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Nucleotide Sequence Analysis of an Endo-Xylanase Gene(*xynA*) from *Bacillus stearothermophilus*

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A gene (*xynA*) encoding the endo-xylanase (E.C.3.2.1.8) from *Bacillus stearothermophilus* was cloned in *E. coli*, and its complete nucleotide sequence was determined. The *xynA* gene consists of a 636 base pairs open reading frame coding for a protein of 212 amino acids with a deduced molecular weight of 23,283 Da. A putative signal sequence of 27 amino acid residues shows the features comparable with the *Bacillus* signal sequences; namely, the signal contains a positively charged region close to the N-terminus followed by a long hydrophobic string. The coding sequence is preceded by a possible ribosome binding site with a free energy value of -16.6 kcal/mol and the transcription initiation signals are located further upstream. The translation termination codon (*TAA*) at the 3' end of the coding sequence is followed by two palindrome sequences, one of which is thought to act as a terminator. The *xynA* gene has a high GC content, especially in the wobble position of codons (64%). Comparison of the primary protein sequence with those of other xylanases shows a high homology to the xylanases belonging to family G.

Xylan, a major component of plant hemicellulose, is second only to cellulose in natural abundance and is now regarded as a usable biomass that is convertible to biofuels, chemicals and many other valuable compounds (7).

Unlike cellulose, xylan is a complex polymer consisting of a β-D-1,4-linked xylopyranoside backbone substituted with acetyl, arabinosyl and glucuronosyl side chains. Complete enzymatic hydrolysis of xylan, therefore, requires the cooperative actions of a range of xylanolytic enzymes including endo-β-1,4-xylanases, β-xylosidases, α-arabinofuranosidases, α-glucuronidases, and esterases (34).

In this regard, studies of the enzymatic degradation of xylan have been started, and we isolated a *Bacillus stearothermophilus* strain capable of producing all the key enzymes necessary for xylan biodegradation (26).

Recently we have cloned in *E. coli*, two distinct genes for β-xylosidases (23), two for α-arabinofuranosidases (8), and two for acetylxylan esterases (15, 16) in addition, a gene for xylanase (3) from the *B. stearothermophilus* strain, and one of the β-xylosidase genes was subsequently sequenced (22). In this report, we present the complete nucleotide sequence of the 2,386bp fragment containing the endo-xylanase gene (*xynA*) from *B. stearothermophilus*.

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Key words: nucleotide sequence, endo-xylanase, *Bacillus stearothermophilus*

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Chemicals

E. coli JM109 (*rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17, *sup*E44, *rel*A1, (*lac-pro*AB), F, *tra*D36, *pro*AB, *lac*IqZM15) (37) was used as a host for the plasmids used in this work and propagated at 37℃ in Luria broth or on Luria agar supplemented with 50 mg of ampicillin per liter. Plasmid pMG12 (3) is a recombinant pBR322 into which the *xynA* gene isolated from a genomic library of *B. stearothermophilus* has been cloned. As subcloning vectors, phagemid pUC118/119 (32) were used. Restriction enzymes were used according to the manufacturers' instructions.

Oat spelts xylan, agarose, acrylamide, and sodium carbonate were purchased from Sigma Chemical Co., St. Louis, Mo. All restriction enzymes, T4 DNA ligase, T4 DNA polymerase, and terminal transferase were obtained from Promega Corporation, Madison, Wis. or New England Biolabs, Beverly, MA.

DNA Isolation and Transformation

Plasmid DNA and single-stranded phagemid DNA were isolated as described by Maniatis *et al.* (19). Transformation and selection of the xylanase positive subclone were performed as described earlier (3).

Construction of Overlapping Deletion Subclones

In order to subclone the xylanase gene into phagemid pUC119, plasmid pMG12 was digested with *Hin*dIII, and the 4.0 kb insert DNA fragment containing *xynA* gene was isolated. The isolated insert DNA was then ligated with *Hin*dIII-digested pUC119 to give pMG119A and pMG119B, both of which have the same insert but in the reverse orientation.

