CHARACTERIZATION OF A DNAZYME FOR THE DETECTION OF *LEGIONELLA PNEUMOPHILA* IN COOLING TOWER WATER

By MEGHAN ROTHENBROKER, BHSc

A Thesis Submitted to the School of Graduate Studies in Partial Fulfilment of the Requirements for the Degree Master of Science

McMaster University MASTER OF SCIENCE (2019) Hamilton, Ontario (Biochemistry and Biomedical Sciences)

TITLE: Characterization of a DNAzyme for the Detection of *Legionella Pneumophila* in Cooling Tower Water
AUTHOR: Meghan Rothenbroker, BHSc (McMaster University)
SUPERVISOR: Professor Yingfu Li
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LAY ABSTRACT

Legionella pneumophila is a deadly water-borne bacterial pathogen that causes Legionnaires' disease - a severe form of pneumonia. Numerous Legionnaires' disease outbreaks have occurred, with the most common source of exposure to *L. pneumophila* coming from contaminated cooling towers. Presently, bacterial culturing is used to determine if a cooling tower is contaminated with *L. pneumophila*, however this process can take up to 10 days to complete. To address this delay, we plan to develop a rapid paper-based test for *L. pneumophila* detection in cooling tower water using DNAzymes. DNAzymes are small, catalytically-active single-stranded DNA molecules that demonstrate target-specific enzymatic activity. We have isolated a DNAzyme that can specifically detect *L. pneumophila* and characterized its properties. In the future we plan to incorporate this DNAzyme into a field-appropriate paper-based test which would play a key role in managing Legionnaires' disease outbreaks.

ABSTRACT

Ineffective bacterial monitoring in water systems represents a danger to public health and can result in costly disease outbreaks. Of interest is *Legionella pneumophila*, a deadly water-borne bacterial pathogen that causes Legionnaires' disease - a severe form of pneumonia. The Center for Disease Control stated that reported cases of Legionnaires' disease have quadrupled since 2000 and ranks *L. pneumophila* as the number one cause of waterborne disease outbreaks in the United States. This threat is expected to increase given an aging population who are more susceptible to *L. pneumophila* infection and rising global temperatures that can promote *L. pneumophila* growth. Presently, Public Health agencies recommend bacterial culturing for the detection of *L. pneumophila* in environmental samples, however, this process can take up to ten days to complete. Consequently, there is a delay between sample collection and subsequent *L. pneumophila* detection, creating an opportunity for a Legionnaires' disease outbreak to occur. There is a great need to develop a field-appropriate device that can provide early-stage detection of *L. pneumophila* in water as a means of mitigating Legionnaires' disease outbreaks. We propose the use of DNAzymes for the development of such a device. DNAzymes are small, catalytically-active single-stranded DNA molecules that demonstrate target-specific enzymatic activity. We have successfully isolated an RNA-cleaving fluorescent DNAzyme (RFD) specific for the detection of *L. pneumophila* using *in vitro* selection. Thorough characterization of the DNAzyme has revealed key structural features influencing kinetics, specificity and sensitivity. In addition, the ability of the DNAzyme to function in cooling tower water, and conservation of the DNAzyme target across *Legionella* bacteria, has been investigated. In the future we plan to incorporate this RFD into a field-appropriate paper-based device

which would play a key role in managing infectious diseases and preventing large-scale outbreaks.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Yingfu Li for his thoughtful guidance and support on this project. In addition, I would like to thank my committee members Dr. Lori Burrows, Dr. Carlos Filipe, and Dr. Lesley MacNeil for their insights and suggestions. I would also like to thank my fellow Li lab members for all their help, whether it be a quick chat about my results or a thorough demonstration of how to carry out an unfamiliar protocol. Specifically, I want to thank Dr. Erin McConnell for her mentorship as I navigated my experiments and her leadership in ensuring this project stayed on task. I would also like to thank my friends and family for supporting me through this journey.

Finally, I would like to thank NSERC for the funding that made this project possible, in addition to our industrial collaborators TGWT for their active participation in this project.

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LIST OF ABBREVIATIONS AND SYMBOLS

DNA-based enzymes	DNAzymes
RNA-cleaving fluorogenic DNAzymes	RFDs
Crude extracellular mixture	CEM
Crude intracellular mixture	CIM
Denaturing polyacrylamide gel electrophoresis	dPAGE
Random region of library	N_{40}
Adenosine ribonucleotide	‘rA’ in blue
Fluorescein-dT	‘F’ in green
DABCYL-dT	‘Q’ in red
6-FAM (fluorescein)	‘FAM’ in green
γ-[32P]	‘P’ in orange
Buffered yeast extract	BYE
Selection buffer	SB
Quenching buffer	QB
Polymerase chain reaction	PCR
Observed rate constants	k_{obs}
Maximum cleavage yields	Y_{max}
Marker	M
Un-cleaved DNAzyme	unclv
Cleaved DNAzyme	clv
Colony forming units	cfu
Optical density	OD_{600}

DECLARATION OF ACADEMIC ACHIEVEMENT

In vitro selection of Lp DNAzyme was conducted by lab technician Jim Gu. Re-selection was performed by former Li lab student Suraj Gopinathbirla. Initial secondary structure and truncation investigations were done by Jim and Suraj. Conservation analysis of re-selection data was performed by Dr. Erin McConnell. Cooling tower water samples were collected and provided by TGWT Clean Technologies (Montreal, Canada), ProAsys (Pennsylvania, New Jersey, Ohio, USA), Chemco Products Company (California, USA). Dr. Alex Ensminger selected and provided the following strains for initial investigations of the DNAzyme target: *Legionella micdadei*, *Legionella dumofii*, *Legionella pneumophila* (Philly -1 CDC), and *Legionella longbeachae.* After discussing the results, Dr. Ensminger selected and provided the following *Legionella pneumophila* isolates for further investigation of the DNAzyme target: Philadelphia-1, Paris, Toronto 2005, Lens and 130b. All *Legionella* bacteria from the Ensminger lab were cultured by Dr. Malene Urbanus. All kinetic, specificity, sensitivity, and truncation tests were set up, run, and analyzed by me. I visited our industry collaborators TGWT in Montreal to conduct the cooling tower water experiments as well as collect cooling tower water myself, with help from Mélanie and Étienne (TGWT employees). All cooling tower water tests were set up, run, and analyzed by me. I travelled to Toronto to investigate the DNAzyme target further, all tests were set up, run, and analyzed by me.

1 Introduction

1.1 *Legionella pneumophila* and Legionnaires' Disease

Ineffective bacterial monitoring in water systems represents a danger to public health and can result in costly disease outbreaks. Of particular interest is *Legionella pneumophila*, a deadly waterborne bacterial pathogen that causes Legionnaires' disease, a severe form of pneumoniae[1]. Inhalation of droplets containing *L. pneumophila* is thought to be the primary mode of disease transmission. Once inhaled, *L. pneumophila* multiply intracellularly in human macrophages by avoiding phagosome-lysosome fusion[2–5]. The first official report of a Legionnaires' disease outbreak took place in July of 1976 at the annual American Legion convention in Philadelphia where 34 of 221 cases were fatal[6]. In contrast to 1976 where the causative agent behind the disease was unknown, the dangers of *L. pneumophila* and its role in causing Legionnaires' disease is now better understood. Despite this knowledge and the implementation of prevention measures, Legionnaires' disease outbreaks continue to arise, impacting the lives of many. The CDC has stated that reported cases of Legionnaires' disease have quadrupled since the year 2000 and ranks *Legionella* as the number one cause of waterborne disease outbreaks in the United States with direct healthcare costs ranging from $101 to $321 million annually[7,8]. Furthermore, a CDC report on waterborne disease outbreaks associated with environmental exposures to water in the US between 2013-2014 stated that *Legionella* was responsible for 63% of outbreaks, 94% of hospitalizations and all deaths[9]. The study also revealed that human-made water systems, including infrastructure intended for water storage or recirculation

(cooling towers), were the source of *Legionella* contamination responsible for the outbreaks.

Although all *Legionella* species have the potential to be pathogenic, *L. pneumophila*, specifically serogroup 1, is responsible for the large majority of cases of Legionnaires' disease[7,10,11]. The current 'gold standard' for *L. pneumophila* detection in water systems recommended by Public Health agencies around the world is bacterial culturing; however, this method is technically challenging and can take up to 10 days to confirm contamination[8]. This delay between sample collection and subsequent *Legionella* detection creates an opportunity for a Legionnaires disease outbreak to occur. Advancements in PCR-based detection methods have decreased detection time but are not without their own challenges. Sample preparation for PCR often requires several steps and the method itself can be inhibited by the components of real-world samples[8]. There is a great need to develop a field-appropriate assay that can provide early stage detection of *L. pneumophila* in water as a means of mitigating Legionnaires' disease outbreaks.

1.2 DNAzymes

DNA, makes RNA, makes protein; this is the Central Dogma of molecular biology. In the early 1980's however, it was revealed that nucleic acids, like proteins, had the ability to function as biological catalysts. In 1989, Thomas Cech and Sidney Altman were awarded the Nobel Prize in Chemistry "for their discovery of catalytic properties of RNA". Their discovery of RNA-based enzymes, or ribozymes, showed for the first time that RNA shared with proteins the ability to catalyze complex biochemical reactions[12,13]. The discovery of natural ribozymes motivated researchers to search for synthetic

ribozymes, as well as DNA-based enzymes (DNAzymes). Unlike ribozymes, there is no evidence that DNAzymes exist in nature. This is likely due to that fact that the natural state of DNA is primarily double-stranded and consequently simple, whereas single-stranded DNA can form complex three-dimensional structures characteristic of enzyme active sites[14]. The search for man-made DNAzymes was made possible with the development of the *in vitro* selection technique by the groups of Larry Gold, Jack Szostak and Gerald Joyce[15–17]. Since the discovery of the first-ever DNAzyme by Breaker and Joyce in 1994[18], numerous DNAzymes capable of catalyzing a wide variety of chemical reactions have been isolated and reported[19–22]. RNA-cleaving fluorogenic DNAzymes (RFDs) are a special class of DNAzymes that simultaneously link catalysis with fluorescence. These DNAzymes are designed to cleave a single RNA linkage embedded in an otherwise all DNA (single-stranded) sequence. Unique to this design is that the RNA is flanked by fluorophore and quencher modified nucleotides. In the absence of the target, the DNAzyme remains inactivated (i.e. intact) and the proximity of the quencher to the fluorophore essentially quenches its fluorescence. However, in the presence of the target, the DNAzyme is activated (i.e. cleaves) and the quencher is separated from the fluorophore, resulting in a significantly enhanced fluorescent signal. RNA-cleaving fluorogenic DNAzymes (RFDs), and DNAzymes in general, are well suited for bacterial detection applications[23–28].

Here we report an RFD that can be used as a molecular probe for the detection of *L. pneumophila*. Using *in vitro* selection, we isolated for the first time a catalytic DNA sequence, termed Lp1, that demonstrates activity towards the crude extracellular

mixture (CEM) of *L. pneumophila*. Thorough characterization of Lp1 has revealed key structural features influencing kinetics, specificity and sensitivity. In addition, the ability of the DNAzyme to function in cooling tower water, and the conservation of the DNAzyme target across *Legionella* bacteria, has been investigated. Numerous assays incorporating DNAzymes as the signal detection element have been reported, and as such could be easily applied to our DNAzyme for the development of a facile assay for the early-stage detection of *L. pneumophila* in cooling tower water as a means of mitigating Legionnaires' disease outbreaks.

