Experimental and supporting data

A simple DNAzyme-based fluorescent assay for *Klebsiella pneumoniae*

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MATERIALS AND METHODS

Materials

DNA oligonucleotides: The Random DNA library and fluorogenic substrate (FS) were purchased from Keck Oligo Synthesis Facilities, Yale University (New Haven, CT). The forward PCR primer (FP), two reverse PCR primers (RP1 and RP2), ligation template (LT) and the DNAzymes after in vitro selection were obtained from Integrated DNA technologies (IDT; Coralville, IA). The sequences are shown in Figure S1A. RDL, FP, RP1, RP2 and LT were purified by 10% dPAGE before use. The DNA library contains 80 nucleotides (nt) including a random sequence domain of 50 nt (denoted by N50) in the center and two constant regions of 16 nt and 14 nt at the 5' and 3' ends, respectively. Each random position, N, represents a 25% probability of A, C, G or T nucleotides. The 28-nt FS contains a riboadenosine nucleotide (rA) that serves as the cleavage site. rA is flanked by a fluorescein-dT (F) and a dabcyl-dT (Q). RP1 and RP2 are two reverse primers used in PCR. RP2 contains a poly-A tail (A20) at the 5' end separated by a hexaethyleneglycol spacer (L). The spacer prevents the poly-A tail from being amplified and thus make the antisense strand 20 nucleotides longer than the sense strand (DNAzyme) which allows us to easily purify the desired sequence by denaturing polyacrylamide gel electrophoresis (dPAGE) (more details are provided below in the in vitro selection procedure).

Enzymes and Chemicals. T4 DNA ligase and T4 polynucleotide kinase (PNK) including their respective buffers were purchased from Thermo Scientific. Unless otherwise noted, all other chemicals were purchased either from Bioshop Canada or from Sigma-Aldrich and used without further purification. Water used in this work was double-deionized (ddH_2O) and autoclaved.

Bacterial strains.

The bacterial strains used in this study are listed in Table S1 and S2. They were cultured maintained, and cell lysates prepared at the University of California, Irvine. Lysates were sent to McMaster University in Canada for in vitro selection and assay development. Other bacteria such as *E. coli* (EC), *B. subtilis* (BS), *L. monocytogenes* (LM) are routinely

maintained in the lab at McMasters University lab as were *C. difficile* (CD) and *F. nucleatum* (FN) which were provided by Yingfu Li lab.

Preparation of Crude Extracellular Mixtures (CEMs). The bacterial strains used in the in vitro selection are listed in **Table 1** below. Crude extracellular mixtures (CEMs) and crude intracellular mixtures (CIMs) from the above mentioned bacteria were prepared as follows: Each of the bacteria were grown in in 5 mL tryptic soy broth (TSB) with continuous shaking at 37 ^{0}C and 250 rpm until the OD of the culture reached ~2. The cells were pelleted by centrifugation at 11,000 x g for 5 min at room temperature. The supernatants were collected in fresh tubes. The cell pellets were resuspended in 1 mL PBS including lysozyme (1 mg/mL) and incubated at 37 ^{0}C for 1 h to lyse the cells. The lysed cell suspension was mixed with the CEM and passed through 0.2 micron molecular cut-off filter discs, aliquoted into microcentrifuge tubes (100 µL each) and stored at -20 ^{0}C until use. The combined CEM and CIM (termed as CEM-CIM) was used as complex target because we assume that some bacteria may have unique extracellular markers that could be a target for DNAzyme.

In Vitro Selection (schematic illustration of in vitro selection is provided in Figure S1)

Library preparation and selection (step I). For the first round of selection, 1,000 pmol of DL was ligated to FS as follows: DL was phosphorylated in 100 µL reaction volume using T4 Polynucleotide Kinase (PNK, 20 units) for 45 min at 37 ^{0}C in 1x T4 polynucleotide Kinase buffer A (PKB; Thermo scientific) in the presence of 1 mM ATP. The reaction was stopped by heating at 90 ^{0}C for 5 min. Equivalent amounts of FS and LT (1,000 pmol each) were added to this solution and the mixture was heated at 90 ^{0}C for 40 s and cooled to room temperature (RT) for 20 min. Then, 30 µL of 10x T4 DNA ligase buffer (T4LB; Thermo Scientific), 30 µL of PEG4000 and 5 µL (25 U total) of T4 DNA ligase (T4DL) were added. The volume was adjusted to 300 µL with ddH_2O, mixed by pipetting and incubated at room temperature (RT) for 1 h.

