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Characterization of the *xaiF* Gene Encoding a Novel Xylanase-activity-increasing Factor, XaiF

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Abstract The DNA sequence immediately following the *xynA* gene of *Bacillus stearothermophilus* 236 [about 1-kb region downstream from the translational termination codon (TAA) of the *xynA* gene] was found to have an ability to enhance the xylanase activity of the upstream *xynA* gene. An 849-bp ORF was identified in the downstream region, and the ORF was confirmed to encode a novel protein of 283 amino acids designated as XaiF (xylanase-activity-increasing factor). From the nucleotide sequence of the *xaiF* gene, the molecular mass and pI of XaiF were deduced to be 32,006 Da and 4.46, respectively. XaiF was overproduced in the *E. coli* cells from the cloned *xaiF* gene by using the T7 expression system. The transcriptional initiation site was determined by primer extension analysis and the putative promoter and ribosome binding regions were also identified. Blast search showed that the *xaiF* and its protein product had no homology with any gene nor any protein reported so far. Also, in *B. subtilis*, the *xaiF* *trans*-activated the xylanase activity at the same rate as in *E. coli*. In contrast, *xaiF* had no activating effect on the co-expressed β-xylosidase of the *xylA* gene derived from the same strain of *B. stearothermophilus*. In addition, the intracellular and extracellular fractions from the *E. coli* cells carrying the plasmid-borne *xaiF* gene did not increase the isolated xylanase activity, indicating that the protein-protein interaction between XynA and XaiF was not a causative event for the xylanase activating effect of the *xaiF* gene.

Key words: Characterization, *xaiF* gene, a novel xylanase-activity-increasing factor, XaiF

Xylan, a component of plant biomass next abundant to cellulose, has a complex structure based on the β-D-1,4 linked xylopyranoside backbone. The backbone structure is substituted to varying degrees depending on the sources with acetyl, arabinosyl, and glucuronyl side chain groups. Therefore, the complete enzymatic hydrolysis of xylan requires the cooperative actions of a range of xylanolytic enzymes including β-1,4-xylanase, β-xylosidase, α-arabinofuranosidase, esterase, and α-glucuronidase [29]. However, xylanase constitutes the most important hydrolytic agent for the hemicellulose component, and has many potential applications in biotechnological processes.

Xylanases have been isolated and characterized from various microorganisms including fungi, yeasts, and bacteria. In our laboratory, a bacterium possessing a highly efficient xylanolytic system has been isolated and identified as a strain of *Bacillus stearothermophilus* [19]. The genes encoding xylanase [2], β-xylosidases [17, 22], acetyl xylan esterases [9, 10], and α-arabinofuranosidase [7] have been isolated from the genomic DNA of the *B. stearothermophilus* 236, and cloned in the *E. coli* strains. The nucleotide sequence of the xylanase [3], β-xylosidase [16], and acetyl xylan esterase [12] genes have also been determined.

In the course of work on the *xynA* gene coding for xylanase, we observed that deletion of the region immediately downstream to *xynA* resulted in significantly reduced xylanase activity expressed from the upstream *xynA* gene [3]. This prompted us to analyze the sequence of the downstream region, and we found that there was an ORF on the DNA sequence immediately following the *xynA* gene. The ORF was subsequently confirmed to produce a protein of about 32 kDa that was directly responsible for the enhancement of the XynA activity.

The xyanase activity-increasing protein described above was identified as a novel protein by Blast homology search. Here, we describe the results obtained in this study on the characterization of the novel xylanase activity-increasing factor (XaiF) from the *B. stearothermophilus* 236 strain.

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MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Chemicals

As the host strains for the plasmids used in this work, *E. coli* JM109 (*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ (*lac-proAB*)/F'[*traD36 proAB*$^+$ *lacIqZ* ΔM15]) [30] and *B. subtilis* MW15 (*his nprR2 nprE18 ΔaprA3 ΔeglS102 ΔbglT bglSRV ΔxynA* CmR) [28] were used. *E. coli* BL21 (DE3) pLysS (*hsdS gal ompT dcm* (cI*ts*857, *ind*1, Sam7, *nin*5, *lac*UV5-T7 gene 1) [pLysS Cam^r]) [27] was also used as the host for the T7 expression system, and *Bacillus stearothermophilus* 236 was used for the preparation of total cellular RNA.

Plasmid pMG119B25 which carries about 2.4-kb *B. stearothermophilus* 236 genomic DNA was used for the source of the *xynA* gene [3]. Plasmids, pBluescript KS (-), pBluescript SK (-) (Stratagene, U.S.A.), and pWP18 [5, 26], were used as subcloning vectors.

All enzymes used in this study were obtained from Promega Corporation (Madison, WI, U.S.A.) or New England Biolabs (Beverly, MA, U.S.A.) and used according to the manufacturer's instructions.

General Methods

Standard methods of molecular biology [18] were used unless otherwise specified. Transformation into *E. coli* JM109 and screening of xylanase positive subclones were performed as previously described [2].

Transformation of the *B. subtilis* MW15 cells [5] and measurement of the xylanase and β-xylosidase activities [2, 17] were done according to procedures previously described.

Enzyme Preparation

E. coli cells harboring the relevant plasmid were grown in LB medium supplemented with appropriate antibiotics. The cells were harvested by centrifuging with a Beckman JA10 rotor at 6000 rpm for 20 min at 4°C and washed twice with 50 mM phosphate buffer (pH 7.0). The collected cells were resuspended in the same buffer and sonicated until they were completely lysed. Cell debris was spun down with a Beckman JA20 rotor at 10,000 rpm for 15 min. The supernatant obtained after the last centrifugation was used as the intracellular protein fraction, and the culture fluid as the extracellular fraction.

