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Effects of *Escherichia coli* RraA Orthologs of *Vibrio vulnificus* on the Ribonucleolytic Activity of RNase E In Vivo

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Abstract RraA is a recently discovered protein inhibitor of RNase E that catalyzes the initial step in the decay and processing of numerous RNAs in *Escherichia coli*. In the genome of *Vibrio vulnificus*, two open reading frames that potentially encode proteins homologous to *E. coli*, RraA-designated RraAV1 and RraAV2, have respectively 80.1% and 59.0% amino acid identity to RraA. The authors report that coexpression of RraAV1 protein in *E. coli* cells overproducing RNase E rescued these cells from growth arrest and restored their normal growth, whereas coexpression of RraAV2 protein further inhibited the growth of *E. coli* cells, whose growth was already impaired by overproduction of RNase E. Analyses of the steady-state level of various RNase E substrates indicated that the coexpression of RraAV1 more efficiently inhibited RNase E action than coexpression of RraA, and consequently resulted in the more increased abundance of each RNA species tested in vivo. The inhibitory effect by RraAV2 coexpression on RNase E was observed only in the case of *trpA* mRNA, indicating the possibility of RNA substrate-dependent inhibition of RraAV2 on RNase E. The findings suggest that these regulators of ribonuclease activity have both a conserved inhibitory function and a differential inhibitory activity on RNase E-like enzymes across the species.

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Introduction

The instability of bacterial mRNA is believed to play an important role in the rapid regulation of gene expression responding to physiologic needs in the cell. Among many factors involved in these processes, RNase E (Rne), an endoribonuclease, is shown to play a major role in the degradation and processing of many RNA transcripts in *Escherichia coli* [2–4, 6–10]. The interacting proteins together with RNase E form a complex termed the "degradosome," which includes polynucleotide phosphorylase (PNPase), the RhlB RNA helicase, the ATP-generating enzyme enolase, the chaperon proteins DnaK and GroEL, and polyphosphate kinase (for a review see Carpousis [1]).

The cellular level and activity of RNase E in *E. coli* are tightly controlled. Findings have shown that forced depletion or overproduction of RNase E is detrimental for cellular growth [6, 13, 14]. By modulating decay of its own mRNA, RNase E autoregulates its synthesis, thus maintaining the enzyme expression at a relatively stable level [5, 11].

The endonucleolytic activity of RNase E also is controlled by protein inhibitors RraA and B (regulator of ribonuclease activity A and B) [2, 9]. 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].

Although 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. *Vibrio vulnificus*

contains an ortholog of *E. coli* RNase E and two open reading frames, designated RraAV1 and RraAV2 (GenBank accession numbers EU918605 and EU918606, respectively), respectively showing 80.1% and 59% identity to the amino acid sequence of RraA. It also contains another open reading frame showing 65% similarity to the amino acid sequence of RraB.

In the current study, using a genetic system that takes advantage of the *rne*-deleted *E. coli* cells, whose viability is maintained by the expression of Rne protein from plasmid, we investigated whether these *V. vulnificus* homologs of RraA proteins can modulate the ribonucleolytic activity of RNase E in vivo.

Materials and Methods

Strains and Plasmids

Plasmids pKAN6B and pKAN6-RraA have previously been described [13]. Plasmids pKAN6-RraAV1 and pKAN6-RraAV2 were constructed by ligating polymerase chain reaction (PCR) DNA digested with *Nde*I and *Xba*I restriction enzymes into the same sites of pKAN6. We synthesized PCR DNA fragments containing coding regions of RraAV1 and RraAV2 using primers vvRraA 3018F (5′-TTCATATGGAATACAACACTTCAGC-3′) and vvRraA3018R (5′-TTTCTAGATTAATCGAGCAG CTCTGGCTCT-3′) for RraAV1 and primers vvRra A0648F (5′-TTCATATGTGGCAAAAATTTTCTCA-3′) and vvRraA0648R (5′- TTTCTAGATTAGAATTGAAGC AACTCTT-3′) for RraAV2, with the genomic DNA from *V. vulnificus* MO6-24/O [12] as a template.

