Functional Characterization of the C-Terminus of YhaV in the *Escherichia coli* PrlF-YhaV Toxin-Antitoxin System^S

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Received: March 8, 2018
Revised: April 1, 2018
Accepted: April 5, 2018

First published online
April 12, 2018

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^SSupplementary data for this paper are available on-line only at <http://jmb.or.kr>.

pISSN 1017-7825, eISSN 1738-8872

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Bacterial programmed cell death is regulated by the toxin-antitoxin (TA) system. YhaV (toxin) and PrlF (antitoxin) have been recently identified as a type II TA system in *Escherichia coli*. YhaV homologs have conserved active residues within the C-terminus, and to characterize the function of this region, we purified native YhaV protein (without denaturing) and constructed YhaV proteins of varying lengths. Here, we report a new low-temperature method of purifying native YhaV, which is notable given the existing challenges of purifying this highly toxic protein. The secondary structures and thermostability of the purified native protein were characterized and no significant structural destruction was observed, suggesting that the observed inhibition of cell growth in vivo was not the result of structural protein damage. However, it has been reported that excessive levels of protein expression may result in protein misfolding and changes in cell growth and mRNA stability. To exclude this possibility, we used an [³⁵S]-methionine prokaryotic cell-free protein synthesis system in vitro in the presence of purified YhaV, and two C-terminal truncated forms of this protein (YhaV-L and YhaV-S). Our results suggest that the YhaV C-terminal region is essential for mRNA interferase activity, and the W143 or H154 residues may play an analogous role to Y87 of RelE.

Keywords: Toxin-antitoxin system, YhaV, PrlF, truncation, circular dichroism, *Escherichia coli*

Introduction

Most bacteria have a toxin-antitoxin system (TA) that regulates cell growth and death in response to various stressful conditions [1, 2]. To date, six types of TA systems have been characterized according to how antitoxins regulate the function of their cognate toxins [3]. An antisense RNA antitoxin can regulate a toxin's translation by binding to the mRNA of its cognate toxin (type I) [4–6] or to the toxin protein directly (type III). The type I system is composed of an mRNA antitoxin and a protein toxin. Hok-Sok, LdrD-RdID, and SymE-SymR were reported as type I TA systems. In type IV TA systems, an antitoxin does not bind to a toxin, but this antitoxin has the opposite function of its related toxin. In type V, an antitoxin protein has RNase activity that is specific to its cognate toxin, and an antitoxin in type VI induces the degradation of its toxin

as an adapter protein between a toxin and a protease. The type II systems are the most studied TA system and are divided into two groups on the basis of their ribosome dependency for their enzymatic activity; ribosome-dependent or ribosome-independent [1]. Among them, toxins that cleave mRNA and inhibit cell growth are called mRNA interferases [1]. This system is composed of two proteins, a stable toxin and an unstable antitoxin capable of neutralizing toxins. When bacteria are under stress, unstable antitoxins are rapidly degraded by proteases (*e.g.*, an ATP-dependent protease, Lon, or ClpX family) [7]. Therefore, toxins of TA systems bind to DNA, RNA, or protein targets and inhibit cell growth through various mechanisms, including multidrug tolerance, biofilm formation, and cell persistence mechanisms [8–10]. In the *E. coli* MazEF TA system, the MazF toxin is a ribosome-independent mRNA interferase that inhibits bacterial cell growth by cleaving

mRNA at A[^]CA (^ indicates the cleavage site) [11]. In addition, the tertiary structures of MazEF indicate that MazF forms a heterohexameric complex with the antitoxin MazE (MazF₂-MazE₂-MazF₂) [12]. Another ribosome-independent TA system of *E. coli*, MqsR-MqsA, involves the toxin MqsR, which induces the inhibition of cell growth by mRNA cleavage at GCU or GUA [13]. Compared with ribosome-independent mRNA interferases, the target mRNA recognition sites of ribosome-dependent mRNA interferases are relatively short and most are codon-related. One well-characterized ribosome-dependent mRNA interferase, the RelE toxin in the RelBE TA system, binds to the A site of the ribosome and cleaves mRNAs at UA[^]G and UA[^]A sequences of termination codons, leading to the inhibition of cell growth [14–16]. An NMR study that analyzed the structure of the toxin RelE indicated that the endoribonuclease active site locates primarily to the C-terminus [17]. Furthermore, other ribosome-dependent TA systems, YoeB-YefM [18], HipA-HipB [19], and YafO-YafN [20], are also reported to bind to the ribosomes of *E. coli* 30S or 50S ribosomal subunits and have their mRNA interferase activities as toxin-ribosome-mRNA substrate [14, 21]. Previously, PrIF-YhaV was classified as a type II TA system, based in part on homology to the ribosome-dependent YoeB and RelE families [22]. Ribosome profiling analysis of the toxin YhaV revealed that the expressed toxin YhaV bound to the 50S ribosomal subunit and inhibited bacterial growth [23]. The RelE toxin is a well-known global inhibitor of translation, and structural analysis suggests that the active site of RelE toxin is located in the C-terminal amino acid region [24, 25].

