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COMMUNICATION

An Unintentional Discovery of a Fluorogenic DNA Probe for Ribonuclease I

Dingran Chang, Thomas Chang, Bruno Salena and Yingfu Li*

Abstract: Ribonuclease I belongs to a class of non-specific endoribonucleases and plays many important roles in a variety of biological and cellular processes. While their ubiquitous nature and high activity contribute to the well-known problem of RNase contamination in experimentation, their abundance in bacteria can potentially be leveraged as a biosensor target. As a result, there is substantial interest in generating a specific and reliable probe for RNase detection for a variety of purposes. To that end, we report on our unintentional discovery of the RNase I probe RFA13-1 isolated through *in vitro* selection with the crude extracellular mixture from *Clostridium difficile* contaminated with *Klebsiella aerogenes* as a selection target. Characterization of RFA13-1 reveals that it exhibits high sensitivity to *Escherichia coli* RNase I with a detection limit of 1.39 pM. Furthermore, RFA13-1 also shows high specificity to RNase I produced only in select bacteria from the *Enterobacteriaceae* family. As a result, this probe offers a simple tool for RNase I detection with potential applications in RNase functional studies, ribonuclease contamination monitoring, and bacterial detection.

Ribonucleases are a class of ubiquitous enzymes that play important roles in many biological processes such as gene expression and regulation[1], genome replication and maintenance[2], host defense[3], stress response[4], and viral strategies of infection[5]. However, while ribonucleases are important for cellular function, the presence of RNase contamination represents a major problem in experiments involving RNA as they may damage precious samples and invalidate the experimental outcomes. Therefore, there is substantial interest in monitoring RNase activity in connection to various applications.

Ribonuclease I (RNase I) is a nonspecific endoribonuclease belonging to the RNase T2 superfamily and is found primarily in the periplasmic space of cells[6]. In the presence of EDTA, ~99% of the total RNase activity in *Escherichia coli* is due to RNase I[7]. Currently, the biological role of RNase I remains incompletely understood. Early reports have proposed a role for the enzyme in scavenging extracellular RNA to supply nutrients for cells[8]. However, the physiological substrate(s) in this process remains unclear. A recent study has found that RNase I can be inhibited by helix 41 of the *E. coli* 16s rRNA which provides a physiological benefit for the host cells in coping with the potential cytotoxicity of this enzyme[9]. In addition, cytoplasmic RNase I was shown to be involved in the degradation of mRNA and rRNA to yield 2',3'-cyclic nucleotide monophosphates (2',3'-cNMPs)[10]. This study has also demonstrated that RNase I and 2', 3'-cNMPs levels play an important role in controlling biofilm formation[10]. However, the biological function of periplasmic RNase I is still unclear. To be noted, RNase I has only been identified in the α- and γ-subdivisions of the Proteobacteria phylum exclusively[11]. This allows this enzyme act as a potential biomarker for bacteria from these subdivisions, including pathogenic bacteria like *Salmonella enterica* and *E. coli*.

A specific and reliable probe for RNase I detection is not only important for biological function studies of this enzyme but can also be employed for nuclease contamination monitoring and bacterial detection. Traditionally, gel electrophoresis and capillary electrophoresis are the predominant means of studying enzyme activity. However, these methods have significant limitations as they often require radioisotope-labeled DNA substrates and can only provide discontinuous enzyme kinetic analysis[12]. Several innovative approaches have been proposed based on the detection of fluorescent or electrochemical signals, colorimetric changes, or surface plasmon resonance[13]. There are also commercial kits for fluorescent monitoring of RNase activities, such as RNaseAlert™ Lab Test Kit. However, none of these methods are designed to specifically detect RNase I.

SELEX (or *in vitro* selection) is an *in vitro*, test-tube selection method that begins with a large pool of randomized DNA or RNA sequences that are subjected to multiple rounds of selective pressure[14]. Only the sequences that are capable of performing a desired function, such as binding to a specific target, are isolated,

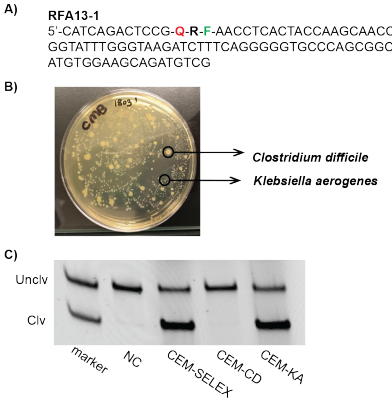

Figure 1. A) The sequence of RFA13-1. Q: Dabcyl-dT; R: adenine-ribonucleotide; F: fluorescein-dT. B) Colonies of *C. difficile* and *K. aerogenes* on an agar plate. C) Responses of RFA13-1 to CEM used in selection(CEM-SELEX), or produced by *C. difficile* (CEM-CD), and *K. aerogenes* (CEM-KA). RFA13-1 was incubated with each CEM for 1 hour, followed by dPAGE analysis. NC: negative control (RFA13-1 in selection buffer).

