J. Microbiol. Biotechnol. (2008), **18**(8), 1353–1356

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

© The Korean Society for Microbiology and Biotechnology

Functional Implications of the Conserved Action of Regulators of Ribonuclease Activity

Yeom, Ji-Hyun[1], Eunkyoung Shin[1], Hayoung Go[1], Se-Hoon Sim[1], Maeng-Je Seong[2], and Kangseok Lee[1]*

[1]*Departments of Life Science and* [2]*Physics, Chung-Ang University, Seoul 156-756, Korea*

Received: February 9, 2008 / Accepted: March 5, 2008

RNase E (Rne) plays a major role in the decay and processing of numerous RNAs in *E. coli*, and protein inhibitors of RNase E, RraA and RraB, have recently been discovered. Here, we report that coexpression of RraA or RraB reduces the ribonucleolytic activity in *rne*-deleted *E. coli* cells overproducing RNase ES, a *Streptomyces coelicolor* functional ortholog of RNase E, and consequently rescues these cells from growth arrest. These findings suggest that the regulators of ribonuclease activity have a conserved intrinsic property that effectively acts on an RNase E-like enzyme found in a distantly related bacterial species.

Keywords: Degradosome, RNase E, RNase ES, RraA, RraB

The instability of bacterial mRNA is believed to play an important role in the rapid regulation of gene expression responding to physiological needs in the cell. Among many factors involved in these processes, an endoribonuclease, RNase E (Rne), has been shown to play a major role in the degradation and processing of a large number of RNA transcripts in *E. coli* (for review see [1]).

RNase E protein has three functionally distinct domains: the N-terminal region, which contains the enzyme's catalytic domain; an arginine-rich central region, which has strong RNA binding ability; and a C-terminal half that provides a scaffold for the interaction of multiple proteins. The interacting proteins together with RNase E form a complex termed the "degradosome", and include polynucleotide phosphorylase (PNPase), the RhlB RNA helicase, the ATP-generating enzyme enolase, the chaperon proteins DnaK and GroEL, polyphosphate kinase, and poly(A) polymerase (for review see [1]).

Previous work aimed at understanding the role of RNA decay in the control of gene expression in *Streptomyces coelicolor* demonstrated that an *S. coelicolor* endoribonuclease, named RNase ES (Rns), which cleaves substrates at or near sites attacked by RNase E [3, 10], can functionally substitute for Rne in *E. coli* [10]. However, despite the similarities between RNase E and RNase ES, these enzymes can accurately process 9S rRNA from just their own bacteria, indicating that these ancient enzymes and the rRNA segments that they attack appear to have coevolved [6–8, 14–16].

The cellular level and activity of RNase E in *E. coli* are tightly controlled. It has been shown that forced depletion or overproduction of RNase E is detrimental for cellular growth [5, 17]. RNase E autoregulates its synthesis by modulating decay of its own mRNA, thus maintaining the enzyme expression at a relatively stable level [4, 12]. The endonucleolytic activity of RNase E is also controlled by protein inhibitors, RraA and B (regulator of ribonuclease activity A and B) [2, 11]. They interact with RNase E at separate sites within the RNase E and exert dramatic and distinct effects on the composition of the degradosome. The combined action of the two proteins differentially alters mRNA decay in a transcript-specific manner [2].

Whereas protein sequences homologous to RraA are widely distributed among Archaea, proteobacteria, and plants, RraB homologs are found only in *γ-Proteobacteria*, suggesting that these proteins may have a more specialized role in modulating RNA degradation. *Streptomyces coelicolor* contains a functional ortholog of *E. coli* RNase E and also two open reading frames (ORFs), showing 39% and 32% similarity to the amino acid sequence of RraA, respectively, whereas it does not contain an ORF similar to RraB.

In the present study, using a genetic system that takes advantage of the ability of RNase ES to functionally complement RNase E in *rne*-deleted *E. coli* cells [10], we investigated whether these *E. coli* protein inhibitors can modulate the ribonucleolytic activity of RNase ES, which is structurally dissimilar to *E. coli* RNase E by having the catalytic domain of the protein near its center.