Here, to evaluate the function of the C-terminus of YhaV, which is a conserved region in YhaV homologs, we purified the native YhaV protein and C-terminal truncated proteins of various lengths (without denaturing) for the first time. By determining the secondary structures and enzyme activities of full-length YhaV and various C-terminal truncated forms, we show that the YhaV C-terminal residues from 131 to 145 are involved in mRNA interferase activity.

Materials and Methods

Bacterial Strains, Plasmids, and Primers

Varying strains of *E. coli* (BW25113Δ*prlF*yhaV, DH5αΔ*prlF*yhaV, and BL21(DE3)Δ*prlF*yhaV) were used in this analysis [23]. Various versions of YhaV (yhaVΔ241-465, Δ271-465, Δ301-465, Δ331-465, Δ361-465, Δ391-465, Δ406-465, Δ421-465, Δ436-465, or Δ451-465) were amplified by PCR using pBAD24-yhaV as the template with the primers listed in Table 1. These fragments and pBAD vector were digested by NdeI and XhoI DNA restriction enzymes. Purified inserts were then cloned into the pBAD24 vector with DNA ligase [23]. The yhaVΔ436-465 encoding YhaV-L and Δ391-465 encoding YhaV-S genes were also separately cloned into the pET28 vector (Novagen, USA) to express the C-terminal truncated YhaV proteins, following the protocol as described above. All constructs were confirmed by DNA sequencing. All the primers used in this study are listed in Table 1.

Measuring the Toxicity of YhaV and C-terminal Truncated Forms of YhaV

The pBAD24-*prlF*, -yhaV, -*prlF*yhaV, -yhaVΔ436-465, or -yhaVΔ391-465 were transformed into *E. coli* BW25113Δ*prlF*yhaV. The transformants were streaked onto M9 agar plates in the presence or absence of 0.2% arabinose and grown at 37°C. The growth

Table 1. Primer designations and sequences.

Primer	Sequence
YhaV-F	5'-TATACATATGGATTTTCCACAAAGGGTTA-3'
YhaV(Δ81-154)-R	5'-TATACTCGAGTCAATTTTCCCAGAGCCT-3'
YhaV(Δ91-154)-R	5'-TATACTCGAGTCAAGCACCAAATTTTACC-3'
YhaV(Δ101-154)-R	5'-TATACTCGAGTCAACTATAACGAAAGAAG-3'
YhaV(Δ111-154)-R	5'-TATACTCGAGTCACCATCCCAGAATGATG-3'
YhaV(Δ121-154)-R	5'-TATACTCGAGTCAGTAGGTGCGCAGAGTG-3'
YhaV(Δ131-154)-R	5'-TATACTCGAGTCAGAATACGGTATAGGCA-3'
YhaV(Δ136-154)-R	5'-TATACTCGAGTCATTTTAACATTTTGCTG-3'
YhaV(Δ141-154)-R	5'-TATACTCGAGTCAAGGAGGATGTCCTCTT-3'
YhaV(Δ146-154)-R	5'-TATACTCGAGTCAGGTTTCCCAGTCGGCA-3'
YhaV(Δ151-154)-R	5'-TATACTCGAGTCATGTTTCTCGGGTGAGG-3'
ompF-F	5'-CTTTGGTCTGGTTGATGGCC-3'
ompF-R	5'-ACTTCAGACCAGTAGCCCCAC-3'

curves were measured using *E. coli* BW25113 Δ *prlF**yhaV* cells harboring pBAD24, pBAD24-*yhaV*, -*yhaV* Δ 436-465, or -*yhaV* Δ 391-465 in M9 liquid medium at 37°C in the presence of 0.2% arabinose.

