli* HB101 (pYS55) cells by the method of Duvall *et al.* (7). The concentration of RNA was normalized on the basis of its A_{260} (16).

S1 Nuclease Mapping

To prepare a probe, plasmid pYS55 was digested with *Hin*dIII-*Fnu*4HI and dephosphorylated with calf intestinal alkaline phosphatase. The fragment (231 bp) was electroeluted from a 5% polyacrylamide gel and the 5'-ends were ^{32}P labeled by using [γ-^{32}P]ATP (Amersham Corp.) and T4 polynucleotide kinase (Boeringer Mannheim Biochemicals) as described by Maniatis *et al.* (16). S1 nuclease mapping was performed by the method of Gilman and Chamberlin (9). The single strand probe was annealed to total RNAs (50 μg) isolated from *Bacillus* sp. YC-335 and *E. coli* HB101 (pYS55) at the stage of late logarithmic phase, and digested with S1 nuclease. The S1 nuclease resistant DNA fragments were resolved by a 6% polyacrylamide gel electrophoresis.

Nucleotide Sequence Accession Number

The nucleotide sequence of the xylanase gene has been submitted to the EMBL Data Library under Accession Number X59059.

RESULTS

Sequencing Strategy

The structure of the plasmid pYS55 is shown in Fig. 1. The nucleotide sequence of insert DNA was determined by the strategy outlined in Fig. 1.

All of the sequenced region was read from at least two subclones as shown by the arrows in the lower part of the Fig. 1, and the results were analyzed by computer matching.

Nucleotide Sequence of *xynY* Gene

The nucleotide sequence of 1,531 bp covering the entire xylanase gene and its flanking regions is shown in Fig. 2. The nucleotide sequence of the xylanase gene has an open reading frame of 1,062 bp which translates to the amino acids shown below the nucleotide sequence. Since the other two reading frames contain numerous stop codons, we concluded that the open reading frame translated in Fig. 2 encodes pre-xylanase, and named it *xynY* (EMBL accession No: X59059).

The encoded 354 amino acid polypeptide had a calculated molecular weight of 38,915 dalton and its pI was 5.9.

To identify the promoter region of the xylanase gene,

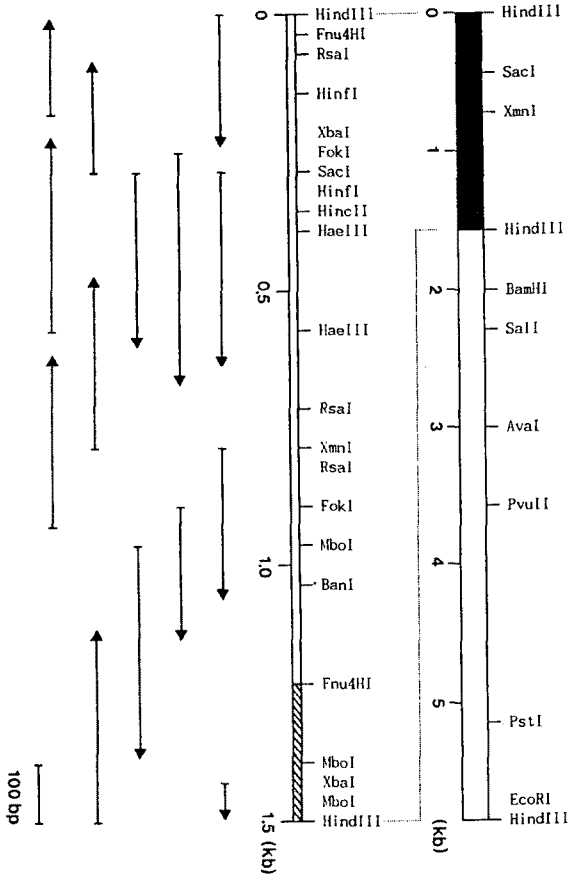

Fig. 1. Restriction map of plasmid pYS55 and strategy of nucleotide sequencing.
pYS55 is a hybrid plasmid consisting of the 1.5 kb *Hin*dIII fragment of *Bacillus* sp. YC-335 DNA (filled bar) and pBR322 (open bar). Fine mapping of the region containing the xylanase gene and the sequencing strategy are enlarged. The arrows show the direction of reading from inserts in M13mp19. The hatched region is the 231-nucleotede *Hin*dIII-*Fnu*4HI probe used for S1 nuclease mapping.