To resolve the question of whether *E. coli* regulators of ribonuclease activity, RraA and RraB, can modulate the

**Corresponding author*
Phone: 82-2-820-5241; Fax: 82-2-822-5241;
E-mail: kangseok@cau.ac.kr

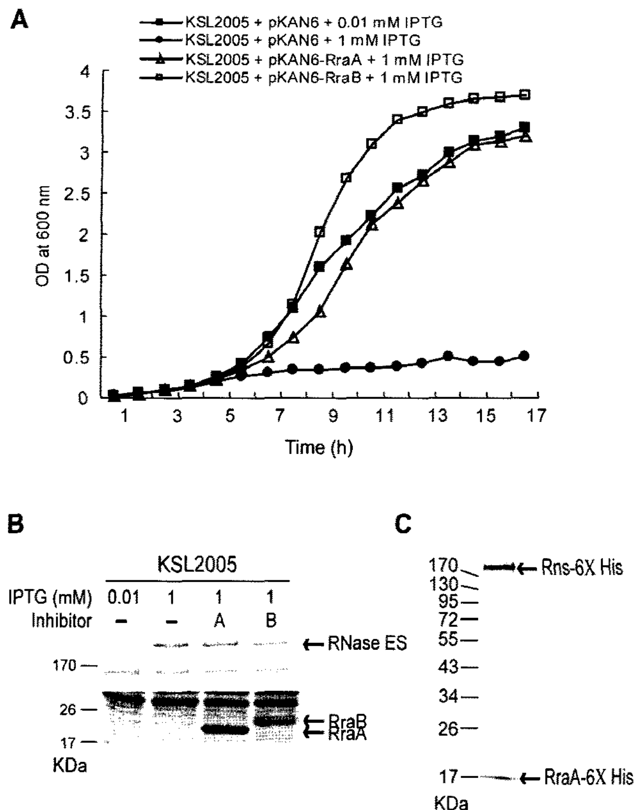

Fig. 1. Effects of overproduction of RNase ES and coexpression of RraA or RraB on *E. coli* growth.
A. Effects of coexpression of RraA or RraB, in *E. coli* cells overproducing RNase ES, on growth. The cultures of KSL2005 cells harboring pKAN6, pKAN6-RraA, or pKAN6-RraB, grown in LB containing 10 μM IPTG medium and 0.2% arabinose and no additional IPTG (KSL2005+pKAN6+10 μM IPTG) or 1 mM IPTG (KSL2005+pKAN6+1 mM IPTG, KSL2005+pKAN6-RraA+1 mM IPTG and KSL2005+pKAN6-RraB+1 mM IPTG), were added to the cultures at OD_{600}=0.1, and growth was monitored by analyzing cell density (absorbance at 600 nm) at indicated time intervals. **B**. Expression profiles of RNase ES, RraA, and RraB. Culture samples from the growth curve in log phase (OD_{600}=0.6) were harvested to obtain total protein, and the proteins were separated by SDS-PAGE and stained with Coomassie blue. **C**. Western blot analysis of KSL2005 harboring pKAN6-His-RraA. The culture was grown to OD_{600}=0.1 in the presence of 10 μM IPTG, 1 mM IPTG and 0.2% arabinose were then added to the culture and further grown to the OD_{600}=0.6, and harvested to obtain total protein. The membrane was probed with anti-His monoclonal antibody (a 1:1 mixture of tetra His and RGS His antibody purchased from Qiagen). Plasmids pKAN6, pKAN6-RraA, and pKAN6-His-RraA have previously been described [17]. Plasmid pKAN6-RraB was constructed by ligating PCR DNA digested with BglII and EcoRI into the same site of pKAN6. PCR DNA containing the coding region of RraB was synthesized using two primers, RraB 5' (5'-CCATGAATTCATATGGCAAACCCGG-AACAA-3') and RraB 3' (5'-TCTAGATCTTTAGTGGCGAACTCCGTC-3').

