Video Article

Detection of Bacteria Using Fluorogenic DNAzymes

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Abstract

Outbreaks linked to food-borne and hospital-acquired pathogens account for millions of deaths and hospitalizations as well as colossal economic losses each and every year. Prevention of such outbreaks and minimization of the impact of an ongoing epidemic place an ever-increasing demand for analytical methods that can accurately identify culprit pathogens at the earliest stage. Although there is a large array of effective methods for pathogen detection, none of them can satisfy all the following five premier requirements embodied for an ideal detection method: high specificity (detecting only the bacterium of interest), high sensitivity (capable of detecting as low as a single live bacterial cell), short time-to-results (minutes to hours), great operational simplicity (no need for lengthy sampling procedures and the use of specialized equipment), and cost effectiveness. For example, classical microbiological methods are highly specific but require a long time (days to weeks) to acquire a definitive result.[1] PCR- and antibody-based techniques offer shorter waiting times (hours to days), but they require the use of expensive reagents and/or sophisticated equipment.[2-4] Consequently, there is still a great demand for scientific research towards developing innovative bacterial detection methods that offer improved characteristics in one or more of the aforementioned requirements. Our laboratory is interested in examining the potential of DNAzymes as a novel class of molecular probes for biosensing applications including bacterial detection.[5]

DNAzymes (also known as deoxyribozymes or DNA enzymes) are man-made single-stranded DNA molecules with the capability of catalyzing chemical reactions.[6-8] These molecules can be isolated from a vast random-sequence DNA pool (which contains as many as 10^{16} individual sequences) by a process known as "*in vitro* selection" or "SELEX" (systematic evolution of ligands by exponential enrichment).[9-16] These special DNA molecules have been widely examined in recent years as molecular tools for biosensing applications.[6-8]

Our laboratory has established *in vitro* selection procedures for isolating RNA-cleaving fluorescent DNAzymes (RFDs; **Fig. 1**) and investigated the use of RFDs as analytical tools.[17-29] RFDs catalyze the cleavage of a DNA-RNA chimeric substrate at a single ribonucleotide junction (R) that is flanked by a fluorophore (F) and a quencher (Q). The close proximity of F and Q renders the uncleaved substrate minimal fluorescence. However, the cleavage event leads to the separation of F and Q, which is accompanied by significant increase of fluorescence intensity.

More recently, we developed a method of isolating RFDs for bacterial detection.[5] These special RFDs were isolated to "light up" in the presence of the crude extracellular mixture (CEM) left behind by a specific type of bacteria in their environment or in the media they are cultured (**Fig. 1**). The use of crude mixture circumvents the tedious process of purifying and identifying a suitable target from the microbe of interest for biosensor development (which could take months or years to complete). The use of extracellular targets means the assaying procedure is simple because there is no need for steps to obtain intracellular targets.

Using the above approach, we derived an RFD that cleaves its substrate (FS1; **Fig. 2A**) only in the presence of the CEM produced by *E. coli* (CEM-EC).[5] This *E. coli*-sensing RFD, named RFD-EC1 (**Fig. 2A**), was found to be strictly responsive to CEM-EC but nonresponsive to CEMs from a host of other bacteria (**Fig. 3**).

Here we present the key experimental procedures for setting up *E. coli* detection assays using RFD-EC1 and representative results.

Video Link

The video component of this article can be found at https://www.jove.com/video/3961/

Protocol

1. Preparation of Chemical Solutions

1. 0.5 M ethylene diaminetetraacetic acid (EDTA): In a 2 liter (L) plastic beaker, weigh 186.1 g EDTA (EM Science) and add 800 milliliter (mL) of autoclaved deionized-distilled water (ddH_2O). Adjust the pH to 8.0 using NaOH pellets (EM Science). Make the final volume to 1 L using ddH_2O. Transfer the solution to glass bottles, autoclave and store at 4 °C.