transcrption initiation site was first determined by S1 nuclease mapping. *Bacillus* sp. YC-335 and *E. coli* HB 101 harboring plasmid pYS55 RNAs were isolated and hybridized to the 231 bp *Hin*dIII-*Fnu*4HI fragment labeled on the 5'-terminus. As shown in Fig. 3., protected fragments of 209 nucleotides (lane 1) and 215 nucleotides (lane 2) were obtained with RNAs isolated from *Bacillus* sp. YC-335 and *E. coli* HB101 (pYS55), respectively. From this autoradiogram, transcription initiation site in *E. coli* HB101 harboring plasmid pYS55 was determined as 'A', −134 position from the translation start codon. But transcription initiation site was determined as 'G', −128 position from the translation start codon, in *Bacillus* sp. YC-335.

Two putative −10 promoter sequences (5'-TATGAT-

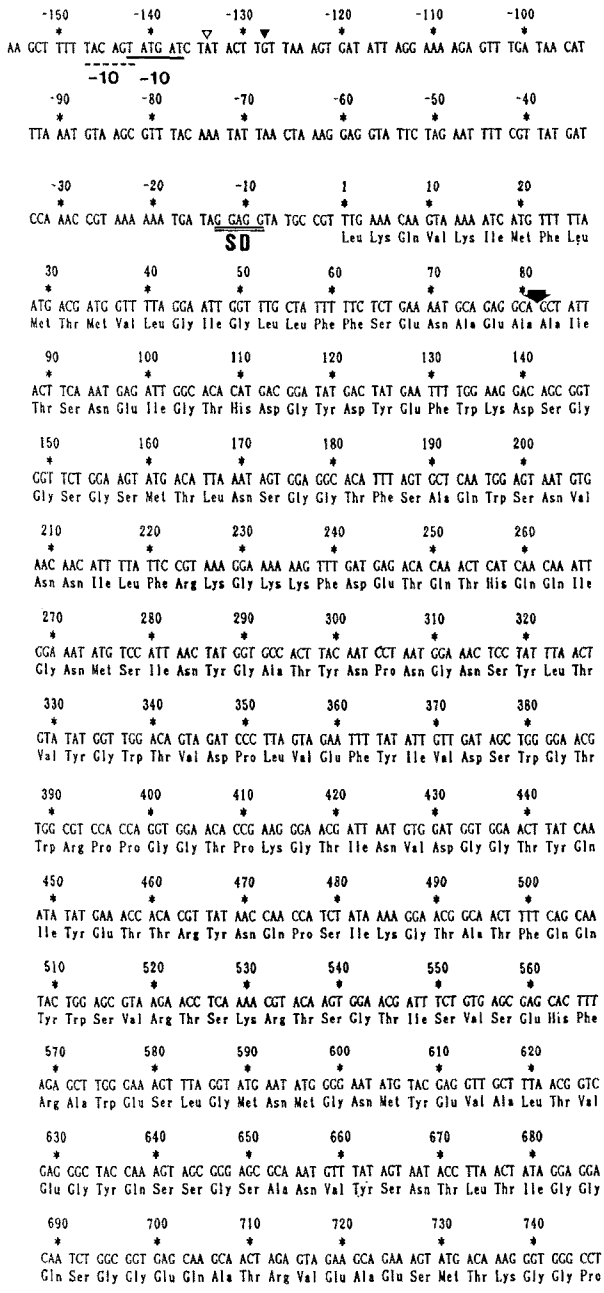

Fig. 2. Nucleotide sequence and deduced amino acid sequence of the xylanase gene of *Bacillus* sp. YC-335.
Numbering of both nucleotides and amino acids starts with the beginning of the coding sequence. The-10 regions of promoters recognized by *Bacillus* sp. YC-335 and *E. coli* HB101 (pYS55) are underlined and dotted-underlined below the sequence, respectively. A blank reverse triangle and filled reverse triangle indicate transcription initiation site recognized by *Bacillus* sp. YC-335 and *E. coli* HB101 (pYS55), respectively. The putative Shine-Dalgarno sequence (SD) is double-underlined. The inverted repeat structure for the transcription terminator is indicated by inverted arrows. The putative position of processing of the signal sequence is shown by an arrowhead.

