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Doctoral Thesis

Functional analysis of RNase HI involved in *rnLAB*
toxin-antitoxin in *Escherichia coli*

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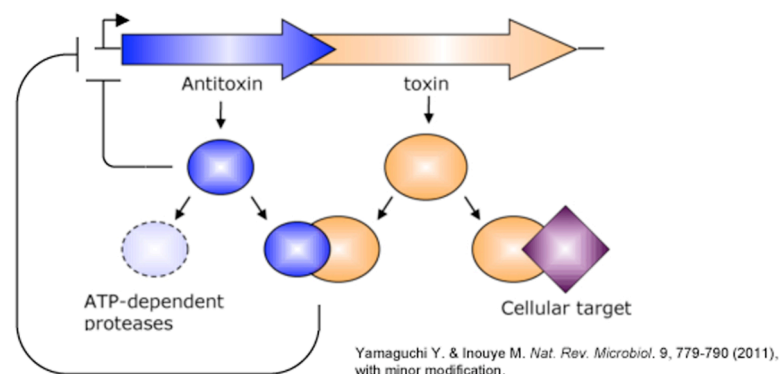
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General introduction

Toxin-antitoxin is a genetic module that is present in plasmids and bacterial genomes and generally encoded together in an operon. In general, antitoxin genes are located upstream of its cognate toxin genes so that the antitoxins are produced before the toxins (Yamaguchi and Inouye, 2011). In most cases, toxins are stable, whereas antitoxins are unstable and constantly expressed. Under normal growth conditions, the activity of toxin is blocked by the antitoxin and toxin-antitoxin operons are negatively regulated at the level of transcription by the antitoxins or toxin-antitoxin complexes. On the other hand, under stress conditions such as acid starvation, high temperature and viral infection, antitoxins are degraded and transcription of the loci increases dramatically (Gerdes *et al.*, 2005). The released toxins attack various cellular targets, which results in impediment of cellular processes. Toxins have various activities, for example, cleavage of free mRNA transcripts (Yamaguchi and Inouye, 2011) and ribosome associated mRNA (Pederson *et al.*, 2003; Zhang and Inouye, 2009), inhibition of ribosome association (Zhang and Inouye, 2011), tRNA synthesis (Germain *et al.*, 2013), or EF-Tu interaction with aminoacylated tRNAs by phosphorylation (Castro-Roa *et al.*, 2013).



Toxin-antitoxin systems are involved in cell physiology, such as programmed cell death that kills a large fraction of a cell population to release nutrients, resulting that surviving cells can grow under nutrient stresses (Engelberg-Kulka *et al.*, 2004), stress response that rapidly adjusts the rates of nutrient and energy consumption to the new and lower levels that match the limited supply (Gerdes *et al.*, 2005; Wang *et al.*, 2011), protection from phage infection (Hazan and Engelberg-Kulka, 2004; Fineran *et al.*, 2009; Koga *et al.*, 2011; Otsuka and Yonesaki, 2012; Sberro *et al.*, 2013), and the formation of bacterial persister cells that are able to survive antibiotic treatment (Maisonneuve *et al.*, 2011; 2013; Amato *et al.*, 2013; Germain *et al.*, 2013).

Toxin-antitoxin loci are surprisingly abundant in the chromosomes of almost all free-living prokaryotes (36 loci in *E. coli* K-12, 38 loci in *Mycobacterium tuberculosis*, and 43 loci in *Nitrosomonas europaea*) (Pandey and Gerdes, 2005). Most of the organisms owning a large number of toxin-antitoxin loci grow in nutrient-limited environments, while obligate intracellular organisms, which proliferate in constant environment conditions, lost the loci (Pandey and Gerdes, 2005). Based on these findings, it is proposed that toxin-antitoxin systems may play important roles during adaptation to environmental stresses.

mlA-mlB is one of the toxin-antitoxin that functions as anti-phage mechanism (Koga *et al.*, 2011). RnlA toxin has an endoribonuclease activity (Otsuka *et al.*, 2007) and is activated in cells infected by T4. When T4 has a mutant in *dmd* gene, encoding Dmd that is a T4 antitoxin against RnlA, activated RnlA results in suppression of propagation of the T4 phage (Kai *et al.*, 1996). On the other hand, the activity of RnlA is repressed by RnlB antitoxin in normal growth cells (Koga *et al.*, 2011).

In previous works, Koga (doctoral thesis 2010) showed that the activity of purified RnlA was much lower than expected from *in vivo* activity, and that RNase HI, which

is responsible for removing the RNA primer in DNA replication, was required *in vivo* for the RnlA activity. This is the first suggestion that the toxin-antitoxin system needs an additional factor. However, a molecular understanding of the mechanism underlying the function of RNase HI remains unclear. Moreover, it remains unknown whether RNase HI is involved in the function of RnlB.

To address these questions, I investigated the posttranslational regulatory mechanisms of RnlA activity (chapter 1) and the interplay between RnlA, RnlB and RNase HI in both T4-infected cells (chapter 1) and T4-uninfected cells (chapter 2).

I demonstrated that RNase HI was an activator for RnlA toxin activity (chapter 1). Recently, we uncovered the crystal structural of RnlA (Wei *et al.*, 2013). RnlA consists of NTD (N-Terminal Domain), NRD (N-Repeated Domain) and DBD (Dmd-Binding Domain). DBD is essential for RNase activity of RnlA and Dmd binding, whereas the roles of two other domains remained unclear. I reported that both RNase HI and RnlB associated with NRD (chapter 2), and that RNase HI was necessary for RnlB to suppress the RnlA activity through interaction with NRD (chapter 2).

The above findings suggest that RNase HI is an essential component of RnlA-RnlB toxin-antitoxin systems.

Chapter 1

RNase HI stimulates the activity of RnlA toxin in *Escherichia coli*

Abstract

A type II toxin-antitoxin system in *E. coli*, *rnlA-rnlB*, functions as an anti-phage mechanism. RnlA is a toxin with the endoribonuclease activity and the cognate RnlB inhibits RnlA toxicity in *E. coli* cells. After bacteriophage T4 infection, RnlA is activated by the disappearance of RnlB, resulting in the rapid degradation of T4 mRNAs and consequently no T4 propagation, when T4 *dmd* is defective; T4 encodes an antitoxin, Dmd, against RnlA for promoting own propagation. Previous studies suggested that the activation of RnlA after T4 infection was regulated by multiple components. Here, I provided the evidence that RNase HI is an essential factor for activation of RnlA. The *dmd* mutant phage could grow on $\Delta rnhA$ (encoding RNase HI) cells, in which RnlA-mediated mRNA cleavage activity was defective. RNase HI bound to RnlA *in vivo* and enhanced the RNA cleavage activity of RnlA *in vitro*. In addition, ectopic expression of RnlA in $\Delta rnlAB \Delta rnhA$ cells has less effect on cell toxicity and RnlA-mediated mRNA degradation than in $\Delta rnlAB$ cells. This is the first example of a factor for activation of a toxin.

Introduction

Toxin-antitoxin (TA) systems are broadly conserved in prokaryotes. Their genes are located in plasmids and chromosomes, and encode a stable toxin plus an unstable antitoxin that antagonizes its toxicity. Generally, genes encoding cognate toxin and antitoxin are contiguous. TA systems are mainly divided into two types; although there are actually five different types of TA systems, only a few examples were discovered in each of type III, IV, or V TA systems (Blower *et al.*, 2009; Masuda *et al.*, 2012; Wang *et al.*, 2012). In the type I system, the antitoxin is a small regulatory RNA that blocks the translation of toxin mRNA (Gerdes and Wagner, 2007; Fozo *et al.*, 2010). In the most characterized type II system, both toxin and antitoxin are proteins and the antitoxin neutralizes the toxin by direct interaction (Gerdes *et al.*, 2005). In general, genes encoding a toxin and an antitoxin are organized in an operon and their expression is auto-regulated at the transcriptional level. When the expression from type II TA loci is impaired by various kinds of stress, such as amino acid starvation, the antitoxin is rapidly degraded by Lon, ClpXP, and/or ClpAP cellular proteases and consequently the level of free toxin is increased (Christensen *et al.*, 2001; Sat *et al.*, 2001; Hayes and Low, 2009). Finally, free toxin inhibits cell growth by blocking DNA replication (Jaffé *et al.*, 1985; Jiang *et al.*, 2002), translation (Liu *et al.*, 2008; Zhang and Inouye, 2011), cell division (Tan *et al.*, 2011), peptidoglycan synthesis (Mutschler *et al.*, 2011), or mRNA degradation (Yamaguchi and Inouye, 2011). TA systems are linked to many roles in cell physiology, including plasmid maintenance (Ogura and Hiraga, 1983; Yarmolinsky, 1995), programmed cell death (Engelberg-Kulka *et al.*, 2004; Nariya and Inouye, 2008), stress response (Gerdes *et al.*, 2005; Wang *et al.*, 2011), protection from phage infection (Hazan and Engelberg-Kulka, 2004; Fineran *et al.*, 2009; Koga *et al.*, 2011; Otsuka and Yonesaki, 2012; Sberro *et al.*, 2013), bacterial persistence against

antibiotics (Maisonneuve *et al.*, 2011; 2013; Amato *et al.*, 2013; Germain *et al.*, 2013) or biofilm formation (Kim *et al.*, 2009; Wang *et al.*, 2011).

We recently discovered two new type II TA systems, *rnIA-rnIB*, encoded by the *E. coli* K-12 chromosome (Koga *et al.*, 2011) and, *lsoA-lsoB*, encoded by a plasmid, pOSAK1, of Enterohaemorrhagic *E. coli* O157:H7 (Otsuka and Yonesaki, 2012), and these TA systems are functional homologs. RnIA and LsoA toxins have mRNA endoribonuclease activities (Otsuka *et al.*, 2007; unpublished data). These toxins are activated after bacteriophage T4 infection, because T4 infection shuts off *E. coli* gene expression, resulting in the disappearance of unstable antitoxins RnIB and LsoB. When a T4 *dmd* mutant infects *E. coli* cells, free RnIA or LsoA degrades most T4 mRNAs at a late stage of infection, leading to a defect in T4 growth (Kai *et al.*, 1996). Thus, these toxins have a role in phage defense. However, when Dmd expresses immediately after T4 infection as found in wild-type infection, the phage can grow normally. T4 Dmd suppresses the RNA cleavage activity of RnIA or LsoA by direct binding, instead of RnIB or LsoB antitoxin (Otsuka and Yonesaki, 2012). Therefore, T4 phage has evolutionally obtained its own antitoxin against bacterial toxins functioning as an anti-phage mechanism. As another important aspect, T4 Dmd antitoxin can inactivate both RnIA and LsoA, although the protection conferred by RnIB or LsoB is limited to its cognate toxin similarly to other cases (Fiebig *et al.*, 2010). This is the first example of a phage with an antitoxin against multiple toxins.

Recently, we determined the crystal structure of RnIA (Wei *et al.*, 2013). RnIA is composed of three independent domains: NTD (N-Terminal Domain), NRD (N-Repeated Domain) and DBD (Dmd-Binding Domain), and is no structural homology to known toxins. DBD is the key domain responsible for RnIA toxicity, RnIA dimerization, and Dmd binding for inactivation of RnIA toxicity.

Although purified RnlA has an endoribonuclease activity (Otsuka *et al.*, 2007), its activity was much lower than expected from *in vivo* activity. However, Otsuka *et al.* (2007) could detect mRNA cleavage activity comparable to *in vivo* activity if a P100 fraction containing ribosomal proteins and ribosome-associated proteins prepared from wild-type *E. coli* was used. When the P100 fraction was divided into an HSS fraction containing ribosome-associated proteins and an HSP fraction containing ribosomal proteins, RNA cleavage activity was almost lost in each fraction, although most RnlA was localized to HSP. Interestingly, RNA cleavage activity was restored when HSS and HSP were mixed. In addition, Otsuka *et al.* (2007) identified some proteins interacting with RnlA by pull-down assay and MS/MS analysis. From these results, we expect that an unknown factor is involved in RnlA-mediated mRNA cleavage activity.

