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MoxT toxin of *Bacillus anthracis* exhibits sequence specific ribonuclease activity

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ABSTRACT

MoxXT module of *Bacillus anthracis* encodes MoxX, a labile protein and MoxT, a ribonuclease. However, mechanism of cleavage of RNA by MoxT has not been explored till date. In the present study, we have demonstrated that MoxT is a sequence specific ribonuclease which recognizes UACAU sequence in ss RNA and cleaves between U and A. Moreover, cleavage of RNA requires 2′ OH group of first residue, i.e. U of UACAU RNA sequence. An interesting finding which makes it distinct from the other MazF family toxins was also observed, i.e. its ability to cleave RNA in DNA–RNA hybrid.

1. Introduction

Toxin antitoxin (TA) systems are ubiquitously present in prokaryotes [1]. Various roles of TA system has also been proposed like stabilization of genomic parasites, antiphage activity [2], programmed cell death, growth control, selfishness, gene regulation [3], biofilm formation [4] and persister cell formation [5,6]. Among five types of TA systems, Type II TA system is a well characterized system. Type II TA system generally exists in operon wherein first gene encodes unstable antitoxin and the latter encodes stable toxin. Antitoxin interacts with toxin to form an inactive complex which abrogates the toxic activity of toxin. Antitoxins contain N-terminal domain for DNA binding and C-terminal domain for toxin binding. Toxins inhibit replication by inactivating DNA-gyrase or translation by cleaving RNA (mRNA, rRNA, tRNA) or elongation by interaction with elongation factor [7].

Bacillus anthracis, a Gram-positive bacterium, causes anthrax and has been classified by NIH as a category A agent on its bioterrorism threat list [8]. In *B. anthracis*, a PemIK chromosomal TA module renamed as MoxXT system [9] has been identified as a type II TA system [10]. MoxXT module encodes MoxX, a labile protein and MoxT, a ribonuclease. MoxX inhibits MoxT toxic activity by forming an inactive complex. MoxT acts as a ribonuclease. Over expression of MoxT inhibits protein synthesis and arrests cell growth. MoxT plays an important role in stress conditions as it found to be up-regulated in different stress conditions [11]. Effect of MoxT ribonuclease activity on cell growth and protein synthesis and, the exact mechanism of cleavage of RNA by MoxT have not been studied till date. One of the applications of the study of TA systems is an antimicrobial strategy where disruption of TA interaction and activation of the toxin would lead to bacterial cell death. In *B. anthracis*, few peptides have been reported as inhibitors of MoxXT interaction [9,11] and activators of MoxT toxicity [12]. Thus, the elucidation of mechanism of MoxT ribonuclease activity is important for the understanding of the effects of MoxT on cell growth, protein synthesis, in stress conditions and in antimicrobial strategy. In the present study, we have characterized the mechanism of RNA cleavage by MoxT.

2. Materials and methods

2.1. Materials

DNA oligos and Chimeric oligos were purchased from Sigma Aldrich (St. Louis, MO) and IDT (Coralville, IA), respectively. T7 large scale transcription kit and Sequenase Version 2.0 DNA sequencing kit were purchased from Promega (Madison, WI) and Affymetrix (Santa Clara, CA), respectively. MS2 phage RNA was purchased from Roche (Basel, Switzerland). T4 polynucleotide kinase was purchased from Thermo Scientific (Waltham, MA). Radioisotopes γ-^{32}P-ATP and γ-^{32}P-dATP were procured from BRIT (CCMB, Hyderabad, India). List of primers have been shown in Table 1.

2.2. Strains and plasmids

Table 2 shows the list of strains and plasmids.

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0006-291X/

Table 1
List of primers.

Name	Sequence (5′–3′)
Primer 1	AGGCACCTTTTCCCACACTATAC
Primer 2	TTTGCAGGACTTCGGTCGAC
Primer 3	GAATTCTTGATTGTAAAACGCGGCGAC
Primer 4	AAGCTTTTAAAAATCTATTAGTCCTAAAC
Primer 5	GGCCGGATCCATGCGCTTACTCGTAGTAGAAG
Primer 6	GGCCAAGCTTTCATTGTTCTTTTAATATATATCC
Primer 7	AGATCTCGATCCCGCGAAATTAATACG
Oligo C	CCCTGAATGTAGACTTCC

2.3. Purification of MoxT

MoxT was purified using method described in Verma et al. [12].

2.4. In vitro primer extension analysis

For primer extension analysis of partially cleaved MS2 phage RNA, the RNA (5 μg) was digested with MoxT (800 ng) in Tris–EDTA (TE) buffer (15 μl) at 37 °C for 15 min. The cleaved RNA was purified with Phenol:chloroform:isoamyl (PCI) extraction and ethanol precipitation. Primer extension of cleaved RNA was performed using superscript II reverse transcriptase (SSII RT) and Primer 1, 2. Products were analyzed on 8% sequencing gel along with sequencing ladder prepared with the same primers using Sequenase Version 2.0 DNA sequencing kit.

For partial cleavage of *bas0540* mRNA by MoxT, mRNA (5 μg) was incubated with MoxT (1 μg) in TE buffer at 37 °C for 15 min. Primer extension of cleaved mRNA was performed using SSII RT and Primer 6.