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750         760         770         780         790         800
 *           *           *           *           *           *
TAC ACC TCT AAT ATT ACT TCT CCT TTT AAC GGA GTT GCT TTA TAT GCA AAT GGT GAT AAT
Tyr Thr Ser Asn Ile Thr Ser Pro Phe Asn Gly Val Ala Leu Tyr Ala Asn Gly Asp Asn

810         820         830         840         850         860
 *           *           *           *           *           *
GTG AGC TTT AAT CAT TCA TTC ACT AAA GCA AAC AGT AGT TTT TCA TTA CGG GGA GCC TCC
Val Ser Phe Asn His Ser Phe Thr Lys Ala Asn Ser Ser Phe Ser Leu Arg Gly Ala Ser

870         880         890         900         910         920
 *           *           *           *           *           *
AAT AAC TCA AAC ATG GCT AGA GTT GAT TTG AGG ATA GGT GGA CAA AAT AGG GGA ACT TTT
Asn Asn Ser Asn Met Ala Arg Val Asp Leu Arg Ile Gly Gly Gln Asn Arg Gly Thr Phe

930         940         950         960         970         980
 *           *           *           *           *           *
TAC TTT GGA GAC CAC TAT CCG GCC GTT TAT ACC ATT AAT AAC GTC AAC CAT GGA ACA GGG
Tyr Phe Gly Asp His Tyr Pro Ala Val Tyr Thr Ile Asn Asn Val Asn His Gly Thr Gly

990         1000        1010        1020        1030        1040
 *           *           *           *           *           *
AAT CAA TTA GTC GAG CTC ATT GTA ACA GCT GAT GAC GGA ACA TGG GAT GCT TAT TTA GAC
Asn Gln Leu Val Glu Leu Ile Val Thr Ala Asp Asp Gly Thr Trp Asp Ala Tyr Leu Asp

1050        1060        1070        1080        1090        1100
 *           *           *           *           *           *
TAT CTA GAA ATA AGG TAG ATT ATA AGT AAA CCC TAA AGA CTG TTA CAG AAT AAA TCT CTT
Tyr Leu Glu Ile Arg ---

1110        1120        1130        1140        1150        1160
 *           *           *           *           *           *
ATT TAA AAA GGT GAA ATG GAA AAA ATC CCA TTT CAC CTT TTC TAT TTT TCT GGG TTC TAC
           ------------>           <------------

1170        1180        1190        1200        1210        1220
 *           *           *           *           *           *
TGT TAA AGC AGT AGA ATC AAC TGT TTG TCT TCG TTT TTA ATT TAA AAA TAA ATA ATT TAT

1230        1240        1250        1260        1270        1280
 *           *           *           *           *           *
AAA ATT TTG AAA CTC AAT TAC TCT TTT TTC GTA AAA TAT AAA AGT ACG AGA TAA AGG GAG

1290        1300        1310        1320        1330        1340
 *           *           *           *           *           *
GTT TAT AAA TAA GTG ACC CAT GCA GCT ATG CAA CTT AAG AAC GTA AAA AAA ACA ATT GGA

1350        1360        1370
 *           *           *
AAA AAA GAG ATT ATT AAA GGT TTA AGC TT
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Fig. 2. Continued.

3′ and 5′-TACAGT-3′) were identified 137 and 142 bp upstream from the ATG codon (Fig. 2). But a −35 consensus sequense was not found. Comparison of putative promoter sequences of the xylanase genes and consensus sequences is Table 1.