A series of nested deletion mutants were generated by the method of Dale *et al.* (5). Single-stranded pMG 119A, pMG119B, and pMG119B25 DNA were annealed with the oligomer RD 20 (5'-CGACGGCCAGTGAATT-CCCC-3', New England Biolabs, Beverly, MA, USA) and then digested with *Eco*RI. The digested DNA was deleted with the exonuclease activity of T4 DNA polymerase from the 3'-end of the inserted DNA and tailed with poly dG by using terminal transferase. Then, the poly dG-tailed DNA was reannealed with RD20, self-ligated, and transformed into *E.coli* JM109. Phagemid DNAs were isolated from the transformants, eletrophoresed, and fractionated.

Measurement of Xylanase Activity

Xylanase activity of the subclones obtained from the above experiment was assayed as described earlier (3).

Nucleotide Sequence Analysis

Fragments of appropriate size prepared from the deletion mutants were sequenced by using the SILVER SEQUENCE DNA sequencing system (Promega Corporation, Madison, Wis.). The Sequencing reaction mixture was prepared according to the suppliers' recommendation, added to each d/ddNTP mix, and amplified with a thermal cycler. The amplified products were analyzed on a 5% polyacrylamide gel containing 8M urea. Preparation of the sequencing gel plate, silver staining of the sequencing gel, and development on Electrophoresis Duplicating Film (EDF) were done as recommended by the suppliers. Sequence data were analyzed by DNASIS program (Hitachi software engineering Co. Ltd., Tokyo, Japan) or by PC/GENE:the nucleic acid and protein sequence analysis software system (IntelliGenetics Inc., Mountain View, Calif.). The nucleotide and amino acid sequences were scanned against the databases available at the National Center for Biotechnology Information (NCBI) of the National Institute of Health (NIH) through Experimental GENINFO (R) BLAST Network Service (Blaster).

Nucloetide Sequence Accession Numbers and References.

The GenBank accession number of the nucleotide sequence data reported in this paper is U15985. The accession numbers for the other sequences used are as follows: *Aeromonas caviae xyn*1, D32065; *Bacillus subtilis xynA*, P18429; *Clostridium acetobutylicum xynB*, M31726 (39); *Bacillus pumilus xynA*, X00660 (9, 18); *Ruminococcus flavefaciens xynA*, Z11127; *Aspergillus kawachii xynC*, S45138 (12); *Trichoderma reesei* C30 *xyn*1 and *xyn*2, S51973 and S51975 (30); *Cochiobolus carbonum xyl*1, L13596; *Bacillus subtilis* PAP115 *xyn*, M36648 (24); *Bacillus circulans xynA*, X07723 (36); *Thermomonospora fusca xynA*, L20093 (13); *Streptomyces lividans xynB* and *xynC*, S68767 and S68769 (17, 25); *Bacillus* sp. YA-14 *xynS*, X59058 (38).

RESULTS AND DISCUSSION

Construction of Deletion Subclones

To locate the exact position of the xylanase gene on the 4.0kb insert DNA of the recombinant phagemids, pMG119A and pMG119B, the insert DNAs were deleted sequentially from the 3' end by using exonuclease activity of T4 DNA polymerase. The nested deletion mutants obtained were tested for their endo-xylanase activities. As shown in Fig. 1.A, any deletion into the 3' end of the linearized pMG119A abolished the endo-xylanase activity. This suggests that the *xynA* gene starts from the