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amplified and subjected to further rounds of selection. This allows one to isolate the sequences that can perform the expected function with the greatest efficiency. This method has also been adopted in the past to understand substrate preferences of nucleic acid interacting enzymes[15]. In this work, we present an RNA-cleaving fluorescent probe isolated through *in vitro* selection that is capable of identifying RNase I with high sensitivity and specificity.

Our discovery of the RNase I probe was unintentional. In our lab, we are interested in isolating RNA-cleaving fluorescent aptazymes (RFAs) for pathogenic bacteria detection[16]. Recently, we attempted to isolate RFA probes targeting a specific strain of *Clostridium difficile*, an intestinal pathogen related to infectious diarrhoea. The crude extracellular mixture (CEM) prepared from *C. difficile* culture was used as a target for selection. The selection was carried out according to our previously established protocols[17]. See Figure S1 for the DNA sequences used in this work. After 13 rounds of selection, the DNA pool was sent for high-throughput sequencing. Several candidate RFA classes were discovered (see Figure S2). The RFA probe with the highest cleavage activity (Class 1) was named RFA13-1 (Figure 1A). However, upon further investigation, it was discovered that RFA13-1 could only be activated by the CEM prepared from a specific *C. difficile* glycerol stock with no activity observed when testing CEM prepared from another C. *difficile* resource. To uncover why this had occurred, we re-cultured *C. difficile* from the initial glycerol stock for selection in cooked meat broth for 48 hours and then spread the diluted bacterial culture on agar plates. After an overnight culture at 37°C, colonies with different appearance and size were observed suggesting that the glycerol stock used may have been contaminated. By utilizing 16S rRNA sequencing analysis, the larger colonies were confirmed to be *C. difficile*, while the smaller colonies were identified as *Klebsiella aerogenes,* a Gram-negative pathogenic bacterium (Figure 1B). The CEM from *C. difficile* (CEM-CD) and *K. aerogenes* (CEM-KA) were then prepared separately and tested with RFA13-1. As shown in Figure 1C, CEM-KA generated a cleavage signal, while CEM-CD did not. This experiment suggests that RFA13-1 exclusively targets *K. aerogenes* instead of *C. difficile*.

We subsequently investigated the response of RFA13-1 to CEM produced by different bacteria. Eleven bacteria from the *Enterobacteriaceae* family and eleven bacteria from other families were chosen for this experiment. Detailed information surrounding these bacteria are summarized in Table S2. Eight out of eleven bacteria from the *Enterobacteriaceae* family were able to activate RFA13-1, whereas none of the other bacteria were able to induce cleavage (see Figure 2).

To discover the targets in the CEM that activate RFA13-1, we first treated the CEM-KA with proteinase K and found that the protease-treated CEM was no longer able to activate RFA13-1 (Figure 3A). This observation suggests that the responsive target is a protein. We then treated the CEM-KA with two protein-based RNase inhibitors, NxGen RNase inhibitor (RI-NG) and SUPERase RNase inhibitor (RI-SP), to examine whether the observed cleavage was generated by RNases that may exist in the CEM. As shown in Figure 3B, the cleavage activity was fully inhibited by adding RI-SP. In contrast, the addition of RI-NG did not affect the cleavage activity. We compared the targets of RI-NG and RI-SP and found that RI-SP can inhibit RNases A, B, C, I, and T1, whereas RI-NG is only able to inhibit RNase A, B and C. Since RNase T1 is a fungal ribonuclease which is not expressed by bacteria, we then suspected that RNase I is the likely target of RFA13-1. It is also important to note that RI-SP also inhibited the cleavage activity of RFA13-1 towards all tested bacteria (Figure S3).