Measurement of Plasmid Copy Number

The culture was grown to an OD_{600} of 0.1 in the presence of 10 μmol/l isopropylthiogalactoside (IPTG), and 0.1%, 0.002% arabinose, and 1 mmol/l IPTG except KSL2003 + pKAN6, 10 μmol/l IPTG (no additional IPTG added) were added to the culture. They were further grown to an OD_{600} of 0.8 and harvested to obtain plasmid DNA. Plasmids were digested with *Not*I restriction enzyme, which is a unique site in pLAC-RNE2 as well as pKAN6, pKAN6-RraA, pKAN6-RraAV1, and pKAN6-RraAV2. The digested plasmid DNA was electrophoresed in 1.0% agarose gel and stained with ethidium bromide. The plasmid copy number was calculated relative to a concurrently present pSC101 derivative (pLAC-RNE2), the replication of which is independent of Rne, by measuring the molar ratio of the pLAC-RNE2 plasmid to the ColE1-type plasmid (pKAN6, pKAN6-RraA, pKAN6-RraAV1, pKAN6-RraAV2).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

The procedure for RT-PCR analysis has been described previously [13, 14].

Results and Discussion

Effects of Coexpressed RraAV1 or RraAV2 on the Growth of *E. coli* Cells Overproducing RNase E

To resolve the question whether *V. vulnificus* homologs of *E. coli* RraA can modulate the ribonuclease activity of *E. coli* RNase E, we used an *E. coli* strain (KSL2003) in which a chromosomal deletion in *rne* was complemented by a plasmid-borne *rne* gene coding RNase E under the control of an IPTG-inducible *lac*UV5 promoter (pLAC-RNE2) [7, 8]. Addition of 1–10 μmol/l IPTG to cultures of KSL2003 allowed the synthesis of C-terminally hexahistidine-tagged full-length RNase E, which supported the survival and growth of this *rne* deletion mutant, whereas KSL2003 cells grew at significantly reduced rates when RNase E was overproduced in the presence of 1 mmol/l IPTG (Fig. 1a). In the presence of 1 mmol/l IPTG, KSL2003 cells overproduced Rne at about seven times the level of Rne produced in KSL2003 cells in the presence of 10 μmol/l IPTG (Fig. 1b, c) [13].

Using this characteristic of KSL2003, we tested the ability of RraAV1 and RraAV2 to restore the growth of KSL2003 cells whose growth was inhibited by overproduction of RNase E in the presence of 1 mmol/l IPTG. To coexpress RraAV1 in KSL2003 cells, a compatible kanamycin resistance (Km^r) plasmid expressing RraAV1 or RraAV2 under the control of the arabinose-inducible promoter (pKAN6-RraAV1 or RraAV2) was introduced into KSL2003 cells. The resulting transformants were grown in the presence of 10 μmol/l arabinose at various concentrations (0.001–0.1%) to determine optimal concentrations of arabinose that result in expression levels of RraAV2 and RraA similar to that of RraAV1, which was lowest when the synthesis was maximally induced with 0.1% arabinose (data not shown).

The optimal concentration of arabinose for expression of RraA and RraAV2 was 0.002%. Whereas KSL2003 cells overproducing both Rne and RraAV1 proteins in the presence of 1 mmol/l IPTG and 0.1% arabinose were able to grow at rates similar to those of the KSL2003 cells harboring an empty vector (pKAN6) grown in the same medium containing 10 μmol/l IPTG and 0.1% arabinose, coexpression of RraAV2 in KSL2003 cells overproducing RNase E further inhibited the growth of the cells whose growth was already impaired by overproduction of RNase