2. 10× Tris-borate EDTA solution (10× TBE; 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 7.5): Weigh 432 g of Tris-base (BioShop Canada) and 220 g of boric acid (BioShop Canada), and add each to a 4 L plastic beaker. Measure 80 mL of 0.5 M EDTA (pH 8.0) and add to the beaker. Add ddH_2O to a final volume of 4 L. Thoroughly mix the solution with a magnetic stir bar until components are completely dissolved. Transfer the solution to glass bottles, autoclave and store at 4 °C.
3. 10% denaturing polyacrylamide gel stock: To a 4 L plastic beaker, add 1681.7 g of urea (BioShop Canada), 400 mL of 10× TBE, 1 L of 40% acrylamide/bisacrylamide (29:1) solution (BioShop Canada). Adjust the volume to 4 L with ddH_2O. Dissolve the urea with stirring. Transfer the solution to 1 L amber glass bottles and store at 4 °C. (Caution! Acrylamide should be handled with gloves, mask, goggles and lab coat because it is a neurotoxin prior to polymerization).
4. 2× gel loading buffer (2× GLB): To a 200 mL glass beaker, add 44 g of urea, 8 g of sucrose (BioShop Canada), 10 mg of bromophenol blue (BioShop Canada), 10 mg of xylenecyanol FF (Sigma-Aldrich), 400 µL of 10% sodium dodecyl sulfate (SDS; BioShop Canada), and 4 mL of 10× TBE. Adjust the final volume to 40 mL with ddH_2O and dissolve the solids with mild heating (50 °C) and stirring with a magnetic bar. Transfer 1 mL aliquot into 1.5 mL microcentrifuge tubes and store at 4 °C. Based on our experience, it is necessary to heat 2× GLB briefly at 90 °C prior to use because 2× GLB solidifies under the storage condition.
5. 1 M Tris-HCl (pH 7.5): Weigh 12.1 g of Tris-base in a 200 mL glass beaker. Add 60 mL of ddH_2O and dissolve the solids by stirring with a magnetic stirrer. Adjust the pH to 7.5 using 1 M HCl (Sigma-Aldrich). Make up the volume to 100 mL with ddH_2O and transfer to a glass bottle and autoclave. Store at 4 °C.
6. 5 M NaCl: In glass beaker weigh 58.4 g of NaCl (BioShop Canada) and dissolve with 150 mL of ddH_2O. Adjust the volume to 200 mL with ddH_2O. Transfer the solution to a glass bottle, autoclave and store at 4 °C.
7. DNA elution buffer: In a glass beaker, mix 2 mL of 1 M Tris-HCl (pH 7.5), 8 mL of 5 M NaCl and 0.4 mL of 0.5 M EDTA (pH 8.0). Adjust the volume to 200 mL with ddH_2O. Autoclave and store at 4 °C.
8. 2× reaction buffer (2× RB; 100 mM HEPES, 300 mM NaCl, 30 mM $MgCl_2$): To a 50 mL Falcon tube (BD Falcon), add 1.2 g of HEPES (BioShop Canada), 0.88 g of NaCl and 0.30 g of $MgCl_2$•$6H_2O$ (EMD Chemicals) and 30 mL of ddH_2O. Mix by shaking mildly until components are completely dissolved. Adjust the pH to 7.5 by adding 10 N NaOH solution and adjust the final volume to 50 mL with ddH_2O. Purify the solution using a syringe-driven filter unit (0.22 µm, Millipore) and store at 4 °C.
9. Luria Bertani (LB) Broth: In a beaker, weigh 20.0 g of LB powder (Sigma-Aldrich) followed by the addition of 1 L of ddH_2O. Thoroughly mix the solution with a magnetic stir bar, transfer the solution to a conical flask. Autoclave the solution and store at room temperature.
10. 1.5% LB agar: Weigh 1.5 g of agar (BioShop Canada) in a 250 mL flask and add 100 mL of liquid LB. Autoclave the mixture and store at room temperature.
11. Agar plating: Melt the LB agar in a microwave and cool the solution to ~50 °C. Pour the solution into Petri dishes (Fisher Scientific) under a flame. In our experience, 100 mL of LB agar can yield 5-6 plates.

2. Construction of RFD-EC1 and RFSS1 by Template Mediated Enzymatic Ligation

RFD-EC1 (**Fig. 2A**) is the featured DNAzyme. It consists of the catalytic sequence EC1 and the substrate sequence FS1 (indicated by black and green lines in **Fig. 2A**). RFSS1 (**Fig. 2A**) is a scrambled version of RFD-EC1 where the catalytic sequence EC1 is partially shuffled into SS1 but the FS1 portion remains unchanged. RFD-EC1 and RFSS1 were made by template mediated enzymatic ligation of the oligonucleotide FS1 with oligonucleotide EC1 or SS1 in the presence of LT1 as the ligation template (see the inserted box in **Fig. 2A**). The procedure for conducting the ligation reaction is provided below. FS1 was obtained from Keck Oligo Synthesis Facilities at Yale University, deprotected and purified by gel electrophoresis following a previously established protocol.[17-24] EC1, SS1 and LT1 were purchased from Integrated DNA Technologies and purified by gel electrophoresis.