Purification of ligated FS-DL (step II). The DNA molecules in the reaction mixture from step I were isolated by ethanol precipitation and the ligated FS-DL molecules were purified

by 10% dPAGE. This DNA pool was dissolved in 200 µL of 1x selection buffer (1x SB) (50 mM HEPES, pH 7.5, 150 mM NaCl, 15 mM $MgCl_2$, and 0.01% Tween 20).

Negative selection (step III). Fifty microliter of CEM-CIM of control bacteria (EC, LM, CD and BS) was mixed with 50 µL of 2x SB and added to the FS-DL pool of step II (Total volume becomes 300 µL). After pipette mixing, the reaction mixture was incubated at room temperature for 2 h. The reaction was quenched by the addition of 30 µL of 3.0 M NaOAc followed by 890 µL of cold ethanol.

Purification of uncleaved FS-DL (step IV) -After ethanol precipitation, the reaction mixture was subjected to 10% dPAGE and the uncleaved FS-DL molecules were isolated.

Positive selection (step V). The purified uncleaved full length DNA pool obtained from step IV was dissolved in 100 µL of 1x SB. Immediately, 100 µL of CEM-CIM of KP in 1x SB (50 µL CEM-CIM-KP mixed with 50 µL 2x SB) was added to the DNA pool. After pipette mixing, the reaction mixture was incubated at room temperature for 60 min. The reaction was stopped by adding 20 µL of NaOAc followed by 590 µL of cold ethanol.

Isolation of cleaved products (step VI). After ethanol precipitation, the reaction mixture was subjected to 10% dPAGE. Before loading the sample in the gel, a marker was prepared by treating a small portion of the FS-DL with 0.25 M NaOH at 90 ^{0}C for 10 min. This was applied in the gel as marker. In the first few rounds of selection, the cleaved band in the gel image is not expected to be clearly visible. Therefore, based on the position of the marker band, a portion of the gel below the uncleaved full length band was excised and the DNA molecules were isolated. After ethanol precipitation the DNA molecules were dissolved in 50 µL ddH_2O.

PCR1 (step VII). PCR was typically conducted in 50 µL volume with the template prepared in step VI including 10 µL of the isolated DNA molecules from step VI, 0.5 µM each of FP and RP1, 200 µM dNTPs (dATP, dCTP, dGTP and dTTP), 1x PCR buffer (75 mM Tris-HCl, pH 9.0, 2 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH_4)_2SO_4$) and 2.5 units of Thermus thermophilus DNA polymerase (Biotools, Madrid, Spain). The amplification was conducted using the following thermocycling setups: one cycle of 94 ^{0}C for 1 min; 13 cycles of 94 ^{0}C for 30 s, 50 ^{0}C for 45 s and 72 ^{0}C for 45 s (the numbers of amplification cycles between

different selection rounds were adjusted, typically between 13 and 15 cycles, to achieve full amplification assessed by 2% agarose gel electrophoresis); one cycle of 72 ^{0}C for 1 min.

PCR2 (step VIII). Because of the requirement of large amount of DNA molecules, a second PCR was conducted using PCR1 product as template. In this case, 1 µL of the PCR1 product was diluted to 20 µL, 1 µL of which was used in PCR2 using FP and RP2 primers following the same amplification setup for PCR1. Note that the numbers of amplification cycles between different selection rounds were adjusted, typically between 13 and 15 cycles, to achieve full amplification (assessed by 2% agarose gel electrophoresis).

Purification of the sense (DNAzyme) strand (step IX). The PCR product was concentrated by ethanol precipitation and subjected to 10% dPAGE. The DNA band of the sense strand (shorter sequence, bottom band) was excised and the DNA was eluted and stored at -20 ^{0}C as dry pellet until use.