RNA Isolation and Northern Blot Analysis

E. coli BW25113 Δ *prlF**yhaV* cells harboring pBAD24, pBAD24-*yhaV*, -*yhaV* Δ 436-465, or -*yhaV* Δ 391-465 were cultured in M9 medium with 0.2% glycerol at 37°C. When the culture's OD₆₀₀ value reached 0.4, the expression of YhaV, YhaV-L, or YhaV-S was induced by adding arabinose to a final concentration of 0.2%. Aliquots of cell cultures were taken at 0, 1, 3, and 6 h. Total RNA was extracted by using Trizol (Thermo Fisher Scientific, USA) following the manufacturer's protocol. Northern blot analysis was carried out as previously described with modified probes, ompF-F and ompF-R, that were labeled with biotin-14-dCTP [26]. All probes used in this study are shown in Table 1.

Protein Expression and Purification

To express and purify YhaV, YhaV-L, and YhaV-S, pET28-*yhaV*, -*yhaV* Δ 436-465, and -*yhaV* Δ 391-465 were transformed into *E. coli* BL21(DE3) Δ *prlF**yhaV*. When the OD₆₀₀ value of the cultures reached 0.5, 0.5 mM isopropyl- β -D-1-thiogalactoside was added to induce YhaV, YhaV-L, and YhaV-S protein expression. The cultures were incubated at 18°C for 6 h. N-Terminally His₆-tagged YhaV, YhaV-L, and YhaV-S were purified using Ni-NTA-agarose (Qiagen, Germany) following the manufacturer's protocol. Additionally, C-terminally His₆-tagged YhaV was purified with Ni-NTA-agarose followed by the solid-phase refolding method with 6 M guanidine-HCl from cells harboring pColdIII-*prlF**yhaV*. All purified proteins were analyzed by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) followed by Coomassie stain. For western blot analysis, purified proteins were resolved on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The blots were probed

with a polyclonal anti-histidine tag antibody (#2365; Cell Signaling Technology, USA).

Circular Dichroism (CD) Analysis of YhaV and C-Terminal Truncated Forms of YhaV

Far-UV CD measurements were carried out using an automated Chirascan CD spectrometer (Applied Photophysics, UK). The spectra were recorded over a wavelength range of 190–260 nm using a 0.5-mm path length from 25°C to 85°C at 10°C intervals to determine the secondary structure of YhaV, YhaV-L, and YhaV-S. To determine the melting points of YhaV, YhaV-L, and YhaV-S, the temperature was increased by 0.2°C stepwise from 25°C to 90°C. Purified proteins were desalted by dialysis solution equilibrated with 10 mM sodium phosphate (pH 7.8), and then adjusted to 0.3 mg/ml protein concentration for CD analysis. The background CD spectrum of the buffer was subtracted from the CD spectra of the protein samples.

Effect of C-Terminal Truncated Forms of YhaV on Protein Synthesis in Prokaryotic Cell-Free Systems

A prokaryotic cell-free protein synthesis assay was performed with the *E. coli* T7 S30 extract system (Promega, USA), following the manufacturer's protocol. Different concentrations (100, 500, and 1,000 nM) of YhaV, YhaV-L, and YhaV-S were added to the reaction mixtures, following a previous protocol [23]. The pellets were analyzed by SDS-PAGE followed by autoradiography.

Results

The Sequence of YhaV Is Highly Similar to Ribosome-Dependent mRNA Interferases Harboring Active Residues in the C-Terminal Region

The *prlF*-*yhaV* operon is located at 70.59 min on the *E. coli* K12 chromosome. YhaV and PrlF have 154 and 111

Fig. 1. Schematic diagram of the study constructs and protocols.