In previous work, Koga (doctoral thesis 2010) searched homologs of RnlA using BLAST, and found that some of them included an RNase HI domain that is fused to the RnlA homolog. *E. coli* RNase HI has an endoribonuclease activity to cleave RNA in a DNA-RNA duplex (Miller *et al.*, 1973) and is responsible for removing the RNA primer in DNA replication (Itoh and Tomizawa, 1980). In addition to DNA replication, RNase HI is also involved in DNA repair and transcription (Tadokoro and Kanaya, 2009; Drolet, 2006; Cerritelli and Crouch, 2009). In this study, I demonstrated that RNase HI was essential for activation of RnlA after T4 infection, and enhanced the RNA cleavage activity of RnlA *in vitro* and the toxicity of RnlA *in vivo*. This is the first example of an activator for toxin activity.

Materials and Methods

Phages bacteria and plasmids: Wild-type bacteriophage T4 is T4D. The *amSF16* mutant contains an amber mutation in the *dmd* gene (Kai *et al.*, 1996). *E. coli* K-12 strain MH1 (*sup⁰ araD139 hsdR ΔlacX74 rpsL*), TY0802 (MH1 *ΔrnlAB*), TY0807 (MH1 *araD⁺*) and TY0809 (TY0807 *ΔrnlAB*) were described previously (Koga *et al.*, 2011). MH1 and TY0807 were used as a wild-type. JW0204 (BW25113 *ΔrnhA::kan*), JW0178 (BW25113 *ΔrnhB::kan*), and JW2669 (BW25113 *ΔrecA::kan*) were purchased from National BioResource Project (NIG, Japan). TY0824 (MH1 *ΔrnhA::kan*), TY0827 (TY0807 *ΔrnlAB ΔrnhA::kan*) and TY0829 (MH1 *ΔrnhA*) were described previously (Koga *et al.*, 2011). TY0860 (TY0807 *ΔrnhB::kan*) was constructed by T4 GT7 phage transduction of *ΔrnhB::kan* from JW0178 into TY0807.

pHSG415r-*rnlAB*, pBAD33-F-*rnlA*, pBAD24-*rnlA*, pHU102, pQE80L-*rnlA*, pMK61, pQE80L-*rnhA*, and pHSG415r-*rnhA* were described previously (Otsuka *et al.*, 2007; Koga *et al.*, 2011; Koga 2010 doctoral thesis).

RNA purification, primer extension analysis and RNA cleavage assay in vitro: Isolation of total RNA, RNA cleavage reactions using cell extracts and primer extension analysis were performed as described (Kai *et al.*, 1996; 2002; Otsuka *et al.*, 2007). In the RNA cleavage assay, 0.25 pmol of fluorescent RNA (5'-AGUAUAUGAAGAAAAUCAAC-(FAM)-3') (GeneDesign), which corresponds to a part of T4 *soc* RNA sequence (+197 to +217), indicated amounts of RnlA, RNase HI and Dmd were mixed in 5 μl of the reaction buffer containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 2 mM MgCl₂, and 0.05 U of RNase Inhibitor (Life Technologies). After incubation for 60 min at 30 °C, RNA was separated through a 20% polyacrylamide gel and detected with an FLA-7000 image analyzer (Fujifilm).

Pull-down and Western blot analysis: The S30 fraction of cell extracts

were prepared as described (Nirenberg 1963; Stanley and Wahba 1967) and aliquots containing 0.2 mg protein were mixed with 20 μ l of Ni-NTA agarose beads (QIAGEN) by end-over-end rotation overnight at 4 °C. The agarose beads were washed four times with 1 ml of TMCK buffer (10 mM Tris-HCl (pH 7.5), 10 mM Mg(OAc)₂, 30 mM KCl, 0.5 mM DTT) containing 20 mM imidazole. Bound proteins were suspended with 20 μ l of SDS-PAGE sample loading buffer, followed by western blotting analysis as described previously (Koga *et al.*, 2011).

Purification of His-tagged RNase HI, His-RnlA and His-Dmd:

His-tagged RNase HI was purified as described previously with minor modifications (Kanaya *et al.*, 1983). $\Delta rnlAB \Delta rnhA::kan$ cells harboring pQE80L-*rnhA* were grown at 37 °C to a density of 4×10^8 cells ml⁻¹ in 300 ml of LB medium. After IPTG was added to a final concentration of 0.5 mM, the cells were cultured for 4 hr, harvested and washed with lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole). The cells were suspended with 6 ml of lysis buffer, and disrupted at a duty of 30% for 5 min using Tomy sonicator UD-201. The lysate was centrifuged at 15,000 rpm for 20 min and the supernatant was mixed with 1 ml of Ni-NTA Superflow (QIAGEN) by end-over-end rotation overnight at 4 °C. The mixture was packed into a column and washed with 300 ml of wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole). His-tagged RNase HI was eluted with 3 ml of elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole), dialyzed against buffer A (50 mM Tris-HCl pH 7.5, 10 mM β -mercaptoethanol, 1 mM EDTA, 10% (V/V) glycerol) and then loaded onto a DEAE-cellulose (DE-52, Whatman) column. His-tagged RNase HI was in the flow through fraction and was loaded onto phosphocellulose P11 (Whatman) column. His-tagged RNase HI was eluted at 0.3-0.6 M NaCl, dialyzed with buffer A, and loaded onto Heparin Sepharose (GE Healthcare) column. His-tagged RNase HI was eluted at 0.05-0.3 M NaCl, dialyzed

against buffer A, and stored at -20 °C. His-tagged RnlA and Dmd were purified as described (Otsuka *et al.*, 2007).

Results

Effects of RNase HI and RNase HII on growth of T4 phage: RnlA homologs in 20 bacterial species were found from proteobacteria to firmicutes (Koga 2010 doctoral thesis). Koga (doctoral thesis 2010) showed that deletion of *rnhA* rendered the cells able to support the normal growth of a *dmd* mutant, like $\Delta rnlAB$ cells, and that RNase HI might be involved in RnlA activity. *E. coli* has another type of RNase H, RNase HII (encoded by *rnhB*). RNase HII is also an endoribonuclease to cleave RNA in a DNA-RNA duplex, although the similarity of amino acid between RNase HI and RNase HII is low (Itaya. 1990). However, the involvement of RNase HII in RnlA activity has not been defined. Thus, I first investigated whether RNase HII was also concerned with RnlA activity. When the T4 *dmd* gene is defective, RnlA is activated after infection and results in shutting off T4 gene expression by rapidly degrading mRNAs to block T4 propagation (Kai *et al.*, 1996; Otsuka and Yonesaki 2005). To examine the effect of RNase HII on RnlA activity, I examined the growth of wild-type T4 or *dmd* mutant on plates seeded with wild-type, $\Delta rnlAB$, $\Delta rnhA$, or $\Delta rnhB$ cells (Fig. 1). Consistent with previous data, *dmd* mutants grew normally on $\Delta rnlAB$ and $\Delta rnhA$ cells, while $\Delta rnhB$ cells did not support the growth of a *dmd* mutant. These results strongly suggested that RNase HI, but not RNase HII, is necessary for RnlA activity after T4 infection.

Effect of RNase HI on the RnlA RNase activity: The growth of a *dmd* mutant on $\Delta rnhA$ cells should reflect the decrease of the RnlA RNase activity. The possibility that the expression of RnlA is repressed by a deletion of *rnhA* could be eliminated, because I confirmed that disruption of *rnhA* slightly increased the transcription of *rnlA* (Fig. 2). In order to confirm the effect of RNase HI on RnlA activity, T4 late-gene *soc* mRNA was examined by primer extension analysis, because RnlA-mediated endoribonucleolytic degradation has been extensively

characterized (Kai *et al.*, 2002). In agreement with previous reports (Kai *et al.*, 2002; Otsuka *et al.*, 2005), cDNA corresponding to the full length of *soc* mRNA after a *dmd* mutant infection in wild-type cells was significantly reduced and RnlA-specific cleavages at nucleotide positions 135, 144, 145, 153, 172, 185 and 207 from the 5' terminus were detected (Fig. 3), whereas full-length mRNA was increased and these cleavages completely disappeared in $\Delta rnlAB$ cells. As expected, all cleavages by RnlA also disappeared in $\Delta rnhA$ cells, indicating the loss of RnlA-mediated RNA cleavage activity by disruption of *rnhA*.

In previous work, the effect of RNase HI was also examined for the RNase activity of RnlA *in vitro* (Koga 2010 doctoral thesis). S30 fractions prepared from *E.coli* cells, *soc* RNA synthesized *in vitro*, and/or purified RNase HI were incubated, and then *soc* RNA was analyzed by primer extension analysis. In previous experiments using S30 fractions from wild-type cells, RnlA-specific cleavages were detected at 135, 153 and 207, which were identical to those detected *in vivo* (Otsuka *et al.*, 2007). In this experiment, Koga (doctoral thesis 2010) could not detect any RnlA-specific cleavages with the S30 fraction from $\Delta rnhA$ cells. However, these cleavages were recovered after the addition of purified RNase HI. From this result, RNase HI is necessary for the RnlA RNase activity.

Association of RNase HI and RnlA: In order to investigate whether RNase HI is physically associated with RnlA, I performed a pull-down experiment. Extracts were prepared from cells harboring pBAD33-F-*rnlA* plus pQE80L-*rnhA* or pHU102 encoding His-tagged RNase G as a control, and His-tagged proteins were pulled down with Ni-NTA beads. In Fig. 4, Flag-RnlA was efficiently recovered by His-RNase HI, but not by His-Rng, suggesting the association of RNase HI and RnlA *in vivo*. I also tried pull-down experiments and mobility shift assays using purified RNase HI and RnlA, but their direct interactions were not detected under our

experimental conditions. This result suggests that an unknown factor may help stabilizing their interaction. This idea would be supported by the observation that, in primer extension analysis, RNase activity of purified RnlA with the aid of RNase HI was significantly stimulated by the addition of S30 extract prepared from $\Delta rnlAB$ $\Delta rnhA$ cells, although purified RNase HI and RnlA alone could not be detected the cleavage activity (Fig. 5).

Effect of RNase HI on RNA cleavage activity of RnlA *in vitro*: To test whether or not RNase HI directly enhances RNA cleavage activity of RnlA, I carried out an *in vitro* RNA cleavage assay with purified RnlA and RNase HI (Fig. 6). To increase the sensitivity for detection of RnlA-specific cleavage, I used 20-nt RNA labeled with fluorescence at its 3'-end as a substrate, whose sequence is the same as a region of T4 *soc* RNA containing a major RnlA-cleavage site at the position 10 (shown by the arrowhead in Fig. 6A). When 100 ng of RnlA were added to the reaction, two significant products by cleavage appeared at positions 10 and 17 (Fig. 6B, lane 4). Because the addition of Dmd, a specific inhibitor of RnlA, eliminated the bands at positions 10 and 17, these cleavages should be specific to RnlA (lane 6). The other weak bands were insensitive to Dmd and might be caused by contamination of other RNases. Cleavages at position 10 and 17 were detected weakly when RnlA was reduced to 50 ng (Fig. 6C, lane 6). However, these cleavage products were prominent with the increasing of RNase HI (lanes 7-10) in the presence of RnlA. I also confirmed that RNase HI alone exhibited no cleavage at positions 10 and 17, in the increasing amount of RNase HI (Fig. 6C, lanes 2-5). These results indicated that RNase HI enhanced RnlA activity *in vitro*.

Effect of endogenous RNase HI on growth of T4 phage in cells exogenously expressing RnlA: Results in Fig. 1 and Fig. 3 indicated that RNase HI is essential for RnlA activity after infection by a T4 *dmd* mutant.

However, the *in vitro* experiment in Fig. 6 and a previous result (Otsuka *et al.*, 2007) demonstrated that RNase HI has a stimulatory effect on the RNA cleavage activity of RnlA, thereby it is not essential for this activity. The endogenous level of RnlA seems very low, because it was undetectable by our western blot analysis with an antibody against RnlA, even when 100 μ g of cell extract were used. These observations suggested that RNase HI might be required for activating a low amount of endogenous RnlA to block T4 gene expression by degrading mRNAs. In other words, when a high amount of RnlA is present, RNase HI may not be necessary for anti-T4 activity of RnlA. To test this hypothesis, we measured the burst size of a *dmd* mutant on wild-type, $\Delta rnlAB$, or $\Delta rnhA$ cells harboring pHSG415r (Takeshita *et al.* 1987), pHSG415r-*rnhA*, or pHSG415r-*rnlAB* containing an *mIA-mIB* operon (Table 1). Since the copy number of pHSG415r is around 5, the amount of RnlA in cells harboring this plasmid would be 5-fold more than in wild-type cells without this plasmid. A *dmd* mutant grew very poorly on wild-type cells harboring pHSG415r, with a burst size only 1.1, while it grew well on $\Delta rnlAB$ or $\Delta rnhA$ cells with a burst size of 107 or 53, respectively. Introducing pHSG415r-*rnlAB* into $\Delta rnlAB$ cells reduced burst size to 0.43. In the presence of pHSG415r-*rnlAB*, $\Delta rnhA$ cells decreased burst size to 16, one-third of that in the presence of vector. This result demonstrates that an excess of RnlA can impair the growth of a *dmd* mutant even in the absence of RNase HI. In contrast, introduction of pHSG415r-*rnhA* into $\Delta rnlAB$ cells exhibited no effect on growth of a *dmd* mutant (burst size of 1.0), which strongly suggested that RNase HI was not the main factor for inhibiting the growth of a *dmd* mutant. The weaker effect of pHSG415r-*rnlAB* on the growth of a *dmd* mutant in $\Delta rnhA$ cells suggested that RNase HI was required for enhancing RnlA activity to block T4 propagation.