2 Materials and Methods

2.1 Synthesis and purification of oligonucleotides.

The sequences of oligonucleotides are listed in

Table **1**. Sequences were purchased as synthetic oligonucleotides from Integrated DNA Technologies (IDT) or Yale. All oligonucleotides were purified by 10% denaturing (8M urea) polyacrylamide ((v/v) 29:1 bisacrylamide:acrylamide) gel electrophoresis (dPAGE) before use. Each random position in LP3 DNA library (N_{40}) represents a 25 % probability of A, C, G or T nucleotide. LP3Z DNA library (N_{40}) was based on the initial selection LP3 round 11 rank 2 sequence (Lp1 DNAzyme) with the variable domain randomized with a 30% mutation rate (A, C, G, or T) for each base position. γ-[32P]-ATP was purchased from Perkin Elmer. SUPERase-In RNAse inhibitor was purchased from Invitrogen. Water was purified with a Milli-Q Synthesis A10 water purification system.

Selection		
Library (LP3)		CAA GCA TGG ACA ATA CCG AGC **N** ATC TTG TCA TCG GAG GCT TAG
Substrate (FQ30)		CTA TGA ACT GAC QTrATF GAC CTC ACT ACC AAG
Template		TAT TGT CCA TGC TTG CTT GGT AGT GAG GTC
Forward primer		CAA GCA TGG ACA ATA CCG AGC
Reverse primer		CTA AGC CTC CGA TGA CAA GAT
Reverse primer with poly T tail		TTTTTTTTTTTTTTT-C18- CTA AGC CTC CGA TGA CAA GAT
Reselection		
Library (LP3Z)		CAA GCA TGG ACA ATA CCG AGC **N** CTT AGT AGC CGA AGT TGC TGA
Substrate (FQ30)		CTA TGA ACT GAC QTrATF GAC CTC ACT ACC AAG
Template		TAT TGT CCA TGC TTG CTT GGT AGT GAG GTC
Forward primer		CAA GCA TGG ACA ATA CCG AGC
Reverse primer		TCA GCA ACT TCG GCT ACT AAG
Reverse primer with poly T tail		TTTTTTTTTTTTTTT-C18-TCA GCA ACT TCG GCT ACT AAG
DNAzymes		
Name	**# bases**	
Lp1	112	CTA TGA ACT GAC QTrATF GAC CTC ACT ACC AAG CAA GCA TGG ACA ATA CCG AGC CTT TCA TTT CAG CCG ATC ATA CCT CAA TGT AGA TAA GCA CAT CTT GTC ATC GGA GGC TTA G
Lp3	112	CTA TGA ACT GAC TrAT GAC CTC ACT ACC AAG CAA GCA TGG ACA ATA CCG AGC CTT TCA TTT CAG CCG ATC ATA CCT CAA TGT AGA TAA GCA CAT CTT GTC ATC GGA GGC TTA G- FAM
Lp5	112	FAM - CTA TGA ACT GAC TrAT GAC CTC ACT ACC AAG CAA GCA TGG ACA ATA CCG AGC CTT TCA TTT CAG CCG ATC ATA CCT CAA TGT AGA TAA GCA CAT CTT GTC ATC GGA GGC TTA G
LpP	112	CTA TGA ACT GAC TrAT GAC CTC ACT ACC AAG PCAA GCA TGG ACA ATA CCG AGC CTT TCA TTT CAG CCG

		ATC ATA CCT CAA TGT AGA TAA GCA CAT CTT GTC ATC GGA GGC TTA G
4TFP	99	FAM- CTA TGA ACT GAC TrAT GAC CTC ACT ACC AAG CT TTT AGC CTT TCA TTT CAG CCG ATC ATA CCT CAA TGT AGA TAA GCA CAT CTT GTC ATC GGA GGC TTA G
9TFP	104	FAM- CTA TGA ACT GAC TrAT GAC CTC ACT ACC AAG CT TTT TTT TTA GCC TTT CAT TTC AGC CGA TCA TAC CTC AAT GTA GAT AAG CAC ATC TTG TCA TCG GAG GCT TAG
17TFP	112	FAM- CTA TGA ACT GAC TrAT GAC CTC ACT ACC AAG CT TTT TTT TTT TTT TTT TAG CCT TTC ATT TCA GCC GAT CAT ACC TCA ATG TAG ATA AGC ACA TCT TGT CAT CGG AGG CTT AG
T1	107	FAM- CTA TGA ACT GAC TrAT GAC CTC ACT ACC AAG CA AGC ATG GAC AAT ACC GAG CCT TTC ATT TCA GCC GAT CAT ACC TCA ATG TAG ATA AGC ACA TCT TGT CAT CGG AGG ~~CTT AG~~
T2	102	FAM- CTA TGA ACT GAC TrAT GAC CTC ACT ACC AAG CA AGC ATG GAC AAT ACC GAG CCT TTC ATT TCA GCC GAT CAT ACC TCA ATG TAG ATA AGC ACA TCT TGT CAT C~~GG AGG CTT AG~~
T3	97	FAM- CTA TGA ACT GAC TrAT GAC CTC ACT ACC AAG CA AGC ATG GAC AAT ACC GAG CCT TTC ATT TCA GCC GAT CAT ACC TCA ATG TAG ATA AGC ACA TCT TG~~T CAT CGG AGG CTT AG~~
T4	92	FAM- CTA TGA ACT GAC TrAT GAC CTC ACT ACC AAG CA AGC ATG GAC AAT ACC GAG CCT TTC ATT TCA GCC GAT CAT ACC TCA ATG TAG ATA AGC ACA ~~TCT TGT CAT CGG AGG CTT AG~~
T5	87	FAM- CTA TGA ACT GAC TrAT GAC CTC ACT ACC AAG CA AGC ATG GAC AAT ACC GAG CCT TTC ATT TCA GCC GAT CAT ACC TCA ATG TAG ATA A~~GC ACA TCT TGT CAT CGG AGG CTT AG~~
CACAT	112	FAM- CTA TGA ACT GAC TrAT GAC CTC ACT ACC AAG CA AGC ATG GAC AAT ACC GAG CCT TTC ATT TCA GCC

		GAT CAT ACC TCA ATG TAG ATA AGT TTT TCT TGT CAT CGG AGG CTT AG
S1T	97	FAM- CTA TGA ACT GAC TrAT GAC CTC ACT ACC AAG CTT TTA GCC TTT CAT TTC AGC CGA TCA TAC CTC AAT GTA ~~G~~ATA AGC ACA TCT TGT~~C~~ ATC GGA GGC TTA G
S2T	95	FAM- CTA TGA ACT GAC TrAT GAC CTC ACT ACC AAG CTT TTA GCC TTT CAT TTC AGC CGA TCA TAC CTC AAT GTA~~GA~~ TAA GCA CAT CTT G~~TC~~ ATC GGA GGC TTA G
S3T	93	FAM- CTA TGA ACT GAC TrAT GAC CTC ACT ACC AAG CTT TTA GCC TTT CAT TTC AGC CGA TCA TAC CTC AAT GTA~~GA~~T AAG CAC ATC TT~~GTC~~A TCG GAG GCT TAG
TL1	93	FAM- CTA TGA ACT GAC TrAT GAC CTC ACT ACC AAG CTT TTA GCC TTT CAT TTC AGC CGA TCA TA ~~CCT CAA~~T GTA GAT AAG CAC ATC TTG TCA TCG GAG GCT TAG
MET1	77	FAM-CTG ACT rATG ACC TCT TAG CCT TTC ATT TCA GCC GAT CAT ACC TCA ATG TAG ATA AGC ACA TCT TGT CAT CGG AGG CT
MET2	63	FAM-CTG ACT rATG ACC TCT TAG CCT TTC ATT TCA GCC GAT GAT AAG CAC ATC TTG TCA TCG GAG GCT
MET3	55	FAM-CTG ACT rATG ATT TTT CAT TTC AGC CGA TGA TAA GCA CAT CTT GTC ATC GGA GGC T
MET4	55	FAM-CTG ACT rATG ACC TCT TAG CCT TTC ATT TCA GCC GAA AGC ACA TCT TTC GGA GGC T
MET5	67	FAM-CTG ACT rATG ACC TCT TAG CCT TTC ATT TCA GCC GAT CGT AGA TAA GCA CAT CTT GTC ATC GGA GGC T
MET6	59	FAM-CTG ACT rATG ACC TCT TTT TTA GCC TTT CAT TTC AGC CGA AAG CAC ATC TTT CGG AGG CT
MET7	101	FAM-CTA TGA ACT GAC TrAT GAC CTC ACT ACC AAG CTT TTT TAG CCT TTC ATT TCA GCC GAT CAT ACC TCA

		ATG TAG ATA AGC ACA TCT TGT CAT CGG AGG CTT AG
MET8	57	FAM-CTG ACT rATG ACC TCT TAG CCT TTC ATT TCA GCC GAT AAA GCA CAT CTT TCG GAG GCT
MET9	61	FAM-CTG ACT rATG ACC TCT TTT TTA GCC TTT CAT TTC AGC CGA TAA AGC ACA TCT TTC GGA GGC T
MET10	55	FAM-CTG ACT rATG ACC TCT TAG CCC TTC ATT TCA GCC GAA AGC ACA TCT TTC GGG GGC T
MET11	61	FAM-CTG ACT rATG ACC TCT TCT AAG CCC TTC ATT TCA GCC GAA AGC ACA TCT TTC GGG GGC TTA G
MET12	51	FAM-CTG ACT rATG ACC TCT TAG CCC TTC ATT TCA GCC AAG CAC ATC TTG GGG GCT
MET13	59	FAM-CTG ACT rATG ACC TCT TTT TTA GCC CTT CAT TTC AGC CGA AAG CAC ATC TTT CGG GGG CT
MET10.1	55	FAM- CTG ACT rATG ATC TCT TAG CCC TTC ATT TCA GCC GAA AGC ACA TCT TTC GGG GGC T
MET10.2	55	FAM- CTG ACT rATG ACT TCT TAG CCC TTC ATT TCA GCC GAA AGC ACA TCT TTC GGG GGC T
MET10.3	55	FAM- CTG ACT rATG ACC CCT TAG CCC TTC ATT TCA GCC GAA AGC ACA TCT TTC GGG GGC T
MET10.4	55	FAM- CTG ACT rATG ACC TTT TAG CCC TTC ATT TCA GCC GAA AGC ACA TCT TTC GGG GGC T
MET10.5	55	FAM- CTG ACT rATG ACC TCC TAG CCC TTC ATT TCA GCC GAA AGC ACA TCT TTC GGG GGC T
MET10.6	55	FAM- CTG ACT rATG ACC TCT CAG CCC TTC ATT TCA GCC GAA AGC ACA TCT TTC GGG GGC T
MET10.7	55	FAM- CTG ATT rATG ACC TCT TAG CCC TTC ATT TCA GCC GAA AGC ACA TCT TTC GGG GGC T
MET10.8	55	FAM- CTG ACC rATG ACC TCT TAG CCC TTC ATT TCA GCC GAA AGC ACA TCT TTC GGG GGC T

MET10.9	55	FAM- CTG ACT rATG ACC TCT TAG CCC TTC ATC TCA GCC GAA AGC ACA TCT TTC GGG GGC T
MET10.10	55	FAM- CTG ACT rATG ACC TCT TTT TTT TTC ATT TCA GCC GAA AGC ACA TCT TTC GGG GGC T

Table 1. Sequences used. Sequences are written 5'-3'. Abbreviations include: N_{40} (N bold), adenosine ribonucleotide (rA in blue), fluorescein-dT (F in green), DABCYL-dT (Q in red), 6-FAM (fluorescein) (FAM in green), γ-[32P] (P in orange). Underlined bases in the DNAzymes denote the substrate sequence. Bases colored red in 4TFP – CACAT sequences represent changes from the original parent sequence of 112 bases. Bases with a strikethrough in 4TFP – CACAT sequences were cut from the original parent sequence of 112 bases. Bases colored red in S1T-TL1 sequences represent the 4T's of 4TFP. All sequences from S1T-TL1 were based on 4TFP (99 bases), not the original parent sequence of 112 bases. Bases with a strikethrough in S1T-TL1 sequences were cut from the 4TFP sequence. All MET sequences were also based on 4TFP, while all MET 10.1-10.10 sequences were based on MET10 (55 bases).

