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Invited Mini Review

Substrate specificity of bacterial endoribonuclease toxins

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Bacterial endoribonuclease toxins belong to a protein family that inhibits bacterial growth by degrading mRNA or rRNA sequences. The toxin genes are organized in pairs with its cognate antitoxins in the chromosome and thus the activities of the toxins are antagonized by antitoxin proteins or RNAs during active translation. In response to a variety of cellular stresses, the endoribonuclease toxins appear to be released from antitoxin molecules via proteolytic cleavage of antitoxin proteins or preferential degradation of antitoxin RNAs and cleave a diverse range of mRNA or rRNA sequences in a sequence-specific or codon-specific manner, resulting in various biological phenomena such as antibiotic tolerance and persister cell formation. Given that substrate specificity of each endoribonuclease toxin is determined by its structure and the composition of active site residues, we summarize the biology, structure, and substrate specificity of the updated bacterial endoribonuclease toxins. [BMB Reports 2020; 53(12): 611-621]

INTRODUCTION

Bacteria grow in constantly changing environments that often limit growth or threaten their survival. To adapt or survive in such fluctuating environments, bacteria need to slow down their growth rate by multiple ways including redistribution of metabolic pathways and nutrient transport, shutdown of translation, or alteration of gene expression of components involved in translation, replication of the genome, and division of the cell walls (1). The ability to adapt to unfavorable environments is largely dependent on how bacteria quickly respond to environmental stimuli and modulate gene expression to adjust the bacterium's growth in a newly given environment.

Toxin-antitoxin systems are one of such genetic elements that directly regulate bacterial growth in response to a variety of cellular stresses including nutrient limitation, SOS response, heat shock, bacteriophage infection, and antibiotic treatment (2, 3). Toxin-antitoxin systems consist of two genes encoding the toxin and its cognate antitoxin, respectively. Toxin proteins arrest bacterial growth by inhibiting DNA gyrase, degrading messenger RNAs, or modifying the ribosomal components, most of which disrupt translation process (4-15). Considering that translation is the most energy-demanding process and global translation efficiency is one of the major limiting factors for bacterial growth rate (16, 17), it is not surprising that the toxins are predominantly involved in inhibiting protein synthesis. Among these toxins, the majority of toxin proteins are endoribonucleases degrading mRNAs (2, 3, 18, 19). The endoribonuclease toxins recognize and cleave defined mRNA sequences depending on its structures and residues at the active sites (20). In this review, we summarize the current understanding of bacterial endoribonuclease toxins focusing on the substrate specificity of the toxins and underlying mechanisms.

Classification of toxin-antitoxin systems

Toxin-antitoxin systems are classified into six different types depending on how the antitoxin recognizes and antagonizes the toxin protein (3). For example, in type I toxin-antitoxin system, the antitoxins are antisense RNAs that bind to the translation initiation regions of the toxin genes and inhibit translation of the toxin mRNAs. By contrast, type II antitoxins are proteins and inhibit toxin's activity by directly binding to the toxin proteins with different molecular ratios (toxin : antitoxin ratio of 2 : 2, or 4 : 2) (18). Type III antitoxins are similar to type I antitoxins in a sense that they are small noncoding RNAs. However, unlike type I antitoxins, type III antitoxin RNAs directly bind to the toxin proteins and inhibit the toxin's activity instead of blocking translation of the toxin genes by base-pairing (Fig. 1).

Type II ribosome-dependent endoribonucleases

RelB-RelE: RelB-RelE is one of the most well-studied toxin-antitoxin operons encoding a toxin RelE and an antitoxin RelB. Expression of the *relBE* operon is induced by amino acid and glucose starvation (21) and the activation is mediated by Lon protease degrading RelB antitoxin, which also acts as a repressor of the *relBE* operon (22). The autorepressor activity of RelE is largely dependent on the molecular ratio between RelE and RelB. At a low RelE:RelB ratio, the RelE-RelB complex binds to the promoter and represses *relBE* expression, but at a high RelE:RelB ratio, RelE is released from the complex, further increasing *relBE* expression by removing the RelE-RelB

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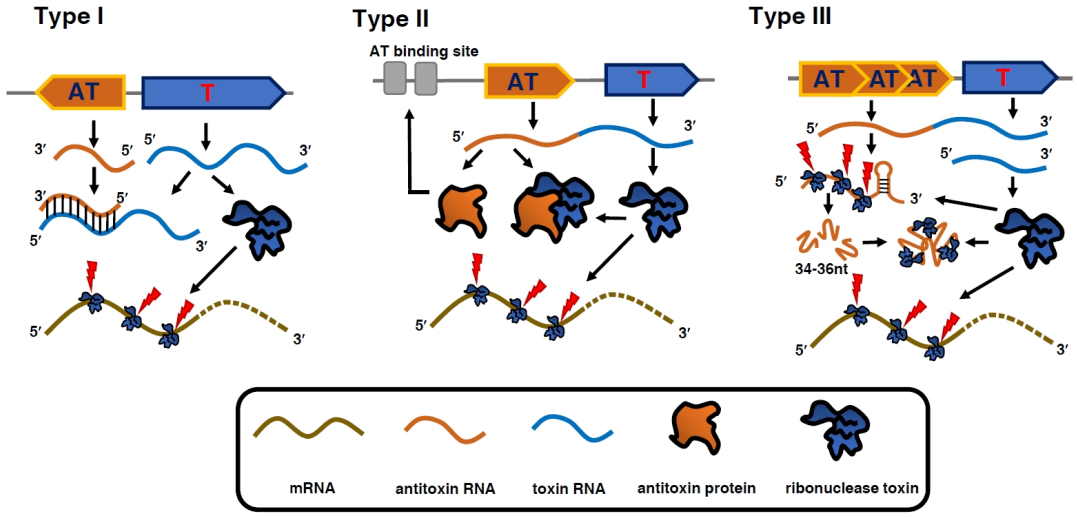