Effect of endogenous RNase HI on cell toxicity and RNA-degrading

activity of RnlA in *E. coli* cells: RnlA activity in the absence of RnlB inhibits bacterial growth (Koga *et al.*, 2011). To test the contribution of RNase HI to RnlA toxicity, we compared the survival of $\Delta rnlAB$ or $\Delta rnlAB \Delta rnhA$ cells, after RnlA was ectopically expressed (Fig. 7). Cells harboring pBAD24-*rnlA* were cultivated to middle-log phase, treated with 0, 0.002 and 0.02% L-arabinose for 1 hr to induce RnlA, and plated to measure colony forming units. $\Delta rnlAB$ cells exhibited an L-arabinose concentration-dependent reduction of cell viability from 100% to 0.24% and 0.04%, respectively. Although the cell viability was also decreased in $\Delta rnlAB \Delta rnhA$ cells after RnlA induction, from 100% to 23% and 3.3%, the efficiency of cell viability was significantly increased compared with $\Delta rnlAB$ cells. Thus, endogenous RNase HI significantly enhanced RnlA-mediated cell toxicity.

Discussion

In this study, I developed a line of evidence indicating that RNase HI is involved in the activity of the RnlA toxin. First, a T4 *dmd* mutant was able to grow on $\Delta rnhA$ cells as well as on $\Delta rnlAB$ cells (Fig. 1). Second, RNase HI is necessary for RnlA-specific RNA cleavages *in vivo* and *in vitro* (Fig. 3). Third, RnlA was associated with RNase HI *in vivo* (Fig. 4). Interestingly, purified RNase HI enhanced the cleavage activity of RnlA (Fig. 6). From these results, I concluded that RNase HI was an activating factor of RnlA toxicity. Finally, the results in Fig. 6 showing that endogenous RNase HI increased cell toxicity and the RNase activity of RnlA in *E. coli* cells supported this conclusion.

RNase H is an endoribonuclease that can cleave RNA in a DNA-RNA duplex, and was originally identified as the enzyme responsible for removing the RNA primer, allowing the completion of newly synthesized DNA in DNA replication. Later, some reports demonstrated that *E. coli* RNase HI was also involved in DNA repair and transcription (Tadokoro and Kanaya, 2009; Drolet, 2006; Cerritelli and Crouch, 2009). RNase H is conserved in all organisms and *E. coli* RNase H has been well characterized. *E. coli* has two types of RNase H, namely RNase HI (*rnhA*) and RNase HII (*rnhB*). RNase HII is distinguished from RNase HI by the lack of amino acid sequence similarity (Itaya, 1990). Also, RNase HI and HII differ in divalent metal ion preferences and specificity in activity (Keck and Marqusee, 1996; Ohtani *et al.*, 2000). Sequence alignment between RnlA and RNase HI/HII exhibited no homologous region. In this study, a T4 *dmd* mutant could grow on $\Delta rnhA$ cells but not on $\Delta rnhB$ cells (Fig. 2B), indicating that the activation of RnlA after T4 infection depends on RNase HI but not on RNase HII. Thus, RNase HI has a role in toxin activation in addition to its roles in DNA replication, repair and transcription.

When *rnhA* was deleted, a *dmd* mutant could propagate (Fig. 1) and RnlA-specific

RNA cleavage activity was abolished (Fig. 3). As described above, RNase HI is involved in DNA replication and transcription. This suggested that the deficiency of RNase HI might indirectly affect RnlA activity. To eliminate this possibility, I first confirmed that there was no reduction in the amount of *rnlA* transcript when *rnhA* was deleted (Fig. 2). The amount of *rnlA* transcript in $\Delta rnhA$ cells is slightly higher than in wild-type cells. Second, we could detect RnlA-specific RNA cleavage activity in an S30 extract from $\Delta rnhA$ cells when purified RNase HI was added (Koga 2010 doctoral thesis), strongly suggesting the direct effect of RNase HI on RnlA activity. Moreover, a *dmd* mutant could propagate normally in $\Delta rnhA \Delta recA$ cells (Koga 2010 doctoral thesis), in which the SOS response was constitutively repressed.

Koga (doctoral thesis 2010) examined whether two RNase HI derivatives with mutations in active site residues (E48Q or D70E) is necessary for RnlA activation after phage infection or not. These two amino acids correspond to metal-binding sites and their substitutions result in the loss of cleavage activity of RNase HI on DNA-RNA hybrids (Tadokoro and Kanaya, 2009). While T4 *dmd* mutant could not propagate on $\Delta rnhA$ cells expressing wild-type RNase HI, mutated RNase HI could not complement disruption of *rnhA*. Although I need to confirm the expression level or the loss of RNase HI activity of these mutated RNase HI, this result suggests that these two amino acids in RNase HI are necessary for RnlA activation after T4 infection, and may be required for interaction to RnlA. From this, there are two possibilities as mechanisms of RnlA activation by RNase HI. One is that interaction of RnlA with RNase HI may increase the affinity for RNAs. The isoelectric point of RnlA (6.4) is lower than the basic pI value of other toxins (pI 11.0 in HicA, pI 10.0 in RelE and pI 8.4 in MazF). That is, RnlA may have a weaker affinity for RNA than other toxins. As RNase HI is a basic protein with the pI 9.0, RnlA may efficiently degrade RNAs by interaction with RNase HI. The other possibility is that both RNase HI and RnlA catalyze the RNA degradation. Although RNase HI alone cannot cleave single-stranded RNA (ssRNA), the RNase HI that interacted with RnlA may lead to conformation change and cleavage of

ssRNA.

As far as I know, a factor activating the toxin had not been identified previously. Further investigations of the mechanism of RNase HI activation and of whether RNase HI can enhance the activity of other toxins may provide novel insights into the molecular basis for regulating toxin activity.

	pHSG415r	pHSG415r- <i>rnhA</i>	pHSG415r- <i>rnLAB</i>
wild-type	1.1 ± 0.6	2.6 ± 0.8	0.92 ± 0.6
$\Delta rnLAB$	107 ± 17	78 ± 2.4	0.43 ± 0.0
$\Delta rnHA$	53 ± 10	1.0 ± 0.2	16 ± 1.1

Table 1. Effect of *rnhA* on growth of a *dmd* mutant in cells exogenously expressing RnIA.

Wild-type, $\Delta rnLAB$, or $\Delta rnHA::kan$ cells with pHSG415r, pHSG415r-*rnhA* or pHSG415r-*rnLAB* containing the *rnIA-rnIB* operon were grown in LB medium until OD₆₀₀ reached 0.6, and infected with a T4 *dmd* mutant at a multiplicity of infection of 0.1 at 30 °C. At 8 min, the cells were diluted 10⁴-fold with fresh LB and further incubated for 70 min. After cells were lysed with chloroform, the total number of progeny phages was determined by plating. The burst size is the ratio of the number of progeny phages to the number of input phage. Each value is the average burst size ± standard deviation obtained from at least two independent experiments.

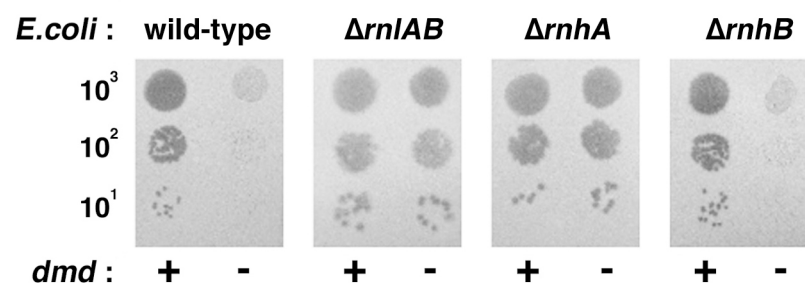


Figure 1. Effect of *rnhA* on growth of T4 phage.

A solution containing wild-type T4 (+) or *dmd* mutant (-) was serially 10-fold diluted as indicated on the left, and 1 μ l of each solution was spotted onto a plate seeded with wild-type, $\Delta rnlAB$, $\Delta rnhA::kan$, or $\Delta rnhB::kan$ cells. Plates were incubated at 30 °C overnight.

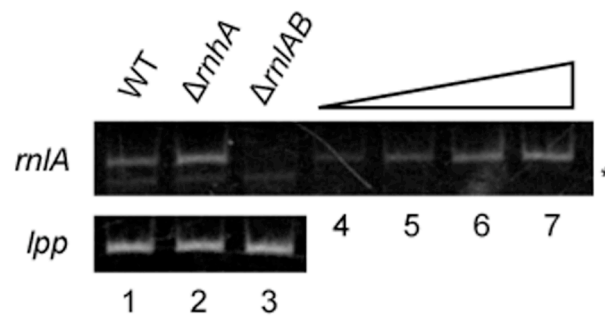


Figure 2. Effect of *rnhA* on *rnlA* transcription.

Wild-type (lane 1), $\Delta rnhA::kan$ (lane 2), and $\Delta rnlAB$ (lane 3) cells were grown to mid-log growth phase in LB medium and total RNAs were extracted. RT-PCR analyses for *rnlA* and *lpp* mRNAs were performed as described in our previous paper (Otsuka *et al.*, 2010). Various amount of pBSNO carrying the sequence from *rnlA* to *rnlB* were used as template to demonstrate a semi-quantitative profile of PCR conditions; lane 4, 2 pg; lane 5, 4 pg; lane 6, 8 pg; lane 7, 16 pg. Asterisk indicates non-specific band.

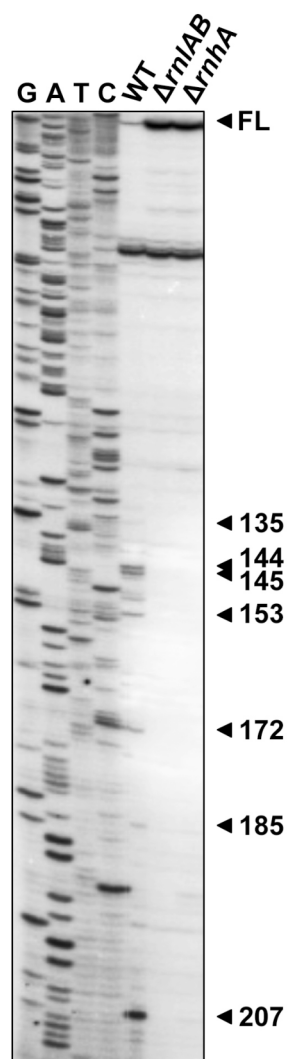


Figure 3. Effect of RNase HI on RnlA RNase activity.

Total RNAs extracted from wild-type, $\Delta rnlAB$, or $\Delta rnhA::kan$ cells infected with *dmd* mutant phage were analyzed by primer extension with a primer for T4 *soc* RNA. FL corresponds to full-length *soc* RNA. Bands marked with the position of the nucleotide 3' of the cleavage site correspond to RnlA-specific cleavage products.

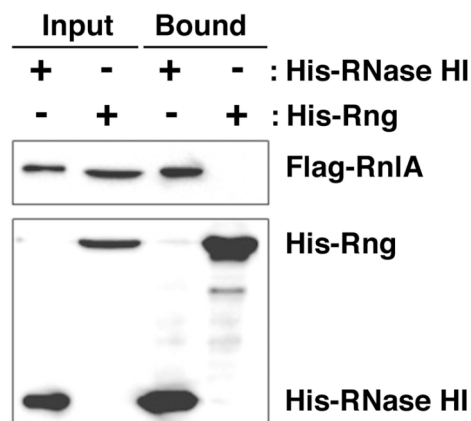


Figure 4. Association between RnlA and RNase HI *in vivo*.