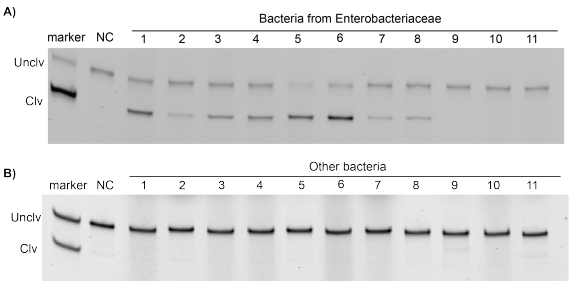

Figure 2. A) Responses of RFA13-1 towards CEM prepared from bacteria from the family of *Enterobacteriaceae*. Lane 1-11: *K. aerogenes, K. pneumoniae, E. aerogenes, E. cloacae, S. enterica, E. coli, S. sonnei, S. flexneri, Y. ruckeri, H. alvei,* and *S. fonticola.* B) Responses of RFA13-1 toward various other bacteria. Lane 1-11: *A. lwoffii, L. pneumophila, O. grignonese, B. diminuta, A. xylosoxidans, F. nucleatum, E. faecium, L. monocytogenes, B. subtilis, C. difficile,* and *A. orientalis.* Incubation time: 1 hour.

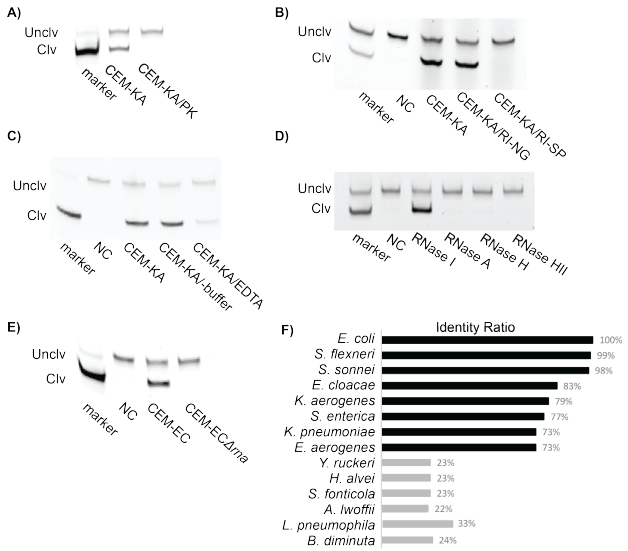

Figure 3. Identification of the activator of RFA13-1. A) Responses of RFA13-1 to protease-treated CEM-KA. PK: proteinase K. B) Responses of RFA13-1 to RNase inhibitor-treated CEM-KA. RI-NG: NxGen RNase inhibitor. RI-SP: SUPERase RNase inhibitor. C) Responses of RFA13-1 to CEM-KA in the absence of reaction buffer or in the presence of reaction buffer with EDTA. D) Responses of RFA13-1 to various RNases. E) Responses of RFA13-1 to CEM-EC and CEM-EC*Δrna*. Incubation time: 1 hour. f) Comparison of the identity of RNase I from different bacteria. The *E. coli*-RNase I protein sequence had served as the subject sequence during Blast.

To test our hypothesis, we examined the metal ion dependence of RFA13-1 as RNase I does not require any divalent cations for activity. We tested the activity of RFA13-1 with CEM-KA in the absence of reaction buffer or in the presence of reaction buffer with EDTA, a chelating agent that can sequester divalent metal ions. As shown in Figure 3C, cleavage activity was still observed in both cases, although an activity reduction was observed in the

presence of EDTA. This reduced activity could have been caused by DNA probe misfolding in the absence of divalent metal ions. These results suggest that metal ions are not necessary for the cleavage reaction of RFA13-1, and supports the idea that the target of RFA13-1 is RNase I.

We then investigated the responses of RFA13-1 to various RNases including RNase I, RNase A, RNase H, and RNase HII (see Figure 3D). Only RNase I induced the cleavage of RFA13-1 indicating the target is RNase I. This test also suggests that RFA13-1 has a strong ribonuclease-specificity towards RNase I.

We also compared the cleavage activity of RFA13-1 in the presence of CEM from wild type *E. coli* strain K12 (CEM-EC) and an *E. coli* RNase I-knockout strain (CEM-EC*Δrna*). As shown in Figure 3E, CEM-EC generated a cleavage signal while CEM-EC*Δrna* did not. This experiment further confirmed that RNase I is the only target of RFA13-1 and no other molecules from the CEM can trigger the cleavage reaction, signifying a strong specificity of the probe.