2.2 Bacterial strains and culture conditions.

L. pneumophila was cultured from a frozen stock (ATCC® 33152™, 33154™, 33155™) on phosphate buffered charcoal yeast extract (BCYE) agar plates for 3-4 days in a 37°C incubator, as previously described[29,30]. The following bacteria were cultured by Dr. Malene Urbanus: *Legionella micdadei*, *Legionella dumofii*, *L. pneumophila* (Philly -1 CDC), and *Legionella longbeachae. L. dumofii* and *L. micdadei* had OD_{600} of 2 at the time of testing, while *L. longbeachae* and *L. pneumophila* had OD_{600} of 1.2 and 1.6,

respectively. Dr. Urbanus also cultured the following *L. pneumophila* strains: Philly, Paris, 130b, Lens, and Toronto-2005. At the time of testing the OD_{600} were: 2.6 and 1 for Philly, 1.4 for Paris, 1 for 130b and Lens, and 1.4 for Toronto-2005. When visualized under the microscope Lens was filamentous, some filamentation was observed for Philly and 130b, and no filamentation was seen for Paris and Toronto-2005.

2.3 Preparation of CEM from bacterial strains.

After growing on BCYE plates for 3-4 days, a single colony of each *L. pneumophila* strain was inoculated in 5 mL of buffered yeast extract (BYE) until OD_{600} reached ~ 1. The bacterial culture was then transferred into new microcentrifuge tubes and centrifuged at 6000 rpm at 4°C for 5 min. The supernatant, now termed CEM-LP, was recovered and passed through a 0.22 µm filter using a syringe. CEM was aliquoted into microcentrifuge tubes and stored at -80°C until further use. All other bacteria from this study were grown according to their designated growth conditions and CEM was prepared as described. CEM of other bacteria were kindly provided by Dingran Chang.

2.4 *In vitro* selection.

In vitro selection was performed by lab technician Jim Gu, as previously described[24,31–33]. Briefly, 500 pmol of LP3 was phosphorylated (reaction volume: 50µL) with 30 units of T4 polynucleotide kinase (PNK) for 30 min at 37°C in 10x PNK buffer (Thermo Scientific). This was followed by ethanol precipitation. Equimolar LP3T and FQ30 along with ddH_2O were then added to the resuspended pellet and the mixture was heated at 90°C for 1 min and cooled to room temperature for 10 min. Then, 10 µL

of 10X T4 DNA ligase buffer (Thermo Scientific) was added followed by 15 units of T4 DNA ligase (reaction volume: 100 µL) and incubated at room temperature for 2 h The DNA molecules in the mixture were concentrated by ethanol precipitation and the ligated FQ30-LP3 molecules were purified by 10% dPAGE. The purified FQ30-LP3 was dissolved in 125 µL of 2x selection buffer (SB) (100 mM HEPES, pH 7.0, 300 mM NaCl, 30 mM $MgCl_2$) along with equal amounts (33.3 µL) of BYE, CEM-*Pseudomonas aeruginosa*, CEM- *Klebsiella pneumoniae* and the volume was adjusted to 250 µL with ddH_2O. This mixture was incubated at room temperature overnight. After ethanol precipitation, the un-cleaved FQ30-LP3 molecules were purified by 10% dPAGE and the pellet stored at -20°C until further use. Thirteen point three µL of CEM-LP serotypes 1, 2, and 3 were mixed with 50 µL of 2x SB and added to 100 pmol of the un-cleaved FQ30-LP3 molecules (reaction volume: 100 µL). This mixture was incubated at room temperature for 2 h. After ethanol precipitation, the cleaved fragment was purified by 10% dPAGE and used as the template for PCR. The percentage of cleaved FQ30-LP3 was also determined and used to measure the progress of selection. The PCR1 mixture (50 µL) contained 5 µL of the template prepared above, 0.5 µM each of LP3-F and LP3-R, 200 µM each of dNTPs (dATP, dCTP, dGTP and dTTP), 10x PCR buffer (500 mM KCl, 100 mM Tris HCl (pH 9.0 at 25°C), 15 mM $MgCl_2$, 1% Triton X-100) and 2.5 units of *Thermus thermophilus* DNA polymerase (GenScript). The DNA was amplified using the following thermocycling steps: 95°C for 1 min; 8 cycles of 95°C for 45 s, 52°C for 45 s, and 72°C for 45 s. For the PCR2 reaction, 50 µL of the PCR1 product was diluted with ddH_2O to 250 µL in a bulk amplification using primers LP3-F and LP3-R-SP18 and the same protocol for PCR1 for a total of 15 cycles. The LP3 strand was purified by 10%

dPAGE (yield approximately 400-500 pmol) and used for the next selection round. A total of 11 cycles of selection were conducted. The DNA population from round 11 was cloned and sequenced.

2.5 Re-selection.

Procedures were performed similarly to *in vitro* selection. Re-selection was performed by former Li lab student Suraj Gopinathbirla. Typically, 300 pmol of LP3Z was phosphorylated (reaction volume: 100 µL) with non-radioactive ATP (final concentration 10 mM), and 20 U of PNK for 30 min at 37°C in 1x PNK buffer. The reaction was quenched by heating the mixture at 90°C for 10 min. This was followed by ethanol precipitation. Equimolar LP3T and FQ30 along with ddH_2O were then added to the resuspended pellet and the mixture was heated at 90°C for 1 min and cooled to room temperature for 10 min. Then, 20 µL of 10X T4 DNA ligase buffer (Thermo Scientific) was added followed by 20 units of T4 DNA ligase (reaction volume: 200 µL) and incubated at room temperature for 2 h The DNA molecules in the mixture were concentrated by ethanol precipitation and the ligated FQ30-LP3Z molecules were purified by 10% dPAGE. The purified FQ30-LP3Z was dissolved in 50 µL of 2x along with equal amounts (10 µL) of BYE, CEM-*Pseudomonas aeruginosa*, CEM- *Klebsiella pneumoniae* (reaction volume: 100 µL). This mixture was incubated at room temperature overnight. After ethanol precipitation, the un-cleaved FQ30-LP3Z molecules were purified by 10% dPAGE and the pellet stored at -20°C until further use. Ten µL of CEM-LP serotypes 1, 2, and 3 were mixed with 50 µL of 2x SB and added to 100 pmol of the un-cleaved FQ30-LP3Z molecules (reaction volume: 100 µL). This mixture was incubated at room temperature for 2 h for round 1, 1 h for rounds 2-5, and

30 min for rounds 6-10. After ethanol precipitation, the cleaved fragment was purified by 10% dPAGE and used as the template for PCR. The percentage of cleaved FQ30-LP3Z was also determined and used to measure the progress of selection. The PCR1 mixture (50 µL) contained 5 µL of the template, 0.5 µM each of LP3-F and LP3Z1-R, 200 µM each of dNTPs (dATP, dCTP, dGTP and dTTP), 10x PCR buffer (500 mM KCl, 100 mM Tris HCl (pH 9.0 at 25°C), 15 mM $MgCl_2$, 1% Triton X-100) and 2.5 units of *Thermus thermophilus* DNA polymerase (GenScript). The DNA was amplified using the following thermocycling steps: 95°C for 1 min 10-14 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 30 s. For the PCR2 reaction, 60 µL of the PCR1 product was diluted with ddH_2O to 3000 µL in a bulk amplification using primers LP3-F and LP3Z1-R-SP18 and the same protocol for PCR1 for 10-14 cycles. The LP3Z strand was purified by 10% dPAGE (yield approximately 400-500 pmol) and used for the next selection round. A total of 10 cycles of selection were conducted. The DNA population from round 10 was cloned and sequenced.

2.6 Construction of substrate-DNAzyme cis constructs.

The catalytic domain was first phosphorylated with PNK. Typically, 600 pmol of the catalytic domain was combined with 20 units of PNK, 10X PNK buffer, and ddH_2O (reaction volume: 100 µL). The reaction mixture was incubated at 37°C for 30 min, quenched at 90°C for 5 min, then ethanol precipitated with 2.5x volume 100% ethanol. The remaining ethanol was evaporated on a 90°C heat block. The phosphorylated catalytic domain was then combined with 600 pmol of template, 600 pmol of substrate, water, and heated for 1 min at 90°C then allowed to cool at room temperature for 10-15

min. Following this cooling period, 20 units of ligase and 10X ligase buffer was added and the reaction (400 µL, total volume) was incubated at room temperature for 2 h. After incubation the reaction was ethanol-precipitated and purified by 10% dPAGE. Construction of LpP involved a small-scale phosphorylation whereby 5 pmol of the catalytic domain was phosphorylated (reaction volume: 10 µL) with 5 µCi [γ-^{32}P]ATP (Perkin Elmer) and 10 units of PNK in 10x PNK buffer at 37°C for 40 min. Thirty-six µL of ddH_2O and 4 µL of PNK buffer was then added to the mixture resulting in a 50 µL final volume. This mixture was ethanol precipitated with 100% ethanol only and the remaining ethanol was evaporated on a 90°C heat block. The phosphorylated catalytic sequence was then combined with 5pmol of splint, 5pmol of substrate, and water and heated for 1 min at 90°C then allowed to cool at room temperature for 10-15 min. Following this cooling period, 5 units of ligase and 10X ligase buffer was added and the reaction (20 µL, total volume) was incubated at room temperature for 2 h. Remaining steps follow same protocol as described above.

2.7 Cleavage reactions.

Typically, the DNAzyme (1 µL of 1 µM stock) and 2x SB were combined and heated at 90°C for 1 min then allowed to cool at room temperature for 10-15 min. After cooling, 4 µL of CEM was added and the reaction (10 µL, total volume) was incubated at room temperature for a specified period. After the designated incubation time the reaction was terminated by the addition of 2x quenching buffer (QB) containing 60 mM EDTA, 7M urea and loading dye solution. For sequences shorter than 80 bases ethanol precipitation was performed at reaction completion followed by the addition of 2x QB.