B. subtilis MW15 containing a derivative of pWPBR18 was cultured in LB supplemented with 10 μg/ml of kanamycin at 37°C for 12 hr and centrifuged to obtain extracellular enzyme solution.

Construction of pBluescript and pACYC184-derived Plasmids

Plasmid pMG119B25 and pMG119B23 [5] were double-digested with *Hin*dIII and *Eco*RI, and the insert DNAs of about 2.4 kb and 1.0 kb were isolated from the respective plasmids. Then, each of the *Hin*dIII-*Eco*RI insert DNA fragments was ligated with *Hin*dIII-*Eco*RI digests of pBluescript SK (-) to produce pMGB25 and pMGB23 (Fig. 1), respectively.

To construct pMGC25, pMGB25 was digested with *Hin*dIII and *Bam*HI, and the 2.4-kb *Hin*dIII-*Bam*HI insert DNA fragment was cut again with *Pvu*II which produces a blunt end. The *Pvu*II-*Bam*HI fragments which contain the region downstream to the *xynA* gene was ligated with *Sma*I-*Bam*HI digests of pMGB23.

To obtain pMGC25R, pMGC19R, pMGC17R, pMGC11R, and pMGC23R, various sizes of *Hin*dIII-*Eco*RI insert DNA fragments were isolated by digesting pMG119B25, pMG119C19, pMG119C17, pMG119C11, and pMG119B23 with *Hin*dIII and *Eco*RI, and cut again with *Pvu*II. The resulting *Pvu*II-*Eco*RI fragments which contain various sizes of the downstream region were ligated with *Sma*I-*Eco*RI digests of pMGB23.

The *Pvu*II-*Bam*HI and *Pvu*II-*Eco*RI fragments from pMGB25 were ligated with *Sma*I-*Bam*HI or *Sma*I-*Eco*RI digests of pBluescript SK (-) to produce pBSB25D and pBSB25R, respectively.

For constructing pBSB25, pBSC19, pBSC17, pBSC11, and pBSB23 (Fig. 2), each of the *Pvu*II-*Eco*RI fragments from pMG119B25, pMG119C19, pMG119C17, pMG 119C11, or pMG119B23 was ligated with the *Sma*I-*Eco*RI digest of pBluescript KS (-), and for pBSC19R, pBSC17R, pBSC11R, and pBSB23R, the respective

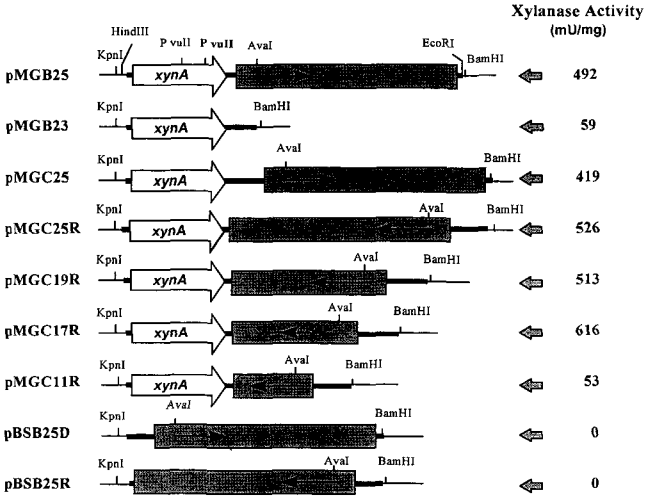

Fig. 1. Effect of the region downstream to the *xynA* gene on xylanase activity.

Xylanase activity from the extracellular fraction of each plasmid-harboring *E. coli* JM109 cell is given on the right. The arrow (⬅) indicates the direction of transcription of the *lacZ'* and amp^R (β-lactamase) genes. The thick line represents foreign DNA and the thin line indicates pBluescript SK (-). The box (■) denotes the region downstream to the *xynA* gene and the arrow in the box shows the orientation of the region.