McLaughlin *et al.* (18) have observed that the ribosome binding sites of Gram-positive mRNAs exhibit extensitive complementarity to the 3′ region of *B. subtilis* 16S rRNA. The putative ribosome binding site of the xylanase gene complementary to the 3′ region of *B. subtilis* 16S rRNA was located 9 base upstream from the unusal translation start codon, TTG (Fig. 2).

Xylanase is an enzyme secreted in *Bacillus* sp. YC-335 and more than 50% of the enzymes synthesized in *E. coli* HB101 harboring plasmid pYS55 pass through the cytoplasmic membrane (39). A hydropathy profile of xylanase shows a hydrophobic region near the NH_2-terminus and this region possibly represents a signal peptide (Fig. 4) showing good conformity with the typical signal peptide structure (27).

Table 1. Comparison of promoter nucleotide sequences of xylanase genes

Organism	Nucleotide sequence*
Consensus sequence	TTGACA TATAAT
Bacillus sp. YC-335	
in *Bacillus* sp. YC-335	AAGCTTTTTACAG**TATGAT**
in *E. coli* HB101 (pYS55)	AAGCTTTT**TACAGT**
B. pumilus	**TT**TCATCTTAGAGATGACAGAA**T**TA**AA**A
B. subtilis	**TTG**G**C**GTTAGTAATTAAGAATGTT**T**TA**AAT**
B. circulans	**TTG**G**C**GTTAGTAATTAAAAATGTT**T**TA**AAT**
Bacillus sp. C-125	GGAT**CA**ACTAAGTTTATTGTTGC**TATAAT**

*Conserved sequence is shown as enlarged letter.

Table 2. Comparison of signal peptides of xylanases

Organism	Amino acid sequence*
Bacillus sp. YC-335	Leu Lys Gln Val Lys Ile Met Phe Leu Met Thr Met Val Leu Gly Ile Gly Leu Leu Phe Phe Ser Glu Asn Ala Glu Ala ↓ Ala
B. pumilus	Met Asn Leu Arg Lys Leu Arg Leu Leu Phe Val Met Cys Ile Gly Leu Thr Leu Ile Leu Thr Ala Val Pro Ala His Ala ↓ Arg
B. subtilis	Met Phe Lys Phe Lys Lys Asn Phe Leu Val Gly Leu Ser Ala Ala Leu Met Ser Ile Ser Leu Phe Ser Ala Thr Ala Ser Ala ↓ Ala
B. circulans	Met Phe Lys Phe Lys Lys Asn Phe Leu Val Gly Leu Ser Ala Ala Leu Met Ser Ile Ser Leu Phe Ser Ala Thr Ala Ser Ala ↓ Ala
Bacillus sp. C-125	Met Ile Thr Leu Phe Arg Lys Pro Phe Val Ala Gly Leu Ala Ile Ser Leu Leu Val Gly Gly Gly Ile Gly Asn Val Ala Ala ↓ Ala
C. thermocellum	Met Ser Arg Lys Leu Phe Ser Val Leu Leu Val Gly Leu Met Leu Met Thr Ser Leu Leu Val Thr Ile Ser Ser Thr Ser Ala ↓ Ala
C. saccharolyticum	Met Arg Cys Leu Ile Val Cys Glu Asn Leu Glu Met Leu Asn Leu Ser Leu Ala Lys Thr Tyr Lys Asp Tyr Phe Lys Ile Gly Ala Ala ↓ Val Thr ↓ Ala ↓ Lys

*Positively charged amino acids are underlined and the possible processing sites are shown by the ↓.

