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Post-transcriptional regulation of the *xynA* expression by a novel mRNA binding protein, XaiF

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Abstract

XaiF, a novel 32 kDa protein encoded by the ORF located in the immediate downstream of the *xynA* gene of *Bacillus stearothermophilus* No. 236, was identified to be the xylanase-specific *trans*-activator. In this study, the positive effect of XaiF was confirmed to be xylanase-specific, and the results from Northern blot and *in vitro* transcription assays showed that the XaiF increased the *xynA* transcripts at post-transcriptional step. Moreover, analysis of the mRNA decay rate led to the assertion that the XaiF functions to stabilize the *xynA* mRNA. Intriguingly, *in vitro* RNA–protein binding assay and analysis using *gst-xynA* 3′-UTR chimeric gene constructs demonstrated that the XaiF stabilizes *xynA* mRNA by direct binding onto the 3′-UTR of the mRNA.

Keywords: *Bacillus stearothermophilus* No. 236; Xylanase; *xynA*; *xaiF*; Post-transcriptional regulation; mRNA binding protein; 3′-Untranslational region; mRNA stability

Xylan, an abundant major hemicellulose component in plant cell wall, has the β-1,4 linked xylopyranoside backbone [1]. The major component of the xylanolytic enzyme is endo-xylanase which has many potential applications in various biotechnological processes [2]. The genes encoding xylanolytic enzymes are subjected to carbon catabolite repression (CCR) in the presence of favorable carbon sources such as glucose. The components involved in CCR have been discovered; a *trans*-acting factor called catabolite control protein A (CcpA), *cis*-acting sequence termed *cre*, and the HPr protein of the phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS) and their molecular mechanisms have been extensively studied [3]. However, the induction or activation mechanism of xylanolytic genes is far less known.

In the previous work, an ORF encoding a novel protein of 283 amino acid residues was identified in the immediate downstream of the *xynA* gene. The gene product was found to significantly enhance the xylanase activity when co-expressed with the *xynA* gene, and then designated as XaiF (xylanase-activity-increasing factor) [4].

The present study on the XaiF regulatory mechanism revealed that XaiF did bind to a *cis*-acting element in 3′-untranslational region (UTR) of the *xynA* transcript, and the binary complex thus formed may protect the *xynA* mRNA from the 3′- to 5′-ribonucleases (RNases)-mediated degradation.

In recent years, mRNA decay has emerged as an important factor in the post-transcriptional regulation of gene expression in bacteria [5]. Of particular note, in bacteria, the 5′-end of transcripts is reported to be a major determinant of mRNA stability and the first cleavage is a rate-limiting step in the decay process [6]. Study on the mRNA 3′-UTR-mediated regulation of gene expression is extremely limited [7] and our present report is conceived to be a

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first example that has been found to govern expression of the bacterial genes involved in polysaccharide degradation.

Materials and methods

Bacterial strains and vectors. *Escherichia coli* XL1-blue or BL21 (DE) was used as the host strain for the plasmids construction or T7 overexpression system, respectively. *Bacillus subtilis* MW15, a mutant strain deficient in alkaline protease and xylanase, was used as the gram-positive host strain. Plasmids pUC119 and pET-23b were used for cloning and protein production, respectively. pACYC184 carrying the origin derived from plasmid p15A is compatible with vector carrying the ColE1 origin. pWPBR, an *E. coli–B. subtilis* shuttle vector and pEBP313 [8], a shuttle vector containing a strong EB promoter cloned from *B. subtilis*, used to investigate effect of the XaiF in *B. subtilis*.

Plasmid construction. The 1.8 kb DNA fragment (*xynA*) and 3.1 kb fragment (*xynA* and *xaiF*) were obtained from pHINC5.5 and introduced into pWPBR to generate pW1.8 and pW3.1, respectively.