ribonuclease activity of a *S. coelicolor* functional ortholog of *E. coli* RNase E (RNase ES), we utilized an *E. coli* strain (KSL2005) in which a chromosomal deletion in *rne* was complemented by a plasmidborne *rns* gene coding RNase ES under the control of an isopropylthiogalactoside (IPTG)-inducible *lac*UV5 promoter (pRNES101) [10]. Addition of 1–10 μM IPTG to cultures of KSL2005 allowed the synthesis of C-terminally hexahistidine-tagged full-length RNase ES that supports survival and growth of this *rne* deletion mutant, whereas KSL2005 cells grew at significantly reduced rates when RNase ES was overproduced in the presence of 1 mM IPTG (Fig. 1A). KSL2005 cells overproduced Rns in the presence of 1 mM IPTG at about five times the level of Rns produced in KSL2005 cells in the presence of 10 μM IPTG (Fig. 1B). Using this characteristic of KSL2005, we tested the ability of RraA and RraB to restore the growth of KSL2005 cells whose growth was inhibited by overproduction of RNase ES in the presence of 1 mM IPTG. To coexpress RraA in KSL2005 cells, a compatible kanamycin resistance (Km^r) plasmid expressing RraA or RraB under the control of the arabinose-inducible promoter (pKAN6-RraA or RraB) was introduced into KSL2005 cells. The resulting transformants that overproduced both proteins, Rns and RraA or RraB, in the presence of 1 mM IPTG and 0.2% arabinose were able to grow at slighly higher or similar rates to KSL2005 cells harboring an empty vector (pKAN6) grown in the same medium containing 10 μM IPTG and 0.2% arabinose (Fig. 1A). A molar ratio of Rns to RraA protein in KSL2005 cells that was sufficient for restoration of normal growth of these cells was assessed by introducing plasmid pKAN6-His-RraA that expresses C-terminally hexahistidine-tagged RraA (RraA-His) into KSL2005 cells and detecting RNase ES and RraA-His proteins using antibody to the His-tag in a Western blot. KSL2005 cells harboring pKAN6-His-RraA expressed an approximately 1:20 molar ratio of RNase ES and RraA (Fig. 1C). Like RraA expressed from pKAN6-RraA, coexpression of C-terminally hexahistidine-tagged RraA was also able to rescue RNase ES overproducing KSL2005 cells from growth arrest (data not shown). A similar molar ratio of Rns to RraB was expressed in KSL2005 harboring pKAN6-RraB under the same culture condition, as judged from the intensity of protein bands in a Coomassie blue-stained gel (Fig. 1B). These results imply that both RraA and RraB are able to effectively modulate aberrantly high RNase ES activity *in vivo* and suggest that the growth inhibition caused by overproduction of RNase ES is due to the increased ribonucleolytic activity in the cell.

To test whether the basis of restoration of normal growth to KSL2005 cells overproducing RNase ES results from the reduced cellular ribonucleolytic activity of RNase ES by coexpression of RraA or RraB, three of known RNase E substrates in *E. coli* that are also cleaved by RNase ES [10] were analyzed for their steady-state level. One of these RNase ES substrates was RNAI, an antisense regulator of ColE1-type plasmid DNA replication, which is cleaved by RNase ES and whose abundance consequently controls the copy number of the plasmid. This property has been used to assess the ribonucleolytic function of RNase E-like enzymes *in vivo* [9, 10]. Induced expression of RNase ES in the presence of 1 mM IPTG in KSL2005 cells resulted in increased copy number of the ColE1-type plasmid pKAN6