2.5. In vivo primer extension analysis

For *in vivo* MoxT cleavage analysis, *moxT* and *bas0540* were cloned into pBAD18 and ptac-PBCSK plasmids using primers 3, 4 and 5, 6, respectively. pBAD18-MoxT and ptac-PBCSK-BAS0540 constructs were co-transformed into *Escherichia coli* BW25113 cells and were grown in M9 media. Cells were grown at 37 °C till OD_{600} reached 0.6 and then, induced by the addition of 1 mM IPTG for the production of *bas0540* mRNA. After 1 h induction, MoxT expression was induced by the addition of 0.2% arabinose at 37 °C. After that 3 ml culture aliquots were collected at different time points (0 min, 10 min, 20 min, 30 min, 40 min, 50 min, 60 min, 1 h 40 min, 2 h 10 min and 4 h). A 3-ml culture aliquot was also collected from cells transformed with only ptac-PBCSK-BAS0540 after 5 h incubation in the same condition as the induced culture. Total RNA was isolated by TRIzol method and primer extension was performed with equal amount of total RNA using SSII RT and Primer 6. Products were analyzed on 8% sequencing gel along with *in vitro* primer extension product.

2.6. In vitro transcription

mRNA transcript of *bas0540* was prepared using T7 large scale transcription kit. For *in vitro* transcription, pET28a-*bas0540* construct was used as a template obtained by PCR amplification of the DNA containing a T7 promoter and *bas0540* gene using primers 7 and 6.

2.7. 5′ and 3′ end analysis of cleaved RNA

For 5′ and 3′ end analysis of MoxT cleaved *bas0540* mRNA, mRNA (1 μg) was digested with MoxT (500 ng) in TE buffer at 37 °C for 15 min. The cleaved RNA was purified by PCI extraction and ethanol precipitation. Purified RNA was subjected to 5′ end labeling with γ-^{32}P-ATP using T4 polynucleotide kinase. The reaction mixture was analyzed on 8% sequencing gel along with *in vitro* primer extension product.

2.8. Cleavage analysis of ss RNA, ds RNA and DNA–RNA hybrid

To analyze MoxT cleavage specificity for single stranded (ss) or double stranded (ds) RNA, a ss chimeric oligo (Oligo A) was synthesized and was used as MoxT substrate. A 14 base ss chimeric oligo (AAGTC**rUrArCrAr**UTCAG) contains 5 RNA bases (UACAU) flanked by 5 DNA bases (AAGTC) on the left and 4 DNA bases (TCAG) on the right side. Oligo A was labeled at its 5′end with γ-^{32}P-ATP using T4 polynucleotide kinase. For cleavage assay, a reaction mixture (10 μl) containing of 5′ labeled oligo A (300 fmol) was incubated with increasing amounts of MoxT (25 ng, 50 ng, 100 ng, 200 ng, 400 ng and 800 ng) in TE buffer at 37 °C for 15 min. The reaction mixture was analyzed on 20% sequencing gel.

To find the effect of MoxT on ds RNA, 5′ labeled Oligo A was annealed to antisense chimeric oligo (Oligo B) to create ds RNA form. For annealing, Oligo A (300 fmol) was incubated with increasing concentrations of Oligo B (15 fmol, 35 fmol, 75 fmol, 150 fmol, 300 fmol, 600 fmol and 800 fmol) and cleavage assay was performed in the presence of MoxT (200 ng) in TE buffer at 37 °C for 20 min.

To find the effect of MoxT on DNA–RNA hybrid, 5′ labeled Oligo A was annealed to antisense DNA oligo (Oligo C) to create DNA–RNA hybrid. For annealing, Oligo A (300 fmol) was incubated with increasing concentrations of Oligo C (15 fmol, 35 fmol, 75 fmol, 150 fmol, 300 fmol, 600 fmol and 800 fmol) and cleavage assay was performed in the presence of MoxT (200 ng) in TE buffer at 37 °C for 20 min. To confirm the formation of DNA–RNA hybrid, RNase H digestion was also performed with the same reaction mixtures.

2.9. Cleavage analysis of mutant oligos

For MoxT cleavage assay with mutant oligos, a reaction mixture (10 μl) containing mutated oligo (300 fmol) was incubated with

Table 2
List of strains and plasmids.

Strains or plasmids	Relevant characteristic(s)	Reference or source
Strains		
E. coli DH5α	SupE44ΔlacU169 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 ϕ80dlacZ ΔM15	Novagen
E. coli BW25113	rrnB3 ΔlacZ4787 hsdR514 Δ(araBAD)567 Δ(rhaBAD)568 rph-1	Lab collection
Plasmids		
pBAD18	MoxT cloning vector, Amp^r	Lab collection
ptac-PBCSK	PBCSK(+) vector containing tac promoter, Cam^r	[25]