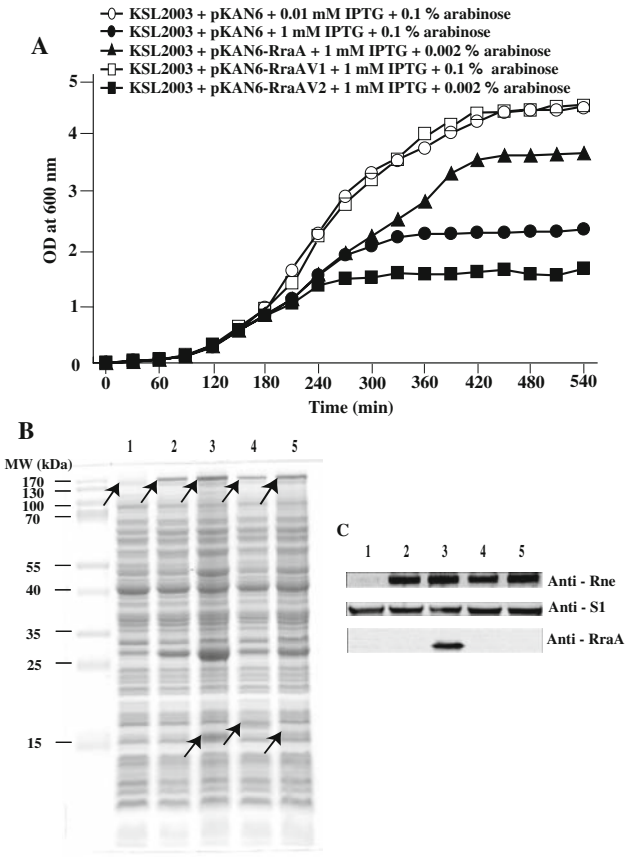

Fig. 1 Effects of RNase E overproduction and coexpression of RraAV1 or RraAV2 on *E. coli* growth. **a** Effects of coexpressed RraAV1 or RraAV2 in *E. coli* cells overproducing RNase E on growth. The cultures of KSL2003 cells were grown (see Materials and Methods section), and the growth was monitored by analyzing cell density (absorbance at 600 nm) at indicated intervals. **b** Expression profiles of RNase E, RraA, RraAV1, and RraAV2. Culture samples from the growth curve in log phase ($OD_{600} = 0.8$) were harvested to obtain total protein. The proteins were separated in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue. Arrows indicate RNase E, RraA, RraAV1, and RraAV2 proteins. **c** Western blot analysis of RNase E, RraA, and S1. The SDS-PAGE was performed in the same way described earlier, and the separated proteins were transferred to the nitrocellulose membrane for probing with monoclonal antibodies to Rne, polyclonal antibodies to RraA, or ribosomal protein S1. (1) KSL2003 + pKAN6 (0.01 mmol/l isopropylthiogalactoside [IPTG], 0.1% arabinose), (2) KSL2003 + pKAN6 (1 mmol/l IPTG, 0.1% arabinose), (3) KSL2003 + pKAN6-RraA (1 mmol/l IPTG, 0.002% arabinose), (4) KSL2003 + pKAN6-RraAV1 (1 mmol/l IPTG, 0.1% arabinose), (5) KSL2003 + pKAN6-RraAV2 (1 mmol/l IPTG, 0.002% arabinose)

E (Fig. 1a). The molar ratio of Rne to RraA, RraAV1, or RraAV2 protein expressed in KSL2003 cells was approximately 1:2, which was assessed by measuring the intensities of the protein bands in Coomassie blue-stained gel (Fig. 1b).

These results imply that RraAV1 was more effective than RraA in modulating aberrantly high RNase E activity to the level for proper processing and decay of all RNA species required to restore normal cellular growth to the cells overproducing RNase E. From these results, it is hard to explain the basis for the detrimental effect of RraAV2 coexpression on the growth of KSL2003 cells overproducing RNase E. A possible explanation for this phenomenon is that coexpression of RraAV2 causes altered substrate specificity of RNase E and, consequently, unbalanced processing and decay of RNase E substrates.