Fig. 1. Bacterial endoribonuclease toxins in the toxin-antitoxin systems. The types of toxin-antitoxin systems are determined by how antitoxins antagonize the endoribonuclease toxins. In type I toxin-antitoxin systems, antitoxins are antisense RNAs and inhibit toxin translation by base-pairing. In type II toxin-antitoxin systems, antitoxins are proteins that directly bind to toxin and neutralize toxin's endoribonuclease activity. Antitoxin alone or toxin-antitoxin complex also binds to the promoter region and represses expression of the toxin-antitoxin operon. In type III toxin-antitoxin systems, antitoxins are small noncoding RNAs that are transcribed as longer transcripts and then processed by the cognate endoribonuclease toxin into 34-36 nt sRNAs. The processed sRNAs bind to the toxin protein and inhibit toxin's endoribonuclease activity.

complex from the operator (23).

RelE is a representative endoribonuclease toxin among bacterial toxin-antitoxin systems. RelE specifically cleaves ribosome-bound mRNAs and has no activity on free mRNAs *in vitro*. RelE induces cleavage of mRNAs between the second and third nucleotides of UAA/UAG stop codons or several sense codons (Py-Pu-G) in the ribosomal A-site (Table 1) (5, 24, 25). RelE has a bacteriostatic effect on growth by quickly inducing cleavage of mRNAs, shutting down translation. The ribosome stalled by RelE can be recycled via tmRNA-mediated trans-translation (26-28). The ribosome-bound RelE structure analysis revealed that RelE occupies the A-site and tightly interacts with 16S rRNA. RelE binding induces conformational change of mRNA of the A site, resulting in RNA hydrolysis between codon positions 2 and 3. The RelE-mediated mRNA cleavage requires the interaction between RelE Tyr87 and the base of the second nucleotide in mRNA codon (Fig. 2A) and the interaction between the bases of the third nucleotide in mRNA codon and C1054 of 16S rRNA (25). These requirements explain why the RelE-mediated mRNA cleavage is ribosome-dependent.

YefM-YoeB: YoeB is a toxin that belongs to a RelE superfamily (29). Unlike other RelE superfamily toxins that are typically monomeric, YoeB toxin binds to the ribosome as homodimer (30). However, the engineered YoeB monomer still retained ribosome-dependent ribonuclease activity despite being unstable during thermal stress (31), suggesting that dimer formation is dispensable for its function but required for its thermal stability. YoeB binds to the 30S subunit of the ribosome and cleaves mRNAs in the A-site (31). A crystal structure of YoeB dimer: 70S subunit showed that one of the YoeB subunits exhibits a close contact with 16S rRNA, 23S rRNA, and mRNA in the A-site whereas the other YoeB subunit is loosely associated with the 30S subunit (31), again supporting that dimer formation is not essential for ribonuclease activity.

Similarly to RelE, YoeB toxin has a broad codon specificity. It cleaves mRNAs between positions 2 and 3 in the UAA stop codon and AAU Asn codon and after the third base of AAA Lys codon (20, 31, 32). It was also reported that YoeB toxin also cleaves between the second and third bases of AAA Lys, CUG Leu, and GCG Ala codons (Table 1) (29). Given that most of mRNA cleavage sites are located close to the AUG initiation codon, it was suggested that YoeB inhibits translation initiation (20). Structural analyses showed that the second nucleotide of mRNA codon in the A-site specifically interacts with YoeB Lys49, while the first and third nucleotides of mRNA codon lack such base-specific interactions (Fig. 2B) (30, 31), which explain the wide range of codon specificity of YoeB.

YafN-YafO: YafN-YafO is a toxin-antitoxin system discovered by a bioinformatics analysis in *E. coli* (8). Genetic organization of *yafN-yafO* is unique in that the *yafN-yafO* genes are a part of *dinB-yafN-yafO-yafP* operon and are transcribed from two promoters, where one promoter is located upstream of the *dinB* gene and the other promoter is upstream of the *yafN* antitoxin gene (33). The *dinB* gene encodes an enzyme required for error-prone DNA repair in SOS response and transcription of the *dinB* gene is induced by DNA damaging agents such as mitomycin C from the upstream promoter of the *dinB-yafN-yafO-yafP* operon (8, 33), suggesting a physiological role of YafO in SOS