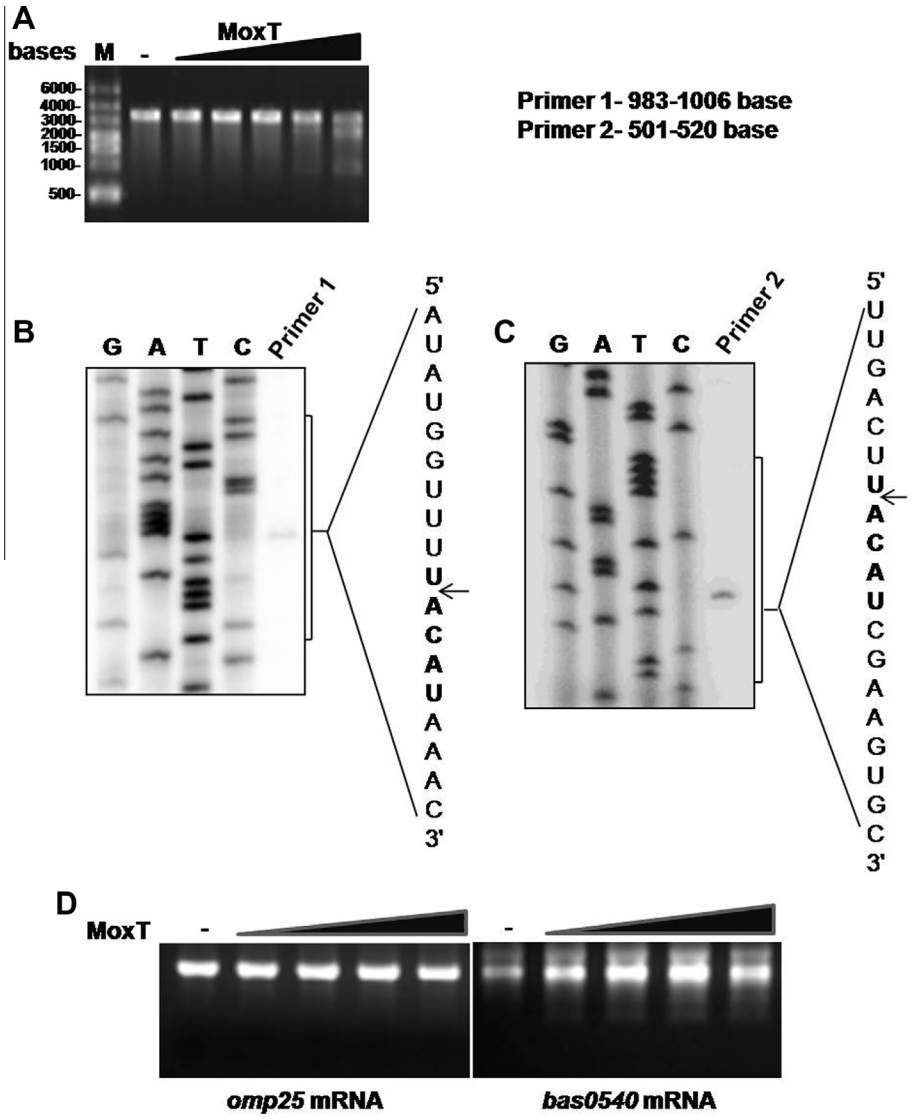

Fig. 1. *In vitro* primer extension analysis of MoxT cleaved RNA. (A) MS2 phage RNA was digested with increasing concentration of MoxT and on the basis of obtained bands, Primer 1 and 2 was designed. (B and C) Primer extension of cleaved MS2 phage RNA was performed. In sequencing gel, lane Primer 1 and 2 shows primer extension product. 5′–3′ sequence around primer extension band has been shown, where recognition sequence is in bold letters and arrows indicate the cleavage site. (D) *bas0540* and *omp25* mRNAs were treated with increasing concentrations of MoxT. *bas0540* mRNA shows degradation with increase in MoxT concentration whereas *omp25* mRNA is showing an intact uncleaved band.

MoxT (200 ng) in TE buffer at 37 °C for 15 min. Reaction mixture was analyzed on 20% sequencing gel.

3. Results

3.1. MoxT cleaves specifically between 5′ U and A at UACAU RNA sequence in vitro and in vivo

MoxT is a ribonuclease [11], however, the exact RNA sequence at which MoxT cleaves is not known. To identify the RNA sequence, MS2 phage RNA was used as MoxT substrate. Partial cleavage of MS2 phage RNA by MoxT, released three fragments of ~2.5 Kb, 1 Kb and <500 bases, indicating that MoxT cleaves MS2 phage RNA at specific sites (Fig. 1A). On the basis of the fragments obtained after MS2 phage RNA cleavage, oligos were designed. Oligo sequence started from 5′ end with 983 base (Primer 1) and 500 base (Primer 2) positions of MS2 phage RNA for primer extension analysis (Fig. 1A). MS2 phage RNA was partially digested with MoxT and cleaved RNA was subjected to primer extension using designed oligos. It was found that MoxT cleaves RNA between 5′ U and A of a common sequence UACAU (Fig. 1B and C). Hence, MoxT recognizes UACAU sequence in RNA and cleaves between 5′ U and A.

Further, to examine whether MoxT cleaves only RNAs that have cleavage specific sequence, two genes were chosen, *bas0540* from *B. anthracis* consisting of UACAU sequence in its mRNA and *omp25* from *Brucella abortus* which lacks the sequence in its mRNA transcript. The mRNA transcripts of *bas0540* and *omp25* were subjected to digestion with MoxT and it was observed that MoxT was able to cleave *bas0540* mRNA whereas *omp25* mRNA was resistant to cleavage (Fig. 1D), since *omp25* mRNA lacks MoxT recognition sequence. Thus, cleavage of specific recognition sequence containing RNA by MoxT was established.