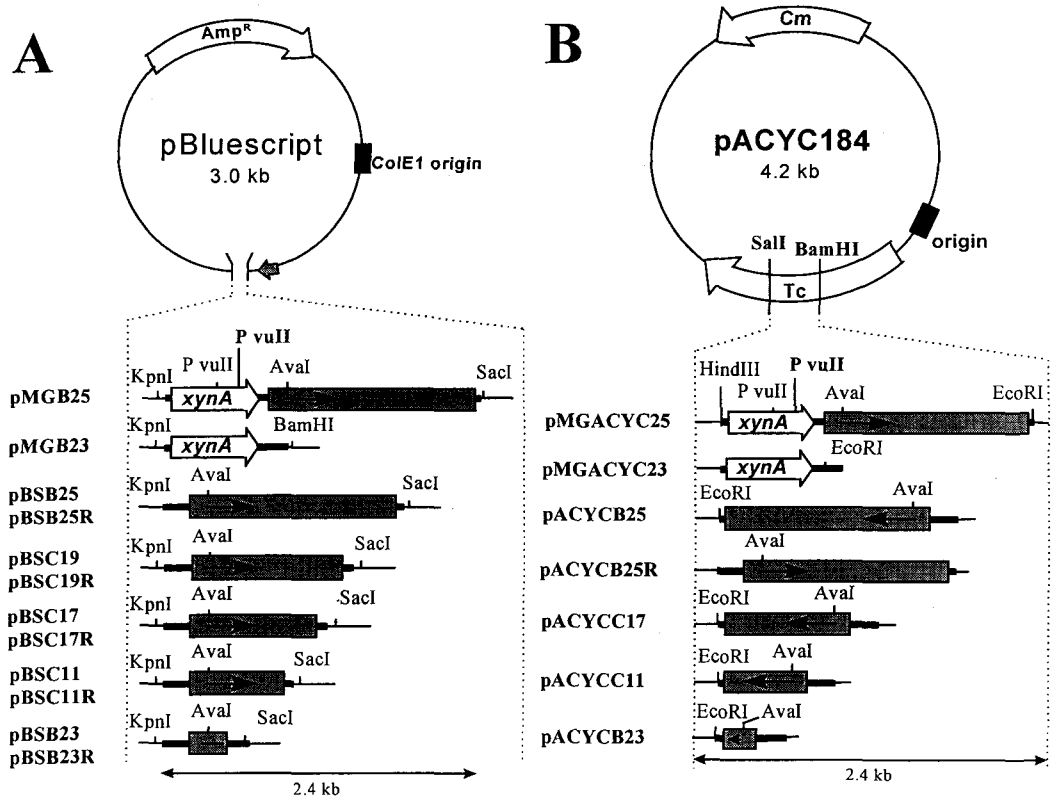

Fig. 2. Structures of pBluescript and pACYC184-derived plasmids.

Construction of the plasmids is described in Materials and Methods. The box (■) denotes the region downstream to the *xynA* gene and the arrow in the box shows the orientation of the region. A. The arrow (⬅) indicates the direction of transcription of the *lacZ'* promoter. The thick line represents foreign DNA. The thin line and circle indicate pBluescript SK (-) (for pMGB25, pMGB23, pBSB25R, pBSC19R, pBSC17R, pBSC11R, and pBSB23R) or pBluescript KS (-) (for pBSB25, pBSC19, pBSC17, pBSC11, and pBSB23). In pBSB25, pBSC19, pBSC17, pBSC11, and pBSB23, the *Kpn*I-*Sac*I fragments have the same orientation with the figure, but in pBSB25R, pBSC19R, pBSC17R, pBSC11R, and pBSB23R, the orientation of the fragments are reversed, i.e., the *Kpn*I site is closest to the *lacZ'*] promoter (⬅). Amp^R means ampicillin-resistance gene. B. Plasmid pACYC 184 carries the origin (■) of replication from plamid p15A, which enables it to co-exist with vectors that carry the ColE1 origin. Cm means the chloramphenicol-resistance gene from Tn*9*, and Tc indicates the tetracycline-resistance gene from pSC101.

*Pvu*II-*Eco*RI fragments were ligated with *Sma*I-*Eco*RI digests of pBluescript SK (-).

To obtain pMGACYC25, pMGACYC23, pACYCB25, pACYCB25R, pACYCC17, pACYCC11, and pACYCB23 (Fig. 2), plasmid pACYC184 was double-digested with *Sal*I and *Bam*HI, and the *Sal*I-*Bam*HI fragments which contain the origin of replication from plasmid p15A were isolated. Then, the fragments were ligated with the various sizes of respective *Sal*I-*Bam*HI insert DNA fragments produced by digesting pMGB25, pMGB23, pBSB25, pBSB25D, pBSC17, pBSC11, and pBSB23 with *Sal*I and *Bam*HI.

Overproduction of XaiF by the T7 Expression System

The *E. coli* BL21 (DE3) pLysS cells transformed with the plasmids encoding *xaiF* were grown in LB supplemented with 100 μg/ml carbenicillin. When the cell density reached A_{600}=0.6~0.8, the cells were induced to produce T7 RNA polymerase by adding IPTG in a final concentration of 0.4 mM and incubated for an additional 2 hours.

Then, cell lysates obtained were analyzed by SDS-PAGE (12% polyacrylamide) as described previously [1].

Construction of the pWPBR18 Shuttle Vector

An *E. coli-B. subtilis* shuttle vector, pWPBR18 was constructed by ligating the *Eco*RI digest of pWP18 with an *Eco*RI fragment of pBR322 (2.3 kb; 2067-4362) obtained by digesting a pBR322-mutant plasmid on which the *Pvu*II site (2064) had been substituted for the *Eco*RI site.

DNA Cycle Sequencing

Plasmid DNA was isolated according to the Qiagen DNA isolation procedure using a Qiaprep spin miniprep kit (Qiagen Inc., Germany). DNA cycle sequencing was performed using ABI PRISM dye terminator cycle sequencing kit (Perkin Elmer Co., U.S.A.) according to the manufacturer's instructions, and the nucleotide sequence was analyzed using the ABI PRISM 310 genetic analyzer.

Preparation of Total RNA from *B. stearothermophilus* 236

B. stearothermophilus 236 was grown in 50 ml of the optimal medium for xylanase production at 45°C and total cellular RNA was isolated from the cells as described earlier [4].

Primer Extension Analysis

Primer extension analysis was performed as described by Hendrickson and Misra [8] with slight modification. About 100 ng of the synthetic oligonucleotide Xn2 (5'-GCCAAAACCTCACCCGAGCC-3'; synthesized by Gibco BRL, USA) was labeled with [γ-^{32}P]ATP (Amersham Corp., U.S.A.) by T4 polynucleotide kinase. The labeled DNA was hybridized with 50 μg of total RNA of *B. stearothermophilus* 236. After hybridization, the nucleic acid recovered by ethanol precipitation was resuspended with 25 μl of reverse transcriptase buffer (50 mM Tris HCl (pH 7.6), 60 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, and 0.4 mM each dNTP). The reverse transcription reaction was started by adding 40 U of AMV (avian myeloblastosis virus) reverse transcriptase (Promega, U.S.A.). The reaction was terminated after incubation at 42°C for 90 min by heating at 70°C for 10 min. After RNaseA treatment and subsequent phenol-chloroform extraction, the nucleic acid was precipitated with ethanol, washed with 70% ethanol, and the dried pellet obtained was resuspended in 6 μl of the TE and formamide loading buffer mixture. The samples thus prepared were heated to 90°C for 3 min and loaded on a sequencing gel along with the sequence standards prepared by the dideoxy nucleotide chain termination method using the Xn2 oligomer as a sequencing primer.