Table 1. Substrate specificity of bacterial endoribonuclease toxins

TA operon	Protein/RNA	TA	Signals/Regulators	Recognition sequence	Reference
Type II ribosome-dependent endoribonuclease toxin					
relB-relE	RelE	Toxin	Amino acid or glucose starvation / Lon protease (*E. coli*)	cleaves mRNA at UA↓G, UA↓A stop codons, and CA↓G Gln sense codon in the ribosomal A site (*E. coli*)	(5, 21, 25)
	RelB	Antitoxin			
yefM-yoeB	YefM	Antitoxin	Lon protease overexpression	UA↓A (stop), AAA↓ (Lys), AA↓U (Asn), AA↓A (Lys), CU↓G (Leu), GC↓G (Ala) Cleaves 3 nt downstream of the AUG initiation codon	(20, 29, 31, 32)
	YoeB	Toxin			
yafN-yafO	YafN	Antitoxin	Amino acid or glucose starvation (*yafN* promoter), UV irradiation, SOS response (*dinB* promoter)	Cleaves 11-13 nt downstream of the AUG initiation codon	(8, 34)
	YafO	Toxin			
dinJ-yafQ	DinJ	Antitoxin	Biofilm formation, SOS response / LexA repressor	AA↓A (Lys) A/G	(36, 38)
	YafQ	Toxin			
higB-higA	HigB	Toxin	Amino acid starvation	Cleaves an adenosine-rich codon including AAA (Lys), ACA (Thr)	(8, 40-42)
	HigA	Antitoxin			
prlF-yhaV	YhaV	Toxin	Unknown	Cleavage mostly occurs between codons but also occurs within codons (between the second and third bases in codons) with a low frequency	(45)
	PrlF	Antitoxin			
Type II ribosome-independent endoribonuclease toxin					
mazE-mazF	MazE	Antitoxin	Amino acid starvation, phage P1 infection (*E. coli*) development / MrpC (*Myxococcus xanthus*), nutrition starvation, antibiotics (*Mycobacterium tuberculosis*)	↓ACA (*E. coli*), U↓ACAU (*B. subtilis*), (G/A)U↓UGC (*Myxococcus xanthus*), U↓ACA (*Deinococcus radiodurans*), U↓ACAU (*Staphylococcus aureus*)	(6, 50-54, 92)
	MazF	Toxin			
chpBI-chpBK	ChpBI	Antitoxin	Unknown	↓ACY or A↓CY (Y: A, U, G)	(57)
	ChpBK	Toxin			
pemI-pemK	PemI	Antitoxin	Unknown	U↓AH or UA↓H (H: A, U, C)	(60)
	PemK	Toxin			
mqsR-mqsA	MqsR	Toxin	Amino acid or glucose starvation	Mostly ↓GCU or G↓CU	(8, 62)
	MqsA	Antitoxin			
hicA-hicB	HicA	Toxin	Sxy competence factor and CRP-cAMP	No specific cleavage site in mRNAs A↓AAC sequences in tmRNA	(7, 69)
	HicB	Antitoxin			
Type I endoribonuclease toxin					
symER	SymE (protein)	Toxin	SOS response	Not determined yet	(70)
	SymR (RNA)	Antitoxin			
Type III endoribonuclease toxin					
antiQ-abiQ	AbiQ	Toxin	Phage infection (*Lactococcal lactis*)	Adenine-rich region within the *antiQ* RNA sequence between 26th and 27thadenines (A↓AAA) in the 35 nt-*antiQ* RNA (*Lactococcal lactis*)	(71, 74)
	antiQ	Antitoxin			
toxI-toxN	ToxN (protein)	Toxin	Phage infection (*Pectobacterium atrosepticum*)	AA↓AU (*Pectobacterium atrosepticum*) A↓AAAA (*Bacillus thuringiensis*)	(75, 76)
	ToxI (RNA)	Antitoxin			

response.

Like RelE, YafO toxin has endoribonuclease activity when it is bound to ribosome (34). YafO binds to the 50S subunit in the 70S ribosome and induces mRNA cleavage. However, unlike RelE and YoeB that cleave mRNAs at the A-site, YafO cleaves mRNAs 11 to 13 nucleotides downstream of the AUG start codon (34). The cleavage location corresponds to the 3′ end of mRNAs that are protected by the 70S ribosome initiation complex (34), indicating that YafO is a ribosome-associated endonuclease inducing mRNA cleavage outside of the ribosome. Interestingly, YafO-mediated mRNA cleavage requires ribosome binding but not translation *per se* because mRNA cleavage occurred even without initiator $tRNA_f^{Met}$ and the introduction of nonsense mutation of the second codon did