To confirm the cleavage of *bas0540* mRNA by MoxT at UACAU sequence, mRNA was digested with MoxT and primer extension was performed. Two products were obtained indicating that MoxT cleaves *bas0540* mRNA at two sites, UACAU and UACGU (Fig. 2A). Hence, RNA cleavage by MoxT is specific to UACA/GU. Comparison

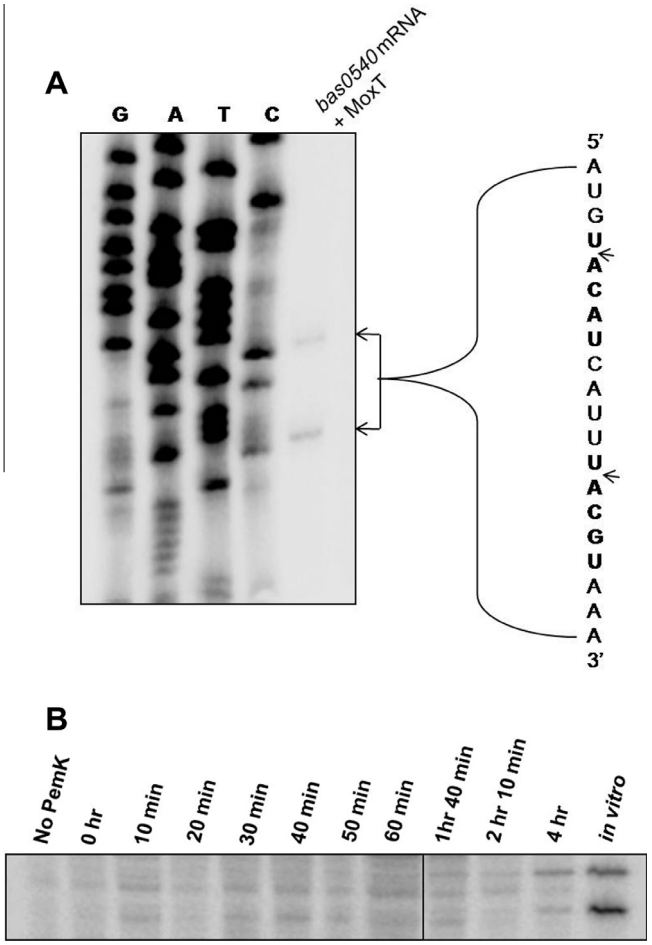

Fig. 2. *In vitro* and *in vivo* primer extension analysis of MoxT cleaved *bas0540* mRNA. (A) Primer extension was performed with MoxT cleaved *bas0540* mRNA. In sequencing gel, lane *bas0540* mRNA+MoxT shows primer extension product. Sequence around both primer extension products have been shown where recognition sequence is in bold letters and arrows indicate cleavage sites. (B) *In vivo* primer extension was performed with the total RNA containing MoxT cleaved *bas0540* mRNA. In sequencing gel, lane No MoxT shows the primer extension products obtained from total RNA isolated from cells transformed with ptac-PBCSK-BAS0540 construct only. Lane 0–4 h shows the primer extension products obtained from total RNA isolated from cells, collected at different time point after the induction of MoxT. Lane *in vitro* shows the primer extension products obtained as in section A.

of these two sequences for the cleavage specificity for MoxT has been shown in Fig. 4 and Table 3.

To analyze whether MoxT cleaves RNA *in vivo* with same specificity, primer extension analysis was performed with total RNA extracted from *E. coli* BW25113 cells after MoxT induction at different time points. Digestion of *bas0540* mRNA was observed after 50 min of induction of MoxT and at 4 h after MoxT induction, primer extension products were obtained similar to *in vitro* primer extension product (Fig. 2B). MoxT exhibited RNA cleavage *in vivo* and *in vitro* in a similar manner. Cells transformed with only ptac-PBCSK-BAS0540 construct did not show degradation of *bas0540* mRNA even after 5 h incubation (Fig. 2B). Therefore, it was concluded that MoxT cleaves RNA with the same specificity *in vivo* and *in vitro*.

3.2. MoxT cleaves RNA and produces 2′, 3′-cyclic phosphate and 5′-OH fragments

Further, to find the mechanism of cleavage of phosphodiester bond by MoxT, 5′ and 3′ end of cleaved *bas0540* mRNA was analyzed. For this analysis, mRNA was digested with MoxT and subjected to 5′ end labeling with γ-^{32}P-ATP using T4 polynucleotide kinase. T4 polynucleotide kinase will be able to phosphorylate RNA fragment only if 5′-OH group is produced on cleavage of mRNA by MoxT. Two products were obtained as in primer extension analysis (Fig. 3A). Hence, MoxT cleaves phosphodiester linkage in RNA and produces 2′, 3′-cyclic phosphate on one side and 5′-OH group on the other.

Table 3
Effect of mutations in UACAU RNA sequence on MoxT ribonuclease activity.