1. Prepare a 100 µM stock solution of FS1, EC1, SS1 and LT1 using ddH_2O. Store them at -20 °C until use.
2. Transfer 5 µL of the FS1 stock solution to two 1.5 µL microcentrifuge tubes marked as Tube 1 and Tube 2. To each tube, add 38.5 µL of ddH_2O and then 5 µL of 10× T4 polynucleotide kinase (PNK) reaction buffer A (MBI Fermentas), which contains 500 mM Tris-HCl (pH 7.6, 25 °C), 100 mM $MgCl_2$, 50 mM DTT, 1.0 mM spermidine. Mix each solution by pipetting (pipette the solution up and down a few times).
3. Add 1 µL of ATP (100 mM; MBI Fermentas) and mix by pipetting.
4. Add 0.5 µL of T4 polynucleotide kinase (PNK; 10 units/µL; MBI Fermentas) and mix by pipetting. Incubate the reaction mixtures at 37 °C for 30 min. Make sure to cover the tubes with aluminum foil to minimize photobleaching of the fluorophore.
5. Quench the reaction by heating at 90 °C for 5 min. Cool the reaction mixtures at room temperature for 10 min.
6. Add 5 µL of the EC1 and SS1 stock solution to Tube 1 and Tube 2, respectively.
7. Add 5 µL of the LT1 stock solution to each tube, mix by pipetting. Heat the reaction mixtures at 90 °C for 1 min and cool to room temperature for 10 min.
8. Add 118 µL of ddH_2O and then 20 µL of 10× T4 DNA ligase buffer (MBI Fermentas), which contains 400 mM Tris-HCl (pH 7.8 at 25 °C), 100 mM $MgCl_2$, 100 mM DTT, and 5 mM ATP. Mix the solution by pipetting.
9. Add 2 µL of T4 DNA ligase (5 units/µL; MBI Fermentas) and mix by pipetting. Incubate the reaction mixtures at room temperature for 1 h.
10. Add 20 µL of 3 M NaOAc (pH 7.0) to each tube, vortex and spin down. Add 500 µL of cold 100% ethanol to each tube, mix the solution by vortexing and place the tubes in -20 °C freezer for 30 min.
11. Centrifuge the mixtures at 11,000 g for 20 min at 4 °C in a refrigerated centrifuge (Allegra X22-R, Beckman Coulter) and carefully remove the supernatant by pipetting.
12. Dry the DNA pellet using a DNA concentrator (Savant DNA Speedvac, Thermo Scientific) for 10 min.
13. Resuspend DNA pellets in 30 µL of 1× gel loading buffer (GLB), briefly vortex and spin down with a benchtop centrifuge (Minicentrifuge, VWR Scientific). The ligated DNA samples are ready for loading onto a 10% dPAGE gel.

3. Preparation of 10% dPAGE Gel

The following steps briefly describe the apparatus of gel electrophoresis and its set-up. For greater details about the apparatus, settings and handling, please refer to our previously published protocols.[30,31]

1. Wash and dry two glass plates, two 0.75 mm spacers and a 16-well comb. Assemble glass plates and spacers with clips and lay horizontally on a flat surface with notched glass plate facing up.
2. Transfer 40 mL of 10% dPAGE mix into a 150 mL beaker. Add 40 µL of tetramethylethylenediamine (TEMED; Bioshop Canada), 400 µL of 10% APS, and mix by swirling with a pipettor.
3. Pour the mixture carefully between the plates and immediately insert the comb. Allow the mixture to polymerize for 10 to 20 min. Polymerization can be confirmed by checking the residual gel mix left in the beaker.
4. Once polymerized, remove the comb gently and rinse the wells with ddH_2O to remove residual gel solution in the wells.
5. Mount the plates onto the gel electrophoresis apparatus with the unnotched rectangular plate facing out and place a metal plate behind the notched plate. The use of the metal plate helps to prevent overheating which can crack the glass plates.
6. Add 1× TBE to the upper and lower chambers of the apparatus. Check to ensure that the wells are filled with the buffer and the lower edge of the gel is immersed in the buffer.
7. Apply a 40 mA (or 750 V) current and pre-run for 10 to 15 min.