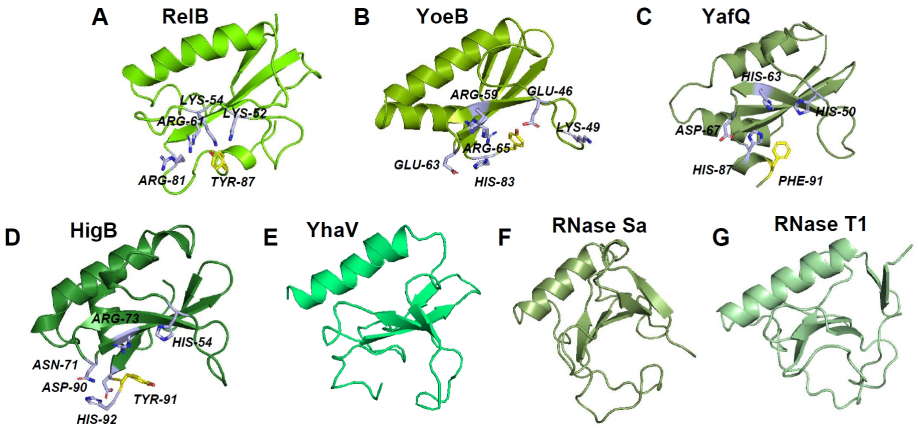

Fig. 2. Type II ribosome-dependent endoribonuclease toxins. Structures of type II ribosome-dependent endoribonuclease toxins. Amino acid sequences for the toxin protein structures were from *Escherichia coli* strain K-12 and *Proteus vulgaris* (for HigB). Amino acid residues involved in general base/acid are indicated in light blue and key residues required for mRNA cleavage are indicated in yellow. (A) RelE monomer (PDB ID: 3KIX). (B) YoeB monomer (PDB ID: 6N90). (C) YafQ monomer (PDB ID: 4Q2U). (D) HigB monomer (PDB ID: 5IFG). (E) YhaV monomer (PDB ID: 2KHE). For comparison, RNase Sa from *Streptomyces aureofaciens* (F) and RNase T1 from *Aspergillus oryzae* (G) were used. (F) RNase Sa monomer (PDB ID: 1NLI). (G) RNase T1 monomer (PBD ID: 1I0V).

not abolish mRNA cleavage (34).

DinJ-YafQ: DinJ-YafQ is another RelBE superfamily toxin-antitoxin system found in *E. coli* (35). YafQ toxin binds to the 50S subunit of the 70S ribosome and induces mRNA cleavage (36, 37). The mRNA cleavage sites of the ribosome-bound YafQ are specific to AAA Lys codon followed by purine (A or G) *in vivo* (36), exhibiting a narrow range of codon specificity among RelE superfamily toxins. Interestingly, purified YafQ has ribonuclease activity without ribosome *in vitro* and cleaves mRNA with lower specificity (36). This is unique given that other RelE-type toxins do not exhibit ribonuclease activity when purified *in vitro*. Such ribonuclease activity on free mRNAs could be due to that YafQ retains several aromatic residues (His50, His87, and Phe91) required for mRNA cleavage (Fig. 2C). Because these aromatic residues are conserved in other RNases including RNase T1 and RNase Sa2 but lacking in other RelE-type toxins (36), it explains *in vitro* ribonuclease activity of the purified YafQ on free mRNAs. His50 and His87 residues of YafQ are required for mRNA cleavage but dispensable for ribosome binding (36, 37). A physiological role of the *dinJ-yafQ* TA system was suggested that the *dinJ-yafQ* TA system is involved in cell death during biofilm formation (38).

HigA-HigB: HigB (The host inhibition of growth B) is a RelE-family endoribonuclease toxin that was originally found in the temperature-sensitive Rts1 plasmid of *Proteus vulgaris* (39). The *higBA* locus was involved in antibiotic resistance and maintenance of the Rts1 plasmid by post-segregation killing (39). The *higBA*-harboring Rts1 plasmid was initially isolated from a *Proteus* clinical isolate that was associated with urinary infection and other chromosomally-encoded *higBA* homologues were also found in several pathogenic bacteria including pathogenic *E. coli*, but not *E. coli* K-12 strain (19). Unlike other toxin genes that lie downstream of the antitoxin genes, the organization of the *higBA* operon is unusual in that the *higB* toxin gene precedes the *higA* antitoxin gene (39).

HigB toxin is a ribosome-dependent RelE-family ribonuclease and cleaves mRNAs between the second and third bases at adenosine-rich codons. Interestingly, although HigB preferentially cleaves AAA Lys and ACA Thr codons, HigB cleaves basically any codon containing adenosine (40). An explanation for the unique selectivity of adenosine was provided by structural analyses of HigB toxin bound to AAA or ACA codons in the A-site of the 70S ribosome (41, 42). HigB does not have a specific interaction with the first position in the codon that allows any nucleotide to be recognized. HigB preferentially interacts with A or C at the second position in the codon, thus A or C being the most effective nucleotide for HigB-mediated mRNA cleavage. At the third position, HigB interacts with C1054 in the 16S rRNA to form an adenosine-specific pocket to accommodate adenosine most efficiently. The HigB Asn71 residue is critical to determine the adenosine specificity of the third position (Fig. 2D) (41). Additionally, His54, Asp90, Tyr91, and His92 residues in the HigB toxin were determined to be critical for the endoribonuclease activity of HigB toxin (Fig. 2D) (42).

PrlF-YhaV: PrlF-YhaV is a toxin-antitoxin system found by a bioinformatics analysis in *E. coli* (43, 44). The *prlF-yhaV* operon encodes PrlF, an antitoxin similar to AbrB transcription factor in *B. subtilis* and YhaV, a RelE-superfamily toxin, respectively (43, 44). PrlF-YhaV forms an unusual heterohexameric complex ($PrlF_2$-$YhaV_4$) *in vitro* that could further form a hexameric dimer ($(PrlF_2\text{-}YhaV_4)_2$ cooperatively when the PrlF-YhaV complex binds to the palindromic DNA sequence of the promoter upstream of the *prlF* gene (44). YhaV toxin has a ribosome-depen-

dent endoribonuclease activity. YhaV binds to the 50S subunit in the 70S ribosome and cleaves mRNAs in a frame-dependent manner (45). The YhaV-mediated mRNA cleavage mostly occurs between codons but also occurs between the second and third bases in codons with a low frequency (45).

