# Functional Conservation of RNase III–like Enzymes: Studies on a Vibrio vulnificus Ortholog of Escherichia coli RNase III

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# Functional Conservation of RNase III-like Enzymes: Studies on a *Vibrio vulnificus* Ortholog of *Escherichia coli* RNase III

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**Abstract** Bacterial ribonuclease III (RNase III) belongs to the RNase III enzyme family, which plays a pivotal role in controlling mRNA stability and RNA processing in both prokaryotes and eukaryotes. In the *Vibrio vulnificus* genome, one open reading frame encodes a protein homologous to *E. coli* RNase III, designated Vv-RNase III, which has 77.9 % amino acid identity to *E. coli* RNase III. Here, we report that Vv-RNase III has the same cleavage specificity as *E. coli* RNase III in vivo and in vitro. Expressing Vv-RNase III in *E. coli* cells deleted for the RNase III gene (*rnc*) restored normal rRNA processing and, consequently, growth rates of these cells comparable to wild-type cells. In vitro cleavage assays further showed that Vv-RNase III has the same cleavage activity and specificity as *E. coli* RNase III on RNase III-targeted sequences of *corA* and *mltD* mRNA. Our findings suggest that RNase III-like proteins have conserved cleavage specificity across bacterial species.

## Introduction

The ribonuclease III (RNase III) proteins are a family of double-stranded RNA (dsRNA)-specific endoribonucleases found in both prokaryotes and eukaryotes (for reviews see [2, 4, 7, 12, 30, 32]). These enzymes require a $Mg^{2+}$ cofactor to perform a ribonucleophilic attack on a phosphodiester bond and produce 5′-monophosphate and a 3′-hydroxyl terminus with a 2-nucleotide overhang at the 3′-end [10, 13, 14, 18, 21].

RNase III proteins have been classified across phylogeny into 4 main classes based on their molecular weight, domain organization, and polypeptide structure [2, 6, 8, 12, 15, 19, 33]. Bacterial RNase III proteins belong to class I, which comprise a single endonuclease domain (endoND) and a dsRNA-binding domain (dsRBD). Class II RNase III, called Drosha, has a dsRBD and two endoNDs, which are commonly referred to as RNase IIIa and RNase IIIb. Class III RNase III, called Dicer, is the largest RNase III and typically contain 2 endoNDs, a dsRBD and an N-terminal DExD/H-box helicase domain, followed by a small domain of unknown function (DUF283) and a PAZ domain [15, 33]. Class IV only includes the mini-III (mini-RNase III) of *Bacillus subtilis*, which has an endoND but no dsRBD [24, 26].

*Escherichia coli* RNase III (Ec-RNase III) was the first dsRNA-specific endoribonuclease described [29]. This enzyme functions as homodimer, in which the two ribonuclease domains form a single processing center [9, 39]. The endoND which carries a highly conserved, 9-amino acid sequence (ERLEFLGDS/A) is sufficient for homodimerization and full enzymatic activity [5, 35]. Ec-RNase III plays a key role in producing functional ribosomal and other RNAs. It first cleaves the 30S primary rRNA transcripts to yield the 17S, p23S, and 9S rRNA precursors for 16S, 23S, and 5S rRNA, respectively [3, 7, 11]. This action of Ec-RNase III has been thought to contribute to the slow-growing phenotype of *E. coli* cells without the *rnc* gene [3, 33, 34]. Ec-RNase III also degrades many mRNAs mostly by cleaving within helical segments of the coding region [3, 15, 18, 19, 27, 33, 34]. Ec-RNase III recognizes its substrates through specific structural and sequence features within a primary reactive epitope consisting of a double-helix of at least 1 full turn (11 bp). However, identified bacterial RNase III substrates showed no conserved sequence [10, 13, 16, 19, 22, 23, 28].

Minho Lee and Sangmi Ahn contributed equally to this work.