The 700 bp DNA fragment possessing the region from the second codon of the XynA to 70 bp downstream of the stop codon and the 1670 bp DNA fragment possessing the 700 bp fragment and the downstream *xaiF* gene were amplified by PCR from pW3.1 with primers EBPxA (5′-AGGATCCTAAGTTAAAGAAGA-3′) and EBPxAR (5′-CAAGCTTCCTTTGATTGAAGG-3′), and primers EBPxA and EBPxFR (5′-CGAAGCTTACCATCCTAACTC-3′), respectively. They were digested with *Bam*HI and *Hin*dIII, and introduced into pEBP313 to generate pEBXA and pEBAF, respectively.

The 680 bp DNA fragment (*xynA* ORF) and the 752 bp DNA fragment (*xynA* ORF and the additional 72 bp downstream region) were amplified by PCR with the EBPxAF as forward primer, and XynAR (5′-GCTAAGCTTCCAAACCGTAAC-3′) or EBPxAR as the respective reverse primer. The PCR products digested with *Bam*HI and *Hin*dIII were ligated with pQE30 to generate pQXA710 and pQXA785, respectively.

Assays for xylanase, β-xylosidase, and α-arabinofuranosidase. Xylanase activity was analyzed by the DNS method as described previously [9,10]. β-Xylosidase and α-arabinofuranosidase activities were determined by the released *p*-nitrophenols as previously described [11,12].

Preparation of polyclonal anti-XynA and anti-XaiF antibodies and Western blotting. Polyclonal antibodies against XynA-his6 and XaiF-his6 were prepared by immunizing New Zealand White rabbits with the purified proteins emulsified in complete Freund's adjuvant as described previously [13]. The *xynA* and *xaiF* ORFs were amplified using primers XynAF (5′-CCGCATATGAAGTTAAAGAAG-3′) and XynAR, and primers XevF (5′-ATCATATGAAGTTAAAGAAGA-3′) and XevR (5′-TAAGCTTCCAAACCGTAACGTT-3′), respectively. The PCR products digested with *Nde*I and *Hin*dIII were cloned into pET23b to produce pETXynA and pETXaiF, respectively. The his6-tagged XynA and XaiF were purified under denaturing conditions using the Ni^{2+}–NTA–agarose (Qiagen) according to the manufacturer's instruction. The SDS–PAGE and Western blotting were performed as described previously [14].

In vitro transcription assay. The 300 bp DNA fragments containing the 200 bp upstream region and part of *xynA* ORF were obtained by PCR. The template DNA was incubated at 37 °C for 20 min in the mixture (50 μl) containing 50 mM Tris–Cl (pH 7.9), 10 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 0.01% Triton X-100, 10 U RNase inhibitor, 2.5 mM ATP, 2.5 mM CTP, 2.5 mM GTP, 0.05 mM UTP, 10 μCi [α-^{32}P]UTP and 0.2 U *E. coli* RNA polymerase holoenzyme (Epicentre Biotechnologies) in the presence of 0.1 μg XaiF-his6 protein. At the denoted time point, 10 μl of reaction mixture was withdrawn, mixed with solution containing 2.5 mM cold UTP and 2.5 μg heparin, and incubated for further 5 min. Following the incubation, the reaction was stopped by adding 5 μl of formamide buffer. Samples were run on a 5% polyacrylamide/7 M urea gel and visualized by autoradiography.

Measurement of the xynA mRNA decay. *Bacillus subtilis* MW15 containing plasmid pW1.8 or pW3.1 was grown in LB medium to mid-log phase. After the addition of actinomycin D (200 μg/ml), cells were harvested at the indicated time points. Total RNA was prepared with RNA ZolB (Teltest, USA) and *xynA* mRNA levels were determined by Northern blotting with radio-labeled DNA probes. The relative intensity of each band was determined by TINA program (Fuji Film Co., Ltd., Japan) and plotted as the percentage of the level at the time zero. The slope of this curve was used to calculate the interval period within which half of the original amount of mRNA had decayed.