Ligation of PCR product to FS (step X) and repetition of steps II-X. The sense DNA strand prepared above (approximately 200 pmol, stored as dried pellet) was ligated to FS as follows: The PCR product from the above step was phosphorylated in 100 µL reaction volume with 10 U of PNK in the presence of 1 mM ATP in 1x PNK buffer for 40 min at 37 ^{0}C. Note that the reaction volume of phosphorylation of PCR product for the subsequent rounds was constantly maintained at 100 µL. The kinase reaction was quenched by heating at 90 ^{0}C for 5 min and cooled down to RT for 20 min. Equal amount of FS and LT (200 pmol each) were added to the PNK reaction mixture, mixed by vortex, heated at 90 ^{0}C for 1 min and cooled to RT for 20 min. Then, 20 µL of T4LB, 20 µL of PEG4000, 4 µL of T4DL were added sequentially and the volume of the reaction was adjusted to 200 µL with ddH_2O (ligations for the subsequent selection rounds were carried out in 200 µL volume). After pipette mixing, the ligation reaction was conducted at RT for 1 h. After ethanol precipitation the ligated DNA product was purified by 10% dPAGE and employed in the second round of selection following the same procedure as described for the first round. Significant amount of cleavage product was obtained at round 11 of selections (Figure 1B) and finally the DNA population of this round was sequenced. Negative selections were carried out at every two rounds of selection to achieve selectivity.

Screening for the most active DNAzyme. The top 11 DNAzymes (sequences and names are shown in Figure S2A) obtained by deep sequencing were chemically synthesized, ligated to FS and tested for cleavage performance. 1,000 nmol of each DNAzyme was individually ligated to FS as described above in the selection procedure (step I). After ligation and purification, the DNAzyme sequences were dissolved in ddH_2O, quantified by nano-drop and stored at -20 ^{0}C until use. The concentration of each was adjusted to 2 µM by diluting with ddH_2O. For the cleavage reaction, 1 µL of each DNAzyme was transferred to individual microcentrifuge tubes followed by addition of 5 µL of 2x SB. Two cleavage reactions with each DNAzyme were conducted: one with reaction buffer alone (control) and another one is with CEM-CIM-KP (test). 4 µL of ddH_2O was added to the control tube and 4 µL of CEM-CIM-KP to the test tube. After pipette mixing, the reaction mixtures were incubated for at RT 30 min. The cleavage reactions were quenched by adding 10 µL of 2x gel loading buffer (GLB) and the reaction mixtures were analyzed by 10% dPAGE. The gel was imaged for fluorescence bands by ChemiDoc fluorescent imager (Bio-Rad, Figure S2B).

Selectivity test. Among the DNAzymes, RFD-KP1, RFD-KP4, RFD-KP6, RFD-KP7 and RFD-KP10 showed adequate cleavage activities and were applied in the selectivity experiment. First, selectivity was tested with bacterial strains (EC*, BS, LM* and *FN*) available in our lab. The bacterial cell lysates were prepared in the same way as described above. The cleavage reactions were carried out in 10 µL volume as follows: 1 µL (2 µM) of each DNAzyme was dispensed in 6 individual microcentrifuge tubes (Numbered by 1, 2, 3, 4, 5, 6) followed by the addition of 5 µM 2x reaction buffer (100 mM HEPES, 300 mM NaCl, 30 mM $MgCl_2$, 0.02% Tween20). Next, 4 µL ddH_2O was added in tube 1 (negative control), and CEM-CIM of KP, EC, BS, LM and FN were added in the consecutive tube 2, 3, 4, 5 and 6 respectively. After pipette mixing, the tubes were incubated at room temperature for 45 minutes. The reactions were quenched by adding 2x GLB and subjected to dPAGE. The gel was imaged using a fluorescent imager (Chemidoc TM, Bio-Rad) (Figure 2A in the main text). The most selective DNAzyme sequence (RFD-KP6) was further tested for specificity with three different KP strains: Carbapenem sensitive (KPS), Carbapenem resistant (KPC) and extended spectrum beta lactamase producing KP (KP-ESBL). The preparation of the cell lysate, cleavage reaction and gel analysis procedures are same as described above.