RESULTS

Influence of the Region Immediately Downstream to the *xynA* Gene on the Xylanase Activity Expressed by the *xynA* Gene

During work on the *xynA* gene from *B. stearothermophilus* 236, we obtained an intriguing result result that deletions of a region downstream to the *xynA* gene resulted in a significant reduction of the xylanase activity expressed from the upstream *xynA* gene [3].

To define a precise region that was critical for the xylanase activity-increasing effect, we constructed a series of 3'-end deletions of the 2.4-kb insert DNA on the pMG119B25 described previously [3], and examined the xylanase activities of the *E. coli* strains carrying the deletion mutant plasmids.

Deletion of the whole downstream DNA produced an approximately 8-fold reduction of the xylanase activity. From the deletion experiments, we also found that a larger than 1.68-kb DNA of the 2.4-kb insert was necessary for retention of the fully enhanced levels of the activity (data not presented).

On the other hand, the *E. coli* JM109 strains harboring only the plasmid bearing the region downstream to *xynA* (*E. coli* JM109/pBSB25D and *E. coli* JM109/pBSB25R) presented no detectable xylanase activities, as illustrated in Fig. 1. In addition, we also observed that the region

Table 1. *Trans*-acting effect of the region downstream to the *xynA* gene on the xylanase activity of the *xynA* gene[a].

pBluescript -derived plasmid	Xylanase activity (mU/mg)[b]							
	PACYC184-derived plasmid							
	None[d]	PACYC184	PMGACYC23	pACYCB25	pACYCB25R	pACYCC17	pACYCC11	pACYCB23
None[e]	-[f]	0	62	0	0	0	0	0
pBS[c]	0	0	56	0	0	-	0	-
pMGB25	432	410	583	469	458	-	431	-
pMGB23	57	58	86	376	396	407	56	57
pBSB25	0	0	357	0	-	-	-	-
pBSB25R	0	0	413	-	0	-	-	-
pBSC19	0	-	395	-	-	0	-	-
pBSC17	0	-	458	-	-	-	-	-
pBSC11	0	-	65	-	-	-	0	-
pBSC11R	0	-	60	-	-	-	-	-
pBSB23	0	-	49	-	-	-	-	-

[a]*E. coli* JM109 cells which contain various plasmids were grown in LB medium supplemented with appropriate antibiotics (ampicillin (50 μg/ml) for pBluescript-derived plasmid and chloramphenicol (35 μg/ml) for pACYC184-derived plasmid) for 12 hr at 37°C.

[b]Xylanase activity (mU/ml) was determined from the extracellular fraction of the *E. coli* JM109 cell in which each of the pACYC184-derived plasmids, carrying the origin of replication from plasmid p15A, co-exists with each of the pBluescript-derived plasmids which carries the ColE1origin. The activity was divided by the concentration of total protein (mg/ml) of each fraction.

[c]Abbreviation of pBluescript SK (-).

[d]Cells containing only pBluescript-derived plasmid.

[e]Cells containing only pACYC184-derived plasmid.

[f]-: Not determined.

downstream to *xynA* exerted its xylanase-increasing ability regardless of its orientation to the upstream *xynA* gene (compare the xylanase activities of *E. coli* strains carrying pMGC25 and pMGC25R in Fig. 1).

Effect of the Downstream Region in the *Trans* Position

To examine whether the downstream sequence can also function in the *trans* position, we constructed several