To characterize the function of the C-terminal region of YhaV, C-terminal truncated forms of YhaV were generated. The secondary structures of the C-terminal truncated forms of YhaV were characterized using circular dichroism. The functions of YhaV's C-terminal region were evaluated using in vivo toxicity and in vitro translation assays.

amino acid residues, respectively. Both *prlF* and *yhaV* are located in the same operon, and the stop codon of *prlF* and the start codon of *yhaV* overlap by one base (Fig. 1). In a previous report, YhaV was aligned to other toxin proteins with known tertiary structures to determine their similarity. With the notable exception of a 20-residue insertion between the first and second α -helices in YhaV, high homology between YhaV and YoeB and RelE from *E. coli* and RelE from *Pyrococcus horikoshii* was observed [22]. To identify the mRNA interferase activity region, we aligned the amino acid sequences of ribosome-dependent mRNA

interferases RelE (PDB ID: 4FXI; Chain A), YoeB (PDB ID: 2A6Q; Chain E), and HigB (PDB ID: 4ZSN; Chain QY or XY) using T-Coffee, a multiple sequence alignment tool (Fig. 2A) [12, 27–33]. Using this approach, we observed that ribosome-dependent mRNA interferases were structurally similar (Fig. 2A). Moreover, tertiary structure alignments were carried out with the magic fit algorithm, a sequence-based structural alignment tool from Swiss-PDB Viewer ver. 4.1.0, and imaged in Jmol 14.6.5 [34, 35]. Interestingly, the secondary structure of MqsR, known as ribosome-independent (PDB ID: 3HI2; Chain B), was similar to

Fig. 2. Sequence alignments and tertiary structure comparisons of YhaV and other TA system toxins. (A) Multiple sequence alignments of YhaV and ribosome-dependent toxins (RelE (PDB ID: 4FXI; Chain A), YoeB (PDB ID: 2A6Q; Chain E) and HigB (PDB ID: 4ZSN; Chain QY or XY)). White and black boxes indicate β -sheets (S) and α -helices (H), respectively, on the basis of the secondary structures of RelE (PDB ID: 4FXI). The asterisk shows 20 elliptical residues, AEVETLKGDPENYQRKAAT. Black circles show active site residues. Similar residues having thresholds of more than 50% in the BLOSUM62 matrix are shaded grey and identical residues are shaded black. (B) Secondary structure of the ribosome-independent toxin MqsR (PDB ID: 3HI2; Chain B). White and black boxes indicate β -sheets (S) and α -helices (H), respectively. (C) Comparison of the tertiary structures of four TA system toxins. Tertiary structure alignments were carried out with magic fit, a sequence-based structural alignment tool from Swiss-PDB Viewer ver. 4.1.0, among RelE (PDB ID: 4FXI; Chain A), YoeB (PDB ID: 2A6Q; Chain E), HigB (PDB ID: 4PX8; Chain A), and MqsR (PDB ID: 3HI2; Chain B). All structures were imaged in Jmol 14.6.5. The N-terminal and C-terminal ends of the tertiary structures are represented by N and C, respectively.

ribosome-dependent mRNA interferases in the N-terminal region, which has a small β -sheet followed by two α -helices. The tertiary structure of MqsR, however, was quite different than the three ribosome-dependent toxins, RelE, YoeB, and HigA (Figs. 2B and 2C) [36]. It is suggested that YhaV, similar to other ribosome-dependent mRNA interferases, also has catalytic residues in the C-terminal region.

YhaV-S Have Reduced Toxicity Compared with Native YhaV and YhaV-L

As the tertiary structure of YhaV has not been reported, we generated YhaV variants with various C-terminal truncations to identify active residues. The cell toxicity effects of 10 C-terminal truncated forms of YhaV were quantified by culturing cells transformed with these constructs on plates at 24°C, 37°C, or 42°C in the presence

Fig. 3. Toxicity and mRNA stability analyses of YhaV and C-terminal truncated forms of YhaV.

(A) Schematic diagram of YhaV and C-terminal truncated forms of YhaVs (YhaV-L and YhaV-S). (B) Toxicity of cells harboring pBAD, pBAD24-*prlF*, -*yhaV*, -*prlFyhaV*, -*yhaV*Δ436-465, or -*yhaV*Δ391-465 on M9-glycerol-casamino acid plates with and without L-arabinose (0.2%) at 37°C for 20 h. (C) Effect of YhaV, YhaV-L, or YhaV-S on *E. coli* BW25113Δ*prlFyhaV* cells harboring pBAD24, pBAD24-*yhaV*, -*yhaV*Δ436-465, or -*yhaV*Δ391-465 on M9-glycerol-casamino acids medium at 37°C for 6 h in the presence of L-arabinose (0.2%). Cell densities were measured for 6 h at 1 h intervals. (D) Northern blot assay was performed to identify the effects of YhaV, YhaV-L, and YhaV-S on cellular mRNA stability. Total RNA was extracted from *E. coli* BW25113Δ*prlFyhaV* cells harboring pBAD24, pBAD24-*yhaV*, -*yhaV*Δ436-465, or -*yhaV*Δ391-465 and Northern blot assay was carried out with labeled *ompF*.