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**Table 1** Strains and plasmids used in this study

| Strains/ plasmids | Description | References or sources |
|---|---|---|
| Strains | | |
| *E. coli* | | |
| DH5α | f80d/*lacZ* DM15 *recA1 endA1 gyrA96 relA1 thi-1 hsdR17*($r_K^-$ $m_K^-$) *supE44 deoR* Δ(*lacZYA-argF*)*U169* | Laboratory collection |
| MG1655 | $F^-$ $\lambda^-$ *ilvG*$^-$ *rfb-50 rph-1* | Laboratory collection |
| MG1655*rnc-14*::△*Tn10* | $F^-$ $\lambda^-$ *ilvG*$^-$ *rfb-50 rph-1 rnc-14*::Δ*Tn10* | [19] |
| *V. vulnificus* | | |
| MO6-24/O | Clinical isolate | [36] |
| Plasmids | | |
| pKAN6B | p15A *ori*, $Km^r$ | [38] |
| pRNC1 | p15A *ori*, $Km^r$, *rnc* under PBAD | [33] |
| pRNCV1 | p15A *ori*, $Km^r$, *rncv* under PBAD | This study |

In this study, we investigated substrate specificity of bacterial RNase III by examining the enzymatic activity of a *V. vulnificus* homolog of RNase III protein (designated as Vv-RNase III, GenBank accession number NC_014965.1) on Ec-RNase III substrates in vitro as well as its ability to complement the ribonucleolytic activity of Ec-RNase III in an *rnc*-deleted *E. coli* strain.

## Materials and Methods

### Strains and Plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. Construction of the *E. coli* strain MG1655*rnc-14*::Δ*Tn10* and plasmids pKAN6B and pRNC1 have been described previously [19, 33, 38]. The plasmid pRNC1 harbors a cloned copy of *rnc* under an arabinose promoter. To construct pRNCV1, the coding region of *V. vulnificus rncv* was amplified using 2 primers, rncv F (5′-TTGCGGCCGCAATAATTTTGTTTAACTTTAAGGAGAGAATTCATATGAATTCTCCAATTGAGAAACTAACCA-3′) and rncv-His R (5′-AAAGATCTAGTGGTGGTGGTGGTGGTGGCCATTGGTTAACTGCTCCA-3′), digested with *Not*I and *Bgl*II and ligated into pKAN6B. Genomic DNA from *V. vulnificus* MO6-24/O [25] was used as a template.

### Protein Purification and Western Blot Analysis

His-tagged Ec-RNase III was purified as previously described [1]. Vv-RNase III was purified from *E. coli* strain MG1655*rnc-14*::△*Tn10* using Ni–NTA resin (Qiagen). The cultures were grown to mid-log phase, induced with 0.2 % arabinose for 2 h, and harvested. The enzyme was eluted from the column using 250 mM imidazole, concentrated, and stored as described previously [1, 6]. The protein concentration was calculated using Coomassie brilliant blue G250 as described [31] with bovine serum albumin (BSA) as a standard. Western blot analysis was carried out as described previously [17, 19, 33].

### Primer Extension Analysis

Primer extension analysis was performed by using total RNA purified with an RNeasy mini kit (Qiagen) and hybridized with a 5′-$^{32}$P-labeled primer for 16S rRNA-rng (5′-CAGCGTTCAATCTGAGCCATGATC-3′). RNA and labeled primers were annealed at 65 °C for 5 min and slowly cooled to 37 °C. The primers were then extended at 42 °C for 1 h using avian myeloblastosis virus (AMV) reverse transcriptase. The extended fragments were separated on 10 % polyacrylamide gels containing 8 M urea.

### In Vitro Cleavage Assay of RNase III Enzymes Substrates

5′-end-labeled *corA* and *mltD* mRNA were synthesized and the in vitro cleavage assay was performed as described previously [19, 20].

## Results and Discussion

### Effects of Vv-RNase III Expression on the Growth of *rnc*-Deleted *E. coli* Cells

The *V. vulnificus* chromosome contains an open reading frame that encodes a putative homolog of Ec-RNase III (designated as Vv-RNase III) that has 77.9 % amino acid identity to Ec-RNase III [25]. First, we tested whether Vv-RNase III has RNase III activity in vivo by using the slow-growing phenotype of *E. coli* cells without *rnc*. To do this, we constructed a plasmid encoding Vv-RNase III (designated as *rncv)* under an arabinose-inducible promoter (pRNCV1) and transformed it into an *rnc*-deleted *E. coli* strain (MG1655*rnc-14*::Δ*Tn10*, designated as MG1655*rnc*).