Real time fluorescence signaling test

Real time fluorescence signal generation was investigated in 96 well plates using a plate reader (Tecan, M200, Bio-Rad). First, 80 µL of RFD-KP6 (50 nM) in 1x SB was dispensed into two wells of the plate. The plate was then placed in the Tecan plate reader and data collected following addition of 20 µL of KPC cell lysate in one well (test) and 20 µL 1x SB in another well (control). The fluorescence data collection was continued for 30 min. The data was processed using Microsoft Excel software.

Microzones Fabrication on Paper

Fabrication of microzomes on paper was carried out following our previous report.[1] In brief, spherical circle (microzones) of ~4 mm diameter were drawn in PowerPoint with 6 mm inter-microzone distance aligned in 8 rows and 12 columns. It was then wax printed on nitrocellulose paper (FF120, GE healthcare) using a Xerox Phaser 8560N printer. The wax-printed paper was heated at 120 °C for 2 minutes to melt the wax into the pores of the paper and provide hydrophobic barriers around the microzones. The paper was stored at room temperature until use.

DNAzyme Immobilization onto Microzones

A 500 µL solution of RFD-KP6 DNAzyme (200 nM, 1 pmol/5 µL) was prepared in 1x RB (50 mM HEPES, 150 mM NaCl, 15 mM, $MgCl_2$, 0.01% Tween 20, pH 7.5) including 0.25 M trehalose (TH), and 8% (w/v) pullulan (PL). Next, 5 µL of this DNAzyme mixture was dispensed onto 21 microzones (3 rows, 7 columns) by BioDot printer and allowed to air dry overnight in the dark.

Cleavage and fluorescence signaling test

Ten microliter CEM-CIM-KP was added onto each of 4 microzones and incubated for 30 min and imaged for fluorescence. As a control to compare the signaling, only buffer was added in another 4 microzones. The fluorescence image was obtained by ChemiDoc TM (Bio-Rad) and quantified by ImageJ software and plotted using Microsoft excel software.

Sensitivity test

To test the sensitivity of the assay, KPC was cultured in TSB overnight and subcultured to fresh media, incubated at 37°C until the culture reached an OD ~2. Next, the bacteria was serially diluted 10-fold in 1 mL phosphate buffer saline (PBS) and the dilutions cultured to establish the colony forming units/mL (CFU/ml). Next, known concentrations of isolates were treated with lysozyme (2 mg/mL) to lyse the cells to release intracellular components. Cleavage reaction were carried out on the paper microzones prepared in the above step. 10 µL of the each concentration, CFU/ml, were dispensed onto each microzone designated for each sample dilution. This was done in triplicate. The reactions were conducted at room temperature for 1 hour and imaged by Chemidoc fluorescent imager (Bio-Rad) and analyzed by ImageJ software.

Testing bacterial isolates

Bacterial isolates were blinded and tested following the same paper-based microzone test described in the above section (Sensitivity Test). The CIM-CEM samples (with ID only without disclosing the name of the bacterial genera and species) were prepared by the Peterson lab at University of California, Irvine and shipped to McMaster University for testing. The DNAzyme, RFD-KP06, was printed in the wax printed paper microzones and air dried. 10 µL of the each bacterial preparation was dispensed onto microzones. After cleavage reactions for 60 min, the paper was imaged for fluorescence using a fluorescent imager (Chemidoc Fluoro ImagerTM, Bio-Rad). The image was analyzed by ImageJ software and plotted using microsoft excel software.