RNA–protein gel mobility shift assay. The 205 bp fragment corresponding to the region +492 to +707 of *xynA* was amplified by PCR using primers Primer3 (5′-CCATCACCTTCAGCAATCACGTGAATGC-3′) and EBPXAR, and cloned into pT7T3α18 to create pT$_7$200RP. The radio-labeled RNA probe was synthesized with a MEGAscript T7 kit (Ambion) using α-^{32}P-ATP (NEN) and the *Hin*dIII-linearized plasmid. The his6-tagged XaiF was purified under native condition using the Ni^{2+}–NTA–agarose according to the manufacturer's instruction. Radio-labeled RNA probe was incubated at 85 °C for 2 min and slowly cooled to room temperature. RNA–protein binding was conducted in reaction mixtures (20 μl) containing 10 mM Tris–Cl (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, 1 μg of yeast tRNA, 10 U RNase inhibitor, 10% glycerol, 20 mM DTT, 10^5 cpm of labeled RNA, and various concentrations of the purified XaiF-his6. The reaction mixtures were incubated at 4 °C for 30 min and then loaded onto the 5% native polyacrylamide gel and analyzed by autoradiography.

Fusion of the xynA 3′-UTR to the 3′-end of GST ORF. Wild-type 3′-UTR of *xynA* was amplified by PCR with primers xynAF and XF-CR3 (5′-AGTGTCGACATAGCTCTGGCT-3′). About 0.7 kb DNA fragments obtained by digesting the PCR product with *Pvu*II and *Sal*I were introduced into the 3′-end of *gst* gene in pGEX4T1 digested with *Sma*I and *Sal*I, and the resulting construct was designated as pGST+3U(WT). The putative stem–loop in the 3′-UTR of *xynA* mRNA was predicted by MFOLD program [15] and deletion of the region was generated by PCR using primers xynA3UF (5′-TTCAATCAAAGGGGAGCTGAC-3′) and xynA3U(Δ22–65)R (5′-AATATTAGCGATGAATTGTTA-3′), and primers xynA3UF and xynA3U(Δ40–65)R (5′-CCGAACGGCCCTGGAACCAAT-3′) for position 22–65 deletion or for position 40–65 deletion, respectively.

Results

Effect of the XaiF on the xylanolytic genes and influence of carbon sources on the XaiF action

In order to determine whether XaiF exerts coordinate regulation on the activation of other xylanolytic enzymes, effect of XaiF on synthesis of the β-xylosidase and α-arabinofuranosidase was examined. When the *xynA* was co-expressed with the *xaiF*, xylanase activity was obtained about 2.7-fold higher than that produced by *E. coli* carrying no functional *xaiF* gene (Table 1). In contrast, in case with the β-xylosidase or α-arabinofuranosidase gene, XaiF showed no recognizable activation of the enzyme activity, indicating that the positive effect of XaiF is xylanase-specific.

Previously, we reported that CCR of the *xynA* is mediated at two catabolite repression elements (*cre*s), *cre*A (nucleotides −137 to −124) and *cre*B (nucleotides +173 to +186) by CcpA [16,17]. Moreover, in *Aspergillus niger*, the two binding sites of AlcR, *trans*-activator for the ethanol regulon, were close to or overlapped with the target site of the catabolite repression protein, CreA, leading to a direct competition for the same region between AlcR and CreA [18].

Accordingly, it is suspected that the XaiF-mediated *trans*-activation may also be ascribed to an antagonistic

Table 1
Effect of XaiF on expression of the xylanolytic enzymes

	Endo-xylanase activity (U/mg) pMG12 (*xynA*)	β-Xylosidase activity (U/mg) pMG1 (*xylA*)	α-Arabinofuranosidase activity (U/mg) pMG11 (*arfA*)
pACYC184 (control)	8.1 ± 0.15	43.95 ± 2.01	3.357 ± 0.385
pACYC-C17 (*xaiF*)	21.87 ± 2.54	45.79 ± 3.08	3.655 ± 0.29