$\Delta rnlAB \Delta rnhA::kan$ cells harboring pBAD33-F-*rnlA* and pQE80L-*rnhA* or pQE80L-*rng* as a control were grown at 30 °C in LB containing 50 $\mu\text{g ml}^{-1}$ ampicillin and 30 $\mu\text{g ml}^{-1}$ chloramphenicol. When the OD₆₀₀ reached 0.5, Flag-RnlA and His-RNase HI/His-Rng were co-induced with 0.05 % L-arabinose and 0.1 mM IPTG for 45 min. S30 extracts from cells were subjected to pull-down with Ni-NTA agarose beads according to the method described in the Experimental Procedures. Input and bound fractions were analyzed by Western blot with anti-Flag (upper panel) or anti-His antibody (lower panel).

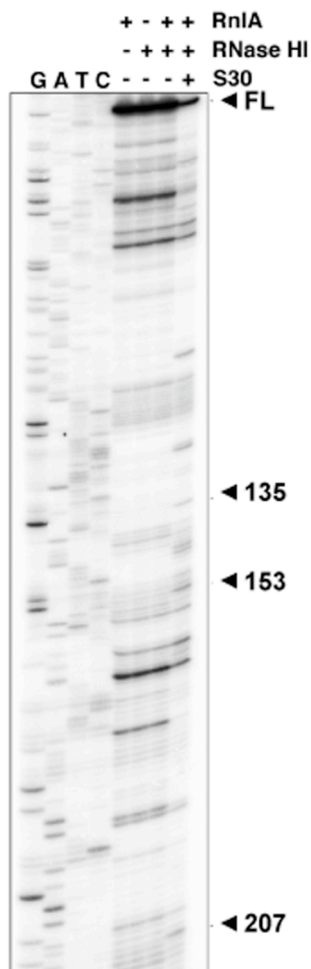


Figure 5. Effect of S30 extracts on RnlA RNase activity. The reaction mixture contained 12 ng of synthesized *soc* RNA, 0 or 20 ng of purified RNase HI, 0 or 20 ng of RnlA, and 0 or 20 μ g of S30 extract from $\Delta rnlAB \Delta rnhA::kan$ cells. After the reaction, *soc* RNAs were analyzed by primer extension. RNase activity of purified RnlA with the aid of RNase HI was significantly detected by the addition of S30 extract prepared from $\Delta rnlAB \Delta rnhA$ cells.

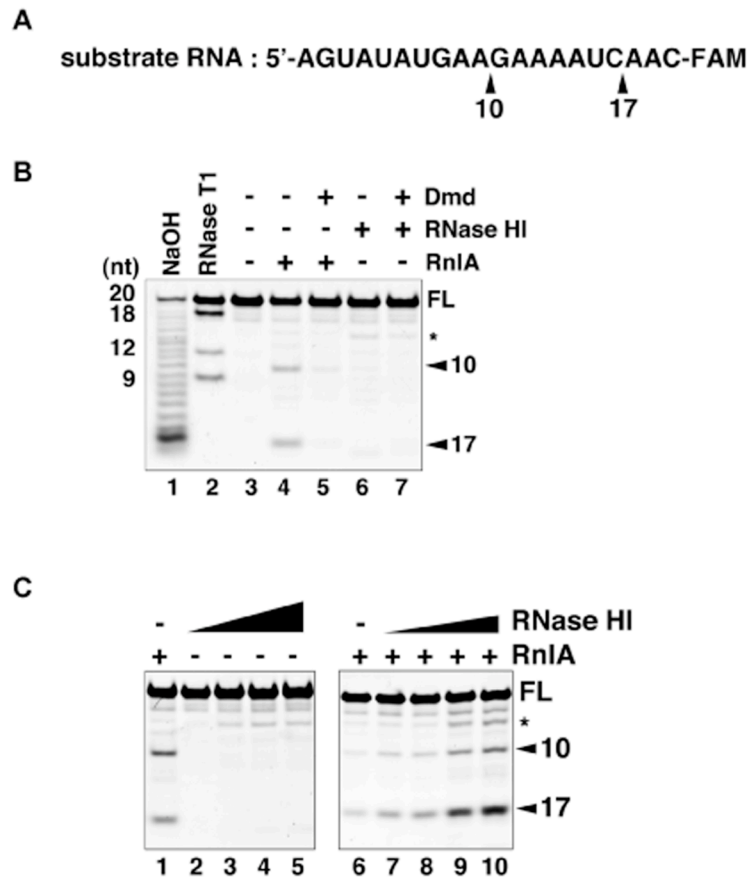


Figure 6. Effect of RNase HI on RnlA cleavage activity.

A. The sequence of the substrate 20-nt RNA is shown. The substrate is labeled with fluorescent FAM at its 3'-end. The cleavage sites of RnlA are indicated by arrowheads.

B. The 20-nt RNA was incubated with the proteins indicated above the figure and the cleavage products were analyzed by polyacrylamide gel electrophoresis as described in the Experimental Procedures. Each reaction mixture contained no protein (lane 3) or 100 ng of RnlA (lane 4), 100 ng of RnlA + 50 ng of Dmd (lane 5), 100 ng of RNase HI (lane 6), 100 ng of RNase HI + 50 ng of Dmd (lane 7). The RNA was partially digested with NaOH (alkaline hydrolysis, lane 1) and RNase T1 (lane 2) which cleaves single-stranded RNA after guanosine residues. Asterisk indicates the band specific to the solution containing RNase HI, which is not affected by Dmd.

C. Each reaction mixture contained 100 ng of RnlA (lane 1), 25 ng (lane 2), 50 ng (lane 3), 100 ng (lane 4), or 200 ng (lane 5) of RNase HI, 50 ng of RnlA (lane 6), 50 ng of RnlA + 6.25 ng (lane 7), 12.5 ng (lane 8), 25 ng (lane 9), or 50 ng (lane 10) of RNase HI.

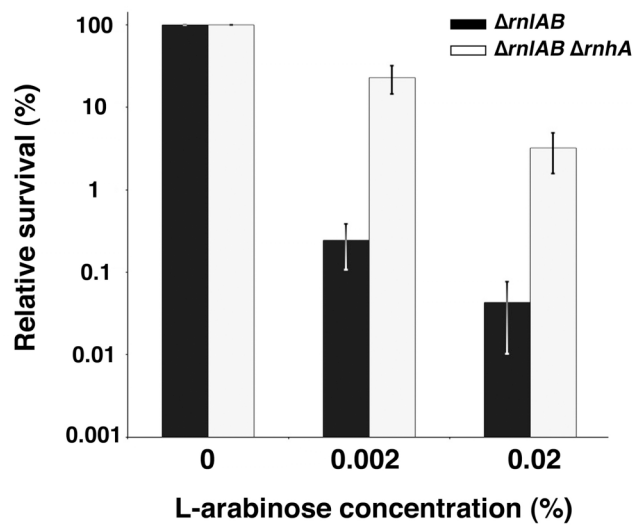


Figure 7. Effect of RNase HI on cell toxicity in *E. coli* cells.

$\Delta rnIAB$ or $\Delta rnIAB \Delta rnhA::kan$ cells harboring pBAD24-*rnIA* were grown in LB medium at 30 °C until OD₆₀₀ reached 0.5. After L-arabinose was added at the final concentration of 0, 0.002 or 0.02 %, cells were cultured for 1 hr. Cell cultures were serially diluted in BS buffer (10 mM K-Pi (pH 7.4), 137 mM NaCl), spread on LB plates, and incubated at 37 °C for 20 hr to count colony forming units. Survival in the absence of L-arabinose is set to 100% and relative survival was plotted. An error bar shows a standard deviation deduced from three independent experiments.

Chapter 2

RnlB antitoxin requires RNase HI to suppress the activity of RnlA toxin in

Escherichia coli

Abstract

Escherichia coli rnlAB genetic module is identified as a toxin-antitoxin related in anti-phage mechanism. *rnlA* encodes a stable toxin protein, RnlA, and *rnlB* encodes a labile antitoxin protein, RnlB. RnlB normally suppresses RnlA activity by binding to it, while, in infection of bacteriophage T4, gene expression from *E. coli* is shut off, leading to disappearance of RnlB. Subsequently, the released RnlA is activated by RNase HI (encoded by *rnhA*) and degrades intracellular mRNAs, resulting in prevention of propagation of T4 *dmd* mutant phage. However, in wild-type T4, T4 Dmd antagonizes RnlA, leading to propagation of wild-type T4. Here, I showed that RNase HI is also required for RnlB to suppress the activity of RnlA. Ectopic expression of RnlA in $\Delta rnhA$ cells showed growth defects regardless of the presence of *rnlB* on the *E. coli* genome. RNase HI bound to NRD, one of three domains composing RnlA, and RnlB also bound to NRD only when RNase HI was present. These results indicate that RNase HI is included in RnlA-RnlB system and required for the interaction between RnlA and RnlB. Thus, it is suggested that RNase HI is an essential component of the RnlA-RnlB toxin-antitoxin system.!

Introduction

Toxin-antitoxin (TA) systems are genetic modules that are present in plasmids and bacterial genomes, and they generally form an operon. Typically, toxins and its cognate antitoxins ordinarily form complexes to neutralize the toxin activity. When cells suffer from stresses such as amino acid starvation and high temperature, the antitoxin is degraded and consequently the level of free toxin is increased. The free toxin attacks a cellular function to inhibit cell division. TA is related to a multitude of role in bacterial physiology, including stress responses, programmed cell death, plasmids maintenance, anti-phage activity and formation of persisters (Yamaguchi and Inouye, 2011; Lewis K, 2009; Gerdes and Maisonneuve, 2012, Schuster and Bertram, 2013).

Koga *et al.*, (2011) discovered a TA system, *rnLAB*, in *E. coli* K-12 strains (Koga *et al.*, 2011). Like other TAs, RnlB antitoxin normally binds to RnlA toxin to suppress the activity. When a bacteriophage T4 infects *E. coli* cells, gene expression from *E. coli* is shut off and RnlB is subjected to degradation by Lon and ClpXP proteases, resulting in liberation of RnlA. However, T4 phage expresses *dmd* immediately after infection and Dmd, instead of RnlB, suppresses RnlA activity (Otsuka and Yonesaki, 2012). Thereby, when a T4 *dmd* mutant infects *E. coli*, activated RnlA degrades most T4 mRNAs (Otsuka *et al.*, 2007), leading to a defect in T4 growth and acting as a potential antagonist of bacteriophage T4 (Kai *et al.*, 1996).

We uncovered the crystal structural of RnlA (Wei *et al.*, 2013). RnlA consists of NTD (N-Terminal Domain), NRD (N-Repeated Domain) and DBD (Dmd-Binding Domain). DBD is essential for RNase activity of RnlA and Dmd binding, whereas the roles of two other domains remain unclear. I also demonstrated that RNase HI, which is encoded by *rnhA* and responsible for removing RNA primers in DNA replication, stimulates the RNase activity and cell toxicity of RnlA (Naka *et al.*, 2014;

chapter 1). However, the roles of NTD and NRD domains remain unclear. In addition, it remains unknown whether RNase HI is involved in the function of RnlB.

To address these questions, I investigated which domains of RnlA were required for the interaction with RnlB and RNase HI. Moreover, I examined the role of RNase HI in the RnlB activity.

Here, I showed that both RNase HI and RnlB associated with NRD, and that RNase HI was necessary for RnlB to suppress the RnlA activity. Thus, RNase HI is involved not only in RnlA activation in T4-infected cells but also in RnlB repression in T4-uninfected cells. From these results, I suggest that RNase HI is an essential component of the RnlA-RnlB toxin-antitoxin system.

Materials and Methods

Bacterial strains: *E. coli* K-12 strain MH1 (*sup⁰ araD139 hsdRΔ lacX74 rpsL*), TY0807 (MH1 *araD⁺*) and TY0809 (TY0807 Δ *mlAB*) were described previously (Koga *et al.*, 2011). TY0826 (TY0807 Δ *mhA::kan*) and TY0827 (TY0809 Δ *mhA::kan*) were described previously (Koga 2010 doctoral thesis; Naka *et al.*, 2014).