Name	Sequence (5′–3′)	Cleavage
Oligo A	AAGTC**rUrArCrArU**TCAG	+ + + + +
SV1	AAGTC**rG**rArCrArUTCAG	–
SV2	AAGTCrU**rG**rCrArUTCAG	–
SV3	AAGTCrUrA**rG**rArUTCAG	+ +
SV4	AAGTCrUrArC**rG**rUTCAG	+ +
SV5	AAGTCrUrArCrA**rG**TCAG	+
SV6	AAGTC**rA**rArCrArUTCAG	–
SV7	AAGTCrU**rU**rCrArUTCAG	–
SV8	AAGTCrU**rC**rCrArUTCAG	–
SV9	AAGTCrUrA**rA**rArUTCAG	–
SV10	AAGTCrUrA**rU**rArUTCAG	–
SV11	AAGTCrUrArC**rU**rUTCAG	–
SV12	AAGTCrUrArC**rC**rUTCAG	–
SV13	AAGTCrUrArCrA**rA**TCAG	–
SV14	AAGTCrUrArCrA**rC**TCAG	–
SV25	AAGTC**rC**rArCrArUTCAG	–
	2′ Deoxy	
SV15	AAGTCrU**ACAU**TCAG	–
SV26	AAGTC**U**rA**CAU**TCAG	–
SV16	AAGTCrUrA**CAU**TCAG	–
SV17	AAGTC**U**rArCrArUTCAG	–
SV18	AAGTCrU**A**rCrArUTCAG	++ +
SV19	AAGTCrUrArC**A**rUTCAG	+ + + +
SV20	AAGTC**UA**rCrArUTCAG	+
	2′ Methyl	
SV21	AAGTC**mU**rArCrArUTCAG	–
SV22	AAGTCrU**mA**rCrArUTCAG	+ + + +
SV23	AAGTCrUrA**mC**rArUTCAG	+ + + +
SV24	AAGTC**mUmA**rCrArUTCAG	–

3.3. MoxT cleaves ss RNA and DNA–RNA hybrid but not ds RNA

To analyze whether cleavage of RNA by MoxT is specific to RNA structure like ss or ds RNA or DNA–RNA hybrid, a 14-base chimeric oligo (Oligo A) containing UACAU sequence was designed and used as MoxT substrate. Oligo A was efficiently cleaved by MoxT in a concentration dependent manner (Fig. 3B). To find the cleavage of ds RNA by MoxT, an antisense chimeric oligo (Oligo B) was annealed with Oligo A in increasing concentrations before the addition of MoxT. After addition of MoxT, cleavage of Oligo A was blocked in a concentration dependent manner (Fig. 3C) which showed that MoxT is not able to cleave ds RNA. Therefore, MoxT is a sequence specific ribonuclease for ss RNA.

To examine the MoxT ribonuclease activity with DNA–RNA hybrid, an antisense DNA oligo (Oligo C) was annealed to Oligo A in increasing concentrations. The DNA–RNA hybrid was subjected to MoxT digestion and it was found that MoxT is able to cleave RNA in DNA–RNA hybrid also (Fig. 3D). RNase H digestion of same annealed reaction mix confirmed the formation of DNA–RNA hybrid (Fig. 3E). Thus, cleavage of RNA by MoxT is specific to ss RNA and DNA–RNA hybrid form.

3.4. Each residue of UACAU in RNA is required for MoxT ribonuclease activity

Cleavage of *bas0540* mRNA by MoxT at two different sites, UACAU and AUCGU, suggest the difference in cleavage specificity

