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MINIREVIEW

Regulation of *Escherichia coli* RNase III activity

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Bacterial cells respond to changes in the environment by adjusting their physiological reactions. In cascades of cellular responses to stresses of various origins, rapid modulation of RNA function is known to be an effective biochemical adaptation. Among many factors affecting RNA function, RNase III, a member of the phylogenetically highly conserved endoribonuclease III family, plays a key role in post-transcriptional regulatory pathways in *Escherichia coli*. In this review, we provide an overview of the factors affecting RNase III activity in *E. coli*.

Keywords: RNase III, mRNA stability, regulator, catalytic mechanism, stress response, osmotic stress

Introduction

Endoribonuclease III (RNase III) was originally identified as a double-stranded RNA specific nuclease in *Escherichia coli* (Robertson *et al.*, 1967). The RNase III family has a conserved nine-residue motif, the so-called RNase III domain. The RNase III family includes mini-RNase III (mini-III) in *Bacillus subtilis*, bacterial RNase III, and eukaryotic Rnt1p, Drosha, and Dicer (Filippov *et al.*, 2000; Lee *et al.*, 2003; Blaszczyk *et al.*, 2004; Ji, 2008; Redko *et al.*, 2008; Court *et al.*, 2013; Nicholson, 2014). RNase III is encoded by the *rnc* gene in *E. coli* that produces a 25.5 kDa polypeptide. It requires divalent ions, preferably Mg^{2+}, for its ribonucleolytic activity (Nashimoto and Uchida, 1985; Watson and Apirion, 1985; Amarasinghe *et al.*, 2001).

RNase III is best known for its role in rRNA and tRNA maturation in *E. coli*. The primary 30S rRNA transcript, which includes all of the rRNA genes, is cleaved by RNase III within the flanking double-stranded regions. This generates the 17S precursor of the 16S rRNA and the p23S precursor of the 23S rRNA (Young and Steitz, 1978; Bram *et al.*, 1980). RNase III is also responsible for the cleavage of the bacteriophage T4 transcript encoding eight tRNAs (Gurevitz and Apirion, 1983). Recent genome-wide analysis studies showed that RNase III is involved in the regulation of gene expression in *E. coli* and other bacteria. Stead *et al.* (2011) showed that approximately 12% of all mRNA species were affected by RNase III and steady-state levels of many transcripts involved in heat shock pathway, iron pathway, and enterobactin production pathway were altered in the absence of RNase III. Microarray analysis also demonstrated that the abundance of several hundred of mRNA species was affected by cellular concentrations of RNase III (Sim *et al.*, 2010). Thus, RNase III has a critical role in the regulation of mRNA stability in *E. coli*.

It is known that RNase III enzymatic activity of can be regulated on multiple levels that include autoregulation by cleavage of its own mRNA message (Bardwell *et al.*, 1989; Matsunaga *et al.*, 1997), upregulation by a bacteriophage kinase-mediated phosphorylation (Rahmsdorf *et al.*, 1974), and downregulation by a protein inhibitor (Kim *et al.*, 2008). Recent studies further uncovered regulatory pathways that modulate RNase III activity upon stress induced by osmotic changes and after an exposure to aminoglycosides (Sim *et al.*, 2010; Kavalchuk *et al.*, 2012; Song *et al.*, 2014).

In this review, we will summarize the molecular mechanisms controlling *E. coli* RNase III activity, which may generally apply to other organisms.

Mechanism of RNase III catalytic action

Endoribonucleolytic activity of RNase III produces 2-nucleotide 3′-OH overhang at the end of dsRNA substrates (for recent reviews, see Court *et al.*, 2013; Nicholson, 2014). The active form of *E. coli* RNase III consists of an endonuclease domain (EndoND) and a double stranded RNA binding domain (dsRBD) combined in a dimer of two 25 kDa polypeptides (Robertson *et al.*, 1967; Blaszczyk *et al.*, 2001). The RNase III dsRBD exhibits an αββββα fold, which is a common form of eukaryotic proteins that recognize dsRNA (Nicholson, 2011). EndoND comprises seven α-helices and a 3_{10} helix. It homodimerizes through hydrophobic interactions, creating a 'ball-and-socket' junction and a large cata-