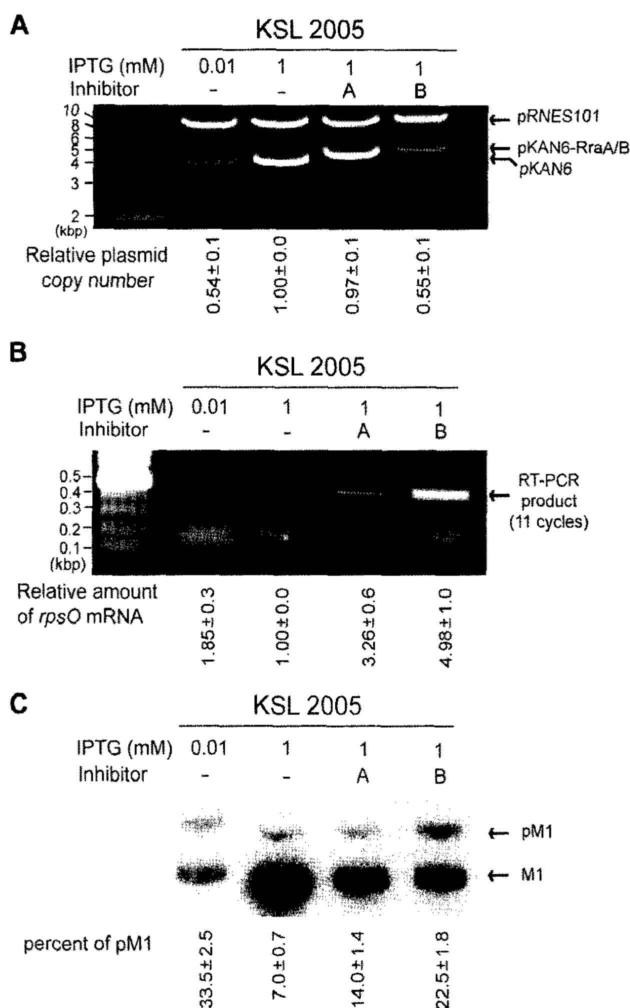

Fig. 2. Effects of coexpression of RraA or RraB on ribonucleolytic activity of RNase ES *in vivo*.

A. Effects of overproduction of RNase ES and coexpression of RraA on copy number of ColE1-type plasmids. Plasmid DNA was isolated from the cultures used in the growth curve (Fig. 1A). Plasmids were digested with HindIII and AflII restriction enzymes, which are unique sites in pRNES101 and pKAN6, pKAN6-RraA, or pKAN6-RraB, respectively. Digested plasmid DNA was electrophoresed in 0.9% agarose gel and stained with ethidium bromide. Plasmid copy number was calculated relative to a concurrently present pSC101 derivative (pRNES101), the replication of which is independent of Rns, by measuring the molar ratio of the pRNES101 plasmid to ColE1-type plasmid (pKAN6, pKAN6-RraA, pKAN6-RraB). Densitometric measurements of bands corresponding to each plasmid were converted to actual ratios after normalizing the values according to the size of ColE1-type plasmids, and are shown at the bottom of the gel. **B.** Effects of overproduction of RNase ES and coexpression of RraA or RraB on abundance of *rpsO* mRNA. The procedure for RT-PCR has previously been described [17]. **C.** Effects of overproduction of RNase ES and coexpression of RraA or RraB on processing of pM1 RNA. The procedure for Northern blot analysis has previously been described [9]. Total RNA was isolated from KSL2005 cells grown to OD_{600}=0.6 in the same way described in the Fig. 1A legend, and RT-RCR or Northern blot were performed. RT-PCR products were electrophoresed in 1.0% agarose gel and the intensity of bands was measured in the same way described above. For Northern blot analysis, RNA samples were separated in a 6% polyacrylamide gel containing 8 M urea. Percentage of pM1 RNA represents the ratio of pM1 to the sum of pM1 and M1 bands hybridized to the probe.

by approximately 2-fold relative to that observed in KSL2005 cells expressing RNase ES in the presence of 10 μM IPTG (Fig. 2A). The plasmid copy number of ColE1-type plasmid (pKAN6-RraB) in KSL2005 cells coexpressing RNase ES and RraB was reduced to levels similar to that of KSL2005 cells producing RNase ES at levels that do not interfere with normal cellular growth (10 μM IPTG), whereas coexpression of RraA under the same culture condition resulted in no significant change in the plasmid copy number of pKAN6-RraA compared with that of pKAN6 in KSL2005 cells overproducing RNase ES (1 mM IPTG). These results show that RraB inhibits RNase ES action on RNAI *in vivo* more effectively than RraA does.