4. Purification of Ligated RFD-EC1 and RFSS1 by 10% dPAGE Gel

1. Following the step of 3.7, rinse wells with 1× TBE using a syringe and a needle.
2. Load the ligation mixture of RFD-EC1 and that of RFSS1 (from 2.13) into 2 wells (one for each) using a pipettor and gel loading tips (Diamed). Apply a 40 mA (750 V) current until the bottom dye (bromophenol blue) is approximately 5 cm above the bottom edge of the plates.
3. Remove glass plates from the gel running apparatus, lie down on a flat bench top and carefully remove the spacers.
4. Carefully remove the top glass plates from the gel and wrap the gel with plastic wrap (try to avoid wrinkles and folding of the wraps on the gel).
5. The ligated products can be visualized either by UV shadowing (260 nm) or by transillumination (360 nm), which will produce a visible DNA band a few centimeters above EC1 or SS1 (100 pmol of EC1 or SS1 can be used as a marker and loaded into a well in the step of 4.2). Mark the desired DNA bands with a marker.
6. Excise the DNA band with sterile razor blades, cut the gel into small pieces and transfer into a fresh 1.5 mL microcentrifuge tube.
7. Crush the gel pieces within the microcentrifuge tube using a sterile pipette tip (200 µL tip size).
8. Add 500 µL DNA elution buffer to each tube and cover them with aluminum foil to protect the fluorophores from light. Vortex the samples for 10 min.
9. Centrifuge the samples at 11,000 g for 4 min at 4 °C in a refrigerated centrifuge (Allegra X22-R, Beckman Coulter) and carefully transfer 350 µL of the supernatant to a fresh 1.5 mL microcentrifuge tube (if needed, a second elution can be done with 350 µL of fresh elution buffer for another 10 min).
10. Add 35 µL (0.1× of the sample volume) of 3 M sodium acetate (NaOAc, pH 7.0) to each tube, mix by vortexing and spin down. Add 900 uL of cold 100% ethanol to each tube. Mix each sample by hand-shaking the tube for a few seconds. Place the tubes at -20 °C for at least 1 h.
11. Centrifuge the samples at 11,000 g for 20 min at 4 °C in a refrigerated centrifuge and carefully remove the supernatant by pipetting.
12. Add 100 µL of cold 70% ethanol and use it to gently rinse the entire inner wall of the tube. Re-centrifuge at 11,000 g for 7 min at 4 °C. Remove the supernatant and dry the pellet using the DNA concentrator for 10 min.
13. Dissolve the DNA pellets in 100 µL of ddH_2O and vortex. Determine the DNA concentration based on the UV absorbance at 260 nm. Store samples at -20 °C until use.

5. Preparation of Bacteria

1. The target bacterial strain *E. coli* K12 (MG1655) and relevant control strains are firstly plated onto LB agar plates from glycerol stocks. Under a flame or within a biological safety cabinet, touch the bacterial glycerol stock with a sterile pipette tip and gently streak onto the plate surface to avoid damaging the LB agar.
2. Invert streaked plates and incubate at 37 °C for 14 h. After incubation, seal the entire perimeter of the plates with Parafilm (Pechiney Plastic Packaging) and store at 4 °C. These plates can be stored for a maximum of 4 weeks.

6. Preparation of Crude Extracellular Mixtures (CEMs)

1. Dispense 2 mL of LB into sterile 14 mL culture tubes (BD Falcon) using a pipette gun (Corning).
2. Using a sterile pipette tip, pick a single colony from an agar plate prepared in the step of 5.2 and insert it into a culture tube. Place tubes in an incubator (New Brunswick Scientific) set at 37 °C, and shake at 250 rpm for 14 h.
3. 1% re-inoculation culture: Dispense 2 mL of fresh LB into 14 mL culture tubes and spike with 20 µL of bacterial cultures prepared in step 6.2. Incubate the tubes at 37 °C with shaking at 250 rpm until each bacterial solution reaches an OD_{600} (optical density measured at 600 nm) of approximately 1. To measure OD_{600}, transfer 1 mL of each culture to a disposable cuvette and measure absorbance at 600 nm with a UV spectrophotometer (Genesys UV 10, Thermo Scientific).
4. Transfer 1 mL of each culture to a new 1.5 mL microcentrifuge tube and pellet cells by centrifugation at 11,000 g for 5 min at room temperature.
5. Transfer the clear supernatant to a fresh 1.5 mL microcentrifuge tube and store at -20 °C if not used immediately.