The cleaved DNA products were separated from the un-cleaved by 10% dPAGE and images of the gel were obtained using a Typhoon 9200 variable mode imager (GE Healthcare). Imaging parameters were set as follows: emission filter: 526 SP Fluorescein, Cy2, AlexaFluor488; laser: blue (488nm); PMT: 400; Focal plane: +3; 200 pixels. The images were analyzed using Image Quant software and the percent cleavage for each DNAzyme was calculated using the following formulas: %Clv = (F Clv/6)/[(F Clv/6)+ FUnclv] for Lp1 and % Clv = (F Clv)/(F Clv + FUnclv) for all other DNAzymes. FClv: volume of cleaved band; FUnclv: volume of un-cleaved band. The quencher molecule can only quench the fluorescence of the fluorophore when in close enough proximity to the fluorophore, approximately 100 angstroms[34]. This interaction is not perfect and therefore some fluorescence is still observed even when the quencher is neighbouring the fluorophore. To account for this, we divide by 6, as previously determined experimentally[35].

2.8 Cooling tower water cleavage reactions.

For the cooling tower water tests, a master mixture containing 0.1 µM of Lp5 DNAzyme and 10x SB was heated at 90°C for 1 min then allowed to cool at room temperature for 10-15 min. Each cooling tower was subject to a positive test containing 5 µL of cooling tower water, 3 µL of CEM-LP serotype 1, 1 µL of Lp5, and 1 µL of 10x SB (10µL total volume) and a negative test containing 8 µL of cooling tower water, 1 µL of Lp5 and 1 µL of 10x SB (10 µL total volume). The positive and negative tests were conducted in duplicate for each cooling tower and were incubated at room temperature for 1 h. After the 1 h incubation period the reaction was terminated via the addition of 2x

QB. The cleaved DNA products were separated from the un-cleaved by 10% dPAGE and images of the gel were obtained using an Amersham Typhoon. Imaging parameters were set as follows: Filter-Cy2, laser- 488nm, Auto PMT, 100 pixels. The images were analyzed using Image Quant software and the percent cleavage for each DNAzyme was calculated using the following formula: % Clv = (F Clv)/(F Clv + FUnclv). Water from each cooling tower was sent for culturing to determine if it contained *L. pneumophila* and a report for each cooling tower detailing the treatment reagents and doses was completed.

2.9 Kinetic analysis of DNAzymes.

All cleavage reactions were conducted in a 400 µL master mixture containing 0.1 µM of DNAzyme. The DNAzyme, 2x SB (200 µL) and water was heated for 1 min at 90°C and cooled at room temperature for 15 min. After cooling CEM (160 µL) was added and the reaction (400 µL, total volume) was incubated at room temperature for specified time points, either: 1, 5, 10, 15, 30, 60, 90, 120, 240, 480, 720 min or 0.5, 1, 2.5, 5, 10, 15, 30, 60, 120 min. Ten µL was withdrawn from the master mixture at each given timepoint in triplicate and quenched with quenching buffer. The cleavage products from a reaction time course were separated by 10% dPAGE and quantified using a Typhoon 9200 variable mode imager (GE Healthcare) and Image Quant software. Imaging parameters were set as follows: emission filter: 526 SP Fluorescein, Cy2, AlexaFluor488; laser: blue (488nm); PMT: 400; Focal plane: +3; 200 pixels. Observed rate constants were determined by curve-fitting the percent cleavage of the DNAzyme in the presence of *L. pneumophila* CEM versus reaction time using Prism (GraphPad, 4.03) where Y = Ymax

[1-e^{-kt}], Ymax represents the maximal cleavage yield and k is the observed first-order rate constant (kobs).

3 Results

3.1 *In vitro* selection produced a DNAzyme that cleaves in the presence of *L. pneumophila.*

A DNA library containing 40 random nucleotides was used to isolate a DNAzyme that can cleave in the presence of *L. pneumophila*. Prior to the start of selection, CEM was prepared for *L. pneumophila* serogroups 1, 2, 3 (LP), *Pseudomonas aeruginosa* (PA) and *Klebsiella pneumoniae* (KP). The DNA library was first incubated with CEM-PA, CEM-KP and BYE at room temperature overnight, representing the negative selection step (Figure 1).

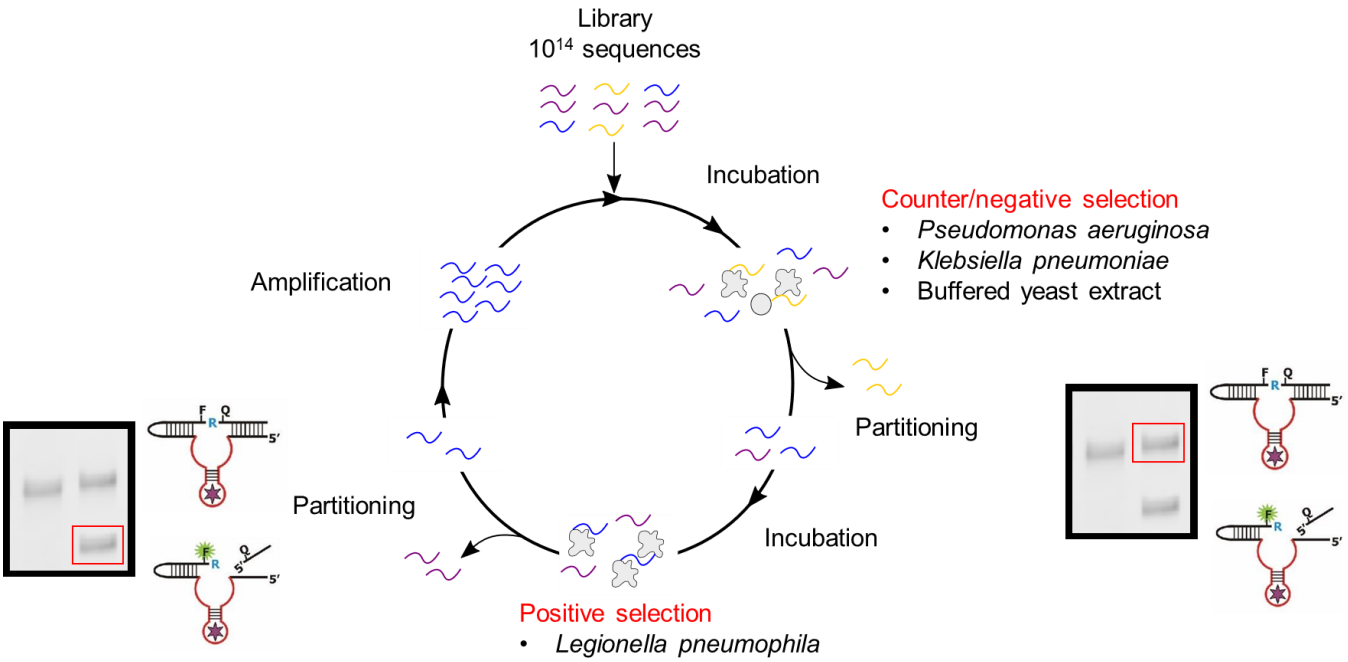


Figure 1. *In vitro* selection schematic for the isolation of a DNAzyme specific for *L. pneumophila*. The selection began with a library containing 10^{14} unique sequences. The library was first incubated in a counter/negative selection step to eliminate any non-specific cleaving sequences. The non-specific sequences were separated from the other sequences in the library by dPAGE, whereby sequences that did not cleave (top band) were excised from the gel and carried forward in the selection. The sequences that did not demonstrate any cleavage activity in the counter/negative selection were then incubated with the desired bacteria, *L. pneumophila*. The sequences that were cleaved by *L. pneumophila* (bottom band) were partitioned from those that do not, again via excision from dPAGE, and amplified. One completion of the circle represents one round of selection. Eleven rounds of selection were conducted, with the negative/counter selection applied at rounds 2, 4, 6, 8, 10.

The un-cleaved DNA molecules were purified and then incubated with CEM-LP for 2 h at room temperature, representing the positive selection step. Following incubation, the cleaved DNA molecules were purified by dPAGE. The negative and positive selection steps constituted the first round of selection. In total, 11 rounds of selection were conducted with the negative selection step included every other round. The 11th DNA pool was sequenced and four DNAzyme classes were discovered. A representative sequence from each class was chosen and the activity of each sequence towards CEM-LP was assessed. After a 1 h incubation at room temperature with CEM-LP a maximum percent cleavage of 15% was observed (data collected by Jim Gu). This DNAzyme demonstrating the highest cleavage activity was named Lp1 and chosen for further investigation.

3.2 Reselection failed to isolate a more active DNAzyme but Lp1 sequence can be truncated.

Mutagenic reselection was performed on the Lp1 sequence to identify more active sequences. Briefly, the DNA library containing the Lp1 sequence was mutated by 30% and the selection conditions were made more stringent in the following ways: the incubation time for the positive selection was reduced to 1 h in round two, and down to 30 min in round six. Similar to the original selection, a negative selection step was employed using CEM-PA, CEM-KP and BYE. Furthermore, the CEM-LP was concentrated 5 times by evaporation to ensure that target concentration was not a limiting factor in the isolation of faster sequences. In total, 9 rounds of reselection were

carried out and the 9th DNA pool was sequenced. Unfortunately, the reselection did not result in any sequences with improved activity over the original Lp1 sequence.

To reduce the size of Lp1 for facilitation in future applications, various sequence truncations were performed (Table 1). Previous data had suggested that removal of the internal fluorophore and quencher (F&Q) modified nucleotides present in Lp1 enhanced DNAzyme activity towards CEM-LP (Figure 2).

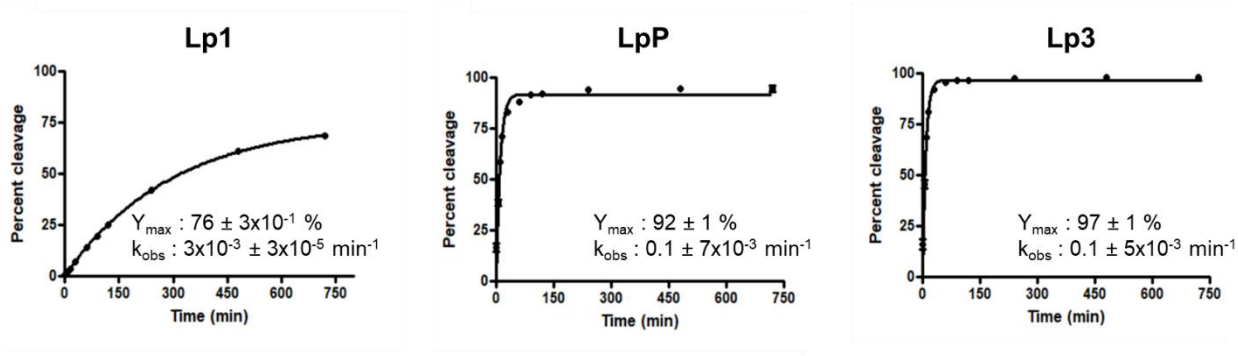


Figure 2. Removal of fluorophore and quencher modifications enhances DNAzyme activity towards *L. pneumophila*. Time course experiments were performed with the DNAzyme in the *cis* conformation. The percent cleavage of the DNAzyme after 1 h incubation at room temperature with CEM-LP was plotted over time and the data was fit using the equation $Y = Y_{max}[1-e^{-kt}]$ with Prism (GraphPad, 4.03). Three trials were performed for each DNAzyme. Observed rate constants (k_{obs}) and maximum cleavage yields (Y_{max}) are reported in the table.