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lytic valley. Crystallographic and modeling studies of RNase III from *Aquifex aeolicus* suggest that highly conserved six negatively charged residues, including E40/D44/D107/E110 and E37/E64, create two potential RNA cleavage sites within the catalytic valley (Blaszczyk *et al.*, 2001). RNase III requires two transition metal ions to stabilize the catalytic valley where it cleaves dsRNA, and Mg^{2+} is the most favored metal ion in the case of *E. coli* RNase III (Campbell *et al.*, 2002). J. Gan group showed that two Mg^{2+} ions interact with negatively charged amino acids and form a hydrogen bond with nucleotide residues of cleavage sites within each catalytic site (Gan *et al.*, 2008). While Mn^{2+}, Ni^{2+}, and Co^{2+} can also support the catalytic activity of RNase III *in vitro*, extracellular levels of Ni^{2+} and Co^{2+} did not significantly alter RNase III activity (Lim *et al.*, 2012).

To explain the manner in which RNase III recognizes its substrate RNAs and cleaves them with high precision, it has been suggested that a possible consensus sequence and secondary structural elements termed distal box (db), middle box (mb), and proximal box (pb) of target RNAs can directly interact with RNase III (Zhang and Nicholson, 1997). A minimal substrate for RNase III should contain 11 bp in order to interact with a set of four RNA-binding motifs (RBMs 1-4). RBM1 and RBM2, located in dsRBD, interact with pb and mb, respectively. RBM3 and RBM4, located in EndoND, are positioned at cleavage sites during RNase III binding to dsRNA (Gan *et al.*, 2006).

Studies by Nicholson's group revealed that the position-specific exclusion of specific Watson-Crick pairing sequences within pb or db affects substrate RNA-RNase III binding affinity or cleavage ability (Zhang and Nicholson, 1997). While previous studies have focused on negative determinants of substrate RNA sequences *in vitro* (Pertzev and Nicholson, 2006), recent studies provided *in vivo* data for determinants