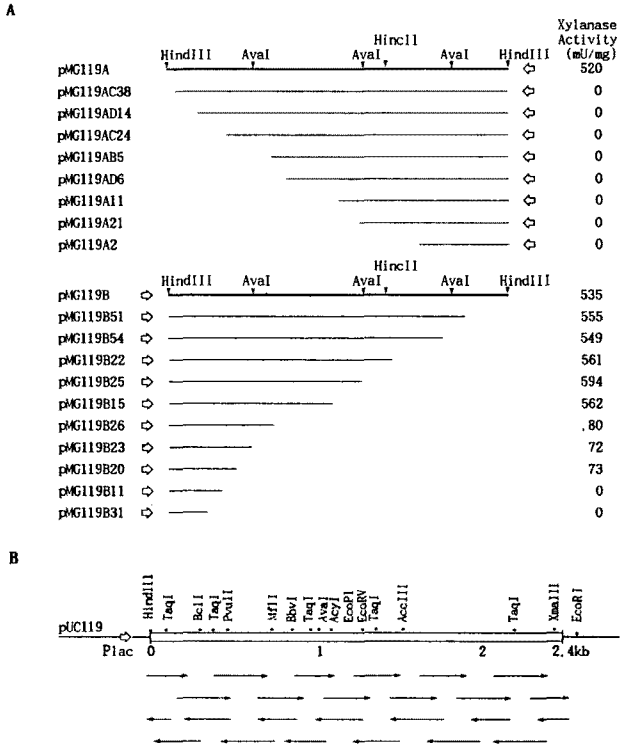

Fig. 1. Subcloning and strategy of nucleotide sequencing.
A. Deletion subclones constructed by deleting the 4.0 kb insert DNAs of pMG119A and pMG119B. Xylanase activities of various subclones are given on the right. The arrow(⇨) indicates the direction and location of the *lacZ'* gene promoter. B. Fine restriction maping of the 2.4 kb insert DNA of pMG119B25 and strategy of nucleotide sequencing. Thin lines correspond to vector phagemid DNAs and box corresponds to the inserted DNA fragment. Arrows(→) indicate the length and direction of sequencing for each fragment used.