Therefore, to assess the sequence truncations at the highest possible activity, a new DNAzyme containing the same full-length sequence of Lp1 but with a 5' fluorophore modified nucleotide rather than internal F&Q modified nucleotides was designed and termed Lp5. Similarly, another DNAzyme containing the same full-length sequence of Lp1 but with a 3' fluorophore modified nucleotide rather than internal F&Q modified nucleotides was designed and termed Lp3 for use in future studies (Figure 3).

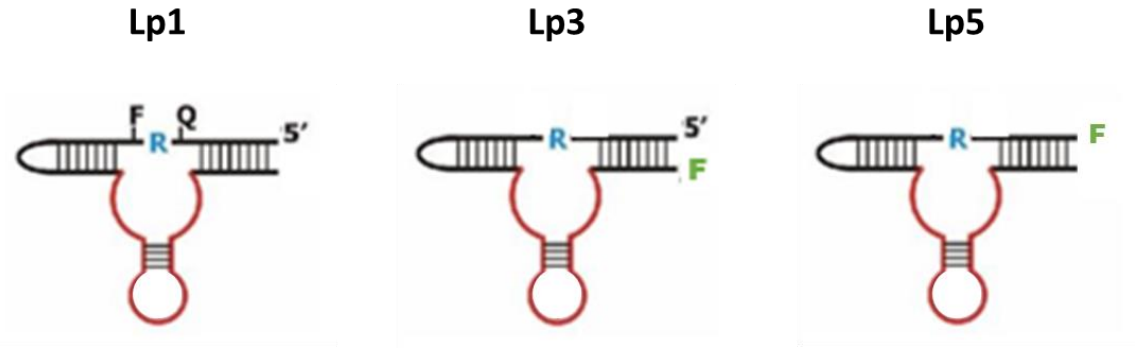


Figure 3. DNAzyme graphics of Lp1, Lp3, and Lp5. Illustration of the three primary DNAzymes used for characterization. Lp1 contains internal fluorophore and quencher modified nucleotides that flank the ribonucleotide. Lp3 and Lp5 contain a 3' and 5' FAM, respectively.

After 1-hour incubation at room temperature with CEM-LP, Lp5 exhibits approximately 90% cleavage, in comparison to the 15% observed with Lp1 under the same conditions. Preliminary truncation of the sequence based on the predicted secondary structure revealed regions which could potentially be minimized. Most notably of all the sequence truncations, 17 bases in the forward primer region could be replaced with 4T's while maintaining the high cleavage activity (90%) observed with the full-length Lp5 sequence. This sequence was named 4TFP and is 99 bases in comparison to the original 112 base Lp1 sequence. Following the reselection, the sequencing data was used to examine the percent conservation of each base position in the 4TFP sequence construct. Using Excel, the top 50 sequence candidates from the reselection were examined. Considering the parent sequence had been mutated by 30%, base positions that were higher than 70% conserved were considered essential, and bases with less than 70% conservation were considered non-essential. With these parameters in mind, several more truncations were rationally predicted, and their activities were assessed using gel electrophoresis. Ultimately, one truncated sequence known as MET10 showed the best activity at 1 h (82%) and was minimized to 55 bases. The predicted secondary structure is shown in Figure 4.

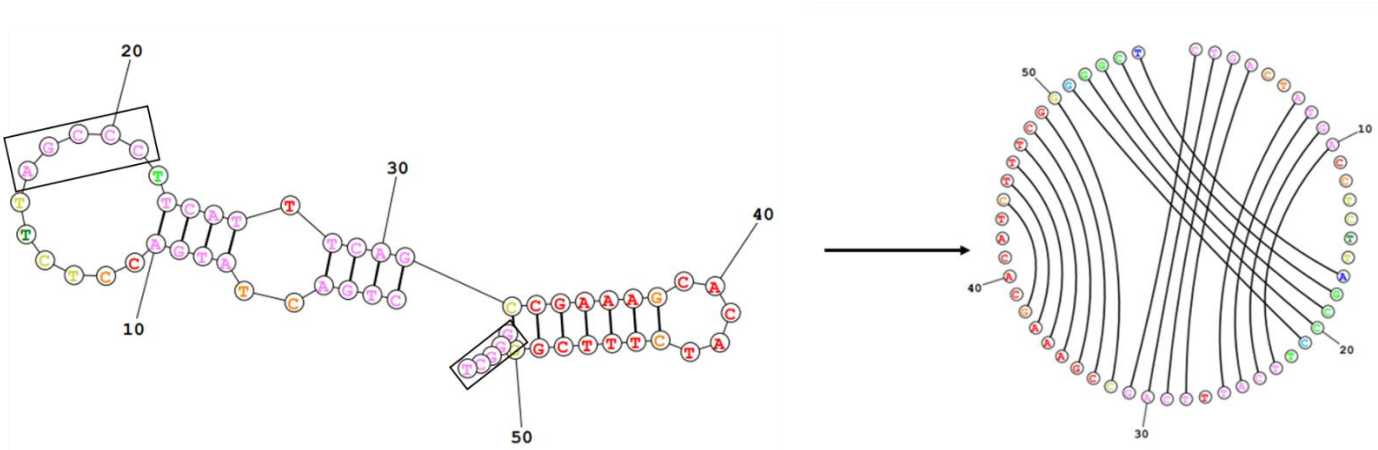


Figure 4. Predicted secondary structure of MET10 contains a pseudoknot. The MET10 sequence was processed in RNAStructure[36] using the default parameters. The predicted secondary structure has a ΔG of -9.4. Colors of bases represent percent probability of accuracy: red (>99%), orange (99>95%), yellow (95>90%), dark green (90>80%), neon green (80>70%), light blue (70>60%), dark blue (60>50%), pink (50%>). A pseudoknot was predicted as shown with the 5 bases at the 3' end (in black box) forming a pseudoknot with 5 bases of the large loop (in black box), suggesting DNAzymes can fold into complex tertiary secondary structures characteristic of enzyme active sites

3.3 Truncated sequences exhibit variable RNA-cleaving activity when incubated with fresh or frozen CEM-LP

The RNA-cleaving activity of the MET truncated sequences incubated with fresh CEM-LP, or frozen CEM-LP that had been stored in the -80°C and thawed prior to use, was assessed following a 1h incubation. The difference in percent cleavage between the frozen CEM-LP and fresh CEM-LP is shown in Figure 5.

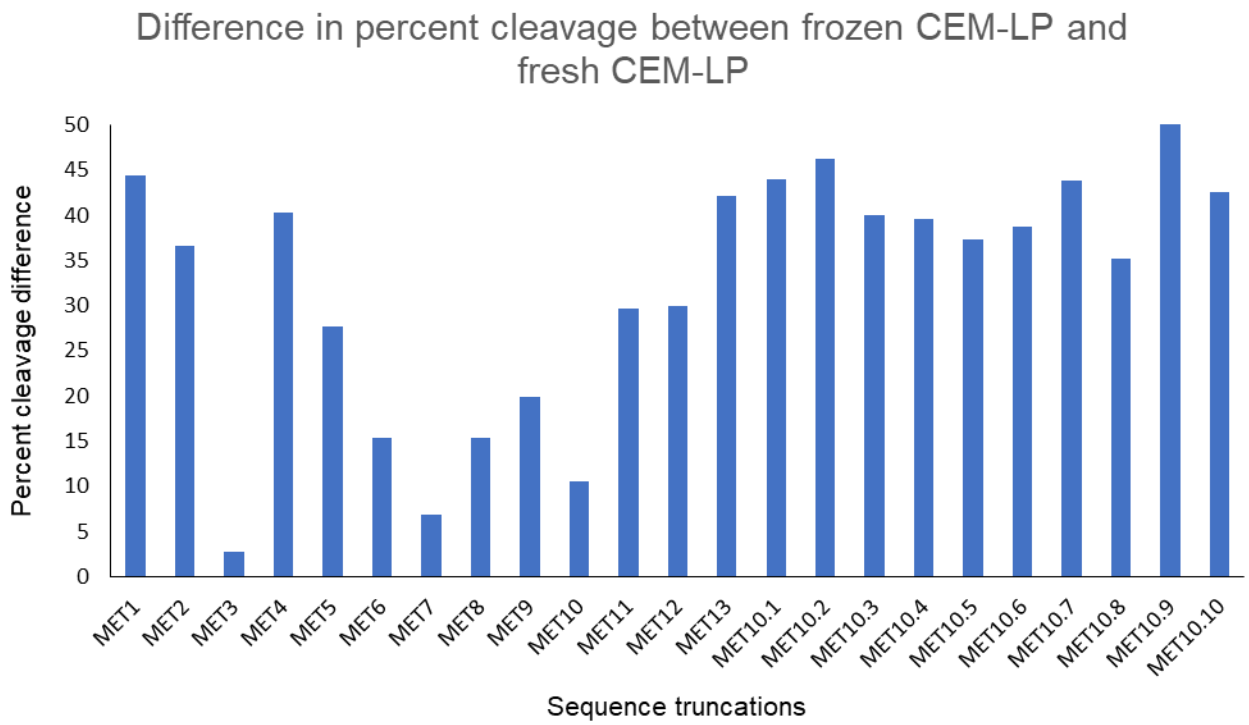
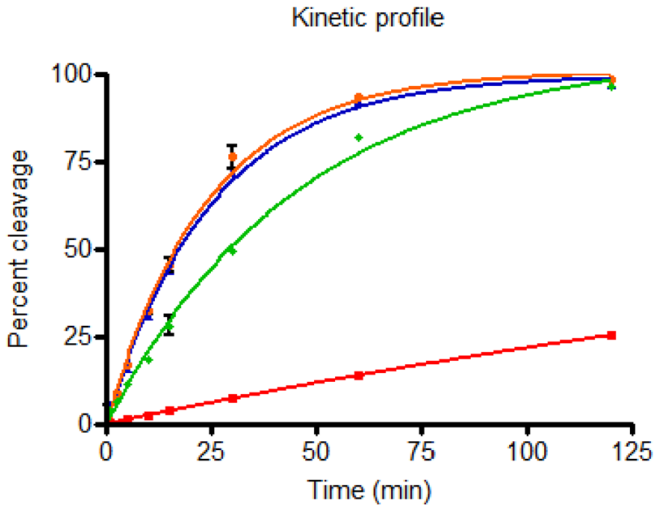


Figure 5. Truncated sequences exhibit increased RNA-cleaving activity when incubated with fresh CEM-LP. The RNA-cleaving activity of each truncation in the presence of fresh or frozen CEM-LP, following a 1 h incubation at room temperature, was determined by gel electrophoresis. Each test was conducted in duplicate, with the average percent cleavage for each truncation in either fresh or frozen CEM-LP collected. The difference in percent cleavage for each sequence truncation was calculated and plotted above using the following formula: average % clv fresh CEM-LP – average % clv frozen CEM-LP.