7. Detection using Fluorescence Spectrophotometer

1. Turn on fluorescence spectrophotometer (Cary Eclipse, Varian Inc) and set up data acquisition parameters with excitation at 488 nm and emission at 520 nm. Readings can be taken every minute for 1 h.
2. Wash 3 quartz crystal cuvettes (Varian Cary) with ddH_2O, followed by 100% ethanol. Dry the cuvettes by flashing nitrogen gas. Label cuvettes C1 (control 1), C2 (control 2) and T (test).

3. Transfer 24 µL of ddH_2O to C1 and 24 µL of CEM-EC to C2 and T. Add 25 µL of 2× RB to each cuvette and place them in the fluorescence spectrophotometer. Start collecting fluorescence data for the first 5 min.
4. Add 1 µL of RFSS1 (from a 5 µM stock solution) to C2 and 1 µL of RFD-EC1 (from a 5 µM stock solution) to T and C1. Mix each solution by pipetting. This must be carefully initiated so that the fluorescence readings are not interrupted. Allow the reaction to continue for the rest of the 1 h acquisition time.
5. Save the data in Excel file format, transfer the data to a personal computer and process data to create a graphical image.

8. Detection by Gel Electrophoresis

The same reaction mixtures prepared in step 7.4 can be used for analysis by gel electrophoresis; alternatively new reactions can be prepared similarly and incubated in 1.5 mL microcentrifuge tubes. In either case:

1. Quench reactions (after 1 h) by adding 5 µL of 3 M NaOAc and 125 µL of 100% ethanol. Mix each solution by vortexing and place the tubes in -20 °C freezer for 1 h.
2. Centrifuge the reaction mixtures at 11,000 g for 20 min at 4 °C and carefully remove the supernatant by pipetting.
3. Dry the pellets using the DNA concentrator for 10 min.
4. Resuspend pellets in 20 µL of 1× GLB by briefly vortexing. Spin down tubes very briefly with a benchtop centrifuge (Minicentrifuge, VWR Scientific). These samples are ready for loading into a dPAGE gel.
5. Prepare a 10% dPAGE gel as described in 3.1-3.7. Load the reaction samples (from step 8.4) into the wells using a pipettor and gel loading tips (Diamed). Apply a 40 mA (750 V) current until the bottom dye (bromophenol blue) is approximately 5 cm above the bottom edge of the plates.
6. Remove glass plates and wash thoroughly with tap water to remove any gel pieces. Wipe plates with a kimwipe (Kimberly-Clark Professional).
7. Scan the gel plate for fluorescence using a Typhoon Scanner (Typhoon 9200, Variable mode, GE Healthcare). Analyze the data using ImageQuant software (Molecular Dynamics).

9. Detection Specificity

1. For testing bacterial specificity, the same procedures described above for culturing *E. coli*, preparing its CEM and conducting a cleavage reaction assay can be performed for a number of different bacterial strains such as *B. subtilis, P. peli, Y. ruckeri, L. planturum, P. acidilactici* (shown in **Fig. 3A**).

10. Single Cell Detection

Prepare a 1 mL *E. coli* glycerol stock of 2 CFU/mL (CFU: colony-forming unit) by serial dilution and confirm CFU concentration by plating.[5] This stock should contain 0.2 CFU/100 µL. Store at -80 °C until use.

1. Prepare 10 culture tubes containing 2 mL of LB.
2. Inoculate each culture with 100 µL of 2 CFU/mL glycerol stock and incubate at 37 °C with shaking at 250 rpm. The use of the entire glycerol stock (1 mL) should yield 10 cultures.
3. Harvest 300 µL from each inoculated culture tube at the following time points: 4, 8, 12, 16 and 24 h. Leave the remaining culture to grow for 24 h.
4. Measure the OD_{600} and precipitate the cells by centrifugation at 11,000 g for 5 min.
5. Transfer the CEMs to fresh 1.5 mL microcentrifuge tubes and store at -20 °C until use.
   Note: Since there may be no detectable OD_{600} for samples harvested at time points 4, 8 and 12, allow the remaining culture to grow for 24 h in order to identify cultures containing bacteria (determined by turbidity and OD measurement). Only one or two of the 10 cultures may contain *E. coli* after inoculation and the remaining tubes will not contain any cells.
6. Use the CEMs recovered from positive cultures (stored at -20 °C) at the designated timepoints to prepare cleavage reactions with RFD-EC1, and then analyze the reaction mixtures using dPAGE gel electrophoresis as described in Section 8.