The *prlF* (protein localization locus F) gene was originally identified as a suppressor locus for defects of protein translocation (46). The *prlF1* allele turned out to have an insertion mutation near the 3′ end of the *prlF* gene that causes premature termination and derepresses *prlF* expression due to loss of DNA binding ability. And the elevated PrlF levels appeared to increase Lon protease activity by a yet unknown mechanism because the suppressive effect of the *prlF1* allele is mediated by an elevated activity of Lon protease (46).

Type II ribosome-independent endoribonucleases

MazE-MazF: MazE-MazF is a toxin-antitoxin system that is well conserved in bacteria and also one of the most-studied examples among bacterial toxin-antitoxin systems. In *E. coli*, the *mazE-mazF* operon is located immediately downstream of the *relA* gene required for stringent response (47). MazE antitoxin binds to MazF toxin as a heterohexameric complex ($MazF_2$-$MazE_2$-$MazF_2$) and neutralizes MazF toxin activity (18). Under stress conditions, the MazE antitoxin is degraded by ClpAP protease and releases MazF toxin (47). The released MazF toxin has a sequence-specific endoribonuclease activity and cleaves upstream of ACA sequences in *E. coli* (4). Given that the ACA sequences are distributed in most of genes, MazF cleaves mRNAs globally and decreases protein synthesis. Among cellular cleavage sites, MazF also cleaves the 3′ end of 16S rRNA including anti-Shine-Dalgarno sequence (48). Thus, it has been proposed that MazF-induced 16S rRNA cleavage creates a specialized ribosome lacking the anti-Shine-Dalgarno sequence and allows to translate leaderless mRNAs (48). However, a recent study argued against this idea and suggested that MazF affects biogenesis and maturation of rRNAs and ribosomal proteins instead of creating ribosome heterogeneity (49).

The RNA substrate specificity of MazF toxin varies in different species. *E. coli* MazF cleaves upstream of ACA sites (4), whereas *Bacillus subtilis* MazF cleaves between U and A of the UACAU recognition sequences (50). Similarly, *Deinococcus radiodurans* cleaves between U and A of the UACA sequences (51). Interestingly, in *Myxococcus xanthus*, a bacterium that undergoes a multicellular development, MazF cleaves mRNAs between two Us of the (G/A)UUGC recognition sequences and the MazF-mediated mRNA cleavage is involved in developmental programmed cell death (52). In addition to bacterial multicellular development, diverse biological functions of MazF were reported. It includes virulence in *Mycobacterium tuberculosis* (53), defense against bacteriophage infection (54), and persister cell formation in *E. coli* (55).

ChpBI-ChpBK: ChpBI-ChpBK is the second MazEF (ChpA)-type toxin-antitoxin system in the *E. coli* chromosome (56). It was originally identified as one of two chromosomal homologues (*chpA* and *chpB*) of the *pemIK* locus required for stable plasmid maintenance. Similar to MazEF, the *chpBI* gene encodes an antitoxin and the *chpBK* gene encodes a toxin (56). ChpBK toxin also has a sequence-specific endoribonuclease activity but the recognition motif is more ambiguous than MazF's because it cleaves mRNA at either the 5′ or 3′ side of the A nucleotide in the ACY recognition sequences where Y could be one of A, U, or G nucleotides (57). Unlike MazF that exhibits a complete inhibition, ChpBK induction inhibits protein synthesis to approximately 40% compared to uninduced control (57).

PemI-PemK: PemI-PemK is a plasmid-encoded toxin-antitoxin system in *E. coli* that is required for stable maintenance of plasmid R100 (58, 59). PemK toxin inhibits protein synthesis by cleaving mRNAs while PemI antitoxin neutralizes PemK to resume protein synthesis (60). PemK toxin is yet another sequence-specific endoribonuclease that cleaves mRNA at the 5′ or 3′ side of the A nucleotide in the UAH recognition sequences where H could be one of C, A, or U nucleotides (60).

MqsR-MqsA: The *mqsR-mqsA* (*ygiU-ygiT*) operon encodes MqsR toxin and MqsA antitoxin protein. The organization of the *mqsR-mqsA* (ygiU-ygiT) operon is non-canonical in a sense that the *mqsR* toxin gene precedes the *mqsA* antitoxin gene (61, 62). In addition to an MqsA-autorepressible promoter upstream of the *mqsR* toxin gene, the *mqsA* antitoxin gene has two additional constitutive promoters in the coding region of the *mqsR* toxin gene, allowing to uncouple transcription of the *mqsR* and *mqsA* genes (63). The MqsR toxin was originally identified as a motility quorum-sensing regulator because an *mqsR* insertional mutant exhibited a decreased autoinducer 2-mediated biofilm formation and reduced motility (64), both of which were controversial (63). In addition to motility and quorum sensing, the biological function of MqsR toxin was also suggested to be involved in persister cell formation (65).