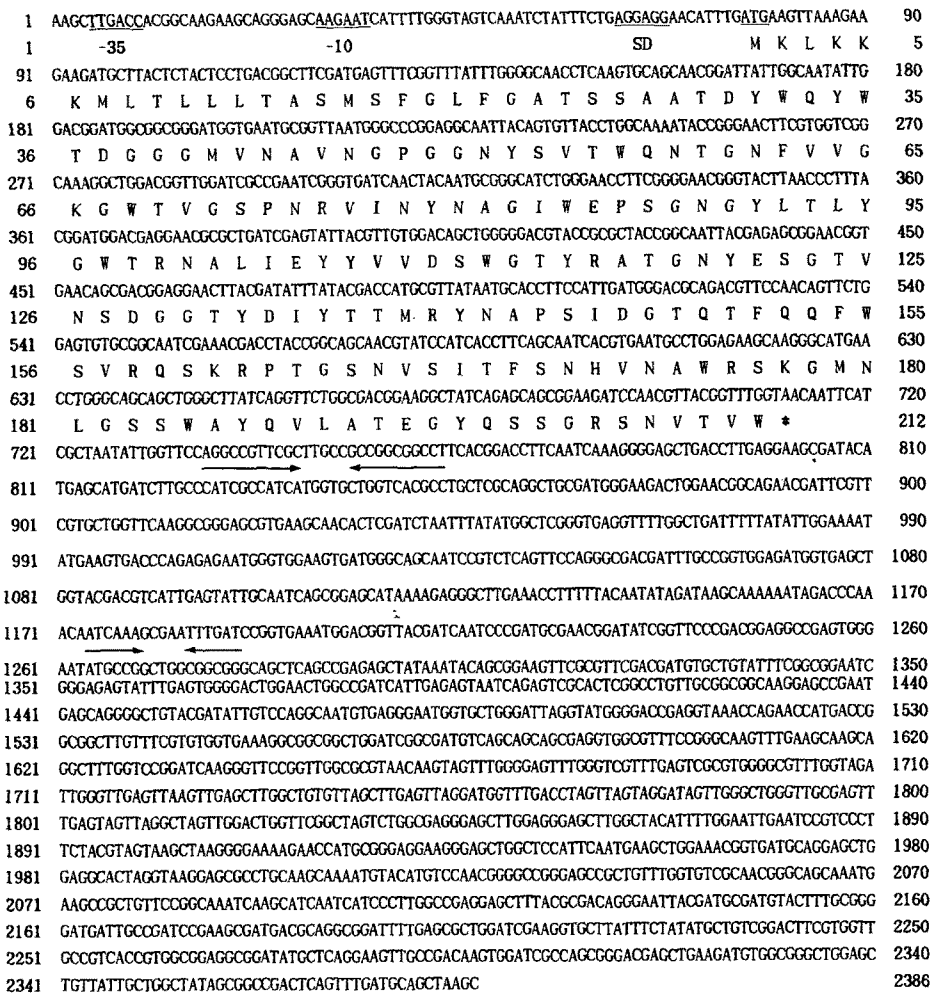

Fig. 2. Nucleotide Sequence of *xynA* and Deduced Amino Acid Sequence of Xylanase A.
The putative promoter, Shine-Dalgarno (SD) and translational initiation sequences are underlined. Two palindromes are indicated by arrows facing each other.

immediate 3′ end of the insert DNA of pMG119A. On the other hand, pMG119B mutants deleted up to pMG119B15 showed full activity. Thus, pMG119B25 and pMG119A were chosen for further deletion experiment in order to obtain the shortened *xynA* gene for subsequent determination of nucleotide sequence.

Nucleotide Sequencing

The deleted insert DNAs obtained by the deletion experiment mentioned above were sequenced by using the SILVER SEQUENCE DNA sequencing system as described in Materials and Methods. The sequence was read from at least two overlapping subclones as shown by the arrows in the lower part of Fig. 1B.

DNA Sequence Analysis

The sequence of 2,386 bp insert DNA in pMG119B25 was shown to cover the entire xylanase gene (*xynA*) and its flanking regions (Fig. 2). The DNA primary structure analysis of the insert DNA identified an open reading frame of 636 bp that encodes 212-amino acid pre-xylanase. The encoded XynA had a calculated molecular weight of 23,283 Da, and its estimated pI was 9.65.

At several bases upstream of the ATG start codon, a deduced ribosome-binding site (SD) sequence was found and it was compared with that of the other genes listed in Table 1. The free energy between the 3′ end of *B. subtilis* 16S rRNA and the SD sequence was calculated to be -16.6 kcal/mol by the method of Tinoco (29). This is lower than the average free energy in Gram positive bacteria, -17.6 kcal/mol (20). The free energy of binding of the 3′ end of *E. coli* 16S rRNA to the SD sequence was -18.8 kcal/mol, which was much higher than that of an average *E. coli* SD sequence, -11.7 kcal/mol (20).

The putative -35 and -10 elements could be identified in the region upstream to the Shine-Dalgarno sequence. These hexanucleotide sequences (TTGACC and AA-

Table 1. Nucleotide sequence of putative ribosome-binding sites of xylanases.

Gene	Nucleotide sequence	△GB[1]	△GE[2]	Reference
B.subtilis α-amylase	AAAAUGAAGGGAGAGG	- 17.2	- 13.6	2
B.subtilis β-lactamase	AACGGAGGGAGACG	- 20	- 17.8	2
B.stearothermophilus xylA	GAACAGGAGGTAATC	- 24.6	- 16.8	22
B.stearothermophilus xynA	TCTGAGGAGGAACAT	- 16.6	- 18.8	This study
E.coli consensus	CTACTGGAGGAAT			2
B.subtilis consensus	AGAAAGGAGGTGATC			2
E.coli 16S rRNA	3′ GAUGACCUCCUUA 5′			2
B.subtilis 16S rRNA	3′ UCUUUCCUCCACUAG 5′			2

[1]GB : The free energy of binding of SD sequences to the 3′ end of *B. subtilis* 16S rRNA, [2]GE : The free energy of binding of SD sequences to the 3′ end of *E. coli* 16S rRNA.

Table 2. Promoter sequences in *Bacillus* sp.