11. Concept and Representative Results

The concept of exploiting an RNA-cleaving fluorescent DNAzyme (RFD) for bacterial detection is illustrated in **Fig. 1**. The RFD cleaves a chimeric DNA/RNA substrate at a lone RNA linkage (blue R) flanked by two nucleotides labeled with a fluorophore (F) and a quencher (Q), respectively. As a bacterium of interest (such as *E. coli*) grows in media, it will leave behind a crude extracellular mixture (CEM). This CEM as a whole is then used in a *in vitro* selection experiment to obtain an RFD that is responsive specifically to the CEM; presumably the RFD interacts with a specific molecule (purple star) in the CEM that is a signature molecule of the bacterium. When the CEM is added to the reaction solution containing the RFD, it triggers the RNA-cleaving activity of the RFD. The cleavage event separates F from Q, resulting in a fluorescent signal that can be detected either using a fluorimeter or by gel electrophoresis.

The experimental validation of the above concept was done with the CEM from *E. coli* (CEM-EC). We obtained 3 RFD molecules via *in vitro* selection, and the most efficient one was designated as RFD-EC1 (**Fig. 2A**).[5] We tested the cleavage activity of RFD-EC1 (along with a mutant sequence named RFSS1) in response to CEM-EC. Both RFD-EC1 and RFSS1 were prepared by enzymatic ligation of the DNAzyme portions to the substrate FS1 (all sequences are shown in **Fig. 2A**). In the fluorescence measurement experiment (**Fig. 2B**), CEM-EC was incubated alone for 5 min, followed by the addition of RFD-EC1 or RFSS1, and by further incubation for 55 more min. The fluorescence intensity of the solution was continuously read every minute and the data was used to calculate relative fluorescence (RF; calculated as the ratio of the fluorescence intensity at time t vs. the fluorescence intensity at time 0). The RF values vs. the time of incubation are plotted as **Fig. 2B**. It was found that RFD-

EC1 produced a high level of fluorescence signal upon the addition of CEM-EC; in stark contrast, RFSS1 did not produce a strong fluorescence signal. Thus, the fluorescence-producing function of RFD-EC1 upon contacting CEM-EC is sequence-specific.

In order to verify that observed fluorescence increases are due to the cleavage of the RNA linkage, we analyzed reaction mixtures by dPAGE. Cleavage of RFD-EC1 is expected to generate two DNA fragments, a 5' fragment retaining the fluorophore and a 3' fragment retaining the quencher. Only uncleaved RFD-EC1 (unclv) and the 5′ fragment (clv) could be detected by fluorescence imaging. The dPAGE result shown in **Fig. 2C** reveals that the reaction mixture of RFD-EC1 and CEM-EC indeed produced the expected cleavage product, while the RFSS1/CEM-EC mixture did not.

The specificity of RFD-EC1 was examined using CEMs collected from several other gram negative and gram positive bacteria and the data is shown in **Fig. 3A**. Only the sample containing CEM-EC (blue curve) produced an increase in fluorescence. The lack of cross-reactivity with CEMs from the other bacteria indicates that RFD-EC1 is highly selective for *E. coli*.

We also examined the time needed for culturing a single *E. coli* cell in order to produce sufficient CEM that can induce the cleavage of RFD-EC1. For this experiment, a *E. coli* sample containing defined CFU (colony-forming units) was adequately diluted to achieve the concentration of 1 CFU/mL. This was followed by mixing 100 µL of the diluted bacterial sample with growth media and culturing it for 4, 8, 12, 16 and 24 h. CEMs were then collected for each timepoint and tested for inducing the cleavage activity of RFD-EC1. The dPAGE result shown in **Fig. 3B** indicates that a culturing time of 12 h is needed.

It is important to note that the initial small signal increase observed in fluorescence measurements after the addition of RFSS1 sequence (as a negative control) to CEM-EC (**Fig. 2B**; red curve) or RFD-EC1 to other bacterial CEMs (**Fig. 3B**; all curves except blue) is attributed to the intrinsic fluorescence of the FRQ module (due to incomplete quenching of F by Q). Thus, it is expected that the addition of F- and Q-labeled sequences would produce an initial fluorescence increase. However, only RFD-EC1/CEM-EC mixtures are capable of producing a high level of fluorescence over time.