We further tested the ribonucleolytic activity of RNase ES on other RNase ES substrates when RraA or RraB was coexpressed, by measuring the abundance of *rpsO* mRNA encoding ribosomal protein S15 and pM1 RNA, the precursor of the RNA component of RNase P [13]. As shown in Figs. 2B and 2C, a nearly analogous effect of RraA or RraB coexpression on the ribonucleolytic activity of RNase ES was observed. KSL2005 cells coexpressing RNase ES and RraA or RraB accumulated approximately three to five times more *rpoS* mRNA and two to three times more pM1 RNA than those in KSL2005 cells overproducing RNase ES only. Once again, RraB inhibited RNase ES action on these RNAs *in vivo* more effectively than RraA did. These results indicate that RraB more strongly affects the ability of RNase ES to attack different target RNA substrates *in vivo* than RraA does, indicating that the inhibitory effect exerted by both the RraA and RraB proteins on RNase ES may not be transcript-specific, and that inhibition of the ribonucleolytic activity of RNase ES by coexpression of RraA or RraB is sufficiently strong enough to override growth arrest induced by RNase ES overproduction.

Previous studies showed that RraA and RraB differentially modulate RNase E activity, resulting in distinct subsets of RNA transcripts whose abundance was specifically increased by each inhibitor [2, 11]. However, the effect of RraA and RraB on RNase ES *in vivo* did not appear to be substrate-specific, and RraB exerted a greater inhibitory effect on RNase ES action than did RraA. The observed difference in the effects of the inhibitors on RNases E and RNase ES might have been due to their mode of action on these RNase E-like enzymes. For instance, it has been previously proposed that alteration of the degradosome composition by binding of RraA and RraB to different sites of RNase E results in modulation of the substrate specificity of RNase E [2]. However, this may not be the underlying mechanism for their action on RNase ES, since RNase ES has to form a multiprotein complex similar to the *E. coli* degradosome in *E. coli*. Instead, it is likely that binding of RraA and RraB

to RNase ES results in direct alteration of the ability of the enzyme to bind and/or cleave target RNAs.

The ability of two *E. coli* proteins to exert an inhibitory effect on RNA decay *via* their interactions with a structurally dissimilar RNase E-like enzyme found in a distantly related bacterial species implies that these protein inhibitors have a conserved function in RNA metabolism, and that modulation of RNA stability may be a common mechanism for global control of transcript abundance in bacteria in response to dynamic changes in the extracellular or intracellular environments.

Acknowledgments

We are particularly grateful to Dr. Stanley N. Cohen for the initiative effort toward this study. This research was supported by the Chung-Ang University Research Scholarship Grants in 2007.