Gene	-35 Region	-10 Region	Spacer length	Reference
B.subtilis σ^{55} consensus	TTGACA	TATAAT	17~19	2
B.subtilis pen	TTGCAT	AATACT	17	2
B.subtilis veg	TTGACA	TACAAT	17	2
B.subtilis tms	TTGAAA	TATATT	17	2
B.stearothermophilus nprT	TTTTCC	TATTTT	18	28
B.stearothermophilus xylA	TTGTTA	CATAAT	13	22
B.stearothermophilus xynA	TTGACC	AAGAAT	19	This study

GAAT) and the spacer length were compared with those of other *Bacillus* promoters (Table 2), and thought to be sufficient for promoting efficient transcription. It has been established previously that *E.coli* RNA polymerase could recognize *B.subtilis* σ^{55} and σ^{37} promoter sequences (4). Thus, the transcription of the *xynA* gene in *E. coli* was thought to occur from its own promoter.

Two pairs of inverted repeat sequences (IRSs) were found in the region downstream to the translation termination codon, TAA. The free energies of the secondary structures formed by those IRSs were calculated as -1 8.4 kcal/mol for the one locating proximal to the termination codon and -2.6 kcal/mol for the distal one. So the first IRS showing the higher free energy was assumed to act as a transcription terminator. But the de-

Table 3. Base composition of each position in the codons of *B.stearothermophilus xynA* gene.

position	Base composition(%)				% G+C
	A	G	C	T	
1st	36	30	13	21	43
2nd	28	28	22	22	50
3rd	14	34	31	21	64
Total	26	31	22	21	53

Table 4. Comparison of GC content of the *B. stearothermophilus xynA* gene with those of other genes of thermophile and methophile.

Strains	Gene	% G+C	% G+C at the third base of codon	Reference
Extreme thermophile	*T.thermophilus*			
	Isopropylmalate dehydrogenase	79	89	14
Moderate thermophile	*B.stearothermophilus*			
	Xylanase(*xynA*)	53	64	this study
	β-xylosidase (*xylA*)	56	63	22
	B.stearothermophilus			
	Neural protease	58	72	28
	α-Amylase	50	57	21
	Tyrosyl-tRNA synthetase	54	70	33
Methophile	*B.subtilis*			
	Neutral protease	44	42	35
	Subtilisin	46	41	27
	B. amyloliquefaciens			
	Neutral protease	46	49	31
	Alkaline protease	49	46	31
	Average of 64 *E.coli* genes	53	55	11

Table 5. Comparison of codon usage frequency*.