3.4 Removing the internal fluorophore and quencher modified nucleotides increased DNAzyme cleavage rate whereas reducing sequence size did not significantly impact cleavage rate.

The kinetic profiles of Lp1, Lp5, 4TFP and MET10 were established to examine how sequence truncation and removal of the internal F&Q modified nucleotides influenced the DNAzyme cleavage rate (Figure 6).



DNAzyme	Y_{max} (%)	K_{obs} (min^{-1})
Lp1	80 ± 4	$3x10^{-3} \pm 2x10^{-4}$
Lp5	99 ± 2	$4x10^{-2} \pm 2x10^{-3}$
4TFP	100 ± 2	$4x10^{-2} \pm 2x10^{-3}$
MET10	106 ± 4	$2x10^{-2} \pm 2x10^{-3}$

Figure 6. DNAzyme MET10 demonstrates comparable cleavage rate to Lp5 and 4TFP. Time course experiments were performed with the DNAzyme in the *cis* conformation. The percent cleavage of the DNAzyme after 1 h incubation at room temperature with CEM-LP was plotted over time and the data was fit using the equation $Y = Y_{max} [1-e^{-kt}]$ with Prism (GraphPad, 4.03). Two trials were performed for each DNAzyme. Observed rate constants (k_{obs}) and maximum cleavage yields (Y_{max}) are reported in the table.

A master mix for each DNAzyme was prepared, with the addition of CEM-LP marking the start time. Aliquots were removed at each timepoint and quenched, then analysed using denaturing polyacrylamide gel electrophoresis (dPAGE). The percent cleavage for each timepoint was calculated and fit to a curve over time. The following rates were determined: $3x10^{-3}$ for Lp1, $4x10^{-2}$ min^{-1} for Lp5, $4x10^{-2}$ min^{-1} for 4TFP, and $2x10^{-2}$ min^{-1} for MET10.

3.5 The RNA-cleaving activity of Lp1 was highly specific for *L. pneumophila*, whereas the RNA-cleaving activity of Lp3 was non-specific for *L. pneumophila* but was improved with the addition of RNase inhibitor.

The recognition specificity of Lp1, and Lp3 were investigated against the CEM of 26 species of bacteria, approximately half of which belong to the *Enterobacteriaceae* family (Figure 7).

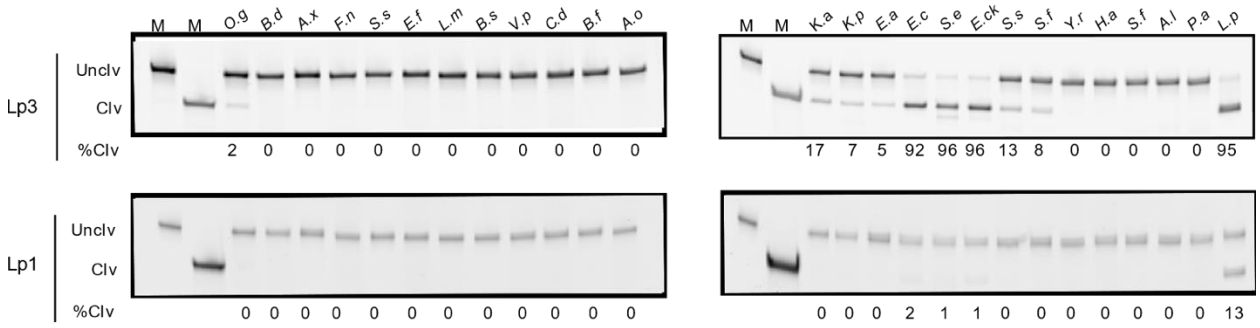


Figure 7. Presence of fluorophore and quencher modified nucleotides affords specificity to Lp1. The RNA-cleaving activity of Lp3 and Lp1 in response to various species of bacteria was determined by gel electrophoresis. Marker lanes comprise the full-length un-cleaved Lp3 or Lp1 sequence, and the cleaved Lp3 or Lp1 sequence, respectively. Lanes 1-26: *Ochrobactrum gringonese, Brevundimonas diminuta, Achromobacter xylosoxidans, Fusobacterium nucleatum, Streptococcus salivarius, Enterococcus faecium, Listeria monocytogenes, Bacillus subtilis, Veillonella parvula, Clostridium difficile, Bacterioied fagillis, Actinomyces orientalis, Klebsiella aerogenes, Klebsiella pneumoniae, Enterobacter aerogenes, Enterobacter cloacae, Salmonella enterica, Escherichia coli k12, Shigella sonnei, Shigella flexneri, Yersinia ruckeri, Hafnia alvei, Serratia fonticola, Acinetobacter lwoffii, Pseudomonas aeruginosa,* and *Legionella pneumophila* respectively. Unclv and Clv denote un-cleaved DNAzyme and cleaved DNAzyme. Incubation time: 1 h.

Following a 1 h incubation at room temperature with the CEM of all 26 species, only CEM-LP was capable of cleaving Lp1. Lp3 however, was cleaved by 8 other CEM, in addition to CEM-LP. To examine whether the observed cleavage was generated by RNases that may exist in the CEM, RNase inhibitor was add ed to CEM-LP and to the CEM of the 8 other species capable of cleaving Lp3 (Figure 8).

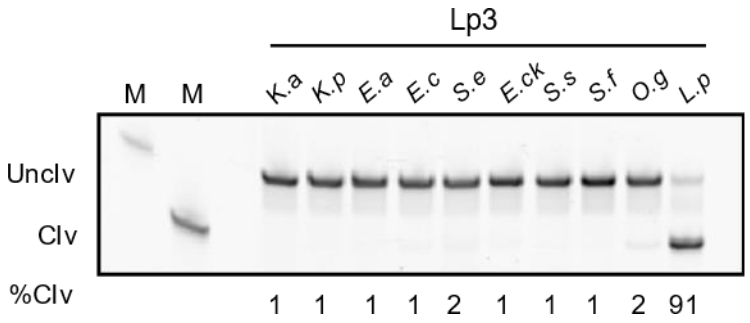


Figure 8. Addition of RNase inhibitor improves specificity of Lp3. The RNA-cleaving activity of Lp3 in the presence of RNase inhibitor towards species capable of inducing non-specific cleavage was determined by gel electrophoresis. Marker lanes comprise the full-length sequence, and the cleaved sequence, respectively. Lanes 1-10: *Klebsiella aerogenes, Klebsiella pneumoniae, Enterobacter aerogenes, Enterobacter cloacae, Salmonella enterica, Escherichia coli k12, Shigella sonnei, Shigella flexneri, Ochrobactrum gringonese*, and *Legionella pneumophila*. Unclv and Clv denote un-cleaved DNAzyme and cleaved DNAzyme. Incubation time: 1 h.

After a 1 h incubation at room temperature with the CEM and RNase inhibitor, only CEM-LP was able to cleave Lp3. The specificity of Lp1 was reassessed, alongside 4TFP and MET10 (Figure 9).

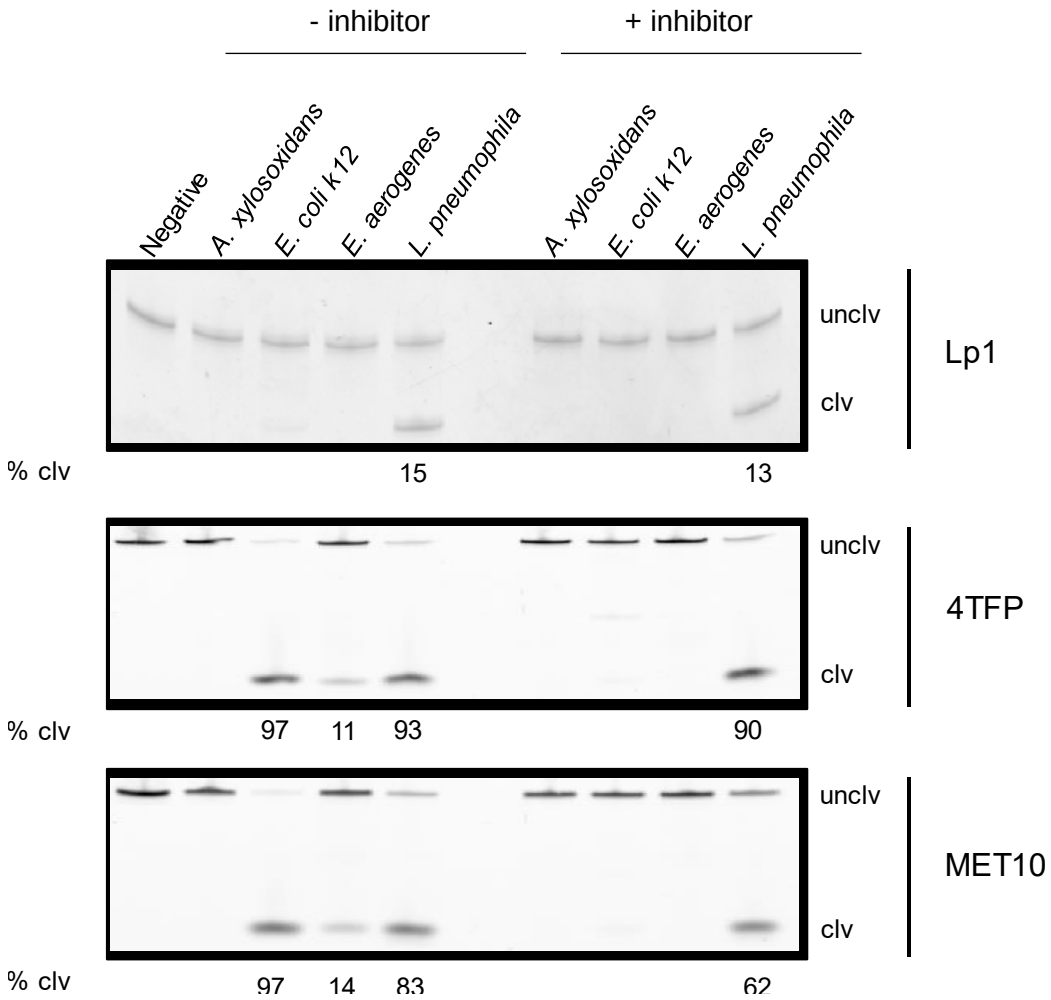


Figure 9. Presence of fluorophore and quencher modified nucleotides enhances the specificity of Lp1 but the addition of RNase inhibitor improves specificity of 4TFP and MET10. The RNA-cleaving activity (% clv) of Lp1, 4TFP, and MET10 in response to various species treated with and without RNAse inhibitor was determined by gel electrophoresis. Lane 1 comprises the full-length un-cleaved sequence, lanes 2-5: *Achromobacter xylosoxidans, Escherichia coli k12, Enterobacter aerogenes, Legionella pneumophila.* Unclv and clv denote un-cleaved DNAzyme and cleaved DNAzyme. Incubation time: 1 h.