References

1. Carpousis, A. J. 2007. The RNA degradosome of *Escherichia coli*: An mRNA-degrading machine assembled on RNase E. *Annu. Rev. Microbiol.* **61:** 71–87.
2. Gao, J., K. Lee, M. Zhao, J. Qiu, X. Zhan, A. Saxena, C. J. Moore, S. N. Cohen, and G. Georgiou. 2006. Differential modulation of *E. coli* mRNA abundance by inhibitory proteins that alter the composition of the degradosome. *Mol. Microbiol.* **61:** 394–406.
3. Hagège, J. M. and S. N. Cohen. 1997. A developmentally regulated *Streptomyces* endoribonuclease resembles ribonuclease E of *Escherichia coli. Mol. Microbiol.* **25:** 1077–1090.
4. Jain, C. and J. G. Belasco. 1995. Autoregulation of RNase E synthesis in *Escherichia coli. Nucleic Acids Symp. Ser.* **33:** 85–88.
5. Jain, C., A. Deana, and J. G. Belasco. 2002. Consequences of RNase E scarcity in *Escherichia coli. Mol. Microbiol.* **43:** 1053–1064.
6. Kim, H.-L., E. Shin, H.-M. Kim, H. Go, J. Roh, J. Bae, and K. Lee. 2007. Heterogeneous rRNA molecules encoded by *Streptomyces coelicolor* M145 genome are all expressed and assembled into ribosomes. *J. Microbiol. Biotechnol.* **17:** 1708–1711.
7. Kim, H.-L., E.-K. Shin, H.-M. Kim, S.-M. Ryou, S. Kim, C.-J. Cha, J. Bae, and K. Lee. 2007. Heterogeneous rRNAs are differentially expressed during the morphological development of *Streptomyces coelicolor. FEMS Microbiol. Lett.* **275:** 146–152.
8. Kim, H.-M., J.-H. Yeom, H.-J. Ha, J.-M. Kim, and K. Lee. 2007. Functional analysis of the residues C770 and G771 of *E. coli* 16S rRNA implicated in forming the intersubunit bridge B2c of the ribosome. *J. Microbiol. Biotechnol.* **17:** 1204–1207.
9. Lee, K., J. A. Bernstein, and S. N. Cohen. 2002. RNase G complementation of *rne* null mutation identifies functional interrelationships with RNase E in *Escherichia coli. Mol. Microbiol.* **43:** 1445–1456.
10. Lee, K. and S. N. Cohen. 2003. A *Streptomyces coelicolor* functional orthologue of *Escherichia coli* RNase E shows shuffling of catalytic and PNPase-binding domains. *Mol. Microbiol.* **48:** 349–360.
11. Lee, K., X. Zhan, J. Gao, J. Qiu, Y. Feng, R. Meganathan, S. N. Cohen, and G. Georgiou. 2003. RraA: A protein inhibitor of RNase E activity that globally modulates RNA abundance in *E. coli. Cell* **114:** 623–634.
12. Mudd, E. A. and C. F. Higgins. 1993. *Escherichia coli* endoribonuclease RNase E: Autoregulation of expression and site-specific cleavage of mRNA. *Mol. Microbiol.* **9:** 557–568.
13. Reed, R. E., M. F. Baer, C. Guerrier-Takada, H. Donis-Keller, and S. Altman. 1982. Nucleotide sequence of the gene encoding the RNA subunit (M1 RNA) of ribonuclease P from *Escherichia coli. Cell* **30:** 627–636.
14. Ryou, S.-M., J.-M. Kim, J.-H. Yeom, H.-L. Kim, H.-Y. Go, E.-K. Shin, and K. Lee. 2005. Species-specific cleavage by RNase E-like enzymes in 5S rRNA maturation. *J. Microbiol. Biotechnol.* **15:** 1100–1105.
15. Shuang, J. L., C. H. Lui, S. Q. An, Y. Xing, G. Q. Zheng, and Y. F. Shen. 2006. Some universal characteristics of intertidal bacterial diversity as revealed by 16S rRNA gene-based PCR clone analysis. *J. Microbiol. Biotechnol.* **16:** 1882–1889.
16. Szatkiewicz, J. P., H. Cho, S. M. Ryou, J. M. Kim, P. R. Cunningham, and K. Lee. 2006. Genetic analysis of a structural motif within the conserved 530 stem-loop of *Escherichia coli* 16S rRNA. *J. Microbiol. Biotechnol.* **16:** 569–575.
17. Yeom, J.-H. and K. Lee. 2006. RraA rescues *Escherichia coli* cells over-producing RNase E from growth arrest by modulating the ribonucleolytic activity. *Biochem. Biophys. Res. Commun.* **345:** 1372–1376.