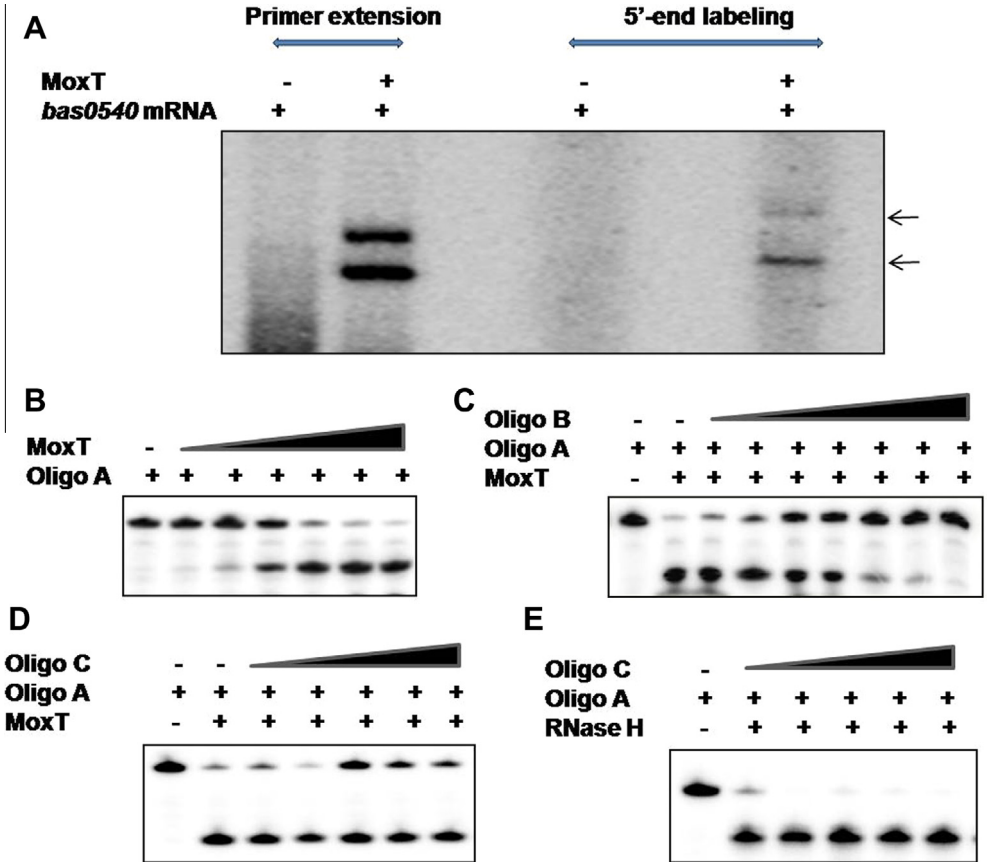

Fig. 3. 5′ and 3′ end analysis of MoxT cleaved *bas0540* mRNA. (A) MoxT cleaved *bas0540* mRNA was subjected to primer extension and 5′ end labeling. In sequencing gel, lane primer extension shows the obtained primer extension products and lane 5′ end labeling shows the similar bands. Arrows indicate the 5′ end labeled products. MoxT cleavage assay with ds RNA and DNA–RNA hybrid. (B) 5′ labeled Oligo A was digested with increasing concentration of MoxT. The appearance of lower band with the increase in concentration of MoxT shows degradation of RNA by MoxT. (C) The annealed 5′ labeled Oligo A and oligo B was treated with MoxT. Disappearance of lower band (cleaved product) indicates the inhibition of MoxT activity with increase in Oligo B concentration. (D) The DNA–RNA hybrid of oligo A and C was treated with MoxT. Appearance of lower band in the presence of Oligo C at high concentration also, indicates the cleavage of DNA–RNA hybrid by MoxT. (E) Digestion of above mentioned DNA–RNA hybrid with RNase H. Appearance of lower band shows the digestion of DNA–RNA hybrid whereas in the absence of Oligo C, Oligo A is intact and resistant to RNase H digestion.

of MoxT with change in the residues of UACAU. To check whether all or few residues in UACAU are important for MoxT activity, each residue of UACAU was mutated with the other remaining 3 nucleotides. MoxT cleavage assay with mutated oligos showed that mutation of 5′ U and A completely blocked the cleavage of oligo substrate by MoxT, however, the other C, A and U mutations exhibited reduced MoxT activity (Fig. 4 and Table 3, SV1–14 and SV25). This confirmed that each residue of UACAU is important for MoxT activity however, 5′ U and A are the most crucial residues for MoxT activity.

3.5. 2′ OH group of 5′U of UACAU is essential for the cleavage of RNA by MoxT

Cleavage specificity to RNA and 5′ and 3′ end analysis of cleaved RNA suggests the role of each sequence as RNA base and requirement of 2′ OH group of first residue, U of UACAU for the MoxT activity. To study the same, 2′ OH group of each residue was modified with deoxy group. MoxT cleavage assay showed that modification of first residue, U completely blocked the MoxT activity which indicated that first residue, U of UACAU is essential for RNA cleavage (Fig. 4 and Table 3, SV17). It was also observed that the first residue alone is not sufficient for the MoxT activity (Fig. 4 and Table 3, SV15). The MoxT activity is dependent on the other three residues also (Fig. 4 and Table 3, SV18–20 and SV26). Moreover, it was observed that MoxT cleaves between U and A at UACAU and both U and A are the important residues for the MoxT activity, however, MoxT is not able to cleave RNA between U and A in the presence of the other three modified deoxy residues (Fig. 4 and Table 3, SV16). Thus, it was concluded that first three residues of UACAU as RNA base are required for the maximum ribonuclease activity of MoxT.

To confirm the involvement of 2′ OH group in RNA cleavage, 2′ OH group of first three residues of UACAU was modified with methyl group. Modified oligos were subjected to digestion with MoxT and it was found that modification of 2′ OH group of the first residue blocked the MoxT activity (Fig. 4 and Table 3, SV21). Whereas, modification of 2′ OH group of first A and C residues had no effect on digestion of RNA by MoxT (Fig. 4 and Table 3, SV22 and SV23). Therefore, 2′ OH group of 5′ U residue in UACAU is essential for the cleavage of RNA by MoxT.

4. Discussion

In type II TA systems, MazEF system is well studied. Toxins belonging to MazEF family, act as sequence specific ribonuclease. In MazEF module, MazF cleaves mRNA specifically in ribosome independent manner [13]. All the characterized MazF orthologs from bacteria and archea are involved in growth arrest by cleaving ss RNA specifically at 3, 5 or 7 base recognition sequences. The recognition sequences, cleavage positions and number of cleavage points are found to be varying in MazF family toxins [13–19].