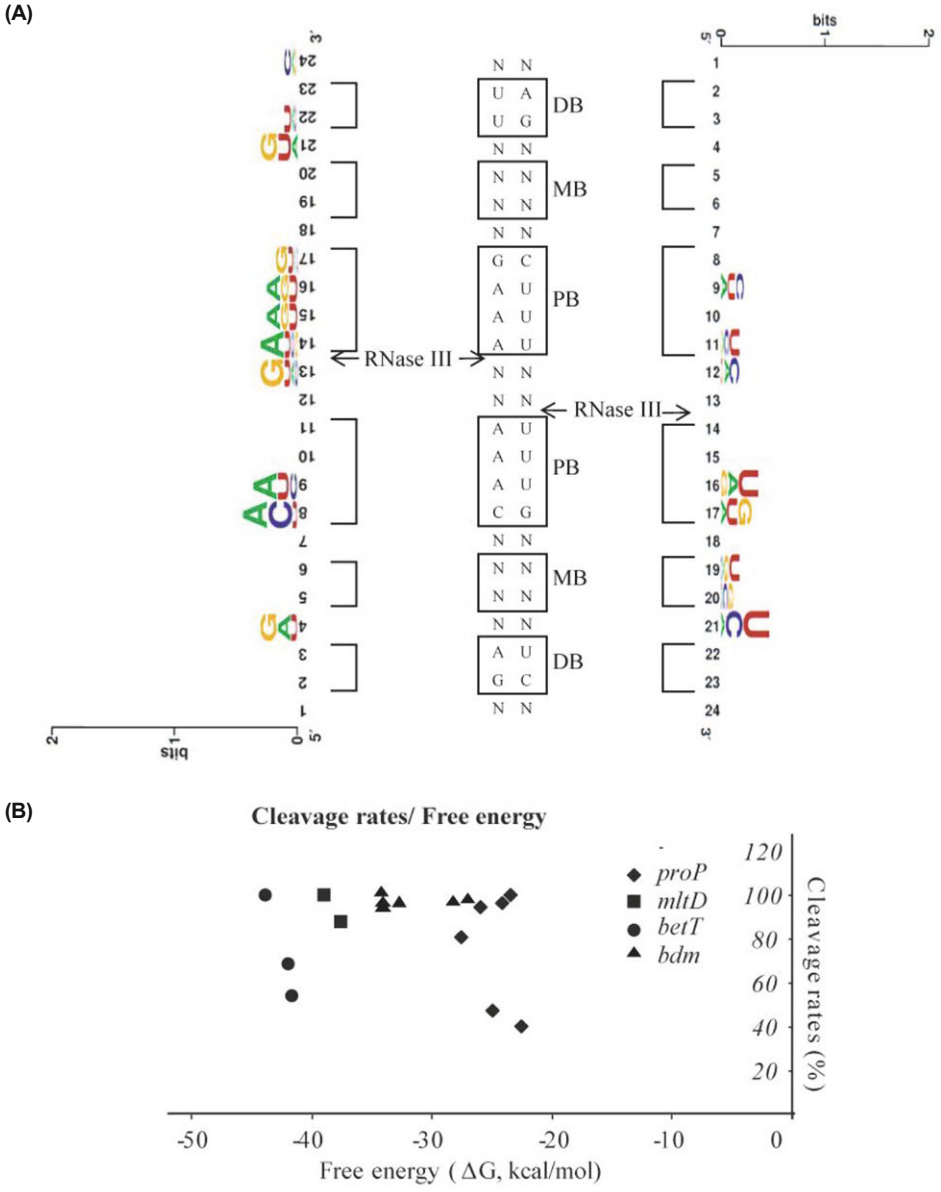

Fig. 1. Analysis of RNase III substrate target sequences *in vivo*. (A) Multiple sequence alignment logo data. WebLogo (http://weblogo.berkeley.edu/) software package was used to identify RNase III cleavage site pattern. The sequence diagram in the middle was from Nicholson (2014). Proximal box (PB), middle box (MB), and distal box (DB) are indicated. Logo data were generated based on RNase III cleavage sites in primary rRNA and mRNA substrates from *bdm*, *betT1*, *betT2*, *corA*, *mltD*, *pnp*, *proP*, *proU*, and *rnc* genes. (B) The relationship between cleavage activity of RNase III towards target mRNAs *in vivo* and free energy of secondary structure formation of hairpins (22 bp stem) encompassing RNase III cleavage sites. The substrates are *bdm*, *betT*, *mltD*, and *proP* mRNAs. Base substitution mutants used were internal loop (IL), bulge (B), stable stem1–3 for *bdm*, C33U and C39U for *betT*, C276U for *mltD*, and C-111U, C-110U/C-111A, C-111G/C-110U/C-111G, C-110A/C-111G for *proP*.

of RNase III cleavage sites by examining *bdm*, *corA*, *mltD*, *proU*, *betT*, and *proP* mRNAs as RNase III substrates (Sim *et al.*, 2010; Kim *et al.*, 2011; Kavalchuk *et al.*, 2012; Lim *et al.*, 2012, 2013; Sim *et al.*, 2014; Lim and Lee, 2015). However, no distinct consensus sequences, which would account for the specificity of RNase III recognition and cleavage process, were observed when *in vivo* RNase III substrates were analyzed (Fig. 1A). In addition, the analysis of free energy required for the formation of the target RNA secondary structure and RNase III cleavage rates among defined wild-type and mutant substrates showed no obvious effects of the free energy on RNase III activity towards these substrates (Fig. 1B).

RNase III-mediated regulation of mRNA stability

pnp and *rnc*

The first example of RNase III-mediated downregulation of gene expression was the *pnp* gene, which encodes polynucleotide phosphorylase (PNPase). Its mRNA is cleaved at two sites located upstream of the start codon by RNase III and the cleavage products become rapidly degraded by PNPase (Regnier and Portier, 1986; Robert-Le Meur and Portier, 1992). RNase III also autoregulates its own transcripts through the site-specific cleavage within the hairpin loop located upstream of the *rnc* coding region (*rncO*), resulting in a rapid degradation of the *rnc* operon message (Bardwell *et al.*, 1989; Matsunaga *et al.*, 1997).