The genes for the xylanolytic enzyme of *B. stearothermophilus* No. 236 were cloned into pUC119 to produce pMG12, pMG1, and pMG11. Each of these plasmids was co-transformed with the compatible plasmid, pACYC184-derived plasmid containing *xaiF* gene (pACYC-C17), into *E. coli* and cells were grown in LB for 12 h at 37 °C. The specific activity of each enzyme was determined as described in Materials and methods, and the values shown are averages ± standard deviation of three independent experiments.

effect against CcpA. To test this hypothesis, *B. subtilis* MW15 which harbors the recombinant plasmid pW1.8 or pW3.1 was grown in media supplemented with different carbon sources. In the presence of xylan, the xylanase activity after 24 h culture was about 5-fold higher than in the culture with glucose in the presence or absence of XaiF (Fig. 1A). Furthermore, level of the XaiF-mediated activation in either xylan or glucose was estimated to be nearly

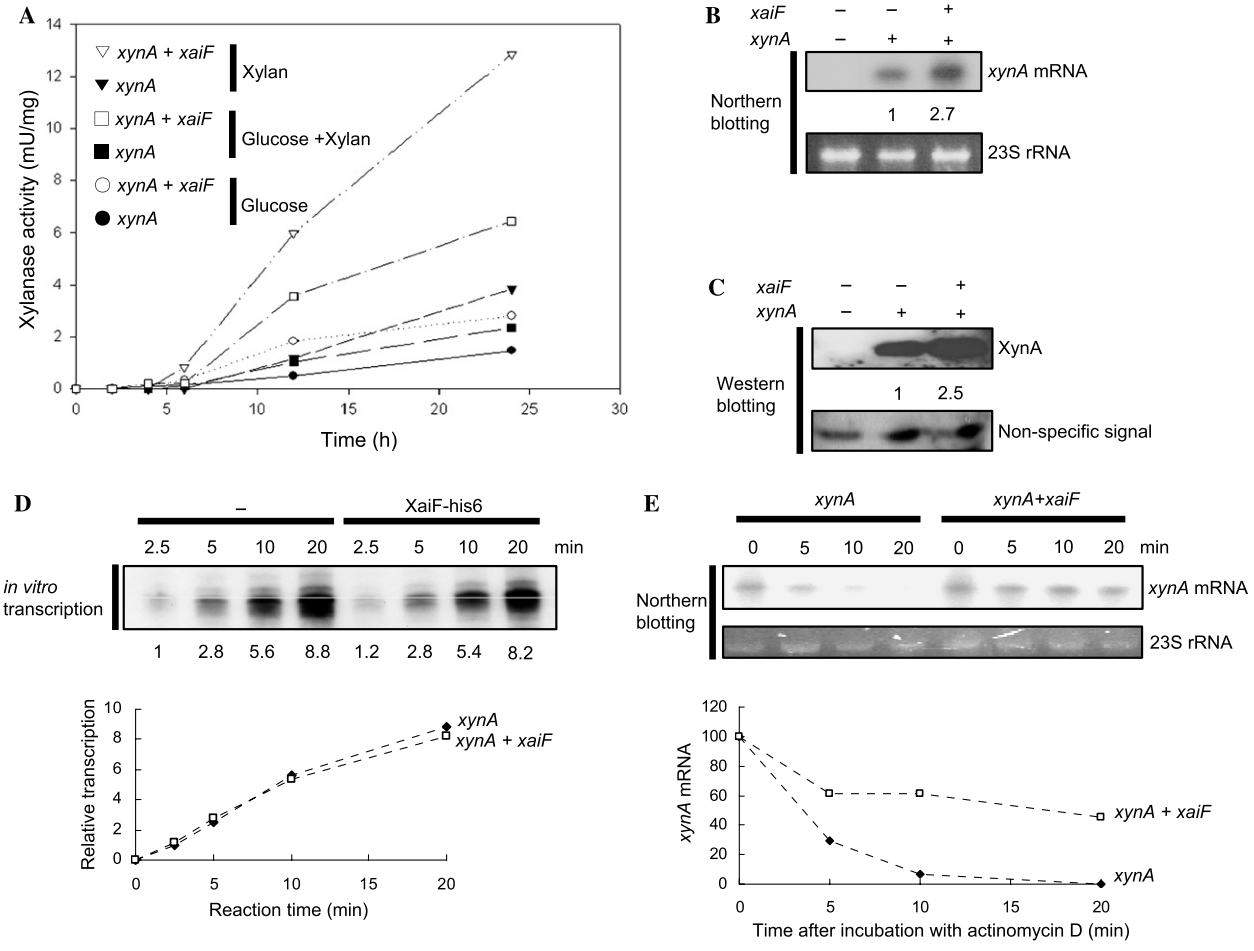

Fig. 1. Positive regulation of the *xynA* gene expression by *xaiF* co-expression. (A) *Bacillus subtilis* MW15 harboring pW1.8 or pW3.1 were grown in media supplemented with different carbon sources at 37 °C. The extracellular enzyme fraction was prepared at the denoted time point and the xylanase activity was measured as described in Materials and methods. (B) *Bacillus subtilis* MW15 was grown to the mid-log phase and the relative level of the *xynA* mRNA was determined by Northern blotting. The relative density was denoted at the bottom. EtBr-stained 23 S rRNA was used as the loading standard. (C) After 24 h cultivation, cells were removed and the extracellular proteins were concentrated. Fifty micrograms of protein was subjected to Western blotting with anti-XynA antibody. The relative density was denoted at the bottom. The non-specific signal was served as loading control. (D) About 300 bp DNA templates