Supporting this view, similar results were observed when RraB, shown to interact with a site on RNase E different from the RraA-binding site and interfere with the cleavage of a set of transcripts different from RraA-specific transcripts [2], was coexpressed in KSL2003 cells overproducing RNase E (J.-H. Yeom and K. Lee, unpublished data). Polyclonal antibodies to RraA were not able to detect RraAV1 and RraAV2 proteins, indicating that these antibodies recognize epitopes of RraA not present in RraAV1 and RraAV2 (Fig. 1c).

Effects of Coexpressed of RraAV1 or RraAV2 on the Ribonucleolytic Activity of RNase E In Vivo

To test whether RraAV1 and RraAV2 are able to modulate the ribonucleolytic activity of RNase E in vivo, five of the known RNase E substrates in *E. coli* [9, 14] were analyzed for their steady-state level. One of these RNase E substrates was RNAI, an antisense regulator of ColE1-type plasmid DNA replication cleaved by RNase E, 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 [7, 8]. Induced expression of RNase E in the presence of 1 mmol/l IPTG in KSL2003 cells increased the copy number of the ColE1-type plasmid pKAN6 approximately 2.5-fold relative to that observed in KSL2003 cells expressing RNase E in the presence of 10 μmol/l IPTG (Fig. 2a). The plasmid copy number of ColE1-type plasmid (pKAN6-RraAV1) in KSL2003 cells coexpressing RNase E and RraAV1 was reduced to levels similar to that of KSL2003 cells producing RNase E at levels that do not interfere with normal cellular growth (10 μmol/l IPTG) or KSL2003 cells overproducing RNase E (1 mmol/l IPTG) and RraA at levels that partially restored cellular growth arrested by RNase E overproduction to KSL2003 cells. However, coexpression of RraAV2 under the same culture condition resulted in no significant change in the plasmid copy number of pKAN6-RraAV2 compared with that of pKAN6 in KSL2003 cells overproducing RNase E (1 mmol/l IPTG). These results show that RraA and RraAV1 effectively inhibited RNase E action on RNAI with similar efficiency and that RraAV2 was not able to inhibit RNase E action on RNAI in vivo.