CODON	#	%AGE	CODON	#	%AGE	CODON	#	%AGE	CODON	#	%AGE
TTT-Phe	1	0.5% (1.3)	TCT-Ser	0	0.0% (1.3)	TAT-Tyr	7	3.3% (1.0)	TGT-Cys	0	0.0% (0.4)
TTC-Phe	5	2.4% (2.2)	TCC-Ser	3	1.4% (1.5)	TAC-Tyr	8	3.8% (1.5)	TGC-Cys	0	0.0% (0.5)
TTA-Leu	3	1.4% (0.7)	TCA-Ser	1	0.5% (0.4)	TAA-***	1	0.5%	TGA-***	0	0.0%
TTG-Leu	0	0.0% (0.9)	TCG-Ser	4	1.9% (0.6)	TAG-***	0	0.0%	TGG-Trp	11	5.2% (0.7)
CTT-Leu	2	0.9% (0.8)	CCT-Pro	3	1.4% (0.5)	CAT-His	0	0.0% (0.7)	CGT-Arg	1	0.5% (3.1)
CTC-Leu	1	0.5% (0.8)	CCC-Pro	1	0.5% (0.3)	CAC-His	1	0.5% (1.2)	CGC-Arg	1	0.5% (2.0)
CTA-Leu	1	0.5% (0.2)	CCA-Pro	0	0.0% (0.7)	CAA-Gln	4	1.9% (1.0)	CGA-Arg	1	0.5% (0.2)
CTG-Leu	4	1.9% (6.8)	CCG-Pro	1	0.5% (2.5)	CAG-Gln	4	1.9% (3.2)	CGG-Arg	2	0.9% (0.2)
ATT-Ile	2	0.9% (2.2)	ACT-Thr	2	0.9% (1.1)	AAT-Asn	10	4.7% (1.0)	AGT-Ser	4	1.9% (0.3)
ATC-Ile	4	1.9% (3.7)	ACC-Thr	8	3.8% (2.4)	AAC-Asn	8	3.8% (2.8)	AGC-Ser	10	4.7% (1.4)
ATA-Ile	0	0.0% (0.2)	ACA-Thr	0	0.0% (0.3)	AAA-Lys	2	0.9% (4.1)	AGA-Arg	2	0.9% (0.1)
ATG-Met	6	2.8% (2.8)	ACG-Thr	12	5.7% (0.8)	AAG-Lys	5	2.4% (1.3)	AGG-Arg	1	0.5% (0.1)
GTT-Val	7	3.3% (2.9)	GCT-Ala	3	1.4% (2.6)	GAT-Asp	4	1.9% (2.5)	GGT-Gly	1	0.5% (3.8)
GTC-Val	1	0.5% (1.2)	GCC-Ala	1	0.5% (2.2)	GAC-Asp	2	0.9% (3.0)	GGC-Gly	11	5.2% (3.1)
GTA-Val	1	0.5% (1.8)	GCA-Ala	4	1.9% (2.3)	GAA-Glu	2	0.9% (4.9)	GGA-Gly	7	3.3% (0.4)
GTG-Val	7	3.3% (2.2)	GCG-Ala	4	1.9% (3.2)	GAG-Glu	2	0.9% (1.8)	GGG-Gly	8	3.8% (0.6)

* The number in parenthesis represents the condon usage frequency of *E. coli* (1). Three asterisks represent translation termination codon.

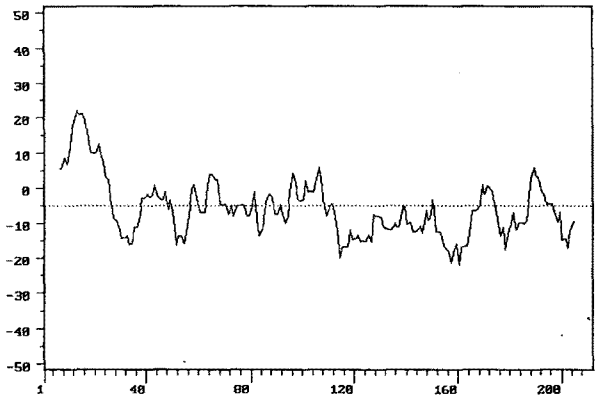

Fig. 3. Hydropathy plot of the pre-XynA.
The abscissa of the panel shows amino acid number. The ordiante shows the average hydrophobicity (positive ordinate) or hydrophilicity (negative ordinate) of amino acid residue.

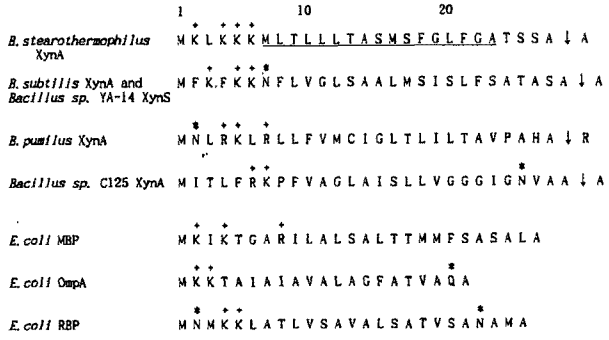

Fig. 4. Comparison of the *B. stearothermophilus* xylanase A signal peptide with signalpeptides of other *Bacillus* xylanases and *E. coli* proteins (maltose-binding protein (MBP), outer-membrane protein A (OmpA), and ribose-binding protein (RBP)).
The predicted membrane-spanning segments are underlined. Positively charged amino acids are indicated by + and polar amino acids are marked by * above their single-letter codes. Possible cleavage sites are shown by the ↓.

letion subclones, pMG119B26, pMG119B23, and pMG 119B20, which contained only the first IRS were found to show much lower xylanase activity than those containing both IRSs. Therefore, the second IRS would be also important for the efficient expression of the *xynA*.