MqsR toxin turned out to be a ribosome-independent endoribonuclease in *E. coli* (62). It cleaves mRNA preferentially at the 5′ or 3′ side of G nucleotide in GCU recognition sequences both *in vivo* and *in vitro* (8, 62). Interestingly, a structural analysis suggested that MqsR toxin is similar to ribosome-dependent RelE-type toxin rather than ribosome-independent MazF toxin because it has a common RNase fold and the location of critical residues (Lys56, Gln68, Tyr81, and Lys 96; Fig. 3E) is well overlapped with RelE toxin (66). The discrepancy between structural prediction and cleavage site determination remains to be solved. In MqsR-MqsA complex, MqsA antitoxin dimer binds to two MqsR toxins (MqsR-$MqsA_2$-MqsR) and antagonizes MqsR toxin (66).

HicA-HicB: The *hicA-hicB* operon was identified in the pilus gene cluster of *Haemophilus influenzae* and named as *hic* (*Haemophilus influenza contiguous*) loci (67). Using bioinformatics analysis, it was later suggested that the *hicA-hicB* operon encodes a toxin-antitoxin system and is distributed widely in bacteria and archaea (68). The *hicA-hicB* operon is a non-canonical toxin-antitoxin system in that the *hicA* toxin gene precedes the *hicB* antitoxin gene, similar to *higBA* and *mqsRA*

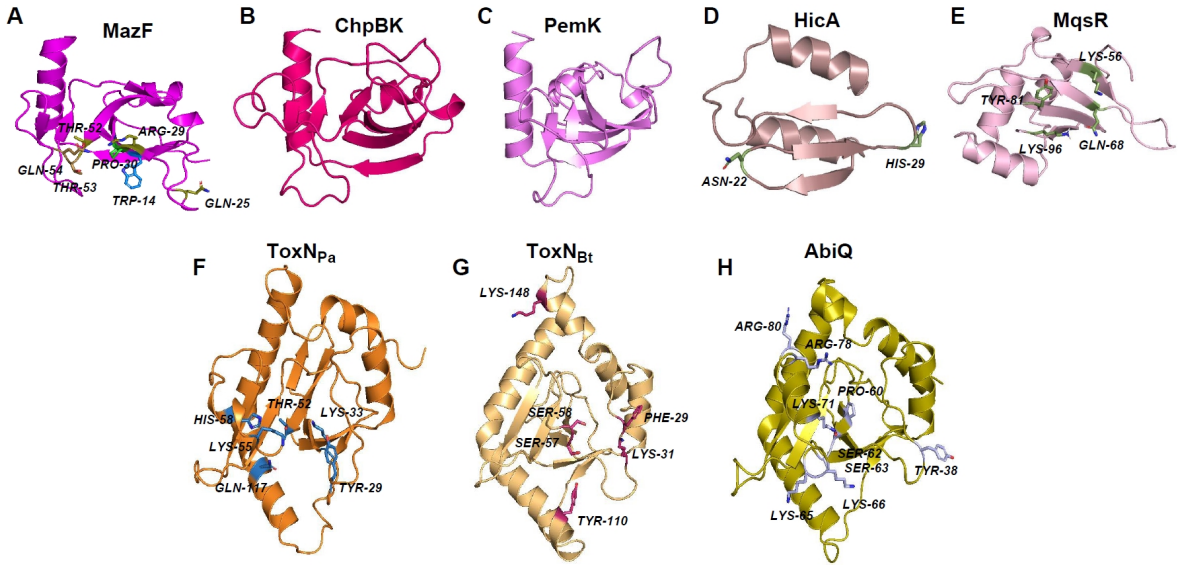

Fig. 3. Type II ribosome-independent endoribonuclease toxins and type III endoribonuclease toxins. Structures of type II ribosome-independent endoribonuclease toxins (A-E) and type III endoribonuclease toxins (F-H). Amino acid sequences for the toxin protein structures were from *Escherichia coli* strain K-12 unless otherwise indicated. (A) MazF monomer (PDB ID: 1UB4). Hydrophilic residues required for interacting with a substrate are indicated in olive green. Pro-30 is required for determining substrate specificity (green) and Trp-14 is also involved in substrate specificity (blue). (B) ChpBK monomer (PDB ID: 1M1F). (C) PemK monomer (PDB ID: 1M1F) of *Staphylococcus aureus*. (D) HicA monomer (PDB ID: 4P78). Two residues required for HicA activity are indicated in green. (E) MqsR monomer (PDB ID: 3HI2). MqsR toxin is the only member of RelE superfamily that has a ribosome-independent endoribonuclease activity. Four key residues are indicated in green. (F) ToxN monomer of *Pectobacterium atrosepticum* (PDB ID: 2XD0). Six residues required for ToxN$_{Pa}$'s activity are indicated in blue. (G) ToxN monomer of *Bacillus thuringiensis* (PDB ID: 4ATO). Six key residues are also indicated in pink. (H) AbiQ monomer of *Lactococcus lactis* (PDB ID: 4GLK). Seven key residues are indicated in pale violet.

operons (7). Moreover, the *hicA* gene encodes a relatively short polypeptide (58 aa in *E. coli*) with a predicted dsRNA-binding fold. The *hicB* gene encodes a hybrid antitoxin with a partial RNase H-fold at the N-terminus and a DNA-binding domain at the C-terminus (7, 68). The operon structure is also unique because it has an additional HicB-independent promoter that is regulated by both Sxy competence factor and CRP-cAMP in *E. coli* (69).