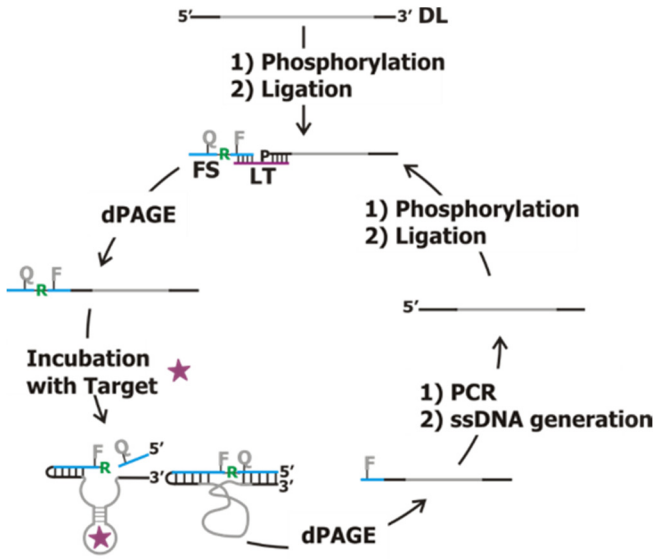

Figure S1. Schematic illustration of the in vitro selection procedure. The library DL is enzymatically ligated to the substrate and purified by denaturing gel electrophoresis (dPAGE). The purified products are mixed with the target CIM-CEM mixture and allowed to react. The reaction mixture is subjected to dPAGE and the cleaved shorter fragments are isolated. The cleaved products are then amplified by PCR and purified by dPAGE. The PCR products are enzymatically ligated to the substrate and applied to the next round of selection.

A

RFD-KP1: **ATGCCATCCTACCAAC**ATATGCAGAGCACAGTCTGGACATATAATTTGACCAAGGCGTCAGGTCCT**GAGCTCTGAACTCG**
RFD-KP2: **ATGCCATCCTACCAAC**CGGCCATCACGGAGGGATTAGCTCATTCTATGGCGACGGGTCCAGTTTGA**GAGCTCTGAACTCG**
RFD-KP3: **ATGCCATCCTACCAAC**CCCAAGTTTGTAGCCTTCTACTAAGCGTCGGTCCTTGGGGCCTCAATTAG**GAGCTCTGAACTCG**
RFD-KP4: **ATGCCATCCTACCAAC**CCTGAAAGTAGTATTAGTCTTGACCTTGTCGTAGGTGAGCTGTTCATTTT**GAGCTCTGAACTCG**
RFD-KP5: **ATGCCATCCTACCAAC**AGCAGGTGCGAAAGAGCTCGGTTCTGGTGCAAACCAAGGCGTCGGTCCCA**GAGCTCTGAACTCG**
RFD-KP6: **ATGCCATCCTACCAAC**CATGACTGGTTTGTACTAAGAGATTTCAGGCATCGCTGCACGTCGTAGGT**GAGCTCTGAACTCG**
RFD-KP7: **ATGCCATCCTACCAAC**ACACAACATTCTCGTTATTGACTTGTTCCAGCATCTTGCACGTCGTAGGT**GAGCTCTGAACTCG**
RFD-KP8: **ATGCCATCCTACCAAC**ACATGCAGAGCACAGTCTGGACATATAATTTGACCAAGGCGTCAGGTCCT**GAGCTCTGAACTCG**
RFD-KP9: **ATGCCATCCTACCAAC**CAAAAGTCCGCTCCTCAGATTGCATCGGTGGGACATCGTATTTCGGATAG**GAGCTCTGAACTCG**
RFD-KP10: **ATGCCATCCTACCAAC**CATGACTGGTTTGTACTAGGAGATTTCAGGCATCGCTGCACGTCGTAGGT**GAGCTCTGAACTCG**
RFD-KP11: **ATGCCATCCTACCAAC**CAATGTACTGGGTGTGTTTGGATGAATCACGCTTCTTGCACGTCGTAGGT**GAGCTCTGAACTCG**

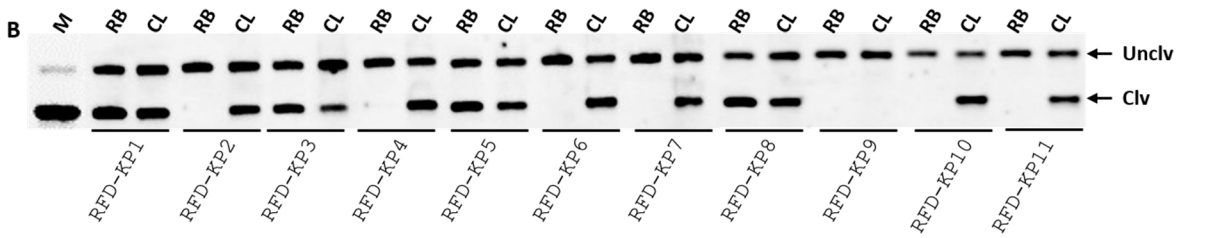

Figure S2. A) Top 11 DNAzyme sequences obtained by deep sequencing. B) Cleavage test of the top 11 sequences. M: marker, RB: reaction buffer, CL: Cell lysate.