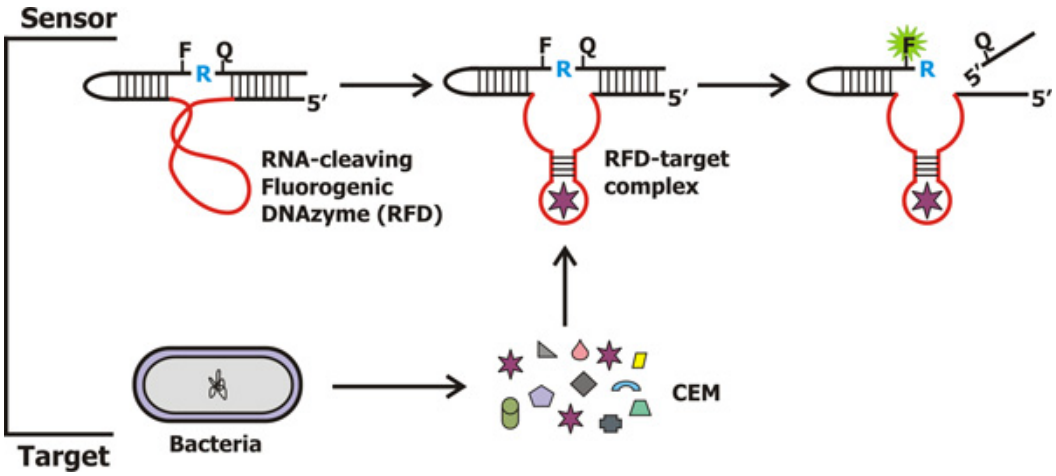

Figure 1. Schematic illustration of the RNA-cleaving fluorescent DNAzyme (RFD) probe that fluoresces upon contact with the crude extracellular mixture (CEM) produced by specific bacterial cells of interest. The RFD cleaves a chimeric DNA/RNA substrate at a lone RNA linkage (blue R) flanked by two nucleotides labeled with a fluorophore (F) and a quencher (Q), respectively. Before the cleavage reaction, the fluorescence level of the RFD is minimal due to the close proximity of F and Q. Upon cleavage, Q departs from F; as a result, a strong fluorescence signal is produced.