Environmental stress-related mRNAs

Several subgroups of RNase III-dependent transcripts encode factors associated with the cellular response to various environmental stresses induced by metal ions, antibiotics, and salts (Sim *et al.*, 2010, 2014; Kavalchuk *et al.*, 2012; Lim *et al.*, 2012; Song *et al.*, 2014; Lim and Lee, 2015). It has been shown that RNase III cleavage activity in the 5′-untranslated region (UTR) downregulates *corA* expression and constitutes a rate-determining step for *corA* mRNA degradation. The *corA* gene encodes a magnesium transporter, which regulated the influx of several divalent metal ions including Mg^{2+}, Co^{2+}, and Ni^{2+} into the cell (Lim *et al.*, 2012). When metal homeostasis is disrupted, the formation of reactive oxygen species (ROS) may be catalyzed, leading to gene mutations (Rouault and Philpott, 2011). The 'open gate' metal ion transporters, such as CorA, are largely involved in metalloregulation; therefore, RNase III-mediated regulatory pathway is considered as one of the important response mechanisms to metal-induced stress. Although the regulatory RNase III activity in response to cobalt/nickel stress has not yet been demonstrated, there is a possibility that other regulatory factors may stimulate *corA* mRNA degradation by RNase III in such conditions. It has recently been shown that expression levels of RNase G negatively correlated with RNase III activity during the development of resistance to aminoglycoside antibiotics in *E. coli* (Song *et al.*, 2014). This regulatory mechanism involved a reduced expression of the *rng* gene that encodes the endoribonuclease RNase G. This effect, mediated by RNase III, caused an accumulation of incompletely processed 16S rRNA and consequently conferred aminoglycoside resistance. RNase III action preceded the RNase G-mediated antibiotic resistance since *rng* gene expression is negatively controlled by RNase III action on *rng* mRNA, which was increased three-fold in natural isolates of streptomycin-resistant *E. coli* cells (Song *et al.*, 2014).

In *Actinobacillus pleuropneumoniae*, enhanced biofilm formation may contribute in response to environmental stress (Li *et al.*, 2014). It also known that σ^B plays an important role in surface-adhered biofilm formation under stress conditions in *Listeria monocytogenes* (Lee *et al.*, 2014). To maintain the cell wall from turgor pressure under high osmotic stress, *E. coli* has many response mechanisms including biofilm formation and accumulation of osmoprotectants. The steady-state levels of *bdm* (biofilm-dependent modulation) mRNA, which is involved in biofilm formation and flagella biogenesis, are regulated by intracellular concentrations of RNase III independently of the RcsCBD phosphorelay regulatory system (Gottesman *et al.*, 1985; Sim *et al.*, 2014; Kim *et al.*, 2015). Experiments *in vivo* and *in vitro* showed that RNase III cleavage sites on *bdm* mRNA are located in the 5′-UTR and that RNase III activity is downregulated in conditions of high salt concentration. With regard to the osmoregulation of RNase III activity, it has been shown recently that three RNase III substrates that encode the synthesis and transport of osmoprotectants are differentially decayed by RNase III depending on the salt concentration in the environment. Under osmotic stress conditions, synthesis and/or transport of osmoprotectants in bacteria are effective for restoring cellular hydration without interrupting normal cellular processes (Yancey *et al.*, 1982; Wood, 2006). Osmoprotectants including glycine betaine, ectoine, proline betaine, choline, and proline are transported into the cell by osmosensor proteins. BetT, ProU, and ProP are representative osmosensor proteins that belong to different transporter families (Sleator and Hill, 2002; Wood, 2006). Expression levels of *betT*, *proU*, and *proP* genes are tightly controlled by RNase III activity depending on osmolarity conditions. The *betT* mRNA encoding a choline transporter is regulated by RNase III activity within a hairpin loop located in the coding region. Unlike the majority of RNase III target mRNAs with conventional cleavage sites, the *betT* RNA has unique two tandem RNase III cleavage sites, which determine mRNA stability (Sim *et al.*, 2014). The *proU* operon contains three genes (*proVWX*) and encodes a binding-protein-dependent osmoprotectant transporter. Glycine betaine and L-proline uptake via the ProU system is increased under osmotic upshift by a combination of transcriptional induction of the nucleoid-associated protein H-NS and housekeeping sigma factor RpoD, as well as posttranscriptional regulation mediated by RNase III and stimulation of ProU protein activity independently of the osmoregulation of the promoter and H-NS-dependent repression (Cairney *et al.*, 1985; Kavalchuk *et al.*, 2012). The *proP* gene has two promoters: P1 and P2. P1 is controlled by RpoD and P2 is dependent on RpoS. RNase III cleavage of the stem-loop within the 5′-UTR determines the stability of *proP* mRNA synthesized from the osmoregulated P1 promoter and, consequently, affects ProP protein levels and proline uptake capacity (Lim and Lee, 2015). These results indicate the existence of an RNase III-

mediated osmoregulatory network that tightly regulates the expression of proteins implicated in the cellular response to osmotic stress in *E. coli*.