As mentioned previously, RNase I has only been identified in the α- and γ-subdivisions of the proteobacteria. Unsurprisingly, from the data presented in Figure 2, the bacteria responsive to RFA13-1 are all from these two divisions. However, not every bacterial species from these divisions are able to trigger the cleavage of the probe. To investigate why this occurred, we then compared the RNase I protein sequence from these bacteria using Protein Blast analysis (*E. coli*-RNase I protein sequence was used as subject sequence and other bacteria's RNase I sequences were used as the query sequence). The sequence identity was calculated and summarized in Figure 3F. We found that the RNase I from RFA13-1-responsive bacteria all had a high sequence identity to *E. coli*-RNase I (≥73%), while RNase I from other bacteria had a low sequence identity (≤33%). These results explain the microbial selectivity of RFA13-1 and suggest that RFA13-1 is targeting specific types of RNase I.

The sensitivity of RFA13-1 was also investigated by using different concentrations of *E. coli*-RNase I. As shown in Figure 4A, RFA 13-1 is able to detect 5 pM of RNase I using gel electrophoresis. We have also measured the detection limit of RFA13-1 using fluorescence assay (see Figure S4). Statistical data analysis suggests a detection limit of 1.39 pM for RFA13-1, which is comparable or even lower than that of many commercial RNase detection kits. However, by removing the part of the selected sequence from RFA13-1, the RNA-cleaving fluorogenic substrate (RFS) alone (see Figure 4B) was only able to detect 500 pM of RNase I using gel electrophoresis. The activity of RFA13-1 and RFS against RNase I was also examined by fluorescence measurement (see Figure 4C). These results suggest that the selected DNA sequence in RFA13-1 is able to significantly improve the RNase I mediated cleavage activity.

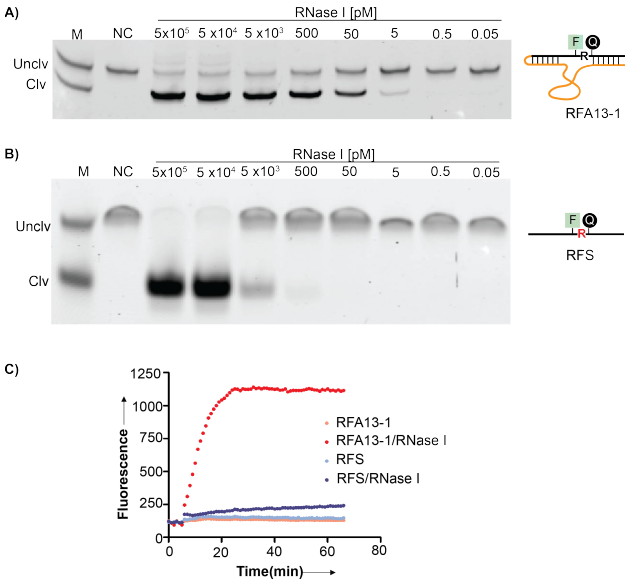

Figure 4. Sensitivity analysis of A) RFA13-1 and B) RFS towards RNase I. RFA13-1 and RFS concentration: 50 nM. Reaction time: 1 hour. C) Signaling profiles of 100 nm RFA13-1 or RFS in the presence or absence of 500 pM RNase I. DNA probe was incubated in selection buffer alone for 5 min, followed by the addition of RNase I and further incubation for 60 min.

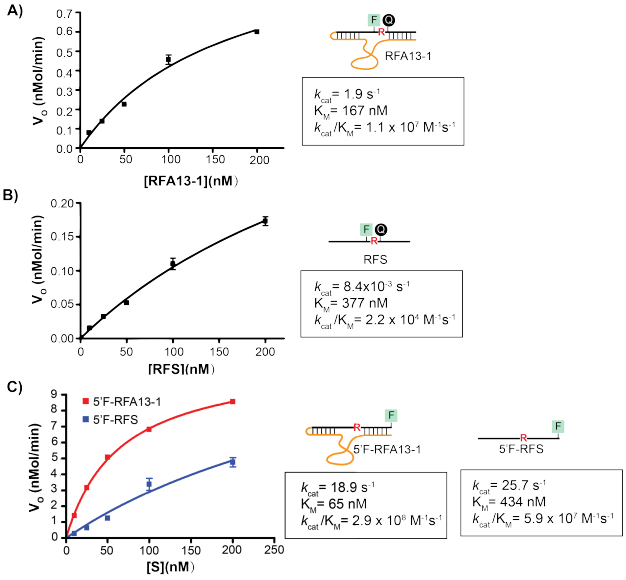

Figure 5. Kinetics analysis of A) RFA13-1, B) RFS, C) 5'F-RFA13-1 and 5'F-RFS towards RNase I. The RNase I concentration is 1 nM for RFA13-1 and 10 pM for RFS, 5'F-RFA13-1 and 5'F-RFS.