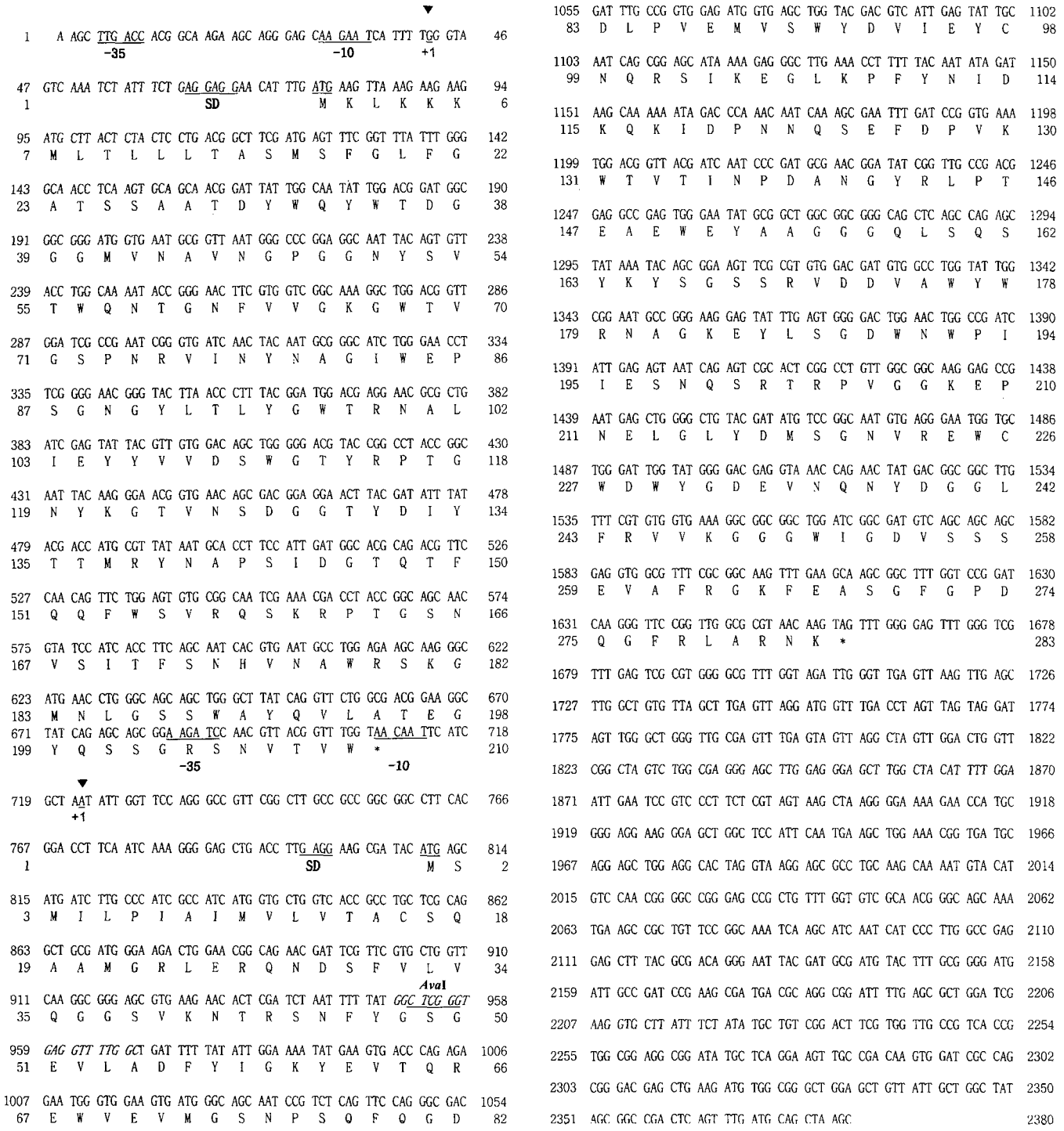

Fig. 3. Nucleotide sequences of the *xynA* and *xaiF* genes, and the deduced amino acid sequences of XynA and XaiF.

The promoter, Shine-Dalgarno (SD), translational initiation sequences, and *Ava*I site are underlined. The transcription initiation sites are indicated as ▼ mark and the synthetic oligonucleotide Xn2 (5'-GCCAAAACCTCACCCGAGCC-3'), which was used to determine the transcription initiation site of the *xaiF* gene by primer extention analysis, are in italic letters.

pBluescript SK/KS-derived plasmids and pACYC184-derived plasmids which were able to co-exist in *E. coli* cells (Fig. 2).

A pBluescript-derived plasmid and a pACYC184-derived plasmid were co-transferred into the appropriate *E. coli* cells, and the xylanase activity was measured from the extracellular fraction of the co-transformed cells. As can be seen in Table 1, the cells harboring pMGB25 and any one of pACYC-derived plasmids illustrated in Fig. 2 produced the same high levels of activity as that shown by the strain carrying pMGB25 alone.

In contrast, when pMGB23 carrying only the *xynA* gene fragment co-existed with the pACYC184-derived plasmids, only the plasmid containing the full downstream sequence resulted in an increased level of the xylanase activity. These results together demonstrate that the downstream region exerts its xylanase activity-increasing effect through a diffusible factor expressed from the region.

The Xylanase Activity-increasing Factor (XaiF)

As described above, the region downstream to *xynA* could function also *in trans*, and thus we determined the nucleotide sequence of the region to search for the presence of an open reading frame (ORF) encoding a potential *trans*-acting protein product.

An 849-bp ORF was found on the downstream DNA sequence, and was expected to encode a protein of 283 amino acids (Fig. 3). The molecular mass and pI value of the protein were deduced from the nucleotide sequence to be 32-kDa and 4.46, repectively. Thus, we named the putative protein product XaiF (the xylanase activity-increasing factor).

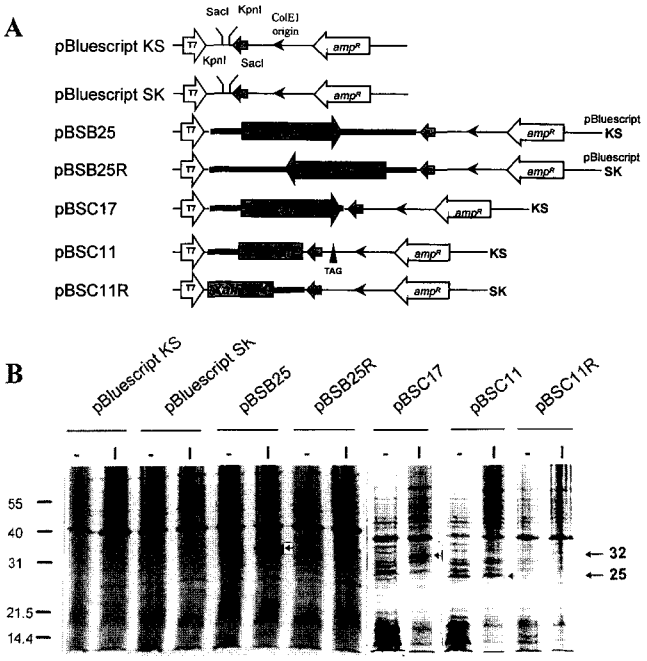

Fig. 4. Overproduction of XaiF.

A. Structures of plasmids used in overproduction of XaiF by the T7 expression system. The arrows, ⬅ and ⇨, indicates the direction and location of the *lacZ'* and T7 promoters, respectively. The translational termination codons on the sequence of pBluescript KS are indicated with ▲. The thick line represents foreign DNA containing *xaiF* gene and the thin line indicates pBluescript KS (-) for pBluescript KS, pBSB25, pBSC17, and pBSC11 and pBluescript SK (-) for pBluescript SK, pBSB25R, and pBSC11R. B. About 32 kDa protein, XaiF, overproduced by the T7 expression system. Protein samples from IPTG treated (|) and untreated (-) cells were run on SDS-PAGE (12% polyacrylamide). Numbers in the left margin indicate the mass of molecular weight markers. Arrows and numbers in the right margin indicate the induced protein bands and their mass (in kDa), respectively.