Plasmids: To construct pBAD24-F-NTD-NRD, pBAD24-F-NTD and pBAD24-F-NRD, DNA fragments were amplified by PCR with pBAD24-*mlA* (Koga *et al.*, 2011) as a template using the primers, YO-17 (5'-CCATGGTACCAGATTACAAGGATGACGACGATAAGACAATCAGGAGTTAC AAAAAC) and YO-145 (5'-AGTGCTGCAGTCAGTAAGTGCGGGCAACCTCCTG), YO-17 and KN-29 (5'-AGTGCTGCAGTCATTCAAAAAGATGATCCGCTAA), and KN-27 (5'-GGAGGAATTCACCATGGATTACAAGGATGACGACGATAAGACCATCAATC CTGCTGAATTTGAG) and YO-145, respectively, digested with *KpnI* and *PstI* for the fragments amplified with YO-17 and YO-145, or YO-17 and KN-27, or with *EcoRI* and *PstI* for those with KN-27 and YO-145, and ligated into pBAD24.

To construct pBAD24-F-*mhA*, a DNA fragment was amplified by PCR with pBR322-*mhA* (Naka *et al.*, 2014) as a template using the primers, KN-13 (5'-CCATGGTACCAATGGATTACAAGGATGACGACGATAAGCTTAAACAGGTA GAAA) and KN-14 (5'-AACTGCAGAACTTAACTTCAACTTGG), digested with *KpnI* and *PstI*, and ligated into pBAD24.

pBAD24-F-NTD-NRD, pBAD24-F-NTD, pBAD24-F-NTD, pBAD24-F-DBD (Wei *et al.*, 2013) and pBAD24-F-*mhA* were treated with *BamHI* and *PstI*, and ligated into pBAD33 to construct pBAD33-F-NTD-NRD, pBAD33-F-NTD, pBAD33-F-NRD, pBAD33-F-DBD and pBAD33-F-*mhA*, respectively.

To construct pBAD33-F-NRD-H-*mlB*, a DNA fragment was amplified by PCR with pMK19 (Koga *et al.*, 2011) as a template using the primers, KN-52 (5'-AGTGCTGCAGATTAAAGAGGAGAAATT) and KN-55 (5'-CCCAAGCTTGTTTAGAAAGAAGATT), digested with *Pst*I and *Hind*III, and ligated into pBAD33-F-NRD.

To construct pQE80L'-*mha*-F, a DNA fragment was amplified by PCR with pQE80L'-*mha* (Naka *et al.*, 2014) as a template using the primers, KN-20 (5'-ACAGGCATGCTTAAACAGGTAGA) and KN-21 (5'-GGTTCTGCAGTTACTTATCGTCGTCATCCTTGTAATCAACTTCAACTTGGA), digested with *Sph*I and *Pst*I, and ligated into pQE80L', which is removed the 6× His-tag region (Koga *et al.*, 2011).

To construct pBAD24-NTD-DBD, DNA fragments were amplified by PCR with pBAD24-*mlA* as a template using the primers, YO-28 (5'-GGAGGAATTCACCATGACAATCAGGAGTTACAAAAAC) and KN-50 (5'-GTTGAACAATATTAGCTTTACCATCCTCTTCAAAAAGATGATCCGC), or YO-28 and YO-145 (5'-AGTGCTGCAGTCAAACAATATATAAGTCC), and digested with *Fok*I. The digested fragment (a DNA fragment amplified by YO-28 and KN-50) was ligated with 3'-terminal DNA fragment digested with *Fok*I (a DNA fragment amplified by YO-28 and YO-145) and the ligated DNA fragment was digested with *Eco*RI and *Pst*I, and ligated into pBAD24.

pBAD33-F-*mlA*, pBAD24-*mlA*, pMK19, pMK33, pHU102, pBAD33-DBD and pUC18-*mlB* were described previously (Koga *et al.*, 2011; Wei *et al.*, 2013).

RNA purification, Northern blotting and RT-PCR analysis:

Isolation of total RNA and northern blotting analysis were performed as described (Kai *et al.*, 1996). RT-PCR was performed as described (Otsuka *et al.*, 2010). For Northern blots, Radioactive probes for *ompA* and *lpp* mRNAs were prepared as

described (Ueno and Yonesaki, 2004). For RT-PCR, primers for PCR were KN-39 (5'-CGGGATCCATGTTTGAAATCACCGG) and KN-28 (5'-GTATCCAGCATGATCCGGCC).

Pull down and Western blotting analysis: Cell extracts were prepared as described (Otsuka *et al.*, 2007) and an aliquot containing 1 mg protein was mixed with 20 μ l of Ni-NTA agarose beads (QIAGEN) by end-over-end rotation overnight at 4°C. The agarose beads were washed four times with 1 ml of TMCK buffer (10 mM Tris-HCl (pH 7.5), 10 mM Mg(oAc)₂, 30 mM KCl, 0.5 mM DTT) containing 20 mM imidazole. Bound proteins were suspended with 20 μ l of SDS-PAGE sample loading buffer, followed by western blotting analysis as described previously (Koga *et al.*, 2011).

Cell viability assay: *mIB* deletion was performed according to Datsenko and Wanner protocol. A DNA fragment was amplified by PCR with pKD3 (Datsenko and Wanner, 2000) as a template using the primers, KN-35 (5'-GAGCGTGGGGAATAATCAAGGACTTATATATTGTTTGAAATCACCGGAATGTGTAGGCTGGAGCTGCTTC) and KN-36 (5'-AAGTTAATATCATGCCAAAAGGGCGAATTCTATACTGGTTTCGTTTAGAAAATGGGAATTAGCCATGGTCC). 500 ng of the amplified fragment was introduced into TY0807 and TY0826 harbouring pKD46 and the cells were added SOC medium, cultured for 1hr, plated on LB plates containing chloramphenicol, and incubated at 30 °C for 20 hr, to count cfu.

Results

RNase HI interacts with NRD: In my previous work, I demonstrated that RNase HI (encoded by *rnhA*) binds to RnlA and stimulates its RNase activity (Naka *et al.*, 2014; chapter 1). RnlA consists of NTD, NRD and DBD (Wei *et al.*, 2013), but the domain involved in interaction with RNase HI has not been defined. I therefore tested which domain of RnlA was required for RNase HI binding.

To do this experiment, I generated a series of plasmids producing Flag-tagged RnlA with part of domains from an arabinose-inducible promoter (hereafter called pBAD33-F-NTD-NRD, pBAD33-F-DBD, pBAD33-F-NTD and pBAD33-F-NRD) and tested them for the interaction of RnlA domains with RNase HI in a pull-down assay (Fig. 1A). The cell extracts were prepared from $\Delta mlAB \Delta rnhA$ cells harbouring pBAD33-F-*rnlA*, pBAD33-F-NTD-NRD or pBAD33-F-DBD together with pQE80L-*rnhA*, and His-RNase HI was pulled down with Ni-NTA beads (Fig. 1B and C). RNase HI again efficiently interacted with full-length RnlA, as described in the chapter 1. In addition, NRD and NTD-NRD, but not DBD and NTD alone, were co-precipitated with RNase HI. These results suggested that NRD was required for the interaction with RNase HI.

RnlB associates with NRD and DBD: RnlB counteracts with RnlA by binding to it (Koga *et al.*, 2011). I next explored the domain of RnlA to which RnlB binds by pull-down analysis using cell extracts prepared from $\Delta mlAB$ cells harbouring pBAD33-F-*rnlA*, pBAD33-F-NTD-NRD or pBAD33-F-DBD and pMK19 (Fig. 2A).

Consistent with the previous report (Koga *et al.*, 2011), I observed that RnlB associated with full-length RnlA. Unexpectedly, RnlB also exhibited interaction with not only DBD but also NTD-NRD. Furthermore, RnlB was efficiently association with NRD alone (Fig. 2B). Taken together, these results indicated that

RnlB bound to both NRD and DBD, in contrast to the fact that Dmd interacts with only DBD (Wei *et al.*, 2013).

Importantly, the interactions of NRD with RNase HI and RnlB were insensitive to RNase A treatment, suggesting that these interactions are not mediated by RNA (Fig. 3).

To examine if which interaction of RnlB with NRD or DBD was necessary to suppress the activity of RnlA, I next assessed the growth of $\Delta rnlAB$ cells harbouring pBAD24-*rnlA* or pBAD24-DBD with the empty vector pJK289 (Kato and Ikeda, 1996) or pMK33 that clones *rnlB* downstream from the *lac* promoter carried on a low-copy-number plasmid (a copy number is around 1). These cells were grown to mid-log phase, and then 0.2% L-arabinose was added to induce full-length RnlA or DBD (Fig. 4A and B). Over-expression of full-length RnlA or DBD in the presence of pJK289 exhibited growth defects, while over-expression of full-length RnlA in the presence of pMK33 allowed to grow normally, as previously reported (Koga *et al.*, 2011). However, a growth defect was observed when the DBD was co-expressed in the presence of pMK33. These results indicated that the activity of full-length RnlA, but not of DBD, was suppressed by RnlB, suggesting that NTD-NRD is essential for suppression of the activity of RnlA. Next, a plasmid expressing NTD-DBD, which is lacking NRD, was constructed (pBAD24-NTD-DBD), and examined for the effect on the growth after induction of NTD-DBD (Fig. 4C). Over-expression of NTD-DBD showed a growth defect in the presence of pMK33 as well as pJK289, suggesting that NRD is essential for the activity of RnlB.

To further validate these results, I investigated whether endogenous RnlB expressing from *E. coli* genome repressed DBD activity (Fig. 5). If indeed NTD-NRD is required for RnlB activity, over-expression of DBD in wild-type cells should exhibit a growth defect regardless of the presence of endogenous RnlB. As a

result, over-expression of full-length RnlA from pBAD24-*rnlA* showed normal growth, whereas over-expression of DBD from pBAD24-DBD exhibited a growth defect, demonstrating that endogenous RnlB did not suppress the activity of DBD. These results supported the notion that NRD was significant for RnlB to inhibit RnlA activity.

Although previous works demonstrated that most toxins were inhibited its activity by the antitoxins directly binding to it (Yamaguchi and Inouye, 2011), RnlB did not bind to RnlA directly (data not shown). This result suggested that unknown factor(s) was involved in the interaction. As demonstrated above, the observation that RNase HI interacts with NRD raised the possibility that RNase HI was involved in RnlB activity.

RnlB is unable to inhibit the activity of RnlA in the absence of RNase HI: Next, I investigated the possibility that RNase HI contributes to RnlB activity. As an excess amount of RnlA can cause the impairment of cell growth even in $\Delta rnlAB$ $\Delta rnhA$ cells (Naka *et al.*, 2014; chapter 1), I examined the effects of over-expression of RnlA on the growth of wild-type and $\Delta rnhA$ cells. Cells harbouring pBAD33-F-*rnlA* were cultured to middle-log phase, then treated with or without 0.2% L-arabinose, and monitored the cell growth (Fig. 6A). The wild-type cells had no effects on cell growth in the absence or presence of L-arabinose. Interestingly, the cells lacking *rnhA* did not grow as rapidly as the wild-type cells when F-RnlA was over-expressed, although *rnlB* gene was present on the genome of $\Delta rnhA$ cells. These results suggested that deletion of *rnhA* led to abrogate RnlB's ability to suppress RnlA activity.

As RnlA has an endoribonuclease activity and RnlA expression in the absence of RnlB results in the degradation of most *E. coli* mRNAs (Otsuka and Yonesaki, 2005; Koga *et al.*, 2011), I next determined the level of some *E. coli* mRNAs in a

time-course manner following the expression of RnlA. Wild-type and $\Delta rnhA$ cells harbouring pBAD33-F-*rnlA* were incubated to mid-log phase and added 0.2% L-arabinose, and sequentially total RNA was extracted and analyzed by Northern blotting analysis. *ompA* and *lpp* mRNAs were significantly reduced at 8 min after induction of RnlA in the $\Delta rnhA$ cells, while, in the wild-type cells, these mRNAs were stable even 32 min after induction of RnlA (Fig. 6B).

This reduction of these mRNAs levels could be due to decreased transcription or increased decay. Thus, to confirm that the decrease in these mRNAs steady-state levels in $\Delta rnhA$ cells was caused by an effect on their rates of degradation, I further measured the stability of these mRNAs (Fig. 6C). Cells containing pBAD33-F-*rnlA* were grown to mid-log phase, and 0.2% L-arabinose was added, followed by rifampicin, a transcriptional inhibitor. In the wild-type cells, the half-lives of *ompA* and *lpp* mRNAs were ~ 10 min and > 50 min, respectively, whereas, in the $\Delta rnhA$ cells, they became unstable, ~ 3.3 min and ~ 7.0 min. These results supported the notion that RnlB fails to inhibit RnlA activity in $\Delta rnhA$ cells.