The signal peptide of pre-xylanase consists of 27 amino acids, 2 of which are basic amino acid residues, Lys, in the region near the N-terminus, and 21 are hydrophobic amino acid residues. The processing site recog-

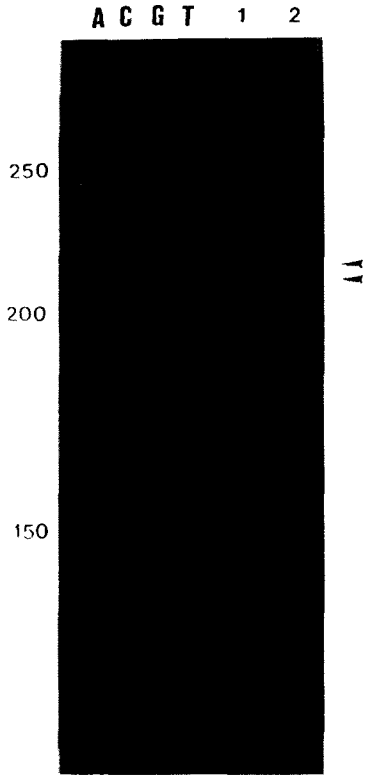

Fig. 3. Transcriptional start site mapping of the *xynY* gene by S1 nuclease analysis.
RNAs were isolated from stationary phase cultures of *Bacillus* sp. YC-335 (lane 1) and *E. coli* HB101 containing plasmid pYS55 (lane 2). The protected bands are shown with the arrows. The sequence ladder of M13mp18 DNA serves as the size marker for the protected band.

nized by signal peptidase could be after Ala-27. The signal peptides of various xylanases are compared in Table 2.

Homology Comparison of Xylanase Primary Structure

Comparison of the predicted amino acid sequence of the xylanase from *Bacillus* sp. YC-335 with those of other xylanases revealed significant similarities between the xylanase from *Bacillus* sp. YC-335 and the xylanases from the following strains (Fig. 5): *B. pumilus* (BP), *Trichoderma hazianum* (TH), *Schizophyllum commune* (SC), *B. subtilis* (BS), *B. circulans* (BC). In the processed enzymes, *Bacillus* sp. YC-335 xylanase showed 65% homology with *B. pumilus* xylanase and 48% homology with *B. subtilis* and *B. circulans* xylanases.

Codon Usage

Codon usage for the pre-xylanase is shown in Table 3. There appears to be a high bias for codon selection, for example, TTT for Phe, TTA for Leu, ATT for Ile,

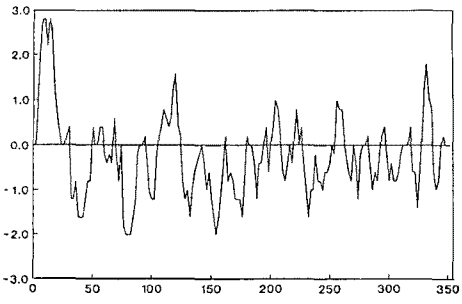

Fig. 4. Hydropathy profile of the xylanase from *Bacillus* sp. YC-335.
The abscissa of the panel shows amino acid number. The ordinate shows the average hydrophobicity (positive ordinate) or hydrophilicity (negative ordinate) of 2 amino acid residues.