Rather than evaluate the cleavage of the DNAzymes against all 26 species, 4 species were chosen as representatives for each class (no activity (<1% cleavage), high activity (>90% cleavage), low activity (<20% cleavage), desired bacteria for detection). The selected 4 species were: *Achromobacter xylosoxidans* which resulted in no cleavage activity by Lp3, *Escherichia coli* K12 which demonstrated high cleavage activity (96%) by Lp3, *Enterobacter aerogenes* which exhibited low cleavage activity (5%) by Lp3, and CEM-LP which is our desired bacteria for detection that demonstrated high cleavage activity (95%) by Lp3. The CEM of these 4 species were incubated for 1 h at room temperature with Lp1, 4TFP, and MET10, both in the absence and presence of RNase inhibitor. Only CEM-LP was capable of cleaving Lp1, while 4TFP and MET10 exhibited the same trends seen with Lp3.

3.6 Lp3 detects 10 cfu/µL after 72 h by dPAGE.

The sensitivity of Lp3 towards CEM-LP by dPAGE was investigated (Figure 10).

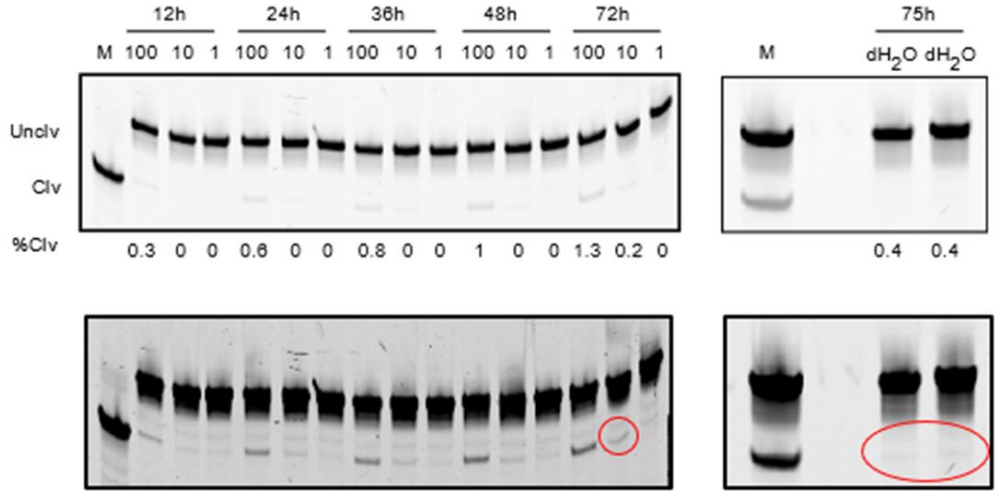


Figure 10. Lp3 was capable of detecting ~10 cfu/µL of *L. pneumophila* after 72 h incubation and is stable at room temperature over 75 h. A cleaved Lp3 was loaded as a control (lane 1). Lp3 was incubated with either 100, 10, or 1 (orders of magnitude) cfu/µL of *L. pneumophila* serotype 1 CEM for various timepoints in hours: 12, 24, 36, 48, 72, after which the RNA-cleaving activity of Lp3 was determined by gel electrophoresis. After 72-hour incubation with *L. pneumophila* serotype 1 CEM at a concentration of 10 cfu/µL a cleavage band is seen. Unclv and Clv denote un-cleaved DNAzyme and cleaved DNAzyme. Lp3 was incubated with water at room temperature over a period of 75 h and RNA-cleaving activity was determined by gel electrophoresis. No significant cleavage band was observed. The contrast for both gels shown was enhanced to facilitate visualization of the cleavage band, or lack thereof (red circles).

After a 12 h incubation at room temperature with CEM-LP, Lp3 can detect ~100 cfu/µL. After a 72 h incubation at room temperature with CEM-LP, Lp3 can detect ~10 cfu/µL. The stability of the DNAzyme at room temperature was also assessed. It was observed that after 75 h incubation at room temperature in water, Lp3 exhibits no cleavage.

3.7 CIM of *L. pneumophila* prepared using liquid nitrogen provided highest RNA-cleavage activity by Lp5 and demonstrated a comparable kinetic profile to CEM of *L. pneumophila.*

A procedure to isolate the crude intracellular mixture (CIM) from *L. pneumophila* without destroying the DNAzyme target was investigated. *L. pneumophila* culture was grown to an OD_{600} of ~ 1, harvested by centrifugation, the supernatant removed, and the pellet resuspended in 150 µL selection buffer. To lyse the pellet and isolate the intracellular mixture, five mechanical methods were used: 1) pass the pellet 3x through a 21 g needle, 2) pass the pellet 3x through a 18g needle, 3) centrifuge the pellet at 10,000g for 10 min, 4) immerse the pellet in liquid nitrogen for 2 min and thaw at 37°C for 10 min, and 5) subject the pellet to 3 cycles of: 15 min at -80°C, 10 min at 37°C (Figure 11).

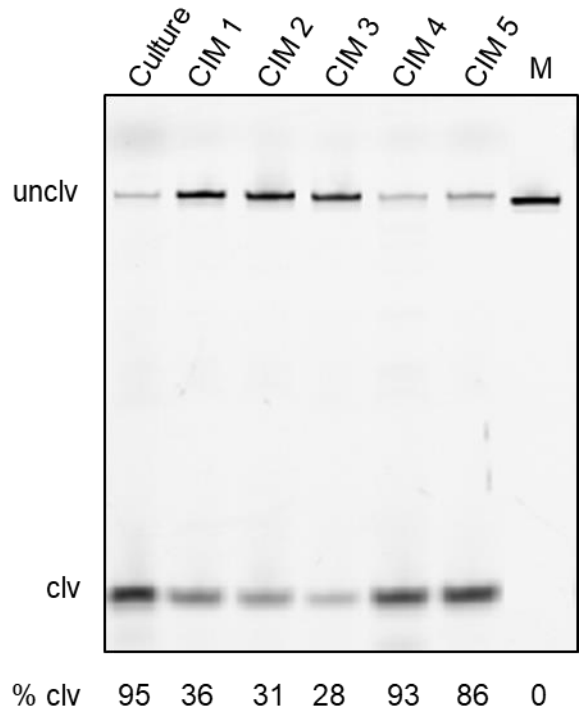
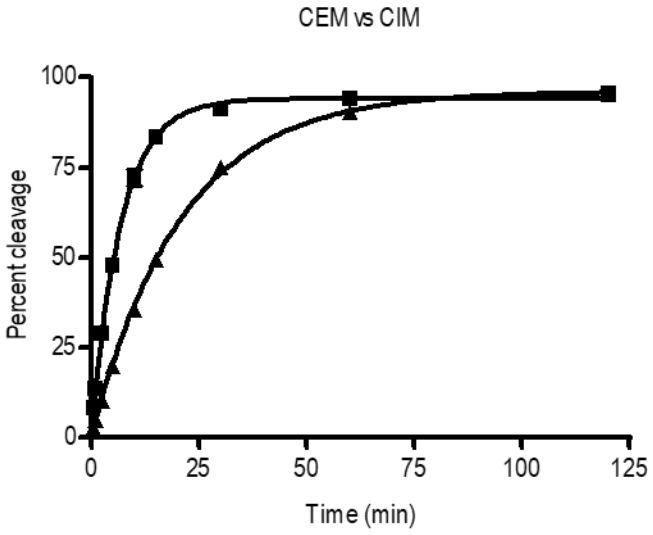


Figure 11. Preparation of *L. pneumophila* CIM using liquid nitrogen resulted in highest RNA-cleavage activity by Lp5. Lanes 1-7: *L. pneumophila* serotype 1 culture, CIM prepared via method 1 (21g needle), method 2 (18g needle), method 3 (centrifugation), method 4 (liquid nitrogen), method 5 (freeze-thaw cycles), or full-length un-cleaved Lp5 sequence (M). Unclv and clv denote un-cleaved DNAzyme and cleaved DNAzyme. Incubation time: 1 h.

Following lysis, the solution was recentrifuged, and the supernatant, now termed CIM collected. After a 1 h incubation at room temperature with each of the five CIM's, Lp5 exhibited the following RNA-cleavage activity: 36% via method 1, 31% via method 2, 28% via method 3, 93% via method 4, and 86% via method 5. Following the determination of an optimal CIM preparation method, the kinetics of *L. pneumophila* CEM and CIM were assessed using Lp5 (Figure 12).



	Y_{max} (%)	k_{obs} (min^{-1})
CEM	94 ± 1	$0.1 \pm 4x10^{-3}$
CIM	96 ± 1	$5x10^{-2} \pm 9x10^{-4}$

■ CEM
▲ CIM

Figure 12. Cleavage rate of Lp5 incubated with CIM is comparable to that of CEM. The percent cleavage of the DNAzyme in the presence of *L. pneumophila* CEM or CIM was plotted over time and the data was fit using the equation $Y = Y_{max} [1-e^{-kt}]$ with Prism (GraphPad, 4.03). Two trials were performed for each CEM and CIM. Observed rate constants (k_{obs}) and maximum cleavage yields (Y_{max}) are reported in the table.

Kinetic profiles were conducted as previously described in 4.3. The following rates were determined: 0.1 for CEM, and $5x10^{-2}$ min^{-1} for CIM. The activity of Lp5 towards *L. pneumophila* culture that has not been manipulated, rather simply grown to an OD_{600} of ~ 1, was also assessed via a 1 hour incubation at room temperature and demonstrated comparable RNA-cleaving activity to the CIM (Figure 11).

3.8 Lp5 DNAzyme cleaved only in cooling tower water spiked with *L. pneumophila*

The ability of Lp5 to maintain its activity in cooling tower water, rather than deionized water, was assessed. After 1 h incubation at room temperature in the presence of over 50 different cooling tower water environments (80% final volume), Lp5 demonstrated no cleavage activity, apart from one cooling tower, however this non-specific cleavage of Lp5 was mitigated after the reagents had the opportunity to circulate through the cooling tower (Figure 13).