As RNase HI has been reported to be involved in DNA replication and transcription (Itoh and Tomizawa, 1980; Drolet, 2006; Cerritelli and Crouch, 2009; Tadokoro and Kanaya, 2009), I next tested whether the elevation of mRNA decay rates was due to increased expression level of RnlA in $\Delta rnhA$ cells. Wild-type and $\Delta rnhA$ cells containing pBAD33-F-*rnlA* were treated with or without 0.2% L-arabinose, and the cell extracts were prepared and analyzed by Western blotting using anti-Flag antibodies (Fig. 7). Western blotting analysis indicated that F-RnlA in both these cells was not detectable in the absence of L-arabinose, on the other hand, F-RnlA was induced in both these cells in the presence of L-arabinose. Unexpectedly, the amount of F-RnlA was decreased by a third in the $\Delta rnhA$ cells as compared with in the wild-type cell. The reduction seen in the cells may cause to

decrease in the replication of plasmids (Kogoma, 1984). Next, I measured the expression level of *rnlB* mRNA in these cells by RT-PCR because the endogenous level of RnlB was undetectable by our Western blotting analysis with an antibody against RnlB, resulted that there was no significant difference in the expression of *rnlB* mRNA between these cells (Fig. 8). From these results, I suggested that the functional disorder of RnlB in $\Delta rnhA$ cells occurs at the posttranslational level.

Interaction of RNase HI with NRD catalyzes the recruitment of RnlB: These data suggested that the absence of RNase HI led to the disruption of interaction between RnlB and NRD. To test this idea, I examined whether RNase HI promoted the ability for RnlB to interact with NRD (Fig. 9). To do this experiment, I constructed a plasmid expressing both Flag-tagged NRD and His-tagged *rnlB* as operons from an arabinose-inducible promoter (pBAD33-F-NRD-H-*rnlB*). Interaction between RnlB and NRD with increasing amounts of RNase HI was examined by pull-down analysis using extracts prepared from cells harbouring pBAD33-F-NRD-H-*rnlB* with pQE80L'-*rnhA*-F or the empty vector pQE80L'. As expected, these results showed that there was hardly recovery of NRD in the absence of RNase HI, whereas increasing amounts of RNase HI were correlated with increase in the NRD binding efficiency to RnlB. These results indicate that RnlB binds to NRD in an RNase HI-dependent manner. Furthermore, I confirmed that RNase HI was pulled-down with NRD-RnlB complex, suggesting that NRD, RNase HI and RnlB form a complex.

I next investigated whether RnlB could bind to RNase HI in the absence of NRD. RnlB and RNase HI or RNase G as a control were expressed in $\Delta rnlAB \Delta rnhA$ cells and examined for the ability to their interactions, but I could not observe their interactions under our experimental conditions (Fig. 10). Collectively, these results indicated that interaction of NRD with RNase HI led to a recruitment of RnlB.

An high amount of RnlB interacts with DBD independently of RNase HI: To investigate if interaction between RnlB and DBD also depends on RNase HI, I next examined for the interaction by pull-down analysis using cell extracts prepared from $\Delta rnlAB \Delta rnhA$ cells harbouring pBAD33-F-*rnlA*, pBAD33-F-NTD-NRD or pBAD33-F-DBD and pMK19 (Fig. 11). In agreement with the above data, RnlB did not interact with NTD-NRD in the absence of RNase HI, which reinforced the notion that RNase HI was required for RnlB to interact with NRD. On the other hand, interaction between DBD and RnlB remained detectable, indicating that the interaction is independent of RNase HI, and that RnlB may repress DBD activity. However, this result contrasts with the observation that RnlB does not repress DBD activity (Fig. 4B). One possible explanation for these results is that there is a difference of an amount of RnlB expression between these experiments (see discussion). Taken together, these results strongly suggested that RnlB associates with NRD in an RNase HI-dependent manner.

Deletion of *rnhA* leads to abolition of endogenous RnlA activity: The observation that RnlB requires RNase HI to suppress the RnlA activity contrasts with the fact that *rnhA* is not essential gene, because RnlA should be active in $\Delta rnhA$ cells, resulting in a growth defect. Actually, disruption of *rnlB* gene into wild-type cells leads to growth defect (Koga *et al.*, 2011), similarly to many others toxin-antitoxin genes (Yamaguchi and Inouye, 2011). A possible explanation for this contradiction is that an *rnhA* deletion cells also abrogate RnlA activity because RNase HI promotes the activity of endogenous RnlA in absence of RnlB (Naka *et al.*, 2014; chapter 1).

To confirm this hypothesis, I tested for ability to formation of colony when *rnlB* gene was disrupted by insertion of unrelated gene into wild-type and $\Delta rnhA$ cells

(Fig. 12) (Datsenko and Wanner, 2000). Wild-type cells almost entirely do not exhibit cell viability (0%) when *rnlB* gene was disrupted, as previously reported (Koga et al., 2011), whereas, in $\Delta rnhA$ cells, the cell viability was significantly recovered as compared with wild-type cells ($\sim 80\%$), in agreement with notion that RNase HI is required for activating endogenous RnlA (Naka *et al.*, 2014; chapter 1). These results could explain a reason why strains deleting *rnhA* were allowed to isolate despite the involvement of RNase HI in RnlB activity.

Discussion

RnlA consists of three domains: NTD, NRD and DBD. DBD is involved in toxic activity and interaction with Dmd, whereas NTD and NRD had not been functionally defined (Wei *et al.*, 2013). I demonstrated that RNase HI (encoded by *rnhA*) stimulates the activity of RnlA toxin by binding to it (Naka *et al.*, 2014; chapter 1). Therefore, in this chapter, I first examined which domains of RnlA are required for interaction with RNase HI, and also explored the domain of RnlA to which RnlB binds. RNase HI interacted with NRD (Fig. 1). Interestingly, RnlB bound to not only DBD, but also NRD (Fig. 2). This result suggested that the repression mechanism of RnlB is different from that of Dmd that interacts with only DBD (Wei *et al.*, 2013). In agreement with this idea, we have observed that the similarity of amino acid between RnlB and Dmd is low (data not shown). Next, I examined if which interactions of RnlB with NRD or DBD are necessary to suppress the activity of RnlA. As a result, NRD was essential for the activity of RnlB (Fig. 4). Although previous works demonstrated that toxins are inhibited its activity by its cognate antitoxins directly binding to it (Yamaguchi and Inouye, 2011), RnlB did not bind to RnlA directly (data not shown). This result suggests that unknown factor(s) is involved in the interaction of RnlB with RnlA. Therefore, the observation that NRD interacts with both RNase HI and RnlB led us to hypothesize that RNase HI is involved in RnlB activity. Interestingly, over-expression of RnlA in cells lacking RNase HI showed a growth defect, with *E. coli* mRNAs being unstable (Fig. 6). Moreover, the interaction between RnlB and NRD was significantly reduced in cells lacking RNase HI (Fig. 9 and 11) and the ability of RnlB to interact with NRD was correlated with increasing amounts of RNase HI (Fig. 9). From these data, I conclude that RnlB suppresses the activity of RnlA via interaction with NRD in an RNase HI-dependent (Fig. 13), suggesting that RNase HI is an essential component

of the RnlA-RnlB toxin-antitoxin system.

In cells lacking RNase HI, the association of RnlB with NRD was significantly reduced, whereas those with DBD remained stable, suggesting that the interaction of RnlB with NRD depends on RNase HI but those with DBD do not (Fig. 11). In our previous experiments, when RnlB was ectopically expressed from high copy vector, pUC18, which was derived from pBR322, together with ectopic expression of DBD in $\Delta mIAB$ cells, the cell was allowed to normally grow (Wei *et al.*, 2013). These results are in contrast to Fig. 4B, in which over-expression of DBD with RnlB in $\Delta mIAB$ cells showed a growth defect. A simple explanation for these results is that there is a difference of an amount of RnlB expression between these experiments. High expression of RnlB might result in inhibition of the activity of DBD by masking the activation site of it without the help of RNase HI, as well as Dmd (Wei *et al.*, 2013). On the other hand, in a low level of RnlB expressed from endogenous gene or from a low copy vector (pMK33), the interaction of RnlB with NRD may be essential to suppress the activity of RnlA (Fig. 4 and Fig. 5).

From these results, I suggest that when the intracellular concentration of RnlB is low, it can not bind to DBD. Therefore, RnlB may have a higher affinity for NRD-RNase HI complex than for DBD. Subsequently the interaction of RnlB with NRD may lead to induce the conformational change of RnlA and increase the interaction efficiency between RnlB and DBD, resulting in the repression of DBD activity. Consistent with this hypothesis, some cases of RNase activity-regulators binding outside of the catalytic region have been reported. For example, RraA and RraB have been shown to interact with a scaffold domain of RNase E, not with catalytic domain, and remodel the structure to repress the activity (Kaberdin and Lin-Chao, 2009; Mackie, 2013).

E. coli MazE-MazF and RelB-RelE toxin-antitoxin complexes, which are

best-characterized, have been disclosed for the crystal structure (Kamada *et al.*, 2003; Li *et al.*, 2009) and, MazE and RelB antitoxins directly inhibit MazF and RelE toxins through binding to the active site, respectively. Dmd binds to only DBD and may repress the activity of RnlA by a similar mechanism of RelB and MazE antitoxins.

Although the association of RnlA with RnlB *in vivo* was detected (Fig. 2 and Koga *et al.*, 2011), we could not detect their direct interaction *in vitro* (data not shown). This result suggests that unknown factor(s) may help stabilizing their interaction in which RnlB associates with NRD and DBD. The unidentified factor(s) involved in the interaction of NRD with RnlB may correspond to unknown factor(s) involved in that of RnlA with RNase HI (discussion in chapter 1) because of that of RnlB with NRD in an RNase HI-dependent manner (Fig. 8 and 10). Namely, the stable interaction of RNase HI with NRD is also required for the association of NRD with RnlB.

In general, the molecular weight of toxin-antitoxins is relatively small (12.1, 11.2 and 10.2 kDa in MazF, RelE and YoeB toxins, 9.4, 9.1 and 9.3 kDa in MazE, RelB and YefM antitoxins, respectively), whereas that of *rnlA* is larger than that of most toxins, 40 kDa, because RnlA consists of three domains (Wei *et al.*, 2013). My results demonstrate that NRD is a regulatory domain of RnlA activity, in which interacts with RNase HI and RnlB. On the other hand, the function of NTD remains unclear, in which unknown factor(s) may bind and regulate the RnlA activity. That is, RnlA may form a network with several factors including RNase HI and RnlB. The regulation of which may be coordinated under various physiological changes. In previous study, Otsuka *et al.*, (2010) demonstrated that IscR, which is a transcriptional factor involved in control of the expression of gene that are necessary for Fe-S cluster formation, negatively regulates transcription of

rnlA. This result may support that RnlA is a member of the network closely related to cellular physiology under iron-starvation conditions.

RNase HI is required to suppress the activity of RnlA, not only for RnlA activation. To our knowledge, it is the first evidence that an antitoxin requires other factor to repress the activity of its cognate toxin. Further understandings as to why RNase HI is required for the RnlB activity may provide a novel insight into functions of toxin-antitoxin system in normal growth cells.