containing the promoter and 5′-end region of the *xynA* gene were mixed with *E. coli* RNA polymerase holoenzyme in the presence or absence of XaiF-his6. The signal density ratio relative to that of the sample without XaiF at the time 2.5 min as a standard was denoted at the bottom. The calculated values were plotted as a function of time and displayed in the lower panel. (E) The decay rate of the *xynA* mRNA in the absence or presence of XaiF was determined as described in Materials and methods. EtBr-stained 23S rRNA was used as the loading standard. The relative *xynA* mRNA levels were plotted as a percentage of the *xynA* mRNA level at the time zero of each sample.

the same as about 3-fold. These results imply that the XaiF-mediated *trans*-activation and CCR of *xynA* are independent and distinct control circuits.

Mechanism for the XaiF-mediated trans-activation of the xynA expression

As the first approach to define the molecular mechanism of the XaiF action, effect of XaiF on the first step in gene expression was examined by measuring the amounts of *xynA* transcript synthesized in the presence or absence of XaiF. Northern blotting (Fig. 1B) and Western blotting (Fig. 1C) revealed that *xynA* expression was increased in the presence of XaiF.

Then, the potential function of XaiF as a transcriptional activator was investigated by the *in vitro* transcription assay. The transcription rate was not increased although XaiF was added in the incubation mixture (Fig. 1D), indicating that XaiF did not work at the transcription level.

The results described above led us to examine the XaiF effect on stability of the *xynA* mRNA by determining the half-life of the mRNA. Intriguingly, half-life of the *xynA* mRNA was increased nearly 4-fold (from 4 to 15 min) with XaiF co-expression (Fig. 1E) strongly implying that XaiF stabilizes the *xynA* transcript working as a post-transcriptional regulator.