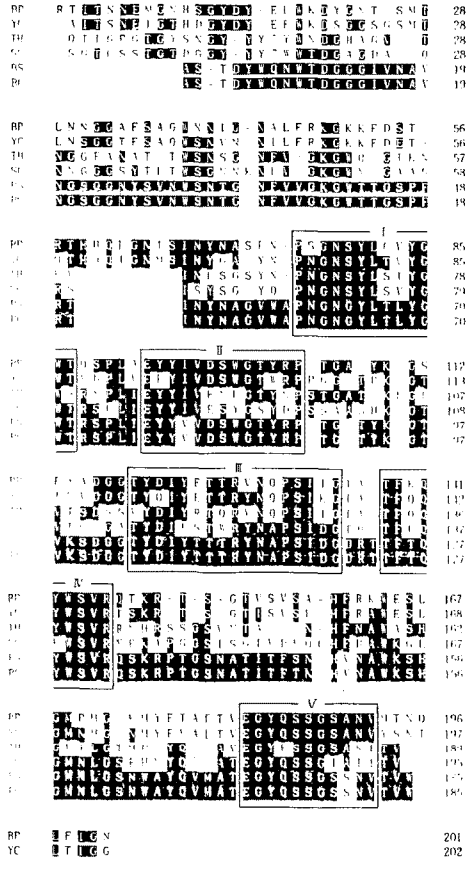

Fig. 5. Alignment of xylanase amino acid sequences from *B. pumilus* (BP), *Bacillus* sp. YC-335 (YC), *Trichoderma harzianum* (TH), *Schizophyllum commune* (SC), *B. subtilis* (BS) and *B. circulans* (BC).
Only the sequences of the processed xylanases are shown and the numbers denote amino acid positions of the proteins. Identical amino acids are in reversed font. If the amino acids are not present in all of the protein sequences compared, they are shown either in stippled boxes or in outlined letters. Dashed lines indicate where it is necessary to introduce gaps for better alignment.

Table 3. Codon usage of pre-xylanase from *Bacillus* sp. YC-335

TTT	Phe	13	3.7%	TCT	Ser	7	2.0%	TAT	Tyr	15	4.2%	TGT	Cys	0	0.0%
TTC	Phe	3	0.8%	TCC	Ser	3	0.8%	TAC	Tyr	6	1.7%	TGC	Cys	0	0.0%
TTA	Leu	13	3.7%	TCA	Ser	5	1.4%	TAA	—	0	0.0%	TGA	—	0	0.0%
TTG	Leu	3	0.8%	TCG	Ser	0	0.0%	TAG	—	1	0.3%	TGG	Trp	8	2.3%
CTT	Leu	0	0.0%	CCT	Pro	3	0.8%	CAT	His	4	1.1%	CGT	Arg	4	1.1%
CTC	Leu	1	0.3%	CCC	Pro	1	0.3%	CAC	His	2	0.6%	CGC	Arg	0	0.0%
CTA	Leu	2	0.6%	CCA	Pro	3	0.8%	CAA	Gln	13	3.7%	CGA	Arg	0	0.0%
CTG	Leu	0	0.0%	CCG	Pro	2	0.6%	CAG	Gln	1	0.3%	CGG	Arg	1	0.3%
ATT	Ile	12	3.4%	ACT	Thr	11	3.1%	AAT	Asn	20	5.6%	AGT	Ser	11	3.1%
ATC	Ile	1	0.3%	ACC	Thr	5	1.4%	AAC	Asn	11	3.1%	AGC	Ser	7	2.0%
ATA	Ile	5	1.4%	ACA	Thr	12	3.4%	AAA	Lys	7	2.0%	AGA	Arg	4	1.1%
ATG	Met	10	2.8%	ACG	Thr	6	1.7%	AAG	Lys	4	1.1%	AGG	Arg	3	0.8%
GTT	Val	7	2.0%	GCT	Ala	8	2.3%	GAT	Asp	8	2.3%	GGT	Gly	12	3.4%
GTC	Val	3	0.8%	GCC	Ala	3	0.8%	GAC	Asp	6	1.7%	GGC	Gly	4	1.1%
GTA	Val	7	2.0%	GCA	Ala	8	2.3%	GAA	Glu	8	2.3%	GGA	Gly	22	6.2%
GTG	Val	4	1.1%	GCG	Ala	0	0.0%	GAG	Glu	8	2.3%	GGG	Gly	4	1.1%

Table 4. Nucleotide sequences of putative ribosome binding sites of xylanase genes

Organism	Nucleotide sequence*	ΔG (Kcal/mol)
16S rRNA 3'	UCUUUCCUCCACUAG	
Bacillus sp. YC-335	$_{U}GA_{U}AGGAGGU_{AUG}\overset{A}{\underset{\vdots}{G}}CGUUUG$	-17.0
B. pumilus	$A_{A}AAAGGAG_{AG}GA_{AU}GACGAAUG$	-12.2
B. subtilis	$UU_{A}U_{U}AGGAGGU_{A}A_{C}AUAUG$	-14.8
B. circulans	$UU_{A}U_{U}AGGAGGU_{A}A_{C}AUAUG$	-14.8
Bacillus sp. C-125	$_{C}GAAAGGAG_{AAUU}U_{GUG}AUG$	-14.2
C. thermocellum	$A_{A}AAAGGAGG_{A}GA_{AAC}AUG$	-17.2
C. saccharolyticum	$AG_{G}AA_{U}GAGGUGUU_{UAAUUGUG}$	-11.2

*Homology with the *B. subtilis* SD sequence is shown as enlarged letter.