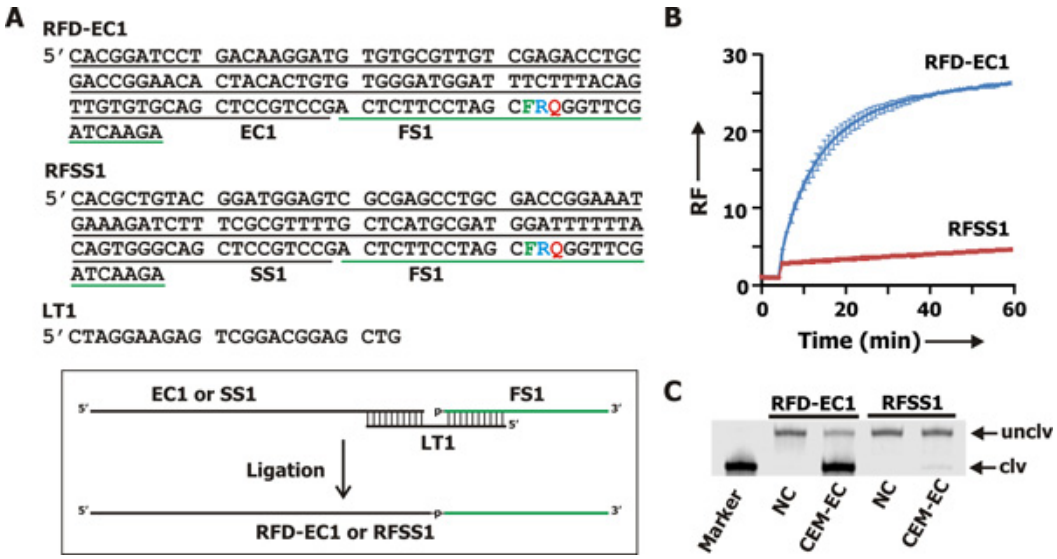

Figure 2. The *E. coli*-sensing RFD. (A) RFD-EC1 is the DNAzyme probe that can be activated by CEM-EC. RFSS1 is a scrambled sequence of RFD-EC1 used as a control. RFD-EC1 and RFSS1 were produced by ligating FS1 with EC1 and SS1, respectively, in the presence of LT1 as the template. F: fluorescein-modified deoxythymidine. Q: Dabcyl-modified deoxythymidine. R: adenine ribonucleotide. (B) Fluorescence signaling profiles of RFD-EC1 and RFSS1 in the presence of CEM-EC. (C) dPAGE analysis of the cleavage reaction mixtures in B (reaction time: 60 min). Pictured is a fluorescence image of the dPAGE gel obtained with by Typhoon scanner. Lane NC: RFD-EC1 or RFSS1 in the reaction buffer alone; Lane CEM-EC: RFD-EC1 or RFSS1 in the reaction buffer containing CEM-EC. Marker: RFD-CE1 treated with 0.25 N NaOH, a procedure known to cause full cleavage of RNA. unclv: uncleaved RFD-EC1. clv: the cleavage fragment containing the fluorophore.

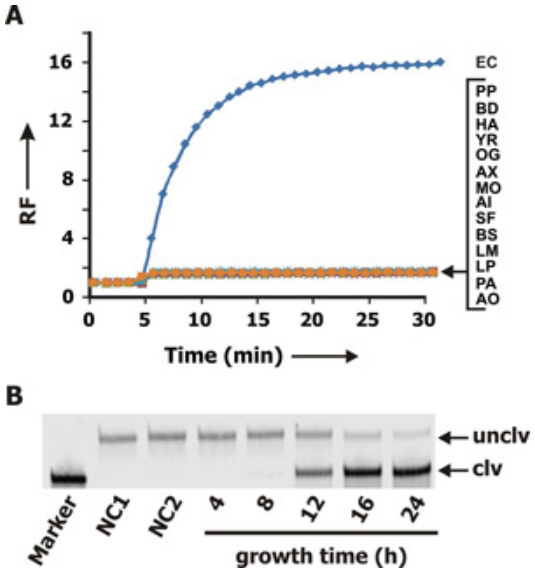

Figure 3. (A) Fluorescence signaling profile of RFD-EC1 in CEMs prepared from various bacterial cells. EC: *Escherichia coli*-K12; PP: *Pseudomonas peli*; BD: *Brevundimonas diminuta*; HA: *Hafnia alvei*; YR: *Yersinia ruckeri*; OG: *Ochrobactrum grignonese*; AX: *Achromobacter xylosoxidans*; MO: *Moraxella osloensis*; AI: *Acinetobacter lwoffi;* SF: *Serratia fonticola*; BS: *Bacillus subtilis*; LM: *Leuconostoc mesenteroides*; LP: *Lactobacillus planturum*; PA: *Pediococcus acidilactici*; AO: *Actinomyces orientalis*. Each CEM sample was incubated for 5 min followed by the addition of RFD-EC1. (B) dPAGE analysis of RFD-EC1/CEM-EC mixtures after a 60-min reaction. Lane NC1: RFD-EC1 in the reaction buffer alone. Lane NC2: RFD-EC1 in the reaction buffer containing CEM-BS (the CEM prepared from *Bacillus subtilis*). The lanes labeled with 4, 8, 12, 16 and 24: RFD-EC1 in the reaction buffer containing CEM-EC taken from the bacterial culture containing a single *E. coli* cell following a growing period of 4, 8, 12, 16 and 24 h, respectively.

Discussion

Most of the common bacterial detection methods today are either slow (classic microbial) or technically demanding (antibody, PCR). Thus, we believe that the next generation of detection tools should cater toward speed and simplicity. To this end, we have created an RNA-cleaving and fluorescence-signaling DNAzyme that can be used to develop simple assays to report the presence of bacteria through the generation of a fluorescence signal. The featured DNAzyme probe, RFD-EC1, is activated by the CEM produced during the growth of *E. coli* in culture media. Since our method uses crude extracellular mixtures of a bacterium as the target of detection and bypasses the laborious target extraction and amplification steps, it can be used to set up very simple, "mix-and-read" type of assays for bacterial detection. The use of our DNAzyme is not restricted to fluorescence based detection method. For example, colorimetric detection using the same DNAzyme system assay can be designed using a previously reported method that exploits rolling circle amplification in conjunction with an organic dye.[32] We foresee the use of DNAzymes for bacterial detection as an attractive avenue to generate new bacterial biosensors with greater operational simplicity.

Disclosures

No conflicts of interest declared.

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