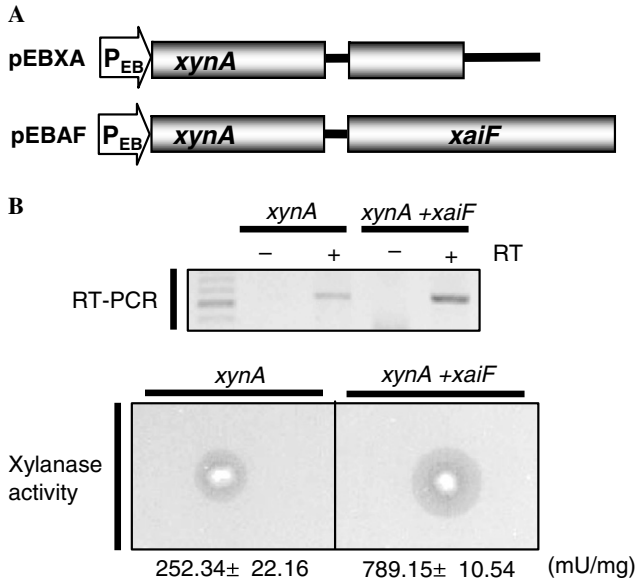

Fig. 2. Effect of XaiF on the *xynA* gene expression from the heterologous EB promoter-*xynA* chimeric construct in pEBAF plasmid. (A) Schematic representation of pEBXA and pEBAF. (B) *Bacillus subtilis* MW 15 harboring each plasmid was grown to the mid-log phase. Total RNA was prepared and the *xynA* mRNA level was determined by RT-PCR (upper panel). To eliminate plasmid DNA contamination, the RT reaction was performed with (+) or without (−) reverse transcriptase. The cells were also picked on LB-agar plate containing 0.5% oat spelt xylan (lower panel). After incubation at 37 °C for 16 h, the clear-zone formed by the secreted xylanase was monitored. Then, the extracellular xylanase activity corresponding to the each sample at the same condition was determined and denoted at the bottom.

Gel mobility shift assay performed with the purified XaiF-his6 and three distinct DNA fragments of the *xynA* including its upstream region did not show any specific DNA–protein binary complex (data not presented). This result demonstrates that XaiF should function quite differently from the DNA-binding transcriptional activator such as XlnR [19,20].

Next, a chimeric *xynA* gene in which the *xynA* promoter was substituted by EB promoter was constructed to investigate the promoter effect on the XaiF action (Fig. 2A). The *xynA* transcript synthesized from EB promoter was as the same rate of increase as that estimated from its original promoter by XaiF (Fig. 2B). Consistently, the *B. subtilis* MW15 bearing pEBAF formed correspondingly larger clear zone on LB plate containing 0.5% oat spelt xylan, indicating the higher xylanase activity than that from the cells harboring pEBXA. This result demonstrates that XaiF does not require the promoter region of the *xynA* for its function.

Binding of XaiF to the 3′-UTR of the xynA mRNA

pQXA710 and pQXA785 were constructed by introducing the *xynA* gene with different 3′-end into pQE30 and