TAT for Tyr, CAA for Gln, and CGT for Arg. There are no codons for Cys.

DISCUSSION

We had reported the cloning of the xylanase gene (*xynY*) from *Bacillus* sp. YC-335 (39). The nucleotide sequence of xylanase gene and its flanking region provide informations on the structure of the enzyme, the regulation of transcription and translation. Xylanase was synthesized in the vegetative growth of *Bacillus* sp. YC-335 and *E. coli* HB101 harboring plasmid pYS55 (data not shown). Therefore, the promoter of xylanase could be recognized by *Bacillus* RNA polymerase containing σ^A subunit. There is good homology between the promoter sequences of *xynY* gene recognized by *Bacillus* RNA polymerase and *E. coli* RNA polymerase and the consensus sequence of a promoter. It is a striking fact that the promoters of xylanase gene (*xynY*) recognized by *Bacillus* RNA polymerase and E. coli RNA polymerase were different. Although *E. coli* RNA polymerase can recognize *B. subtilis* σ^A promoter sequence, there could be subtle difference in the preference of the promoter selection, or *Bacillus* RNA polymerase and *E. coli* RNA polymerase could recognize different sequences where the promoter regions are overlapped.

The inverted repeat (IR) sequence, which can act as a transcription terminator, is found in the downstream from the stop codon. This IR sequence can form a stem-loop structure and the free energy of this secondary structure is calculated as −22.8 Kcal/mol.

McLaughlin *et al.* (18) have proposed that the ribosome binding sites of Gram-positive mRNAs are characteristically able to form highly stable complexes with the 3' terminal region of 16S rRNA, and that the free energies of interaction (ΔG) between ribosome binding sites vary from −14 to −23 Kcal/mol. The ribosome binding site of *xynY* gene was highly homologous to 3' terminal region of 16S rRNA, and the free energy of interaction had a fairly high value of −17.0 Kcal/mol (Table 4). Most of the genes expressed in Gram-positive bacteria use ATG as the translation start codon. But the chloramphenicol acetyltransferase gene of *B. pumilus* (1), the β-lactamase gene of *Staphylococcus aureus* (19), and the middle gene of the *Bacillus* phage SPO1 (19) used TTG as the translation start codon. In the nucleotide

sequence of the *xynY* gene, only TTG was included in the functional initiation codon, because of the long distance from the ribosome binding site or the unusual N-terminal amino acid sequence of the signal peptide. All of the xylanase genes which were sequenced use ATG as a translation start codon except for the xylanase from *C. saccharolyticum* whose translation start codon is GTG (Table 4). The *xynY* gene is very peculiar in that TTG is used as a translation start codon.

Morosoli *et al.* (20) classified 13 xylanase genes into two groups, Type A and B, by comparing their deduced polypeptides sequences. Type A xylanases include xylanases from *Streptomyces lividans, Clostridium thermocellum, Bacillus* strain C-125, *Cryptococcus albidus, Cellulomonas fimi* and *Caldocellum saccharolyticum*. Type B xylanases include xylanases from *S. lividans, B. pumilus, B. subtilis, Schyzophillum commune, Clostridium acetobutylicum*, and *Trichoderma hazianum*.

Our xylanase belongs to type B xylanase, and homologies among *xynY* and type B xylanases were more than 50%. Type B xylanases share five large regions of high homology in their sequences (Fig. 3). There is no homology between type A and B xylanases.

Since a detailed analysis of the hydrolytic properties of the type A and type B xylanases is not yet available, it is not possible to make a correlation between the enzymatic properties and the amino acid sequences. Although the primary sequence of the structural gene is known, very little information is available on the active site of the xylanases and crystallographic data are needed in correlation to the mutagenesis and the chemical modifications.

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