Interestingly, HicA toxin cleaves mRNAs with no sequence specificity (7). Because HicA also cleaves tmRNA at specific AAAC sequences (A^AAC), HicA toxin appeared to be a ribosome-independent endoribonuclease (7). However, the detailed mechanism of RNA recognition by HicA toxin needs to be elaborated.

Type I endoribonuclease toxin

SymR-SymE: SymR-SymE belongs to type I toxin-antitoxin system because SymR is an RNA acting as a 77 nucleotide-long antisense RNA to the *symE* toxin gene. The SymR antisense RNA is transcribed from the translation initiation region of the *symE* toxin gene and thus inhibits synthesis of SymE toxin *in cis* (70). Because the *symE* toxin gene (originally *yjiW*) is induced by SOS response, it was renamed as SymE (SOS-induced *yjiW* gene with similarity to MazE) toxin and the associated antisense RNA (RyjC) was named as SymR (Symbiotic RNA).

SymE toxin appeared to have a ribosome-independent ribonuclease activity given that most of the tested mRNAs were cleaved upon SymE induction (70). However, the specific cleavage sites of SymE toxin have yet to be determined. The predicted structure of SymE toxin is unique in a sense that it is similar to AbrB superfamily, a protein fold typically observed in antitoxins such as MazE (70). It would be interesting to understand how the antitoxin-folded SymE toxin recognizes and cleaves RNA substrates.

Type III endoribonuclease toxins

AbiQ-antiQ: AbiQ was discovered as a phage resistance system that causes abortive infection in *Lactobacillus lactis* (71). Abortive infection is one of phage defense systems whereby a phage-infected bacteria commits suicide to prevent the spread of bacteriophage among bacterial populations (72). The *abiQ* gene was isolated from the plasmid pRSQ900 and encodes a 192 aa protein similar to ToxN type III toxin from *Pectobacterium atrosepticum* (71). The *abiQ* gene is preceded by a 35 nt-tandem direct repeat (2.8 times) and a subsequent rho-independent transcription terminator. This 5′ UTR region is transcribed and processed as 35-nt noncoding RNAs (antiQ), which direct-

ly bind to AbiQ as a triangular heterohexamer ($AbiQ_3$: $antiQ_3$) and neutralize AbiQ toxin (73).

AbiQ toxin has a sequence-dependent endoribonuclease activity that cleaves an adenine-rich sequence within the *antiQ* sequence. It specifically cleaves between 26th and 27th adenines in the A-rich sequence of the 35-nt *antiQ* sequence (A^AAA) (74), whose ribonucleolytic activity seemed to be associated with phage resistance mechanism.

ToxI-ToxN: ToxI-ToxN was initially discovered as a bicistronic operon in a cryptic plasmid from the plant pathogen *Pectobacterium atrosepticum* (previously known as *Erwinia carotovora*) (75). Based on a similarity with AbiQ toxin from *Lactobacillus lactis* (71), ToxN was suggested as a toxin involved in abortive infection (Abi). Indeed, the *toxI-toxN* operon turned out to encode a toxin-antitoxin system that exhibits phage resistance in the presence of a functional ToxN protein. The *toxI* gene encodes an RNA antitoxin and consists of 5.5 identical repeats of a 36 nt sequence followed by a Rho-independent transcriptional terminator sequence that allows only 10% of read-through transcripts. The presence of a Rho-independent transcription terminator between the *toxI* and *toxN* genes appeared to maintain the ratio of ToxI : ToxN at approximately 10 : 1. Similar to AbiQ, ToxN toxin has a MazF-type sequence-specific endoribonuclease activity and processes ToxI transcripts into 36 nt RNAs (76). The processed ToxI RNAs are folded into pseudoknot structures, bind to ToxN toxin, and neutralize endoribonuclease activity by forming an interconnected heterohexameric complex ($ToxN_3$: $ToxI_3$) (77). When ToxN toxins are released from ToxI RNAs, ToxN is likely to cleave other mRNAs resulting in a bacteriostatic effect (75). The mRNA cleavage sites for ToxN were mapped at AA^AU sequences using 5′ RACE analysis (76).

In another plasmid-encoded ToxIN from *Bacillus thuringiensis*, ToxN cleaves mRNA at different recognition sequences (preferentially A^AAAA)(76) as well as ToxI RNAs. ToxN also processes 2.9 repeats of 34 nt-containing ToxI antitoxin RNA into the 34-nt noncoding RNAs and forms a heterohexameric complex with the processed ToxI RNAs ($ToxN_3$: $ToxI_3$).