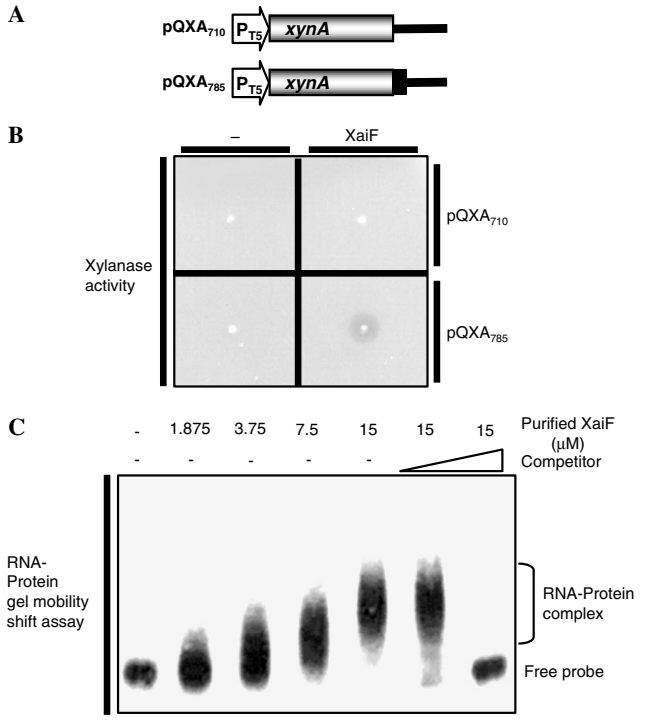

Fig. 3. Binding of XaiF on the 3′-UTR of *xynA* mRNA. (A) Schematic representation of the plasmids containing the *xynA* constructs with different 3′-ends. The additional 3′-UTR region of the *xynA* is presented as solid box. (B) Each plasmid was co-transformed into *E. coli* with the compatible plasmid, pACYC184 or *xaiF* gene-containing pACYC-C17. Cells with different combinations of plasmids were picked on LB-agar containing 0.5% oat spelt xylan and their xylanase activities were analyzed with the clear-zone formation after 16 h of incubation at 37 °C. (C) Increasing amounts of XaiF-his6 were incubated with radio-labeled RNA probe prepared as described in Materials and methods. The RNA–protein complex was analyzed on 5% native polyacrylamide gel. As competitor, about 100-fold unlabeled RNA probe was added to the reaction.

used to investigate the effect of the 3′-end region on the XaiF-mediated *trans*-activiation (Fig. 3A). Among the different plasmid combinations, only the cells carrying pQXA785 and pACYC-C17 formed a large clear zone, suggesting that the XaiF-mediated *trans*-activation might be mediated through its binding to a potential *cis*-acting element in the 3′-UTR of *xynA* mRNA (Fig. 3B).

The stem–loop structures in 5′- and 3′-UTR were reported to act as a barrier for nuclease attack and a target site of the mRNA binding protein [21] and a putative stem–loop structure was suggested at positions 22–65 nt downstream of the *xynA* stop codon. Therefore, we performed RNA–protein mobility shift assay to investigate whether XaiF directly binds to the 3′-UTR of *xynA* mRNA. At concentrations above 3.75 μM of XaiF-his6, the shifted bands were observed which could be explained as a result of the RNA–protein binary complex formation (Fig. 3C). Addition of 100-fold excess of the unlabeled probe results in release of the labeled probes from the complex, strongly suggesting that XaiF *trans*-activates *xynA* expression by binding to 3′-UTR of its transcript and thus stabilizing the mRNA.

Finally, to confirm the importance of the putative stem–loop *cis*-acting element in *xynA* 3′-UTR, the wild-type or mutant form of 3′-UTR of *xynA* mRNA was fused downstream of the *gst* ORF (Fig. 4A) and expression of the reporter gene was analyzed by Western blotting. Just like the case of the *xynA* expression, GSTt which was synthesized from the chimeric *gst* gene fused with wild-type 3′-UTR of *xynA* mRNA was assessed to be higher in the presence of XaiF (Fig. 4B). In contrast, the products synthesized from the original reporter gene or *gst* fused with mutant forms of the 3′-UTR were unaffected by the *xaiF* co-expression. Taken together, these results imply that the stem–loop structure in the 3′-UTR of *xynA* mRNA is important for the XaiF-mediated *trans*-activation.

Discussion

As the regulatory mechanism for the xylanolytic genes, a negative regulation known as CCR was extensively studied, but the induction or activation mechanism for xylanolytic genes is far less reported. A relatively well-characterized example of positive regulators for xylanolytic genes is the transcriptional activators, XlnR and its homologues [19,20]. These activators was a DNA-binding protein, binding to the sequence 5′-GGCTAAA-3′ on the several xylanolytic gene promoters, and activating the transcription of the xylanolytic genes. Recently, XynR was also reported to have DNA-binding activity and act as a positive regulator that activates expression of *xynABD* xylanase gene cluster of the rumen bacterium *Prevotella bryantii* B14 [22].