GC Content

The base composition and the GC content of each position in the codons of the *xynA* gene were shown in Table 3. The total GC content of the *xynA* was 53% and the GC content of the 3rd position of codons was calculated to be 64%. This high GC content of the *xynA*, especially in the wobble position of codons, correlated well with the fact that the *xynA* gene was from a moderate thermophile, *Bacillus stearothermophilus* (Table 4).

Codon Usage

Codon usage for the pre-XynA was presented in Table 5 and averaged codon usage for *E. coli* (1) was also included for comparison. There appeared to be a bias for codon selection, for example, TTC for Phe, ACC and ACG for Thr, TAC for Tyr, AGC for Ser, and GGC and GGG for Gly. The higher GC content of *B. stearothermophilus* compared to that of *E. coli* was clearly evident in the preferred usage of G or C in the wobble position of all codons.

The frequency of Thr, Tyr, and Gly in the pre-XynA, which were 10.4%, 7.1%, and 12.8%, respectively, were found to be considerably higher than the average frequency of *E. coli*(4.6%, 2.5%, and 7.9% for Thr, Tyr, and Gly, respectively). There were no codons for Cys.

Signal Sequence

Hydropathy plot of the pre-XynA shows a hydrophobic

region near the N-terminus, which represents a signal peptide(Fig. 3). The amino acid sequence of the signal peptide was deduced from the DNA sequence of the N-terminal region of the *xynA*. It consists of 27 amino acids, of which 4 residues are basic (Lys) and concentrated in the region near the N-terminus, and the following 21 are hydrophobic amino acid residues.

Signal sequences of some other *Bacillus* spp. genes and *E. coli* genes are compared in Fig. 4. The signal peptides of gram-positive exoproteins including *B. stearothermophilus* XynA carry a higher net positive charge at their amino termini and have the longer hydrophobic region than those of the signal peptides of the *E. coli* envelope proteins. *B. subtilis* has been reported to secrete inefficiently the proteins fused with the signal peptides from *E. coli* (6). But the *E. coli* harboring plasmid pMG12 was shown to secrete efficiently *B. stearothermophilus* xylanase into the culture broth and the periplasmic space (data not shown).

Comparison with Other Xylanases

The nucleotide sequence of the *xynA* and its deduced amino acid sequence were compared with those of other xylanase genes and found to have high homology with several xylanases which belonged to family G as classified