or absence of 0.2% arabinose. YhaV-S and smaller C-terminal truncates lost their mRNA interferase activity (Fig. S1 and Fig. 3B). These effects may be due to either conformational changes or a loss of active residues. Interestingly, YhaV Δ 141-154 was not toxic at 37°C, was slightly toxic at 24°C, and was toxic as full-length YhaV at 42°C, indicating that toxicity is temperature sensitive (Fig. S1). To characterize the function of the C-terminal region of YhaV, we chose three constructs, YhaV, YhaV-L, and YhaV-S. YhaV-S has the largest truncation and was not toxic, whereas YhaV and YhaV-L were fully toxic (Fig. 3A). We tested the ability of these constructs to inhibit growth in the presence of 0.2% arabinose at 37°C. YhaV and the YhaV-L construct resulted in immediate growth inhibition following induction with 0.2% arabinose, whereas YhaV-S had no effect on cell growth (Fig. 3C). If the tertiary structure of the proteins encoded by these constructs is not affected, these results suggest that YhaV's C-terminal amino acid region from 131 to 154 may mediate its enzymatic activity. To evaluate the mRNA interferase activity of YhaV, YhaV-L, and YhaV-S, the stability of mRNA *ompF* in *E. coli* BW25113 Δ *prlF**yhaV* cells harboring pBAD24-*yhaV*, -*yhaV* Δ 436-465 (encoding YhaV-L), or -*yhaV* Δ 391-465 (encoding YhaV-S) were quantified. YhaV-S did not impact *ompF* mRNA stability at the time intervals tested (*i.e.*, 0, 1, 3, and 6 h), whereas *ompF* mRNA bands were absent within 1 h in cells expressing YhaV and YhaV-L, confirming their mRNA interferase capacity. In addition, 16S and 23S ribosomal subunits were stable at all time points, suggesting that the expression of the YhaV toxin has no effect on the formation of 16S and 23S ribosomes (Fig. 3D).

Truncations in the C-Terminus of YhaV Did Not Affect the Secondary Structures and Thermal Stability of YhaV

Full-length YhaV, YhaV-L, and YhaV-S were expressed in *E. coli* BL21(DE3) Δ *prlF*-*yhaV* cells. N-Terminally His₆-tagged YhaV, YhaV-L, and YhaV-S were purified with Ni-NTA agarose (Fig. 4A). Additionally, C-terminally His₆-tagged YhaV was purified with Ni-NTA agarose following the solid-phase refolding method (Fig. 4A). Using *in silico* approaches (*i.e.*, ProtParam and ExPASy), the molecular masses of YhaV, YhaV-L, and YhaV-S were predicted to be 18.7, 17.6, and 15.8 kDa, respectively; the actual molecular mass of each construct was confirmed using SDS-PAGE and western blot analyses (Fig. 4).

In a previous study, the CD spectrum of YhaV renatured from the PrIF-YhaV complex by solid-phase refolding showed values as a typical helical protein that exhibits double minima at 208 and 222 nm. To determine if C-terminal

Fig. 4. Purification of native YhaV, YhaV-L, and YhaV-S proteins.

(A) Purified denatured or native YhaV, YhaV-L, and YhaV-S on SDS-PAGE. Lane 1, YhaV from pColdIII-*prlF**yhaV* purified under denaturing conditions; lane 2, YhaV-S from pET28-*yhaV* Δ 391-465 purified; lane 3, YhaV-L from pET28-*yhaV* Δ 436-465 purified; lane 4, native YhaV from pET28-*yhaV* purified. (B) Western blot of denatured or native YhaV, YhaV-L, and YhaV-S probed with anti-histidine polyclonal antibody.