Differently from these positive regulators, our results led to conclude that XaiF increased the xylanase activity by stabilizing the *xynA* mRNA through its direct binding

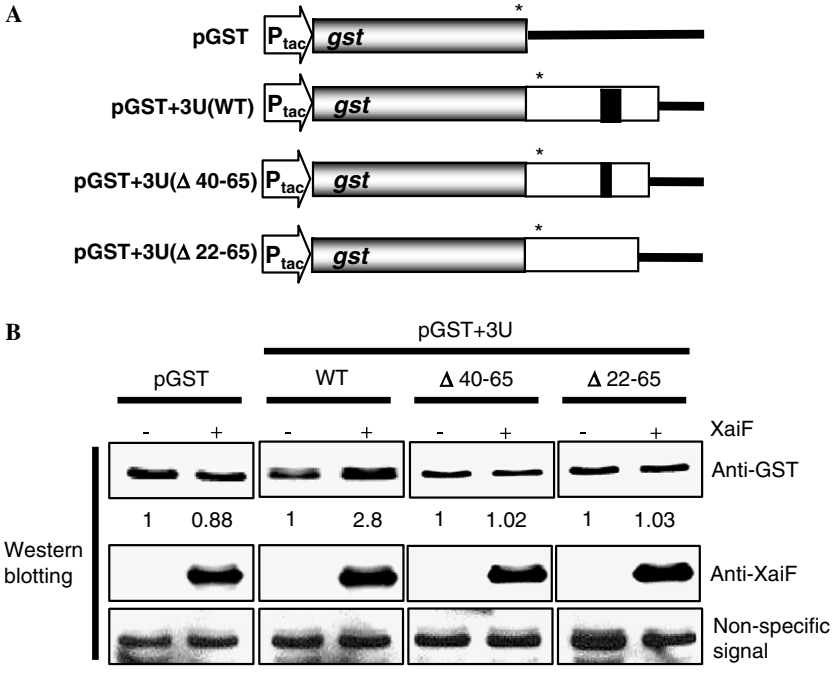

Fig. 4. Effect of XaiF on expression of the GST-*xynA* 3′-UTR chimeric gene. (A) Schematic representation of GST reporter constructs containing the wild-type or mutant form of *xynA* mRNA 3′-UTR. The region corresponding to the putative stem–loop is indicated as solid box and the position of stop codon of each construct is as star symbol. Translation of each fusion construct is estimated to terminate at the stop codon (*), producing chimeric GST-XynA protein. (B) Each plasmid was co-transformed into *E. coli* with the compatible pACYC184 or *xaiF*-containing pACYC-C17 plasmid. After 5 h cultivation in LB at 37 °C, cells with different combinations of plasmids were collected and lysed. Thirty micrograms of total cell lysates were subjected to Western blotting with anti-GST or anti-XaiF antibody.

onto 3′-UTR of *xynA* mRNA, thereby protecting the mRNA from decay by RNase(s).

Several factors known to influence prokaryotic mRNA stability have been identified and two types of secondary structures responsible for mRNA stability were reported to be 5′- and 3′-hairpins [23]. The 3′-hairpins protected the mRNA from degradation by the exonucleases RNaseII and PNPase. For the efficient mRNA protection against RNase, these 3′-hairpins were proposed to require the presence of binding proteins [7]. In bacteria, *E. coli* and *B. subtilis* aconitases are reported to resemble the bifunctional iron-regulatory proteins (IRP1)/cytoplasmic aconitases of vertebrates [24–26].

In this study, the secondary structure prediction of 3′-UTR of *xynA* transcript revealed a putative stem–loop structure and we could confirm that XaiF can directly bind to the putative stem–loop structure in 3′-UTR of the *xynA* transcript, to make the mRNA more secure from the degradation by exonucleases (Figs. 3 and 4). What we presented here is valuable in molecular biologically in that XaiF, a novel mRNA-binding protein is the first example of a regulator of this type that has been found to govern expression of xylanolytic genes. In addition to the aconitase-mediated mRNA stabilization, our results provide another example for the importance of mRNA decay as a critical means of gene expression regulation in prokaryotes as well as in eukaryotes.

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