Overproduction of XaiF

To confirm that XaiF, a 32-kDa putative protein, was indeed produced from the putative *xaiF* gene, we attempted to overexpress the *xaiF* gene by using the T7 expression system.

E. coli BL21 (DE3) pLysS cells carrying one of the pBluescript-derived plasmids illustrated in Fig. 4A were induced by adding IPTG into the culture medium, and the cell lysates, prepared according to the procedure described in Materials and Methods, were analyzed by SDS-PAGE.

As expected, from the control *E. coli* strain harboring pBluescript KS/SK, no protein band of 32-kDa was detected (Fig. 4.B), and the cell lysate from pBSB25R also did not produce the expected protein. In contrast, a protein band of about 32-kDa which was suspected to be XaiF was observed from the lysate prepared from the culture of the *E. coli* strain carrying the pBSB25 plasmid.

Furthermore, from the *E. coli* cells harboring a partially deleted plasmid-borne *xaiF* (pBSC 11), a new protein of about 25-kDa was overproduced instead of the 32-kDa protein described above. This small protein might be a product of translation of the *xaiF* mRNA that had been terminated at the new termination codon (TAG) generated on the pBluescript KS sequence as indicated in Fig. 4A. Taken together, these results indicate that XaiF was indeed produced from the ORF (*xaiF*) on the region downstream to *xynA*.

In addition, the transcription initiation nucleotide of the *xaiF* gene was determined by primer extension analysis using a synthetic oligonucleotide, Xn2 (5'-GCCAAAACCTCACCCGAGCC-3'). The adenine start nucleotide was assigned as the +1 site in Fig. 3 and Fig. 5.

Analysis of the Nucleotide Sequence of *xaiF*

In the upstream region of the transcription initiation site, a putative promoter and a ribosome binding site were also recognized and are indicated in Fig. 3. The -10 (AACAAT) and -35 (AAGATC) elements, and the

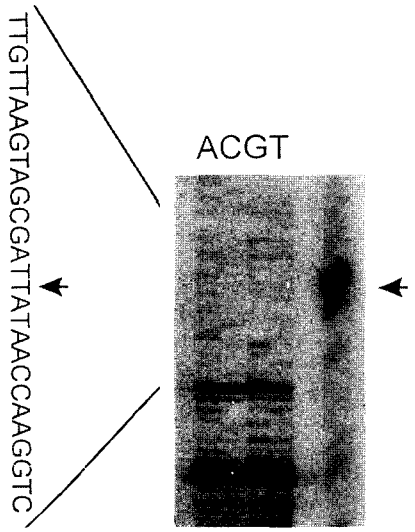

Fig. 5. The transcriptional initiation site of the *xaiF* gene determined by primer extension analysis.

The recombinant plasmid pBSC17R was sequenced using Xn2 (5'-GCCAAAACCTCACCCGAGCC-3') as a primer to determine the length of extension. Primer extension was performed on total RNAs of *B. stearothermophilus* 236 with labeled primer Xn2. The start site of transcription is marked with an arrow.

space length between the two elements were assessed to support efficient transcription. In addition, the free energy between the *xaiF* SD sequence and the 3'-end of *B. subtilis* 16S rRNA was calculated to be -9.4 kcal/mol by the method of Tenoco *et al.* [25]. This free energy value is much lower than the average value (17.6 kcal/mol) for gram-positive bacteria [13]. The total G+C content of the *xaiF* gene was 53% and the GC content of the 3rd position of codons was calculated to be 66%. This high G+C content, especially at the wobble position of codons, is consistent with the genes for the xylan degrading enzymes from the same *B. stearothermophilus* strain, including *xynA* [3] and *xylA* [16].

XaiF, a Novel Protein

Blast homology search for the *xaiF* and its protein product (XaiF) was performed through NCBI BLAST sequence similarity searching (http://www.ncbi.nlm.nih.gov/ BLAST/).

No significant sequence homology between the 849-bp *xaiF* and the other sequence data reported hitherto was detected. From this analysis, we did not get any valuable information for characterizing the *xaiF* gene. Similarly, only the short amino acid sequence of XaiF was found to be homologous with other protein sequences available. Moreover, even the proteins showing a slight homology with XaiF were reported to be unknown or hypothetical ones. Conclusively, the Blast search presented evidence that the XaiF was a novel protein.

Hydropathy Plot of XaiF

The hydropathy plot of XaiF revealed a hydrophobic region near its N-terminus, suggesting that the region might be a signal peptide. Furthermore, prediction of a potential signal sequence that had been done with the PC/GENE program indicated that XaiF had a potential signal sequence of 19 or 22 amino acids. Nevertheless, the potential signal sequence of XaiF was not very compatible with the consensus *Bacillus* or *E. coli* signal sequences [3]. It also has no comparable positively-charged amino acid (s) preceding the hydrophobic region. Therefore, at present, it is not clear whether XaiF is a secretion protein.