The resulting transformants were grown in LB in the presence of 0.2 % arabinose, which resulted in Vv-RNase III expression similar to that of endogenous Ec-RNase III in a parental *E. coli* strain MG1655 (Fig. 1a). These proteins migrated more slowly than their estimated molecular weights (24.75 and 25.41 kDa for Ec-RNase III and Vv-RNase III, respectively). Cells expressing Vv-RNase III grew at rates similar to wild-type (MG1655) cells harboring an empty vector (pKAN6B) grown in LB with 0.2 %

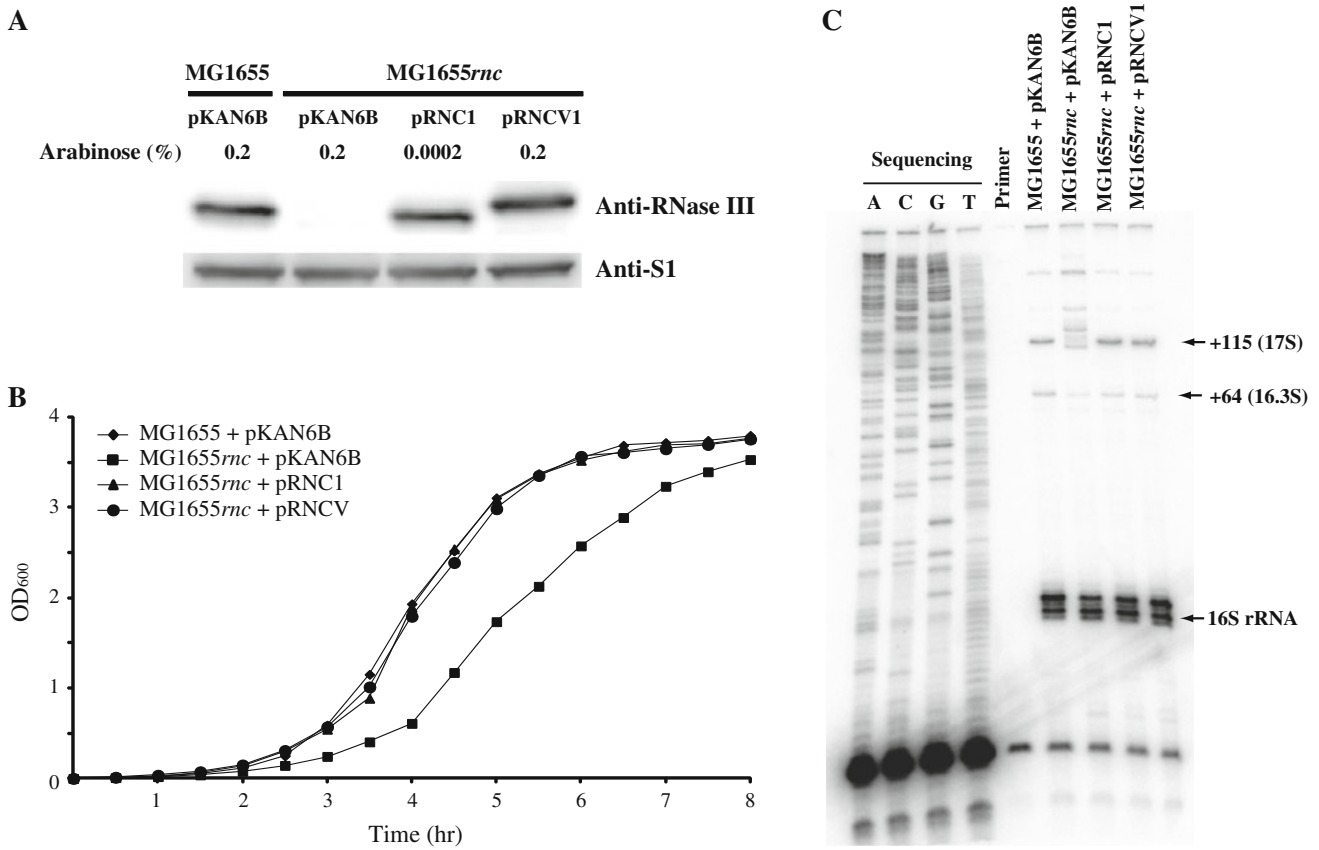


**Fig. 1** Functional complementation of Ec-RNase III by Vv-RNase III in vivo. **a** Expression profiles of Ec-RNase III and Vv-RNase III. The proteins were separated by 12 % SDS-PAGE and analyzed by immunoblotting with polyclonal antibodies to Ec-RNase III. The membrane was probed with anti-Rnc polyclonal antibodies, stripped, and re-probed with anti-S1 polyclonal antibodies to provide an internal standard for quantitative assessment of expression levels of RNase III proteins. **b** Effects of Ec-RNase III or Vv-RNase III expression on the growth of *rnc*-deleted *E. coli* cells. Culture growth was monitored by measuring the optical density at 600 nm ($OD_{600}$) at indicated time intervals. **c** Processing of *E. coli* rRNA by Vv-RNase III. Synthesized cDNA products were analyzed on a 10 % polyacrylamide gel containing 8 M urea. Sequencing ladders were produced using the same primer used in the cDNA synthesis, and PCR DNA encompassing the 16S rRNA gene was used as a template. The cDNA band corresponding to the 5′-end of mature 16S rRNA is indicated by M. 16S rRNA precursors, 16.3S and 17S, are indicated. Culture samples in log-phase ($OD_{600} = 0.7$) were harvested to obtain total protein and RNA

arabinose or MG1655*rnc* harboring the pRNC1 plasmid grown in LB with 0.0002 % arabinose (Fig. 1b). Consistent with a previous report [33], Ec-RNase III expression from pRNC1 in MG1655*rnc* in the presence of 0.0002 % arabinose resulted in Ec-RNase III expression levels similar to those of endogenous Ec-RNase III in MG1655 and resulted in MG1655*rnc* with normal growth rates. These results suggest that Vv-RNase III can functionally complement Ec-RNase III.

Because RNase III processes rRNA, deletion of the *rnc* gene has been suspected to slow the growth of *E. coli* cells. Therefore, we examined whether Vv-RNase III can process 30S transcripts in *E. coli* using primer extension analysis of 16S rRNA. Total RNAs were purified from the strains used to measure growth curves, and the 5′-end of 16S rRNA was analyzed. Similar distributions of 16S rRNA precursors were present in total RNA samples from MG1655 harboring pKAN6B and MG1655*rnc* harboring either pRNC1 or pRNCV1. MG1655*rnc* harboring pKAN6B had various additional 16S rRNA precursors the lengths of which ranged from ~110 to 240 nucleotides (Fig. 1c). These additional 16S rRNA precursors are products of alternative rRNA processing by unknown pathways in the absence of RNase III [11]. These results clearly showed that Vv-RNase III can process rRNA in *E. coli*, which might have restored normal growth to the *rnc*-deleted cells.

## Substrate Specificity of Vv-RNase III In Vitro

Given the in vivo ribonucleolytic activity of Vv-RNase III in *E. coli*, we further tested the substrate specificity and activity of Vv-RNase III on Ec-RNase III substrates