Regulators of RNase III activity

Since ribonucleases play an essential role in almost every aspect of RNA metabolism in prokaryotes and eukaryotes, it is important to understand how their catalytic activity is controlled. Moreover, regulation of the expression of ribonucleases and their activity is an important determinant of steady-state RNA levels in cells and, therefore, constitutes an important means of gene expression control. While it has been known that RNase III activity is affected by various physiological conditions (Nilsson *et al.*, 1984; Georgellis *et al.*, 1993; Chen and Deutscher, 2005; Freire *et al.*, 2006; Sim *et al.*, 2010), our understanding of the mechanisms that regulate RNase III activity is still rather limited.

Autoregulation

The RNase III operon includes the *rnc* and *era* genes encoding RNase III and Era proteins, respectively. RNase III cleaves its own leader RNA, causing instability of its mRNA. Thus, RNase III can repress its own synthesis and also that of Era, because *era* is transcriptionally and translationally coupled to *rnc* expression (Bardwell *et al.*, 1989; Matsunaga *et al.*, 1997). Era function is critical for maintaining cell growth and cell division rate in *E. coli*, so that the regulation of Era levels by RNase III has been proposed as an important feature of the *E. coli* cell cycle (Britton *et al.*, 1998).

In prokaryotic organisms, various ribonucleases, such as PNPase, RNase III, and RNase E, can regulate the degradation of their own mRNA. RNase III autoregulation has been also demonstrated in *Streptomyces coelicolor* (Xu *et al.*, 2008). The Sc-RNase III gene of *S. coelicolor* (antibiotic biosynthesis gene B, *absB*), is transcribed as one of three cistrons within the operon transcript that is cleaved by Sc-RNase III at two sites, one of which is within the RNase III coding sequence. *E. coli* RNase III negatively regulates its own synthesis by cleaving a stem-loop structure within the 5′-UTR of the *rnc* mRNA, causing a rapid degradation of the *rnc* mRNA by other ribonucleases. This region, termed *rncO*, is portable, conferring stability and RNase III regulation to heterologous RNAs (Matsunaga *et al.*, 1996). This autoregulation reduces its own mRNA level approximately 5- to 10-fold and thereby influences the expression of both RNase III and Era.

T7 bacteriophage protein kinase

The first report about a modulator of RNase III activity was by Mayer and Schweiger (1983), who demonstrated that T7 protein kinase positively regulates RNase III in *E. coli*. Bacteriophage T7 expresses the serine/threonine-specific protein kinase T7PK that phosphorylates around 100 different proteins in the infected cell (Rahmsdorf *et al.*, 1974). RNase III undergoes serine phosphorylation in the T7 virus-infected cell, which enhances its catalytic activity by 3–5 fold. Biochemical studies revealed that this phosphorylation facilitates product release, which is the rate-limiting step in the catalytic pathway (Mayer and Schweiger, 1983).

YmdB

RNase III can also be regulated by trans-acting factors. One of well-characterized determinants of RNase III activity is the bacterial macro domain protein YmdB. It has been reported that this highly conserved bacterial protein inhibits RNase III activity *in vivo* (Kim *et al.*, 2008). YmdB expression is induced during the growth of *E. coli* cells at low temperature or entry into the stationary phase. Elevated YmdB expression, in turn, downregulates RNase III activity by direct binding to the region required for dimerization/activation of the enzyme. Cross-linking assays revealed that YmdB interacts with the N-terminal portion of bacterial RNase III polypeptides that includes EndoND, and that the cleavage step is inhibited without affecting the ability of dsRBD to bind dsRNA (Kim *et al.*, 2008).