Effect of the *xaiF* Gene on the β-Xylosidase Activity of the *xylA* Gene

To examine whether the *xaiF* gene was also effective on the β-xylosidase activity, each of the pACYC184-derived plasmids shown in Fig. 2 was co-transferred into the *E. coli* cells carrying pMG1 [16], which contained a ColE1 origin of pUC18 and the entire *xylA* gene encoding the major intracellular β-xylosidase of *B. stearothermophilus* 236. As seen in Table 2, even when pMG1 was co-transfered with pACYCB25 or pACYCB 25R, both of which contain the intact *xaiF*, no significant increase in β-xylosidase activity was recognized, and the levels of the β-xylosidase activity were nearly the same as those for the *E. coli* strains carrying both pMG1 and any one of the plasmids containing no effective *xaiF* genes such as pACYC184, pACYCC11, or pACYCB23.

These results indicate that the *xaiF* gene does not play any role in the production of β-xylosidase activity from the *xylA* gene.

Table 2. Effect of the *xaiF* gene on the β-xylosidase activity expressed by the *xylA* gene[a].

PACYC184 -derived plasmid	β-Xylosidase activity (mU/mg)[b]			
	pUC18		pMG1 (*xylA* gene)	
	Intracellular	Extracellular	Intracellular	Extracellular
None[c]	-[d]	-	328.91	0.10
PACYC184	-	-	324.74	0.09
pACYCB25	0	0	356.26	0.09
pACYCB25R	0	0	359.14	0.12
pACYCC11	0	0	322.25	0.11
pACYCB23	0	0	362.42	0.13

[a]*E. coli* JM109 cells which contain various plasmids were grown in LB medium supplemented with appropriate antibiotics (ampicillin (50 μg/ml) for plasmid pMG1 and chloramphenicol (35 μg/ml) for pACYC184-derived plasmid) for 12 hr at 37°C.

[b]β-Xylosidase activity (mU/ml) was determined from both the intracellular and extracellular fractions of the *E. coli* cell in which each of the pACYC184-derived plasmids, carrying the origin of replication from plasmid p15A, co-exists with plasmid pMG1 which carries the ColE1 origin from pUC18. The activity was divided by the concentration of total protein (mg/ml) of each fraction.

[c]Cells containing only one kind of plasmid (pUC18 or pMG1).

[d]-: Not determined but estimated to be 0 mU/mg.

In-vitro Interactions between XaiF and the Xylanase and β-Xylosidase

In previous studies, we confirmed a prominent synergism between xylanase, β-xylosidase, α-arabinofuranosidase, and acetylxylan esterase in xylan hydrolysis [21, 23]. Hence, the xylanase activity-increasing effect of the *xaiF* gene demonstrated in this study was suspected to be a result of the synergism between XaiF and the xylanase.

To test whether this idea was the case or not, we examined the effects of the intracellular and extracellular protein fractions of the *E. coli* cells, carrying the plasmid borne *xaiF* gene, on the isolated xylanase and β-xylosidase activities. As shown in Table 3, both the intra- and extracellular fractions of the *E. coli* strains carrying pACYCB25 or pACYCB25R have shown no detectable activity-increasing effect on the xylanolytic enzymes. This clearly provides evidence that the protein-protein interaction between XynA and XaiF was not a causative event for the xylanase activating effect of the *xaiF* gene.

Table 3. Effect of XaiF on the xylanase and β-xylosidase activities expressed by *xynA* and *xylA* genes, respectively[a]

PACYC184-derived plasmid		Xylanase activity (mU/mg)[b]		β-Xylosidase activity (mU/mg)[c]	
		None[d]	pMGB23	None[d]	pMG1
None[e]		-	59 (61)	-	330
PACYC184	*In*[f]	0	31 (31)	0	171
	Ex	0	33 (34)	0	158
PACYCB25	*In*	0	32 (34)	0	174
	Ex	0	35 (35)	0	167
PACYCB25R	*In*	0	32 (32)	0	169
	Ex	0	32 (33)	0	176
PACYCC11	*In*	0	30 (32)	0	157
	Ex	0	31 (32)	0	166
PACYCB23	*In*	0	33 (34)	0	162
	Ex	0	34 (34)	0	169

[a]*E. coli* JM109 cells which contain various plasmids were grown in LB medium supplemented with appropriate antibiotics (ampicillin (50 μg/ml) for pMGB23 and pMG1, and chloramphenicol (35 μg/ml) for pACYC184-derived plasmid) for 12 hr at 37°C.

[b]The cells carrying each of the pACYC184-derived plasmids and the cells containing pMGB23 were grown separately. And, 0.25 ml of the extracellular fraction of the cells containing pMGB23 was mixed with the same volume of each of the intracellular and extracellular fractions of the cells carrying each of the pACYC184-derived plasmids. The mixed solution (0.5 ml) was used to measure xylanase activity (mU/ml) and the activity was divided by the concentration of total protein (mg/ml) of each solution. The number in parenthesis means the activity which was measured with oat spelts xylan as substrate.

[c]The cells carrying each of the pACYC184-derived plasmids and the cells containing pMG1 were grown separately. The intracellular fraction of the cells containing pMG1 and each fraction of the cells carrying the pACYC184-derived plasmids were mixed half and half (each 0.05 ml). The mixed solution (0.1 ml) was used to measure β-xylosidase activity (mU/ml) and the activity was divided by the concentration of total protein (mg/ml) of each solution.

[d]Enzyme activity was measured with 0.5 ml (for xylanase) or 0.1 ml (for β-xylosidase) of the fraction from the cell carrying each of the pACYC184-derived plasmids.

[e]To measure xylanase activity, 0.5 ml of the extracellular fraction of the cells containing pMGB23 was used. For β-xylosidase activity, 0.1 ml of the intracellular fraction of the cell containing pMG1 was used.

[f]*In*: Intracellular fraction, *Ex*: Extracellular fraction.