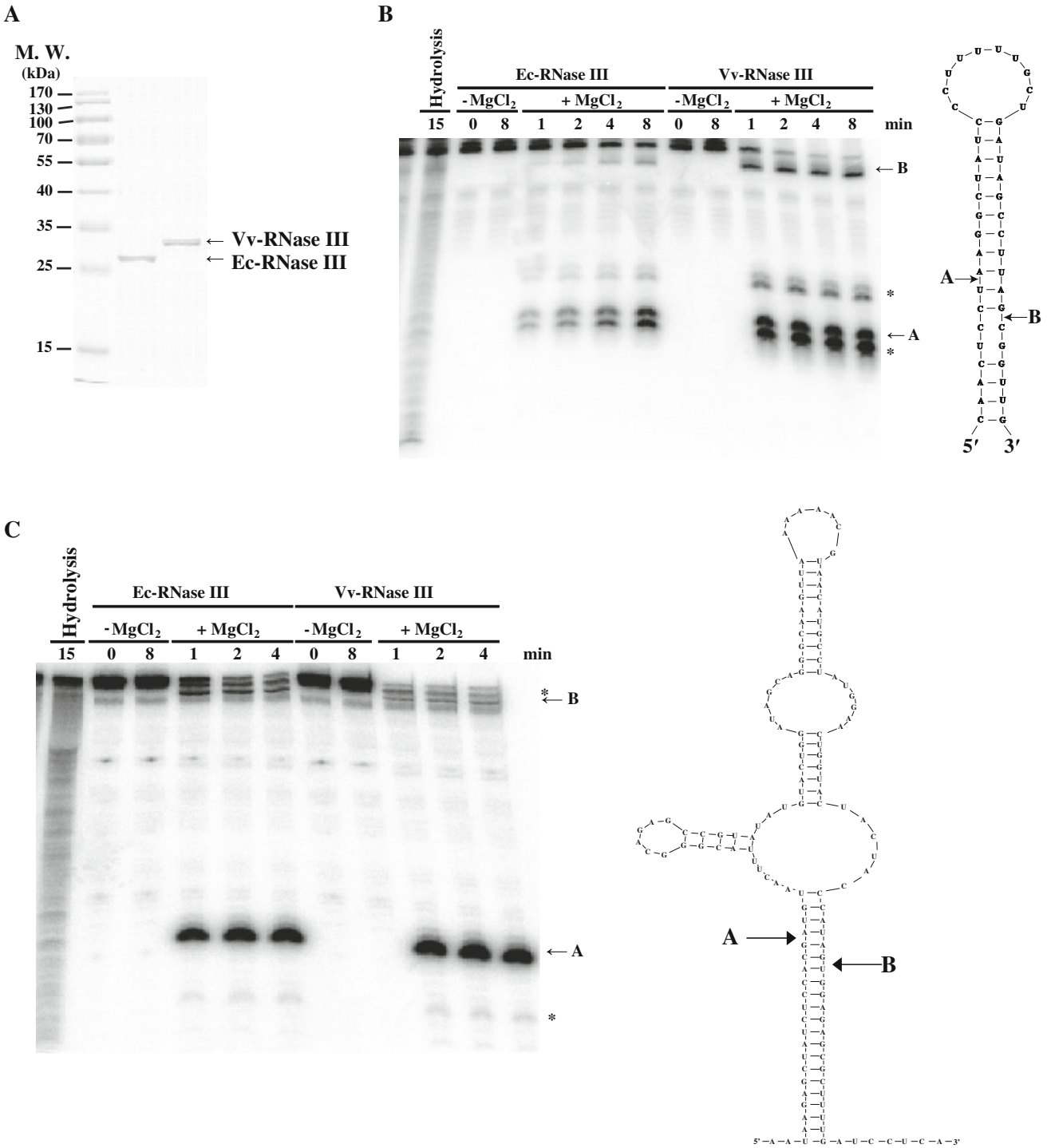


**Fig. 2** In vitro cleavage activity and specificity of Vv-RNase III on Ec-RNase III substrates. **a** Analysis of affinity-purified Ec-RNase III and Vv-RNase III. Purified Ec-RNase III and Vv-RNase III proteins were separated by SDS-PAGE and stained with Coomassie brilliant blue G250. **b**, **c** In vitro cleavage of model hairpin RNAs. One pmol of 5′-$^{32}$P-end-labeled model hairpins derived from *corA* (*B*) or *mltD* (*C*) mRNA was incubated with 5 ng of purified Ec-RNase III or Vv-RNase III in cleavage buffer with (+$MgCl_2$) or without (−$MgCl_2$) $MgCl_2$. Samples were withdrawn at the indicated time intervals and separated on 12 % polyacrylamide gels containing 8 M urea. Cleavage products (*A*, *B*) were identified by using size markers generated by alkaline hydrolysis. The main cleavage products are shown with *arrows*, and other minor cleavage products are indicated with *asterisks*. The secondary structure of model hairpins is deduced using the M-fold (http://mfold.rna.albany.edu) program and shown in the *right* panel

in vitro. Vv-RNase III protein was affinity purified from MG1655*rnc* cells that were conditionally overexpressing Vv-RNase III (Fig. 2a). The ability of the purified Vv-RNase III to cleave model hairpin RNAs, derived from *corA* [19] and *mltD* [20] mRNA, which contain the Ec-RNase III target site, was tested. The cleavage reactions were carried out under single-turnover conditions [19]. Vv-RNase III and Ec-RNase III cleaved 5′-$^{32}$P-end-labeled model hairpin RNAs and produced cleavage products of the same size at comparable rates (Fig. 2b, c). These results show that Vv-RNase III has the same cleavage specificity and activity as Ec-RNase III on these synthetic RNAs. Other cleavage products were also observed. Their size and distribution were similar regardless of which enzyme was used. Generation of other cleavage products is probably due to either the presence of minor RNase III-reactive epitopes in the synthetic RNAs or the intrinsic ability of bacterial RNase III enzymes to degrade long double-stranded RNAs into short duplex products in a largely sequence-independent manner under certain in vitro conditions [37].

The ability of Vv-RNase III to functionally complement Ec-RNase III in vivo and their similar cleavage specificities and activities indicate that RNase III-like enzymes found in different bacterial species have a conserved function in RNA metabolism. This notion suggests that RNA decay and processing by RNase III-like enzymes may be a common mechanism-controlling post-transcriptional regulation of gene expression in bacteria.

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