Kinetics analysis was then performed to investigate why the selected sequence in RFA13-1 could enhance the cleavage activity of RNase I. As shown in Figure 5A and B, a comparison of k_{cat} indicated that RNase I had a ~230-fold greater catalytic turnover towards RFA13-1 than RFS. The K_M value for RFA13-1 was less than half of that for RFS, suggesting a stronger affinity between RFA13-1 and RNase I. As a result, compared to RFS, RFA13-1 serves as a significantly better substrate for RNase I in the cleavage reaction. We also performed kinetics analysis for the probes (5'F-RFA13-1 and 5'F-RFS) which do not have a fluorophore and a quencher beside the cleavage site (see Figure 5C). Compared with the original probes, the catalytic turnover increased by ~10 times for 5'F-RFA13-1 and ~3000 times for 5'F-RFS. The binding affinity against RNase I was also improved for 5'F-RFA13-1, but not for 5'F-RFS. These results suggest that the fluorophore- and quencher- modifications have a role in protecting the ribonucleotide from RNase cleavage as they could significantly decrease the catalytic turnover of RNase I. However,

the selected DNA is able to recover the turnover and also improve the binding affinity between RNase I and the probe.

In summary, we performed an *in vitro* selection experiment for generating *C. difficile* probes, but unintentionally isolated a probe, RFA13-1, targeting RNase I, due to the contamination of the *C. difficile* glycerol stock used for selection. However, despite the unexpected result, RFA13-1 may nonetheless serve as a useful probe as it exhibited high sensitivity being able to detect as low as 1.39 pM of RNase I. RFA13-1 also showed high specificity as it only responds to specific types of RNase I expressed in *Enterobacteriaceae* bacteria. Although RFA13-1 is unable to perform species-specific bacterial identification, it can serve as a general probe to monitor the existence of potentially pathogenic bacteria including *E. coli, S. enterica* and *S. sonnei.* In addition, we believe this RNase I-specific probe can also be employed as a sensitive tool in biological function studies of RNase I.

There are two potential mechanisms responsible for the cleavage reaction of RFA13-1. The first one is that RFA13-1 functions as an aptazyme and the binding of RNase I induces the formation of its active structure triggering the self-cleavage reaction. The second mechanism is that RFA13-1 functions purely as the substrate for RNase I. The EDTA test indicates that RNase I is responsible for some of the observed cleavage as RNase I doesn't require divalent metal ions. However, the presence of EDTA also caused a significantly reduced cleavage activity, suggesting that RFA13-1 may undergo target-binding activation for self-cleavage as well. Further studies are still required to fully decipher the cleavage reaction mechanism of RFA13-1.

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Keywords: • Ribonuclease I • In vitro selection • RNA cleavage • DNA probe • Bacterial pathogen

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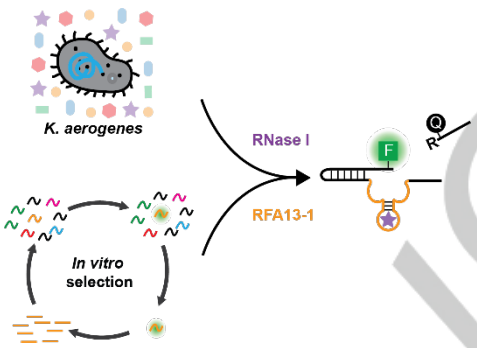
An Unintentional Discovery of a Fluorogenic DNA Probe for Ribonuclease I

A fluorogenic DNA probe, RFA13-1, was isolated through *in vitro* selection against crude extracellular mixture prepared from *Klebsiella aerogenes*. The molecular target of RFA 13-1 has been identified as Ribonuclease I.



*D. Chang, T. Chang, B. Salena and Y. Li***

Page No. – Page No.