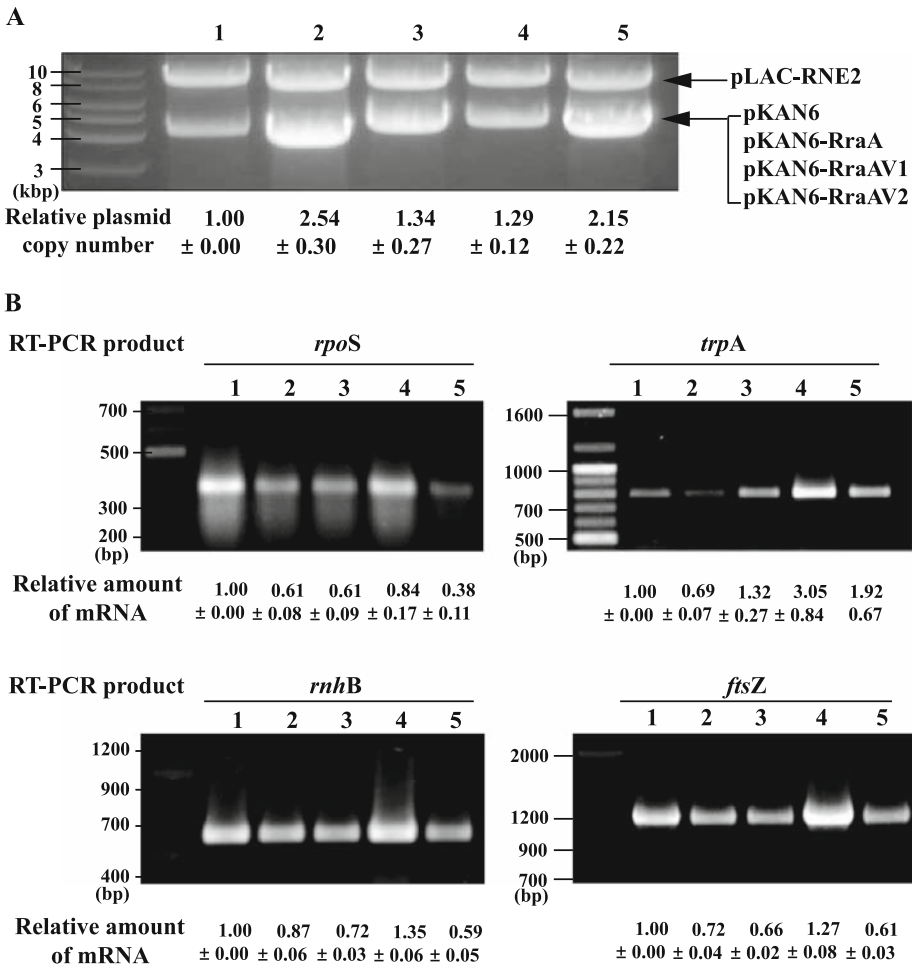

Fig. 2 Effects of coexpressed RraAV1 or RraAV2 on the ribonucleolytic activity of RNase E in vivo. **a** Effects of overproduced RNase E and coexpressed RraAV1 or RraAV2 on the copy number of ColE1-type plasmids. Plasmid DNA was isolated from the cultures used in the growth curve (Fig. 1a), and the plasmid copy number was calculated as described in the Materials and Methods section. The densitometric measurements of bands corresponding to each plasmid were converted to actual ratios after the values were normalized according to the size of ColE1-type plasmids, and are shown at the bottom of the gel. **b** Effects of overproduced RNase E and coexpressed RraAV1 or RraAV2 on the steady-state level of *ftsZ*, *rnhB*, *rpoS,* and *trpA* mRNA. Total RNA was isolated from KSL2003 cells grown to an OD_{600} of 0.8 in the same way as described in Fig. 1a, and reverse transcriptase-polymerase chain reaction (RT-PCR) was performed. The relative abundance of each mRNA is shown at the bottom of the gels. The samples loaded in the lanes numbered 1 to 5 from the cultures used for isolation of plasmid DNA or total RNA are denoted in the legend of Fig. 1. The experiments were repeated at least three times, and standard error of the mean (±numbers) was used to indicate the range of the assay results in the graph

We further tested the ribonucleolytic activity of RNase E on other RNase E substrates [13, 14] when RraAV1 or RraAV2 was coexpressed by measuring the steady-state level of several mRNA species. As shown in Fig. 2b, d, coexpression of RraAV1 more efficiently inhibited RNase E action on these mRNA transcripts than coexpression of RraA, and consequently resulted in the more increased abundance of each RNA species tested in vivo. However, the inhibitory effect by RraAV2 coexpression on RNase E action was observed only in the case of *trpA* mRNA, indicating a possibility of the RNA substrate-dependent inhibition of RraAV2 on RNase E.

The observed difference in the effects of the inhibitors on RNases may have been due to their mode of action on these RNase E-like enzymes. For instance, it was proposed previously that alteration of the degradosome composition by binding of RraA and RraB to different sites of RNase E results in modulation of substrate specificity of RNase E [2]. However, this may not be the underlying mechanism for the action of RraAV1 and RraAV2 on RNase E, as has been shown previously for the mode of RraA and RraB action on RNase ES, a *Streptomyces coelicolor* functional ortholog of *E. coli* RNase E [14]. Considering the degrees of RraAV1 and RraAV2 homology to RraA, it is tempting

to speculate that binding of RraAV1 and RraAV2 to RNase E results in direct alteration of the enzyme's ability to bind or cleave target RNAs. Further studies are needed to identify their mode action on RNase E.

The ability of two *V. vulnificus* proteins to exert an inhibitory effect on RNA decay by an 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 extra- or intracellular environment.

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