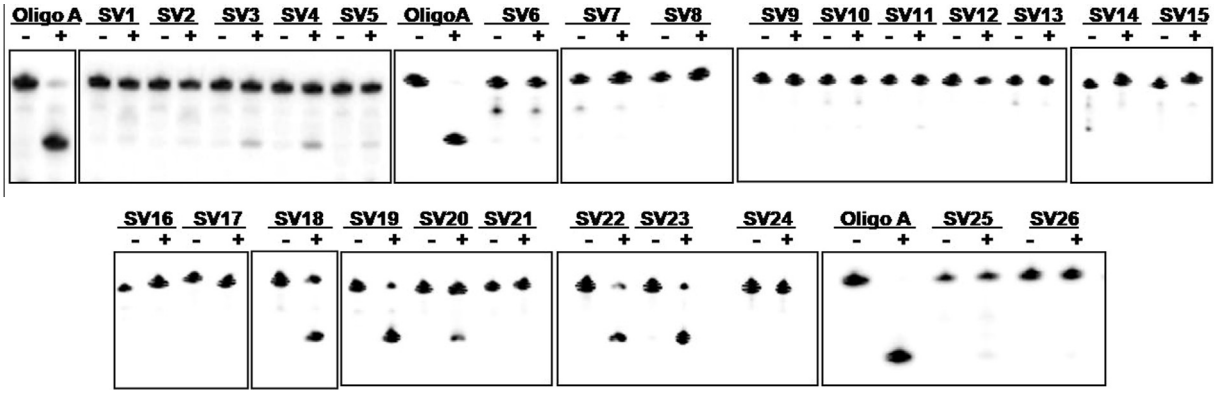

Fig. 4. MoxT cleavage assay with mutant oligos. Oligo A is a MoxT substrate and oligos SV1–26 are mutated and modified. 5′ labeled Oligo A and SV1–26 oligos were digested with MoxT. In sequencing gel lower band indicates the degradation by MoxT.

Likewise, MoxT also inhibits cell growth and protein synthesis. It acts as a ribonuclease [11]. However, the mechanism of RNA cleavage by MoxT is not known. YdcE of *Bacillus subtilis* shares 94% sequence similarity with MoxT, suggests the similar function of MoxT. YdcE is a ribonuclease which cleaves RNA at UACAU sequence [20,21]. Similarly, in the present study, we have demonstrated that MoxT cleaves RNA between U and A at UACAU pentad sequence *in vitro* and *in vivo* which confirmed that MoxT is a sequence specific endoribonuclease for ss RNA. Interestingly, mutational analysis indicated that MoxT is highly specific to UACAU sequence and each residue of UACAU is crucial for the MoxT ribonuclease activity. This sequence specificity for ribonuclease activity of MoxT suggests that MoxT acts as a RNA restriction enzyme as reported in the case of MazF [14]. It has also been reported that MazF family members recognize specific sequences so that its expression should not lead to total mRNA cleavage. Transcripts lacking the toxin recognition sequence are found to be stable in the presence of toxins [15,16,18], and susceptibility of transcript to the toxin is dependent on availability of cleavage sites in the RNA [15,18]. Concurrently, *bas0540* mRNA having MoxT recognition sequence is susceptible to MoxT cleavage and *omp25* mRNA lacking this sequence is resistant to MoxT cleavage. This suggests that MoxT only acts on RNA containing specific sequence UACAU. MoxT cleaves phosphodiester bond and produces a free 5′-OH group and 3′-phosphate end products. The mechanism of RNA cleavage by MoxT is similar to RNase A because in both cases, 2′OH group is required for the cleavage reaction at specific site and this type of cleavage mechanism has been also reported in case of MazF toxin [14]. These characteristics of MoxT suggest that MoxT also belongs to MazF family toxins. One unique characteristic exhibited by MoxT makes it different from MazF, i.e. MoxT cleaves RNA in DNA–RNA hybrid whereas MazF does not [13]. Earlier, MazF family toxins have been reported as "mRNA interferases" as they cleave ss RNA but do not cleave tRNAs or rRNAs [13,16,17,22]. However, recently it has been demonstrated that *E. coli* MazF cleaves 16S rRNA *in vivo* [23]. It has also been reported that MazF from *Mycobacterium tuberculosis* (MazF-mt6) inhibits protein synthesis by cleaving 23S rRNA in the ribosome active center [24]. Here, MoxT cleaves mRNA as well as rRNA efficiently *in vitro*. However, the effect of MoxT on protein synthesis, cell growth and in stress condition is still unclear. Inhibition of protein synthesis may be due to the cleavage of both mRNA and rRNA but the role of cleavage DNA–RNA hybrid is not clear. It can be speculated that cleavage of mRNA, rRNA and DNA–RNA hybrid by MoxT affects cell physiology diversely in different conditions.