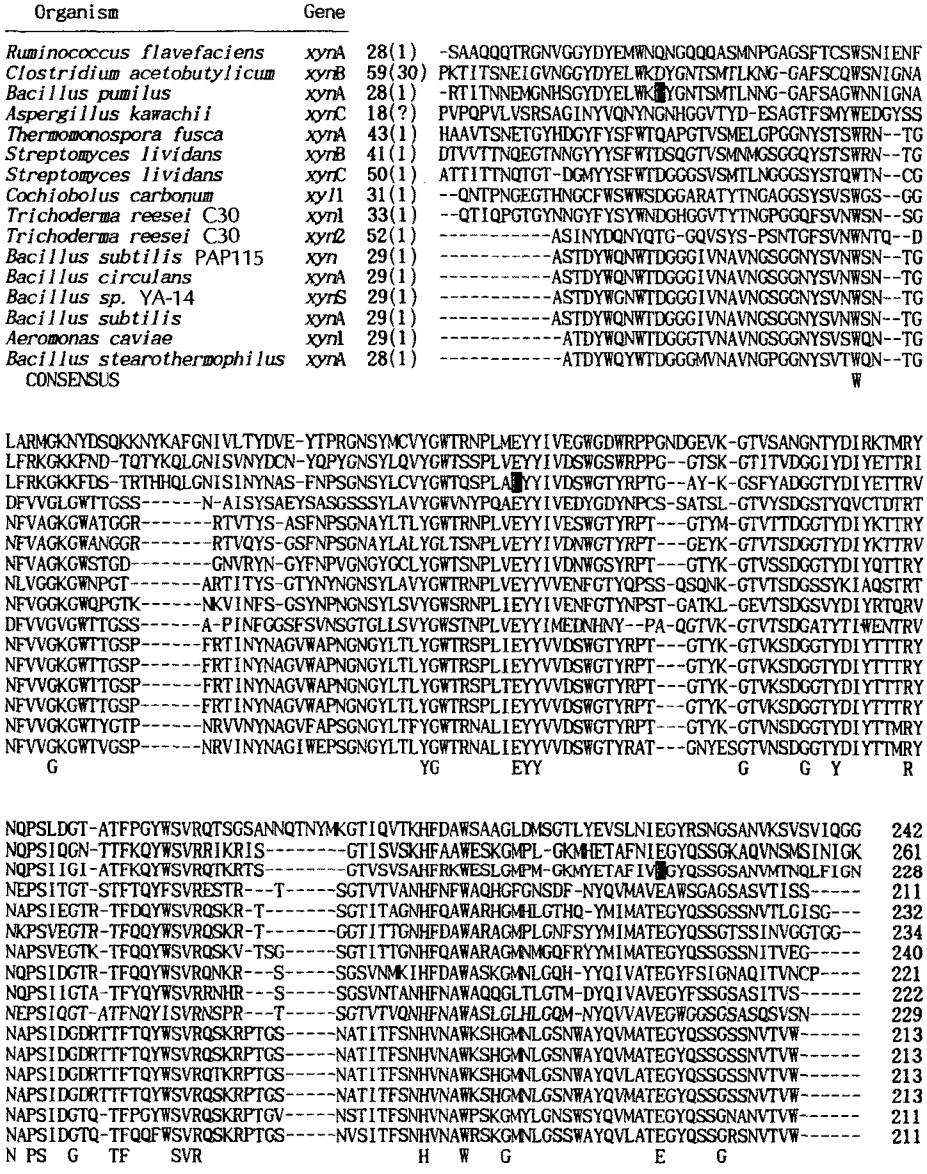

Fig. 5. Amino acid (AA) sequence alignment of the *B. stearothermophilus* xylanase and other family G xylanases.
AA numbers begin with the start codon: the AA numbers from the mature N termini are given in parentheses. Residues shown to be important for the catalytic activity of *B. pumilus* XynA by Ko *et al.* (18) are indicated by D, E. References and GenBank accession numbers are given in Materials and Methods.

by Gilkes *et al.* (10). In the nucleotide sequence, the *xynA* gene showed 73% homology with *Aeromonas caviae xyn*1, 61% with *Bacillus subtilis xynA*, and 60% with *Bacillus* sp. *xynS*.

From the computer comparison of the amino acid sequence of the processed family G xylanases (Fig. 5), XynA was shown to have 88% identity with *Aeromonas caviae* Xyn1, 84% with *Bacillus subtilis* XynA, *Bacillus* sp. XynS and *Bacillus circulans* XynA.

The enzymatic hydrolysis of xylan was thought to occur via the acid catalysis mechanism similar to that for lysozymes. In the *B. pumilus* XynA, two Glu (E-93 and E-182) were found to be essential for its catalytic activity by site-directed mutagenesis (18). Two residues were conserved in all the 16 family G xylanases including *Bacillus stearothermophilus* XynA as seen in Fig. 5. Mutations of the third acidic amino acid residue indicated in Fig. 5. (D21E and D21S) had a less drastic effect but did lower the V_{max} to less than half that of the wild type and increased the K_M by threefold. This residue is conserved in 12 xylanases including *B.stearothermophilus* XynA.

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