A recent study showed that YmdB binds the homodimeric nuclease to form a 1:1 complex that is stabilized by contacts with the RNase III EndoND, specifically within the region comprising residues 120–140 *in vivo* (Paudyal *et al.*, 2015). Using the structure-based integrative computational and experimental approaches, it has been demonstrated that YmdB can bind to RNase III without any major structural rearrangement in either protein (Paudyal *et al.*, 2015). In addition to the regulatory properties of YmdB, the phosphodiesterase activity of YmdB is necessary for biofilm formation (Diethmaier *et al.*, 2014). YmdB can inhibit biofilm formation in a manner similar to that of RpoS, which is a regulator of general stress responses and a biofilm inhibitor (Kim *et al.*, 2015).

Modulation of RNase III activity induced by antibiotic stress

RNase III catalytic activity is affected by an unknown factor during aminoglycoside antibiotic stress (Song *et al.*, 2014). This factor has not been characterized; however, it has been clearly observed that the endoribonucleolytic activity of RNase III towards the *rng* mRNA was increased in *E. coli* isolates that transiently acquired resistance to low levels of aminoglycoside antibiotics. Downregulation of RNase G expression resulted in an accumulation of 16S rRNA precursors containing 3–8 extra nucleotides at the 5′ terminus, which was sufficient to induce aminoglycoside resistance. Biochemical analysis of ribosomes containing incompletely processed 16S rRNA revealed a decreased binding capacity for, and diminished sensitivity to, aminoglycosides. A similar pathway appeared to be present in another gram negative bacterium, *Salmonella enterica* serovar Typhimurium. The exact physiological factor required for increased RNase III activity towards the *rng* mRNA remains unknown.

Osmoregulation of RNase III activity

Under osmotic stress conditions, bacteria have developed signal transduction systems to sense changes in the environment and to control the coordinated expression of genes involved in cellular defense mechanisms (Kennelly and Potts, 1996). When the osmotic pressure in the surrounding environment increases, cells activate osmoregulation systems in order to balance the intracellular and environmental pres-

sures (Wood, 1999).

Previous studies identified the mechanisms of RNase III-mediated regulation of a subgroup of mRNA species including *bdm*, *betT*, *proP*, and *proU* whose protein products are associated with the cellular response to osmotic stress (Sim *et al.*, 2010, 2014; Kavalchuk *et al.*, 2012; Lim and Lee, 2015). These studies documented the role of RNase III activity in the cellular response to osmotic stresses in *E. coli*. Analysis of substrate RNA molecules bound to RNase III by *in vivo* crosslinking and immunoprecipitation of RNase III indicated that downregulation of RNase III cleavage activity under hyperosmotic stress is caused by the decreased RNA binding capacity of RNase III (Lim and Lee, 2015). This observation prompts an investigation of mechanistic causes of the decreased RNA-binding capacity of RNase III. One obvious mechanism could be a rapid accumulation of potassium ions in the cytoplasm in the presence of compatible solutes, such as betaines, amino acids, and sugars in *E. coli* under hyperosmotic conditions (Wood, 1999). The osmoregulatory K^+ uptake is mediated by the Trk and Kdp transporters. Constitutively expressed Trk mediates K^+ uptake with a high K_M value for K^+ (approximately 0.3–3 mM), when extracellular K^+ concentration is above 1 mM (Bossemeyer *et al.*, 1989). Kdp transporter system is expressed from the kdp operon with a low K_M for K^+ (2 μM) (Laimins *et al.*, 1981). Both Trk and Kdp systems are activated within seconds upon