Effect of the *xaiF* Gene on the Xylanase Activity in *B. subtilis*

As in the case of *E. coli*, nearly the same rate (about 8-fold) of the enzyme activation was observed in *B. subtilis* strains containing the plasmid-borne *xynA* and *xaiF* genes. But the *Bacillus* strains produced more than 13-fold higher total activities than the corresponding *E. coli* strains as depicted in Fig. 6.

DISCUSSION

Here, we have shown that the xylanase activity-increasing factor (XaiF) increased the XynA activity (an extracellular enzyme) in *trans* as well as in *cis* positions about 8-fold

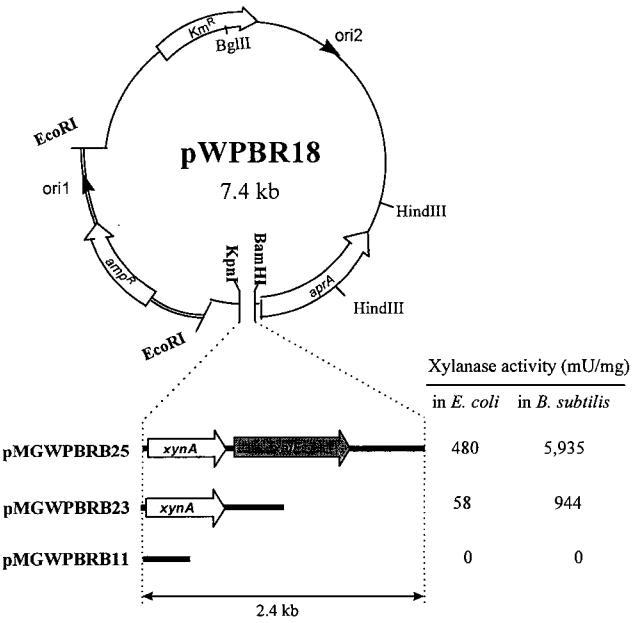

Fig. 6. Effect of the *xaiF* gene on the xylanase acitivity of the *xynA* gene in both of *E. coli* and *B. subtilis* strains.

Each of pMGWPBRB25, pMGWPBRB23, and pMGWPBRB25 was transferred into *E. coli* JM109 and *B. subtilis* MW15. Xylanase activity was determined from each extracellular fraction of the cells grown in LB medium supplemented with appropriate antibiotics (ampicillin (50 μg/ml) for *E. coli* and kanamycin (10 μg/ml) for *B. subtilis*) for 12 hr at 37°C. A shuttle vector, pWPBR18, is composed of a total sequence (5.1 kD; single-lined circle) of pWP18 which is a pUB110 derivative and a part of pBR322 [2.3-kb (2067–4362); double-lined circle]. Ori1 and ori2 are the *E. coli* and *B. subtilis* replication origin, respectively. Km denotes the gene encoded in plasmid pUB110 for kanamycin nucleotidyltransferase. Construction of the plasmids is described in Materials and Methods.

in both *E. coli* and *B. subtilis strains*. The XaiF was found to be a novel protein encoded by an 849-bp ORF (*xaiF*) located on the region immediately downstream to the *xynA* gene of *Bacillus stearothermophilus* 236.

The *xaiF* has displayed no stimulatory effect on the β-xylosidase activity of the *xylA* (an intracellular enzyme) from the same *B. stearothermophilus* strain. In addition, both the intracellular and extracellular fractions of the recombinant *E. coli* cells carrying the *xaiF* gene in their plasmid revealed no detectable effect on xylanase activity of the isolated XynA enzyme, indicating that the direct interaction between the two proteins was not the cause for the xylanase increasing effect shown by the *xaiF* gene. It was also confirmed that XaiF itself did not have any xylanolytic activities. Taken together, the results described above suggest that the *xaiF* gene product may be a *trans*-acting regulator controlling the expression of the *xynA* gene. In *Bacillus subtilis*, the DegS-DegU signaling system that is an example of the two-component signal transduction pathway which has been identified and studied extensively in recent years [6, 14], was reported to control the synthesis rate of a set of both secreted and intracellular enzymes including levansucrase, intra- and extracellular proteases, α-amylase, β-glucanase (s), and xylanase (s).

Takagi *et al.* [24] have described another new regulatory gene, *degT* from *B. stearothermophilus* NCA 1503, that had also exhibited similar pleiotropic mutant phenotypes to those of the mutant DegS-DegU system. DegT was analyzed and found to contain a hydrophobic core region in the N-terminal portion, a consensus sequence for a DNA binding region, and a region homologous to those for transcription activators. From this sequence analysis, they expected that DegT, a membrane protein, could function as a sensor protein and transfer the environmental signal to the targets to activate or to repress transcription of the target genes. Since then, several other regulatory proteins with the specific features of sensor proteins have also been reported [11, 15, 20].

Analysis of the sequence of the XaiF protein has shown it to have some regions comparable to those of DegT even though XaiF showed, in overall structure, no significant homology with DegT and other Deg proteins described above. XaiF has a highly hydrophobic region at its N-terminus which is predicted to form a transmembrane α-helix. Furthermore, in the region between the hydrophobic and hydrophilic regions of the XaiF (amino acid positions 18 to 37), a possible sequence for DNA binding was also found, although there were some deviations from the conserved sequence for the DNA-binding proteins. Nevertheless, a domain homologous to the motif for the transcription activator proteins was not recognized from the amino acid sequence of XaiF.

Based on the observations described above, we hypothesize that like the Deg proteins, XaiF may be a membrane protein that functions as a sensor protein which transfers the signal of environmental stimuli to the regulatory region of the target genes including the *xynA* gene.

To elucidate the mechanism by which XaiF functions *in vivo* in *B. stearothermophilus*, the purification, and characterization, and intracellular localization of XaiF will be performed along with an investigation of the phenotypic changes of the *xaiF*-disrupted mutant strain.

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