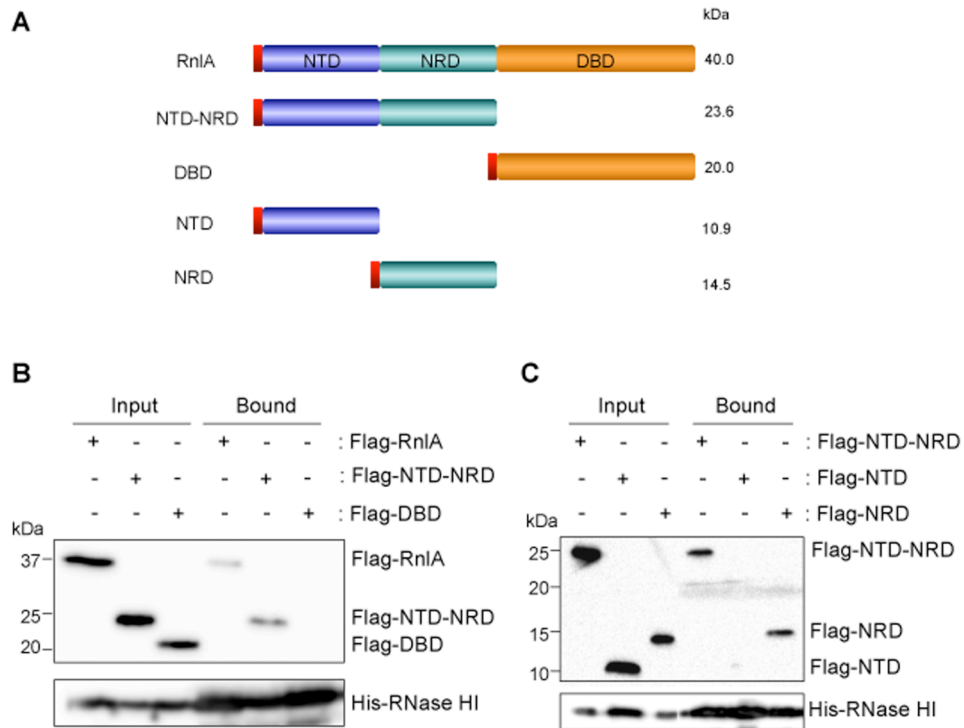


Figure 1. The association of RNase HI with truncated RnlAs.

A. The organization of RnlA is shown at the top. (NTD) N-terminal domain, (NRD) N-repeated domain and (DBD) Dmd-binding domain are indicated. Truncated FLAG-tagged RnlA proteins are shown below. The red boxes represent the FLAG polypeptide. The molecular weight of truncated RnlA proteins are indicated on the right.

B. and C. $\Delta mIAB \Delta mHA$ cells harbouring pQE80L-*mHA* and pBAD33-F-*mIA*, pBAD33-F-NTD-NRD or pBAD33-F-DBD (B), and pBAD33-F-NTD-NRD, pBAD33-F-NTD or pBAD33-F-NRD (C) were grown at 30°C in 100 ml of LB medium containing 50 $\mu\text{g ml}^{-1}$ ampicillin and 30 $\mu\text{g ml}^{-1}$ chloramphenicol. When the OD_{600} reached 0.5, Flag-RnlA proteins and His-RNase HI were co-induced with 0.06 mM IPTG and 0.05% L-arabinose for 45 min. S30 extracts from cells were subjected to pull-down with Ni-NTA agarose beads according to the method described in material and methods. Input and bound fractions were analyzed by Western blot with anti-Flag (upper panel) or anti-His antibody (lower panel).

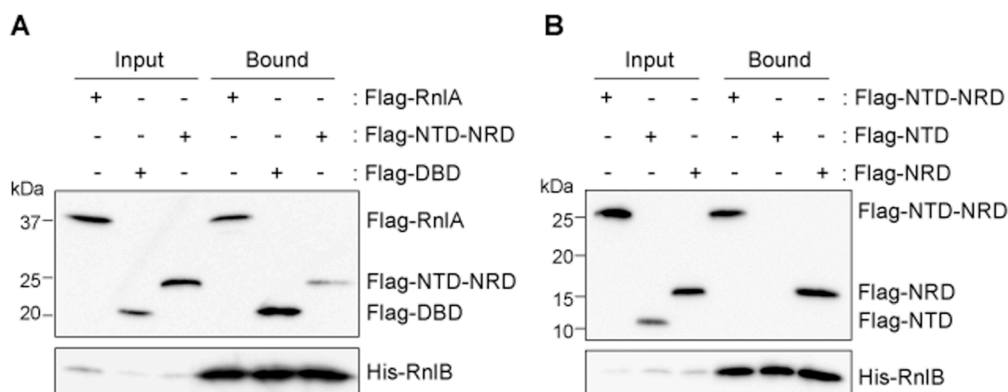


Figure 2. Interaction between RnlB and truncated RnlAs.

$\Delta rnlAB$ cells harbouring pMK19 and pBAD33-F-*rnlA*, pBAD33-F-NTD-NRD or pBAD33-F-DBD (A), and pBAD33-F-NTD-NRD, pBAD33-F-NTD or pBAD33-F-NRD (B) were grown in 100 ml of LB medium containing 50 $\mu\text{g ml}^{-1}$ ampicillin and 30 $\mu\text{g ml}^{-1}$ chloramphenicol to the $\text{OD}_{600} = 0.5$, and then added 0.06 mM IPTG and 0.05% L-arabinose to induce Flag-RnlA variant proteins and His-RnlB. S30 extracts from cells were subjected to pull-down with Ni-NTA agarose beads according to the method described in material and methods. Input and bound fractions were analyzed by Western blot with anti-Flag (upper panel) or anti-His antibody (lower panel).

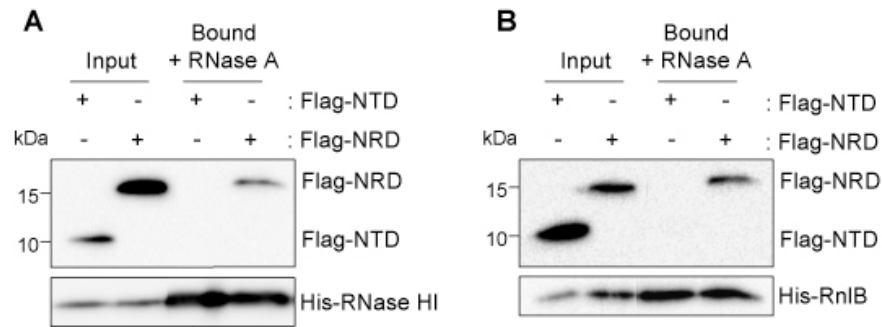


Figure 3. Interaction between NRD and RNase HI or RnlB in the presence of RNase A.

$\Delta mlAB \Delta rnhA$ cells harbouring pQE80L-*rnhA* and pBAD33-F-NTD or pBAD33-F-NRD (A), and $\Delta mlAB$ cells harbouring pMK19 and pBAD33-F-NTD or pBAD33-F-NRD (B) were grown in 100 ml of LB medium containing 50 $\mu\text{g ml}^{-1}$ ampicillin and 30 $\mu\text{g ml}^{-1}$ chloramphenicol to the $\text{OD}_{600} = 0.5$, and then added 0.06 mM IPTG and 0.05% L-arabinose. S30 extracts from cells were treated with 10 $\mu\text{g ml}^{-1}$ RNase A prior to the pull-down analysis. Input and bound fractions were analyzed by Western blot with anti-Flag (upper panel) or anti-His antibody (lower panel).

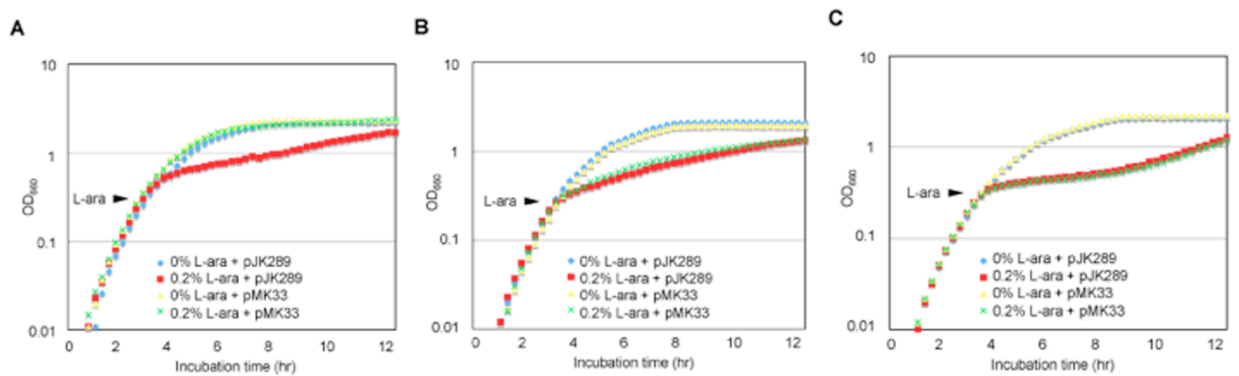


Figure 4. Effects of expression of full-length RnlA, DBD or NTD-DBD with RnlB on cell growth in $\Delta rnlAB$ cells.

$\Delta rnlAB$ cells carrying pBAD24-*rnlA* (A), pBAD24-DBD (B) or pBAD24-NTD-DBD (C) with pMK33 or the empty vector pJK289 were grown in LB medium containing 50 $\mu\text{g ml}^{-1}$ ampicillin and 30 $\mu\text{g ml}^{-1}$ kanamycin to mid-log phase, and added 0 or 0.2% L-arabinose. Cell densities were monitored at 660 nm every 15 min.

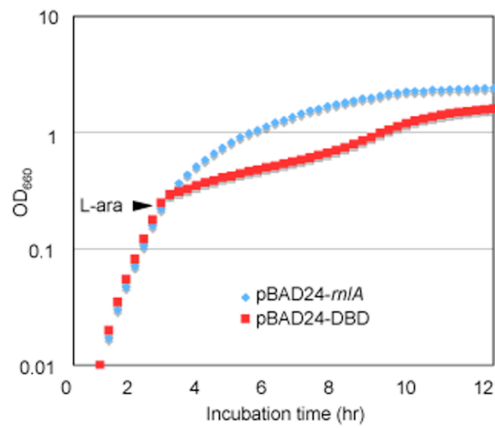


Figure 5. Effects of expression of full-length RnIA or DBD on cell growth in wild-type cells.

Wild-type cells harbouring pBAD24-*rnIA* or pBAD24-DBD were grown in LB medium containing $50 \mu\text{g ml}^{-1}$ ampicillin to mid-log phase and then treated with 0.2% L-arabinose. Cell densities were monitored at 660 nm every 15 min.

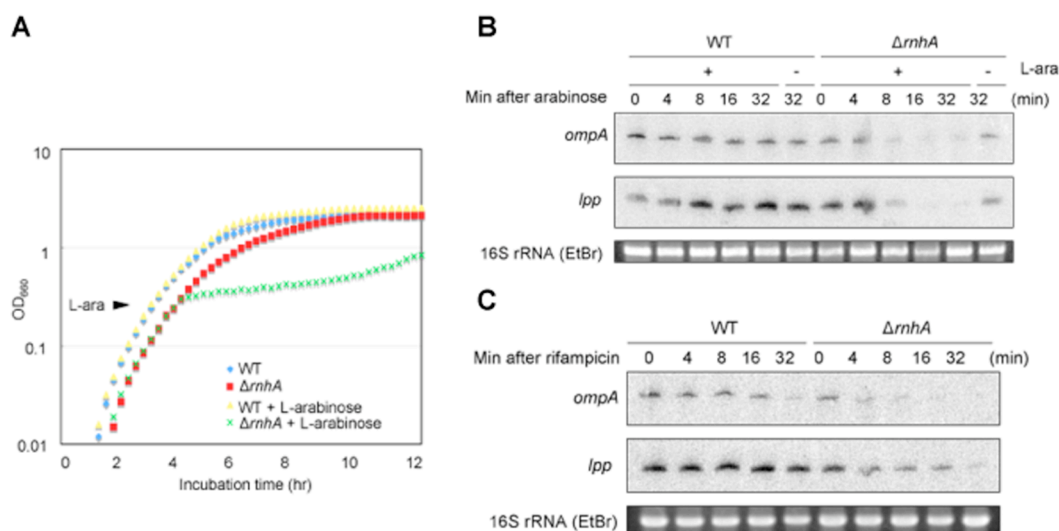


Figure 6. Effects of *rnhA* on the activity of RnlB.

A. wild-type (WT) or $\Delta rnhA$ cells carrying pBAD33-F-*rnlA* were grown in LB medium to mid-log phase and then treated with or without 0.2% L-arabinose. Cell densities were monitored at 660 nm every 15 min.

B. wild-type (WT) or $\Delta rnhA$ cells harbouring pBAD33-F-*rnlA* were grown in LB medium. At the OD₆₀₀ = 0.5, the culture was split and treated with (+) or without (-) 0.2% L-arabinose. Total RNAs were extracted at the indicated times after induction and subjected to Northern blotting analysis using *ompA* or *lpp* probes. Five μ g of total RNAs were applied to 4% polyacrylamide gel containing 7 M urea. 16S rRNA was used as a loading control.