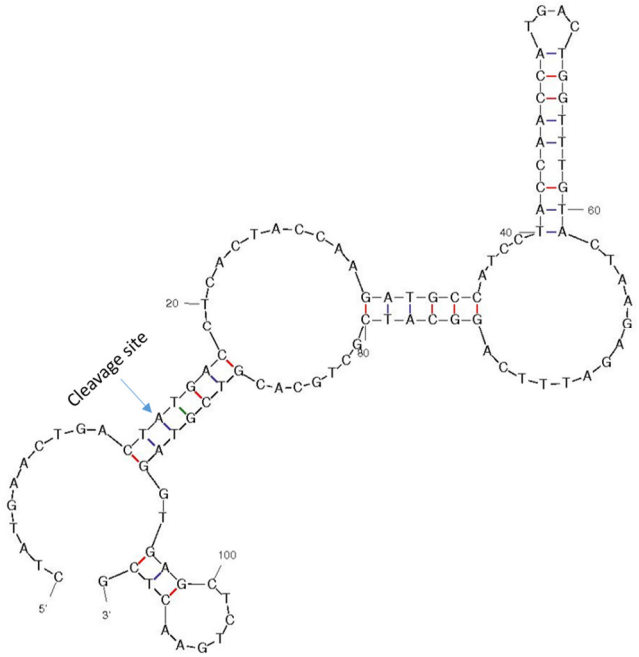

Figure S3. Predicted secondary structure of RFD-KP6 (obtained by mFold: http://unafold.rna.albany.edu/?q=mfold/DNA-Folding-Form)

Table 1: Bacterial strains used in the in vitro selection

Bacterial Strain	ID number	Resistance	Acquired resistance
K. pneumoniae	ATCC13883	Susceptible Control	n/a
K. pneumoniae	ATCC700603	ESBL	SHV-18
K. pneumoniae	AR0034	Carbapenems	IMP
K. pneumoniae	AR0041	Carbapenems	NDM
K. pneumoniae	AR0046	Carbapenems	VIM
K. pneumoniae	AR0049	Carbapenems	NDM
K. pneumoniae	AR0097	Carbapenems	KPC
K. pneumoniae	AR0098	Carbapenems	KPC
K. pneumoniae	AR0112	Carbapenems	KPC-3
K. pneumoniae	AR0113	Carbapenems	KPC-3
K. pneumoniae	AR0120	Carbapenems	KPC-2

Table 2: Bacterial isolates used in DNAzyme performance evaluation

Sample ID	Identification	Source	Carbapenem Resistant
434	*Enterobacter aerogenes*	AR Bank 0007	NO
435	*Klebsiella aerogenes*	AR Bank 0087	NO
436	*Citrobacter freundii*	AR Bank 023	NO
437	*Enterobacter cloacae*	AR Bank 0154	YES
438	*Providencia stuartii*	AR Bank 0026	NO
439	*Klebsiella pneumoniae*	AR Bank 0135	YES
440	*Serratia marcescens*	AR Bank 0027	NO
441	*Enterobacter cloacae*	ATCC 13047	NO
442	*Klebsiella pneumoniae*	ATCC 700603	NO
443	*Klebsiella oxytoca*	AR Bank 0028	NO
444	*Enterococcus faecalis*	ATCC 29212	N/A
445	*Escherichia coli*	AR Bank 0086	NO
446	*Klebsiella pneumoniae*	AR Bank 0076	YES
447	*Klebsiella pneumoniae*	AR Bank 13883	NO
448	*Klebsiella pneumoniae*	AR Bank 0046	YES
449	*Citrobacter koseri*	AR Bank 0025	NO
450	*Staphylococcus epidermidis*	UCI-clinical isolate	N/A
451	*Escherichia coli*	AR Bank 0017	NO
452	*Klebsiella pneumoniae*	AR Bank 0040	YES
453	*Proteus mirabilis*	AR Bank 0029	NO

ATCC, American Type Culture Collection

AR, CDC Antimicrobic Resistance Bank

N/A, not applicable

References

[1] M. M. Ali, C. L. Brown, S. Jahanshahi-Anbuhi, Y. Li, J.D. Brennan, *Sceintific Reports*, **2017**, 7, 12335