truncations of YhaV induce the destruction of the native YhaV structure or result in the loss of enzyme active residues, the secondary structures of purified YhaV, YhaV-L, and YhaV-S were compared with each other using far-UV CD spectrometry. The CD spectrum of purified N-terminally His₆-tagged YhaV, YhaV-L, and YhaV-S were analyzed in a wavelength range of 180–260 nm using a 0.5-mm path length from 25°C to 85°C at 10°C intervals, and the CD spectra of all constructions showed no significant differences in the double minima at 208 and 222 nm (Figs. 5A–5C). Next, we examined how C-terminal truncations of YhaV affects the thermal stability of YhaV. The thermal denaturation of purified N-terminally His₆-tagged YhaV, YhaV-L, and YhaV-S were analyzed at 222 nm by increasing the temperature by 0.2°C stepwise from 25°C to 90°C. The melting points of YhaV, YhaV-L, and YhaV-S were

Fig. 5. Circular dichroism (CD) analysis of YhaV and C-terminal truncated forms of YhaV to confirm their secondary structures and thermostability.

(A–C) The spectra were recorded over a range of 180–260 nm using a 0.5-mm path length from 25°C to 85°C at 10°C intervals to determine the secondary structure of YhaV, YhaV-L, and YhaV-S. (D–F) To determine the melting points of YhaV, YhaV-L, and YhaV-S, temperatures were increased by 0.2°C stepwise from 25°C to 90°C. The background CD spectrum of the buffer was subtracted from the CD spectra of the protein samples.

55°C, 48°C, and 55°C, respectively (Figs. 5D–5F), and the physiological stability of YhaV-L and YhaV-S were not significantly different than full-length YhaV. These results suggest that the C-terminal truncations of YhaV do not induce structural destruction of YhaV, and YhaV-S's loss of toxicity may be the result of a loss of active residues within the C-terminal region.

The C-Terminal Region of YhaV Has mRNA Interferase Activity

It has been reported that changes in cell growth and mRNA stability in vivo might be due to protein misfolding resulting from excessive protein expression [37]. Therefore, we examined the effect of full-length YhaV and the C-terminal truncated forms of YhaV (YhaV-L and YhaV-S) on protein translation using a [³⁵S]-methionine incorporation prokaryotic cell-free protein synthesis system in vitro. We measured incorporation at different concentrations (100, 500, or 1,000 nM) (Fig. 6). The PinPoint Xa vector with the chloramphenicol acetyltransferase gene (CAT) or β -lactamase gene was used in this experiment. Acetyltransferase and β -

lactamase syntheses were inhibited by purified toxin YhaV and YhaV-L at concentrations of 500 or 1,000 nM, whereas YhaV-S had no impact on translation at any concentration (Fig. 6). These results suggest that the C-terminal region of YhaV has mRNA interferase activity.

Discussion

mRNA interferases are enzymes that cleave RNA. These enzymes are classified into two type II classes of TA systems based on their ribosome dependency (*i.e.*, ribosome-dependent and ribosome-independent) [1]. Alignment of the amino acid sequence of YhaV and ribosome-dependent toxins by multiple sequence alignment suggests that YhaV is more closely related to ribosome-dependent toxins (*e.g.*, RelE, YoeB, and HigB) [30–33] compared with the MazF family and MqsR, a well-known group of ribosome-independent toxins [12, 28, 29, 36]. Moreover, the PrIF-YhaV TA system in *E. coli* was classified as a ribosome-dependent mRNA interferase with a polysome profiling assay [23]. Interestingly, there were differences in bound

Fig. 6. Effect of C-terminal truncated forms of YhaV on protein synthesis in prokaryotic cell-free systems.

A prokaryotic cell-free protein synthesis assay was performed using different concentrations (100, 500, and 1,000 nM) of YhaV, YhaV-L, and YhaV-S. Pellets were analyzed by SDS-PAGE followed by autoradiography.

ribosomal subunits among RelE, YoeB, and HigB when characterized by polysome profiling assays and tertiary structure models. YhaV is 154 amino acids in length, whereas RelE, YoeB, and HigB are relatively smaller in size at 95, 84, and 92 amino acids, respectively. YhaV has 20 additional residues between the first and second alpha-helix when compared with secondary motifs of the RelE homolog using amino acid sequence alignments [22]. Regions similar to RelE are related mainly to the interaction with the 16S ribosomal subunit, and thus the additional 20 residues in YhaV may also be involved in ribosomal interactions [17]. The tertiary structure of the RelE:ribosome:RNA substrate complex indicates that the Y87, R61, and R81 residues of RelE are critically involved in its enzymatic activity and that K53 and K54 might be involved in the stabilization of the negatively charged transition state. In the crystal structure of the RelE:ribosome:target RNA complex: (i) RelE binds to ribosome A sites, (ii) the aromatic ring of Y87 and the base ring of the second nucleotide of the target RNA were stacked, and (iii) the third nucleotide of the target RNA was stacked with the 16S ribosomal RNA. Next, Y87 of RelE induced the hydrolysis of the second and third nucleotides conveying RelE's mRNA interferase activity. H83 of another YhaV homolog,