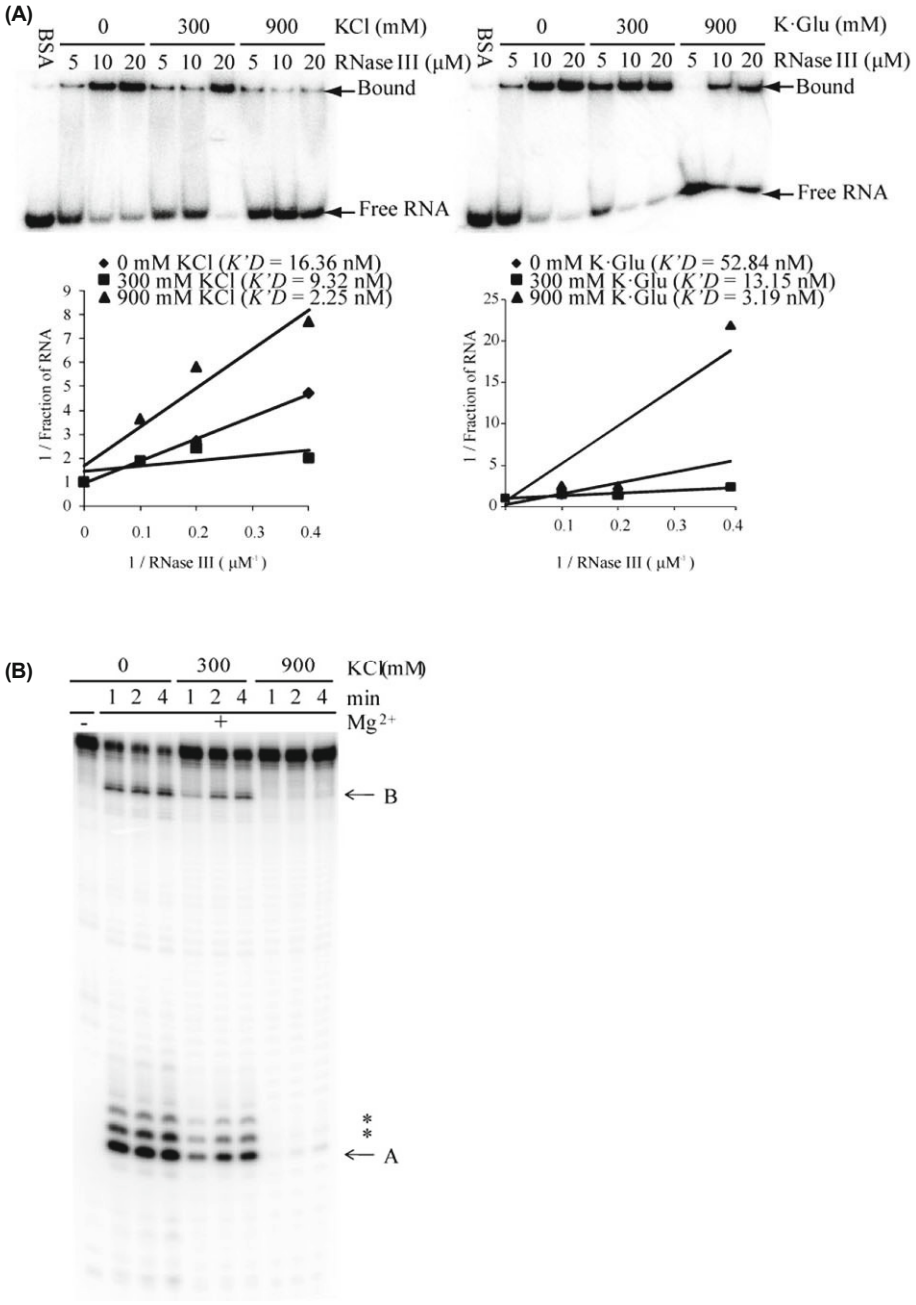

Fig. 2. Effects of K^+ on interactions of RNase III with substrate RNA. (A) Effects of K^+ on RNA binding capacity of RNase III. 5′-end-labeled *proP* model hairpin RNA was mixed with different concentrations of purified RNase III protein (5, 10, 20 μM) under different concentrations of potassium chloride and potassium glutamate (0, 300, 900 mM) and analyzed in a 10% native polyacrylamide gel. Dissociation constants (K_D) were calculated from the plots on the graph. We obtained K_D values from the slope that represents a linear relation between the fraction of bound RNA and the reciprocal of RNase III concentration. (B) Effects of K^+ on the RNA cleavage activity of RNase III. *In vitro* cleavage of the model hairpin RNA under different concentrations of potassium salts. One pM of the 5′-end-labeled *proP* model hairpin RNA was incubated with 0.05 pM of purified RNase III in a cleavage buffer with or without $MgCl_2$ under different concentrations of potassium salts (potassium chloride and potassium glutamate). Samples were taken at indicated time intervals and separated by 10% PAGE in gels containing 8 M urea. Major cleavage products (A and B) are shown and other minor cleavage products are indicated with asterisks.