DISCUSSION

Here we summarized the bacterial toxins with endoribonuclease activity. The toxin components are organized in pairs with its cognate antitoxin components. For example, type II toxin-antitoxin systems consist of toxins and its cognate antitoxin proteins that are organized as bicistronic operons. The expression of the bicistronic operon is mostly repressed by the antitoxin protein, which has a DNA-binding domain for auto-repressor activity and a toxin-binding domain for neutralizing the toxin's activity. Stress conditions including amino acid starvation promote the differential degradation of the labile antitoxins by Lon or ClpAP proteases (78), and the removal of antitoxins results in an increase in the expression of the toxin-antitoxin operon (Fig. 1). The molecular ratio between toxin and antitoxin proteins appears to be tightly regulated because most of the antitoxin and toxin genes are bicistronic and translationally coupled. Generally, the antitoxin gene precedes the toxin gene, which is also likely to ensure an appropriate molecular ratio between toxin and antitoxin proteins. However, the *hicAB*, *higBA*, and *mqsRA* operons have a reverse arrangement, whereby the toxin genes (*hicA*, *higB*, and *mqsR*) precede its cognate antitoxin genes (*hicB*, *higA*, and *mqsA*) (7, 62, 79). Moreover, the *mqsRA* operon has an additional promoter within the *mqsR* gene uncoupling transcription of the *mqsR* and *mqsA* genes (63). However, the biological significance of the reverse arrangement or transcriptional uncoupling is currently unclear. Similarly to most type II toxin-antitoxin systems, type III toxin-antitoxin systems are transcribed as single transcripts and then antitoxin RNAs are processed as mature sRNAs (34-36 nt) by the type III ribonuclease toxin. Because the antitoxin genes have a tandem array of 34 nt or 36 nt-repeat sequences, it seems to guarantee a proper molecular ratio between the processed antitoxin sRNAs and type III ribonuclease toxins (75, 80).

Toxin-antitoxin systems are expressed in response to diverse cellular stresses, including nutrient starvation, stringent response, or exposure to acidic pH (8). In type II toxin-antitoxin systems, these stress conditions preferentially degrade antitoxin proteins by Lon or ClpAP proteases, leading to expression of the toxin genes. In addition to the multiple stress response-mediated antitoxin degradation, SOS response is also suggested to be an inducing signal for several ribonuclease toxin operons includeing the *yafN-yafO*, *dinJ-yafQ*, and *symE-symR* operons. The SOS response-mediated expression of the *yafN-yafO* and *dinJ-yafQ* operons is unique in a sense that they have an additional promoter along with the one regulated by the cognate antitoxins (33, 36). The *yafN-yafO* is a part of the SOS-responsive *dinB-yafN-yafO-yafP* operon, suggesting YafO's additional role in SOS response (33). The *dinJ-yafQ* operon has binding sites for both DinJ-YafQ complex and LexA SOS response repressor (36). The SymE toxin in the *symE-symR* type I toxin-antitoxin operon has a LexA-binding site at its promoter region, suggesting its role in SOS response (70). Given that SymE toxins are degraded by Lon protease, the *symE-symR* type I toxin-antitoxin system is subject to multiple layers of regulation including SymR antitoxin RNA-mediated translational inhibition, SOS response-mediated *symE* induction, and Lon protease-mediated SymE proteolysis (70).

Ribonuclease toxins cleave mRNAs with a diverse range of substrate specificity. RelE ribonuclease toxin requires ribosome to cleave mRNAs and it cleaves mRNA in a codon-dependent manner but with loose codon specificity (5, 25). YafQ ribonuclease toxin is also ribosome-associated but cleaves mRNAs at mostly AAA Lys codon, showing narrow substrate specificity (36, 37, 41). As an opposite extreme, MazF ribonuclease toxin does not require ribosome and cleaves mRNAs in a codon-independent and sequence-dependent manner (4, 6). Considering such diverse substrate specificity, it is not surprising that

the overall sequence similarity of endoribonuclease toxins is low. However, these endoribonuclease toxins have a strikingly similar protein structure depending on toxin types, which raises a question about the factors determining its substrate specificity. Type II endoribonuclease toxins have a common microbial RNase protein fold similar to RNase T1 and RNase Sa2 (Fig. 2) (81-84). SymE type I toxin has a protein fold similar to MazF endoribonuclease (70). Type III endoribonuclease toxins, ToxN and AbiQ, are also homologs of MazF endoribonuclease toxin with additional residues for antitoxin RNA binding (76). And ToxN and AbiQ toxins are structurally similar to each other and superimposable (73, 75, 76). Interestingly, the active site residues of the endoribonuclease toxins are highly variable and thus the amino acid compositions within the active sites appear to determine the substrate specificity. For example, RelE toxin lacks residues required for ribonuclease activity that were found in RNase T1. Instead, 16S rRNA C1054 with the ribosome provides a base required for recognition of specific mRNA codons and its ribonuclease activity (25). By contrast, YafQ ribonuclease toxin harbors active site residues for recognizing mRNAs similar to RNase T1, explaining the ribonuclease activity of the purified YafQ toxin *in vitro* (37). YafQ toxin also retains a patch of basic residues for ribosome binding, being a ribosome-associated ribonuclease toxin (37).

The biological roles of endoribonuclease toxins were suggested to inhibit bacterial growth in response to multiple stressful conditions such as nutrient starvation, SOS response, and bacteriophage infection (2, 3). Such inhibition of bacterial growth contributes to antibiotic tolerance, persister cell formation, biofilm, colonization in the host, and abortive infection (65, 85-89), some of which are controversial (63, 90, 91). To understand the biological role of the endoribonuclease toxins and the underlying mechanisms, mRNA substrate specificity of each toxin needs to be determined in the context of the bacterium's niche and physiology.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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