YeoB, plays a similar role to Y87 of RelE and facilitates YeoB-mediated cleavage of target RNA [21]. In contrast, site-directed mutagenesis of Y87 to F87 did not lead to a dramatic decrease of RelE activity, perhaps because the arginines and lysines within the active sites could compensate for the role of Y87. In this study, the sequence alignments showed that aromatic residues, W143 or H154, in YhaV may play an analogous role to Y87 of RelE. However, site-directed mutagenesis of W143 or H154 to alanine did not show the change of YhaV activity in toxicity test and northern blot using *ompF* mRNA (Fig. S2). The loss of W143 or H154 in YhaV might be compensated by aspartic acid, glutamic acid, or arginine within the active sites, similar to the results of site-directed mutagenesis of Y87 to F87 in RelE.

In this report, to evaluate the C-terminal function of YhaV, we used various lengths of C-terminal truncated forms of YhaV, rather than site-directed mutagenesis, based on the structural analysis of RelE, YeoB, and HigB homologs. Notably, in this report, we present a new low-temperature method for the purification of native YhaV, which was previously very difficult because of its toxicity. Furthermore, we evaluated the secondary structures and thermostability of these natively purified proteins and confirmed that there was no structural destruction, suggesting that the inhibition of cell growth in vivo was not due to structural damage. However, it has been reported that excessive protein expression may result in protein misfolding and changes in cell growth and mRNA stability. To exclude this possibility, we used an [³⁵S]-methionine incorporation prokaryotic cell-free protein synthesis system in vitro with purified YhaV, YhaV-L, and YhaV-S. In this way, we showed that the C-terminal region of YhaV is essential for mRNA interferase activity, and W143 or H154 residues might play a role similar to that of Y87 of RelE.

mRNA interferases may be of interest as (i) possible therapeutic options for the treatment of RNA virus-related diseases and (ii) secondary structural analysis of RNA since they bind specifically to single-stranded RNA and knock out rather than knock down the target gene [38]. However, for their application in knocking out specific genes, further studies are needed to improve the RNA sequence recognition length and the specificity. mRNA interferases can recognize seven nucleotides and cleave target RNAs. Similarly, zinc finger nucleases (ZFN) were developed to cleave specific DNA sequences, and those nucleases originally recognized three nucleotides. The ZFN system has advanced and can now recognize a maximum of 18 DNA nucleotides by the trimerization of three monomers and the addition of FokI, which can be dimerized and

cleave double-stranded DNAs. Moreover, it was revealed that zinc finger protein amino acids within the α -helix (positions -1, +1, +2, +3, +5, and +6) are the conserved amino acids for sequence recognition, and this region can be modified to acquire new sequence specificity [39]. It is likely worth developing mRNA interferases as an RNA cleavage tool similar to ZFN for DNA cleavage. For this purpose, understanding the residues mediating enzymatic activity and RNA sequence recognition might be important. It remains unknown whether the residues that are involved in target sequence recognition and cleavage are distinct or if they overlap. In a previous study, we showed the possibility of engineered mRNA interferases as a specific RNA cleavage tool by exchanging two different predicted recognition loop regions, which resulted in a chimeric protein that recognizes a new sequence and length [27]. In the case of YhaV, important residues for the enzymatic activities are in the C-terminus like its homologs, RelE, YeoB, and HigB. It is possible that the C-terminal region of those mRNA interferases also have target RNA sequence recognition residues. Further studies will be required to determine the tertiary structure of the YhaV:ribosome:target mRNA complex to better characterize the functions of the C-terminal region of YhaV.

Acknowledgments

We thank Dr. Munjin Kwon for critical reading of this manuscript. This work was supported by grants from the KRIIBB Research Initiative Program and the Next-Generation BioGreen 21 Program (No. PJ013686012018), Rural Development Administration, Republic of Korea.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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