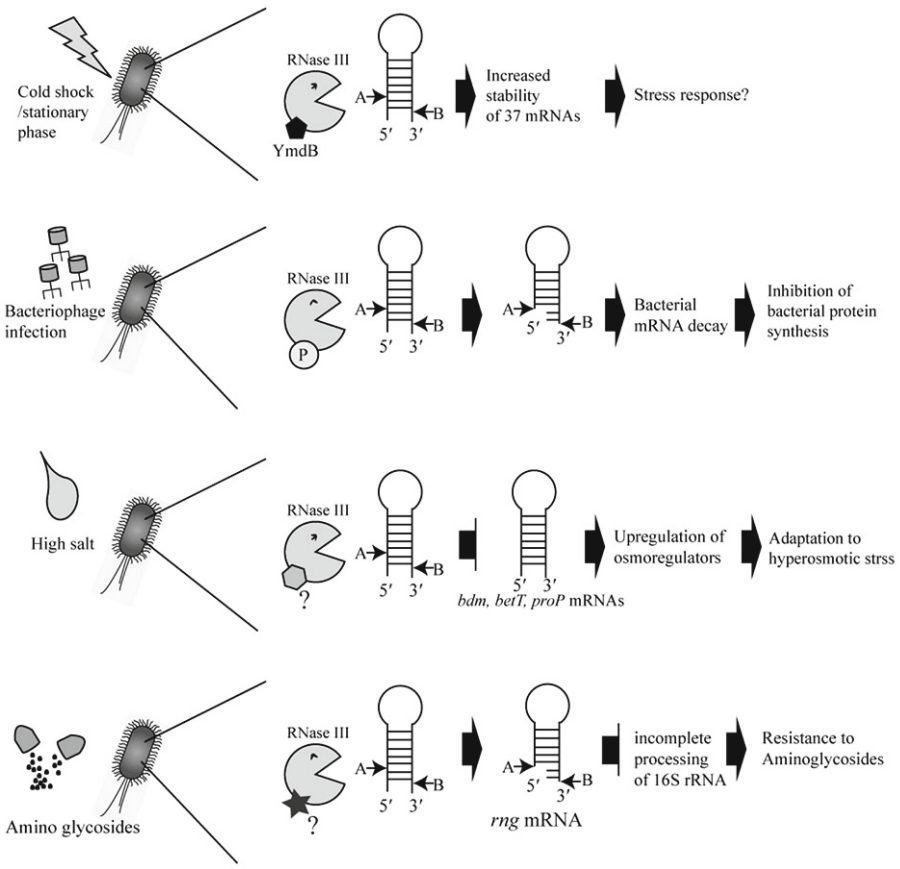

Fig. 3. Schematic representations of RNase III activity during various environmental stresses.

osmotic stress (Wood, 1999). For these reasons, we focused on K^+ as a key osmoregulator. Electrophoretic mobility shift assays (EMSA) revealed that K^+ concentrations comparable to those in *E. coli* cells under hyperosmotic conditions inhibit RNase III binding to a substrate RNA (Fig. 2A). *In vitro* cleavage assays further showed that the cleavage activity correlates with the RNA binding capacity of RNase III that was inversely dependent on K^+ concentration. Whether increased K^+ concentrations in *E. coli* cells upon hyperosmotic conditions are responsible for the decreased RNA binding capacity of RNase III remains to be investigated.

Concluding remarks

In recent years, our knowledge of the regulation of RNA stability has been considerably advanced by discoveries of participating RNases, and analysis of RNA decay and processing pathways involving these enzymes. We have discussed the mechanism of RNase III catalytic activity and RNase III-mediated regulation of mRNA stability in *E. coli*. Although RNase III has long been considered as an rRNA processing enzyme, recent studies showed its active role in modulation of mRNA stability associated with cellular responses to various environmental stresses (Fig. 3). Regulatory mechanisms of this homodimeric complex have not been yet elucidated; however, investigations of RNase III target substrates, including sequence components and secondary structure features, have been accumulating. Experiments revealing RNase III-mediated regulation of environmental stress-related genes provide evidence of the critical role of RNase III in rapid cellular responses to various stress conditions. Although regulation of such genes by RNase III is essential for bacteria to adapt to a wide range of environmental conditions that they encounter in nature, very few reports have addressed the exact mode of substrate recognition and mechanisms underlying the regulation of RNase III activity. Nevertheless, recent molecular and structural studies of RNase III have greatly improved our understanding of this phylogenetically highly conserved enzyme as a global regulator of gene expression.

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