C. wild-type (WT) and $\Delta rnhA$ cells containing pBAD33-F-*rnlA* were treated with 0.2% L-arabinose when OD₆₀₀ reached 0.5, and rifampicin was added at 500 μ g ml⁻¹. Time zero was set 2 min after addition of rifampicin. Total RNAs were extracted at the indicated times after induction and subjected to Northern blotting analysis using *ompA* or *lpp* probes. Five μ g of total RNAs were applied to 4% polyacrylamide gel containing 7 M urea. 16S rRNA was used as a loading control.

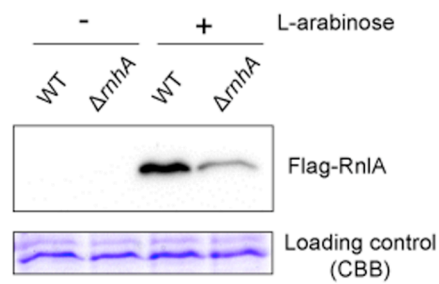


Figure 7. Effect of *mhaA* on expression of RnlA.

Wild-type (WT) or $\Delta mhaA$ cells carrying pBAD33-F-*mIA* were grown in LB medium containing 30 $\mu\text{g ml}^{-1}$ chloramphenicol to mid-log phase and then treated with (+) or without (-) 0.2% L-arabinose. S30 extracts from cells were analyzed by Western blot with anti-Flag (upper panel). Proteins stained by Coomassie Brilliant Blue were used as a loading control (lower panel).

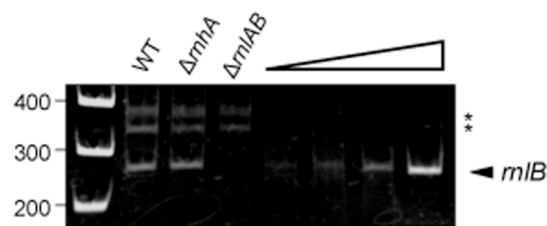


Figure 8. Effect of *rnhA* on *rnlB* transcription.

Wild-type (WT), $\Delta rnhA$ and $\Delta rnlAB$ cells were grown to mid-log growth phase in LB medium and total RNAs were extracted. RT-PCR analyses for *rnlB* mRNA was performed as described in our previous paper (Otsuka *et al.*, 2010). Various amounts of pBSNO carrying the sequence from *rnlA* to *rnlB* were used as template to demonstrate a semi-quantitative profile of PCR conditions; 10 pg, 20 pg, 40 pg and 80 pg. Asterisks indicate non-specific bands.

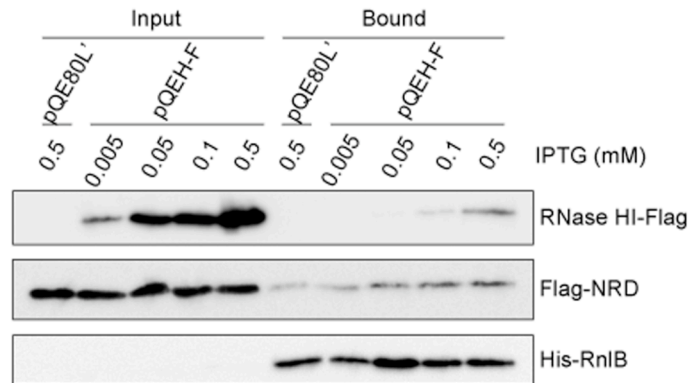


Figure 9. Interaction between NRD and RnlB with increasing amounts of the RNase HI.

Δ *mlAB* Δ *mhA* cells harbouring pBAD33-F-NRD-H-*rnlB* and (pQEH-F) pQE80L'-*rnhA*-F or the empty vector pQE80L' were grown in 100 ml of LB medium containing 50 μ g ml⁻¹ ampicillin and 30 μ g ml⁻¹ chloramphenicol to the OD₆₀₀ = 0.5, and then added IPTG at indicated concentration and 0.1% L-arabinose. S30 extracts from cells were extracted at 30 minutes after induction and subjected to pull-down with Ni-NTA agarose beads according to the method described in materials and methods. Input and bound fractions were analyzed by Western blot with anti-Flag (upper and middle panel) or anti-His antibody (lower panel).

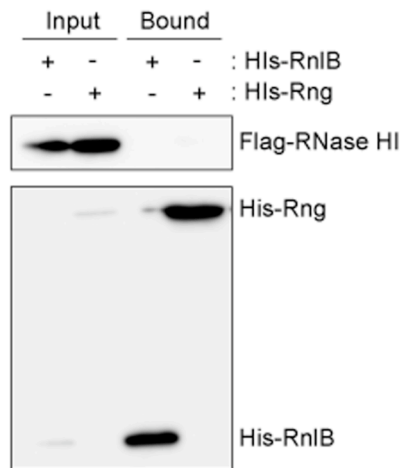


Figure 10. Interaction of RNase HI with RnlB.

$\Delta rnlAB \Delta rnhA$ cells harbouring pBAD33-F-*rnhA* and pMK19 or pHU102 as a control were grown in 100 ml of LB medium containing 50 $\mu\text{g ml}^{-1}$ ampicillin and 30 $\mu\text{g ml}^{-1}$ chloramphenicol to the $\text{OD}_{600} = 0.5$, and then added 0.06 mM IPTG and 0.05% L-arabinose. S30 extracts from cells were subjected to pull-down with Ni-NTA agarose beads according to the method described in materials and methods. Input and bound fractions were analyzed by Western blot with anti-Flag (upper panel) or anti-His antibody (lower panel).

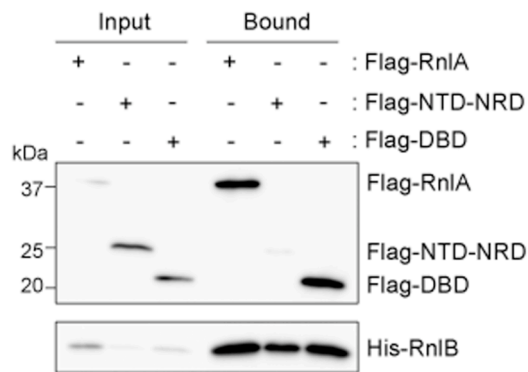


Figure 11. Interaction between RnlB and truncated RnlAs in the absence of *rnhA*. Δ *mlAB* Δ *rnhA* cells harbouring pMK19 and pBAD33-F-*rnlA*, pBAD33-F-NTD-NRD or pBAD33-F-DBD were grown in 100 ml of LB medium containing 50 $\mu\text{g ml}^{-1}$ ampicillin and 30 $\mu\text{g ml}^{-1}$ chloramphenicol to the $\text{OD}_{600} = 0.5$, and then added 0.06 mM IPTG and 0.05% L-arabinose to induce Flag-RnlA variants and His-RnlB. S30 extracts from cells were subjected to pull-down with Ni-NTA agarose beads according to the method described in materials and methods. Input and bound fractions were analyzed by Western blot with anti-Flag (upper panel) or anti-His antibody (lower panel).

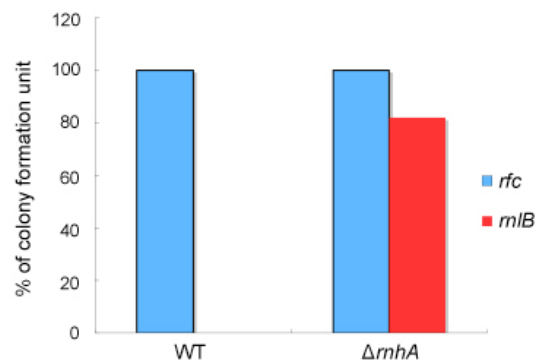


Figure. 12 Effect of deletion of *mnhA* on the activity of endogenous RnlA. Gene disruption was performed according to the method of Datsenko and Wanner. Cell cultures were plated on LB plates containing chloramphenicol, and incubated at 30 °C for 20 hr, to count cfu. Results were normalized to *rfaC*, which encodes ADP-heptose (HepI), as a control (arbitrarily set to 100%).

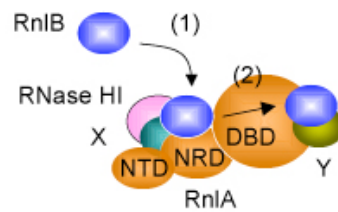


Figure. 13 Working model for RnlB repression of RnlA activity and the role of RNase HI.

Interaction of RNase HI with NRD catalyzes the recruitment of RnlB (1). Subsequently, RnlB interacts with DBD to repress the activity (2). X and Y represent unidentified factors, which is required for the interaction between RnlA and RNase HI (discussion in chapter 1) or RnlB (discussion in chapter 2), respectively.

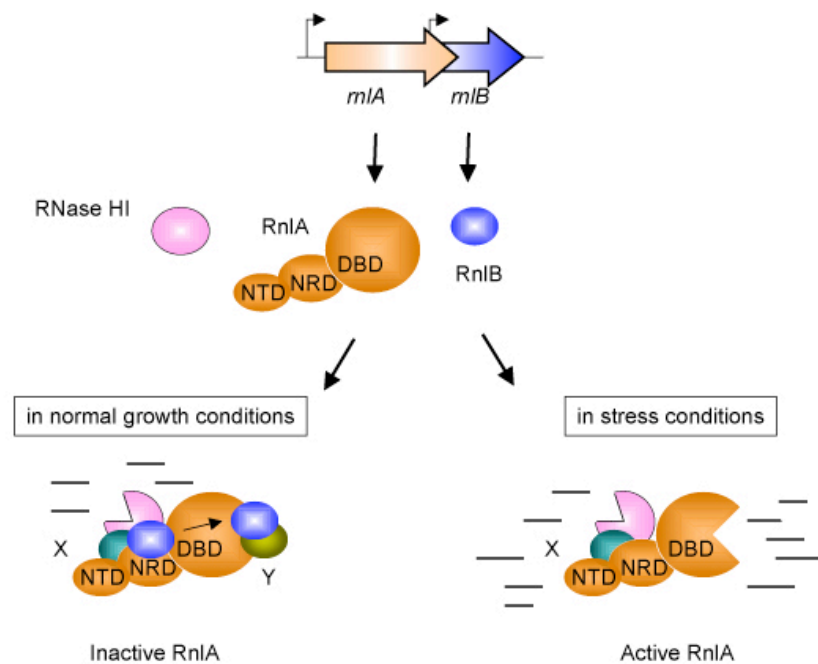
General discussion

My results showed that the activity of RnlA toxin was stimulated by RNase HI through the interaction with NRD (figure below, right), and that RNase HI was required for the repression of RnlA activity by RnlB (figure below, left), which suggests that RNase HI is an essential component of the RnlA-RnlB toxin-antitoxin system.

As toxin-antitoxin systems are generally composed of two components, these results add an intriguing example on the regulatory mechanism of toxin-antitoxin systems. In previous works, several mRNAs and rRNA were stabilized in *mlA* mutant cells, suggesting that RnlA plays a role in normal growth cells (Otsuka and Yonesaki; 2005, Iwamoto *et al.*, 2008). In those cells, the RNase HI interacting with RnlA may function in that decay.

Previous studies have shown that RNase HI is involved in DNA replication and transcription (Tadokoro and Kanaya, 2009). In my study, RNase HI additionally has a role in *mlAB* toxin-antitoxin.

Further investigations of the mechanism of RNase HI activation in RnlA activity and the necessity of RNase HI in RnlB activity will enhance our understanding of the significance of toxin-antitoxin systems.



Working model for a regulatory mechanism of RnlA in normal growth and stress conditions.

The interaction of NRD with RNase HI recruits the binding of RnlB, and subsequently RnlB associates with DBD, resulting in repression of the activity. The RNase HI associating with NRD may be involved in RNA metabolism in normal cells (left panel). On the other hand, under stress conditions, RNase HI activates the RnlA RNase activity (left panel). X and Y represent unidentified factors, which is required for the interaction between RnlA and RNase HI (discussion in chapter 1) or RnlB (discussion in chapter 2), respectively.

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Publications

1. Wei, Y., Gao, Z.Q., Otsuka, Y., Naka, K., Yonesaki, T., Zhang, H., and Dong, Y.H. (2013)

Structure-function studies of *Escherichia coli* RnlA reveal a novel toxin structure involved in bacteriophage resistance.

Mol Microbiol **90**: 956-965.

2. Naka, K., Koga, M., Yonesaki, T., and Otsuka, Y. (2014)

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