Acknowledgments

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References

[1] D.P. Pandey, K. Gerdes, Toxin–antitoxin loci are highly abundant in free-living but lost from host-associated prokaryotes, Nucleic Acids Res. 33 (2005) 966–976.
[2] D.C. Pecota, T.K. Wood, Exclusion of T4 phage by the hok/sok killer locus from plasmid R1, J. Bacteriol. 178 (1996) 2044–2050.
[3] R.D. Magnuson, Hypothetical functions of toxin–antitoxin systems, J. Bacteriol. 189 (2007) 6089–6092.
[4] J.J. Harrison, W.D. Wade, S. Akierman, C. Vacchi-Suzzi, C.A. Stremick, R.J. Turner, H. Ceri, The chromosomal toxin gene yafQ is a determinant of multidrug tolerance for *E. coli* growing in a biofilm, Antimicrob. Agents Chemother. 53 (2009) 2253–2258.
[5] K. Lewis, Persister cells, dormancy and infectious disease, Nat. Rev. Microbiol. 5 (2007) 48–56.
[6] X. Wang, T.K. Wood, Toxin–antitoxin systems influence biofilm and persister cell formation and the general stress response, Appl. Environ. Microbiol. 77 (2011) 5577–5583.
[7] K. Gerdes, S.K. Christensen, A. Lobner-Olesen, Prokaryotic toxin–antitoxin stress response loci, Nat. Rev. Microbiol. 3 (2005) 371–382.
[8] A.S. Khan, A.M. Levitt, M.J. Sage, Biological and chemical terrorism: strategic plan for preparedness and response. Recommendations of the CDC strategic planning workgroup, MMWR Recomm. Rep. 49 (2000) 1–14.
[9] N. Chopra, S. Agarwal, S. Verma, S. Bhatnagar, R. Bhatnagar, Modeling of the structure and interactions of the *B. anthracis* antitoxin, MoxX: deletion mutant studies highlight its modular structure and repressor function, J. Comput. Aided Mol. Des. 25 (2011) 275–291.
[10] S. Agarwal, R. Bhatnagar, Identification and characterization of a novel toxin–antitoxin module from *B. anthracis*, FEBS Lett. 581 (2007) 1727–1734.
[11] S. Agarwal, N.K. Mishra, S. Bhatnagar, R. Bhatnagar, PemK toxin of *B. anthracis* is a ribonuclease: an insight into its active site, structure, and function, J. Biol. Chem. 285 (2010) 7254–7270.
[12] S. Verma, S. Kumar, V.P. Gupta, S. Gourinath, S. Bhatnagar, R. Bhatnagar, Structural basis of *B. anthracis* MoxXT disruption and the modulation of MoxT ribonuclease activity by rationally designed peptides, J. Biomol. Struct. Dyn. (2014).
[13] Y. Zhang, J. Zhang, K.P. Hoeflich, M. Ikura, G. Qing, M. Inouye, MazF cleaves cellular mRNAs specifically at ACA to block protein synthesis in *E. coli*, Mol. Cell 12 (2003) 913–923.
[14] Y. Zhang, J. Zhang, H. Hara, I. Kato, M. Inouye, Insights into the mRNA cleavage mechanism by MazF, an mRNA interferase, J. Biol. Chem. 280 (2005) 3143–3150.
[15] F.P. Rothenbacher, M. Suzuki, J.M. Hurley, T.J. Montville, T.J. Kirn, M. Ouyang, N.A. Woychik, Clostridium difficile MazF toxin exhibits selective, not global, mRNA cleavage, J. Bacteriol. 194 (2012) 3464–3474.
[16] Y. Yamaguchi, H. Nariya, J.H. Park, M. Inouye, Inhibition of specific gene expressions by protein-mediated mRNA interference, Nat. Commun. 3 (2012) 607.

[17] J. Zhang, Y. Zhang, L. Zhu, M. Suzuki, M. Inouye, Interference of mRNA function by sequence-specific endoribonuclease PemK, J. Biol. Chem. 279 (2004) 20678–20684.
[18] L. Zhu, K. Inoue, S. Yoshizumi, H. Kobayashi, Y. Zhang, M. Ouyang, F. Kato, M. Sugai, M. Inouye, Staphylococcus aureus MazF specifically cleaves a pentad sequence, UACAU, which is unusually abundant in the mRNA for pathogenic adhesive factor SraP, J. Bacteriol. 191 (2009) 3248–3255.
[19] L. Zhu, S. Phadtare, H. Nariya, M. Ouyang, R.N. Husson, M. Inouye, The mRNA interferases, MazF-mt3 and MazF-mt7 from *M. tuberculosis* target unique pentad sequences in single-stranded RNA, Mol. Microbiol. 69 (2008) 559–569.
[20] O. Pellegrini, N. Mathy, A. Gogos, L. Shapiro, C. Condon, The *B. subtilis* ydcDE operon encodes an endoribonuclease of the MazF/PemK family and its inhibitor, Mol. Microbiol. 56 (2005) 1139–1148.
[21] J.H. Park, Y. Yamaguchi, M. Inouye, *B. subtilis* MazF-bs (EndoA) is a UACAU-specific mRNA interferase, FEBS Lett. 585 (2011) 2526–2532.
[22] S. Baik, K. Inoue, M. Ouyang, M. Inouye, Significant bias against the ACA triplet in the tmRNA sequence of *E. coli* K-12, J. Bacteriol. 191 (2009) 6157–6166.
[23] O. Vesper, S. Amitai, M. Belitsky, K. Byrgazov, A.C. Kaberdina, H. Engelberg-Kulka, I. Moll, Selective translation of leaderless mRNAs by specialized ribosomes generated by MazF in *E. coli*, Cell 147 (2011) 147–157.
[24] J.M. Schifano, R. Edifor, J.D. Sharp, M. Ouyang, A. Konkimalla, R.N. Husson, N.A. Woychik, Mycobacterial toxin MazF-mt6 inhibits translation through cleavage of 23S rRNA at the ribosomal A site, Proc. Natl. Acad. Sci. U.S.A. 110 (2013) 8501–8506.
[25] P. Singh, D. Park, S. Forst, N. Banerjee, Xenocin export by the flagellar type III pathway in *Xenorhabdus nematophila*, J. Bacteriol. 195 (2013) 1400–1410.