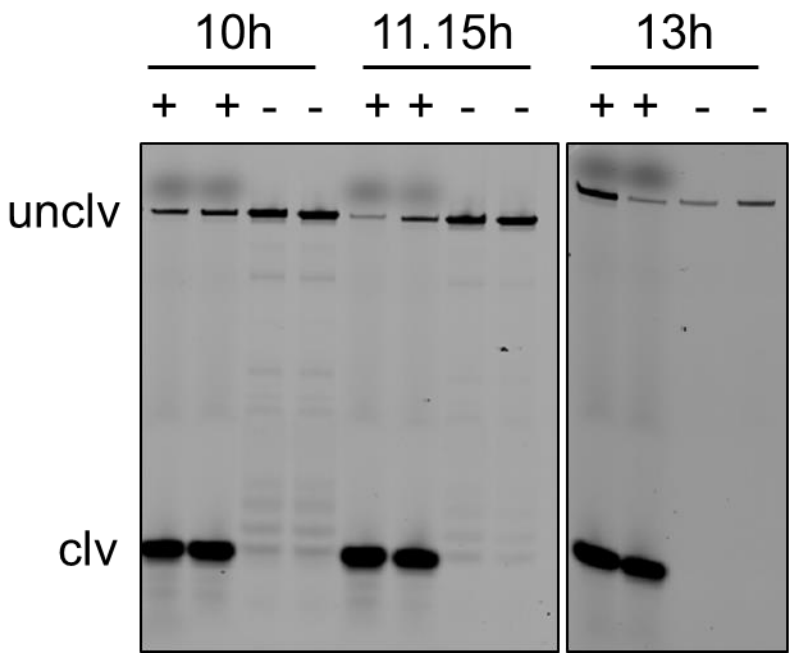


Figure 13. Lp5 demonstrates non-specific cleavage activity in cooling tower water sample not spiked with CEM-LP. The RNA-cleaving activity of Lp5 in the presence of cooling tower with (+) and without (-) CEM-LP was determined by gel electrophoresis. Three water samples from the same cooling tower were collected at different time points: 10 h, 11.15 h, 13 h. Upon arrival it was determined that the cooling tower water contained no treatment reagents and a large dose of oxidizing biocide (hypochlorous acid) was added and a water sample was taken (lanes 1-4). After allowing the biocide to circulate in the cooling tower for a short period of time another water sample was taken (lanes 5-8). Finally, after allowing the biocide to circulate in the cooling tower for a longer period a final water sample was taken (lanes 9-12). All incubations were conducted in duplicate. Unclv and clv denote un-cleaved DNAzyme and cleaved DNAzyme. The contrast for both gels shown was enhanced to facilitate visualization of the bands in the negative (no CEM-LP) lanes. Incubation time: 1 h.

After 1-h incubation at room temperature in the presence of the same 50 plus cooling tower water environments (50% final volume), spiked with CEM-LP, Lp5 was cleaved in all samples.

3.9 Lp5 recognized all *L. pneumophila* strains investigated and did not cleave in the presence of other *Legionella* species.

The RNA-cleaving activity of Lp5 towards other species of *Legionella* was assessed. Following a 1 h incubation at room temperature with the liquid culture of either: *Legionella micdadei*, *Legionella dumofii*, *Legionella pneumophila* (Philly -1 CDC), or *Legionella longbeachae*, only *L. pneumophila* was capable of cleaving Lp5 (Figure 14).

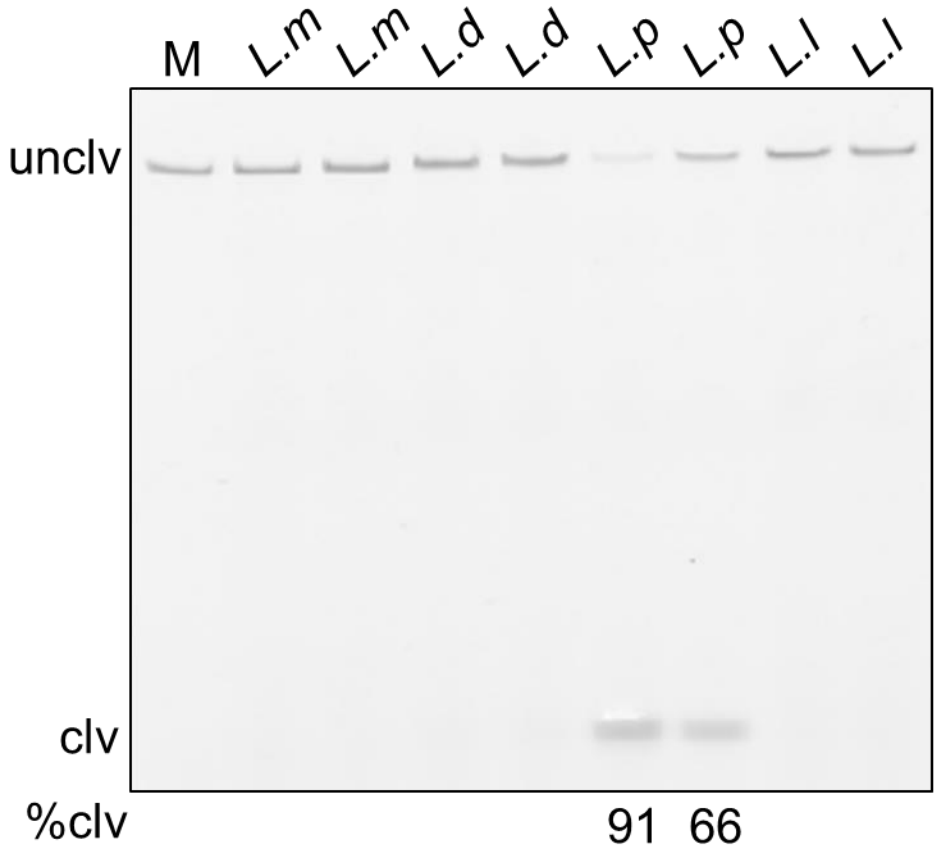


Figure 14. Lp5 only cleaves in the presence of *L. pneumophila*. The RNA-cleaving activity of Lp5 in the presence of other *Legionella* species was determined by gel electrophoresis. Marker lane comprises the full-length sequence. Lanes 1-8: *Legionella micdadei*, *Legionella dumofii*, *Legionella pneumophila* (philly -1 CDC), and *Legionella longbeachae*, tested in duplicate. Unclv and clv denote un-cleaved DNAzyme and cleaved DNAzyme. Incubation time: 1 h.

Following this species test, the RNA-cleaving activity of Lp5 towards other strains of *L. pneumophila* was investigated. After a 1 h incubation at room temperature with the liquid culture of *L. pneumophila* strains: Philly, Paris, 130b, Lens, and Toronto-2005, Lp5 was cleaved by all strains (Figure 15).

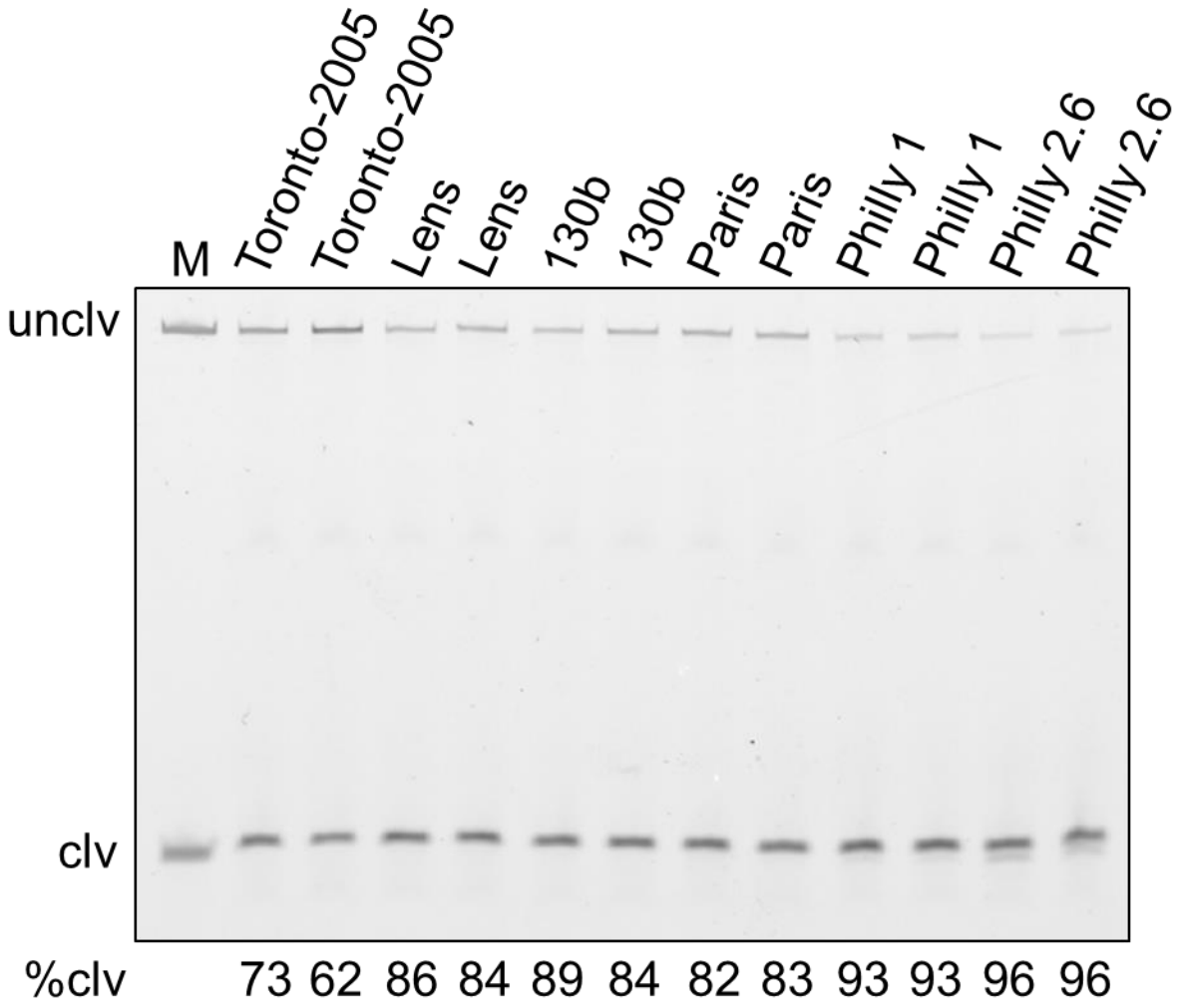


Figure 15. Lp5 cleaves in the presence of all *L. pneumophila* strains investigated.

The RNA-cleaving activity of Lp5 in the presence of other *Legionella* strains was determined by gel electrophoresis. Marker lane comprises the full-length cleaved sequence. Lanes 1-12: *L. pneumophila* str. Toronto-2005, *L. pneumophila* str. Lens, *L. pneumophila* str. 130b, *L. pneumophila* str. Paris, *L. pneumophila* str. Philly grown to OD_{600} 1, *L. pneumophila* str. Philly grown to OD_{600} 2.6 for comparison. All strains tested in duplicate. Unclv and clv denote un-cleaved DNAzyme and cleaved DNAzyme. Incubation time: 1 h.

4 Discussion

4.1 Analysis of DNAzyme secondary structure provides better understanding of essential nucleotides for *L. pneumophila* detection.

The use of a DNA library of over 100 nucleotides in length enables the selection process to access more complex secondary structures and survey a wider sequence composition. However, it is often the case that not all nucleotides present in the isolated DNAzyme sequence are required for catalysis. The removal of nucleotides from the DNAzyme and subsequent assessment of the activity of the shortened DNAzyme towards CEM-LP enabled the identification of nucleotides key for function. The truncations were designed in a systematic way, starting with the removal of nucleotides from the 3' end. Unique structural features such as stems and loops were also the subject of initial investigations. The identification of 4TFP followed such a workflow, whereas the design of all MET truncations was based on the percent composition of each base using the reselection deep sequencing data. Interestingly, MET10, which exhibits 82% cleavage after a 1 h incubation with CEM-LP, differs only from MET4, which exhibits 42% cleavage after a 1 h incubation with CEM-LP, by one base-pair substitution. The MET4 sequence contains an AT base pair, whereas in the MET10 sequence, substitution for a CG base pair resulted in a 40% increase in cleavage activity. When looking at the sequencing data, the A and T bases of the base pair in MET4 were only 30% and 14% conserved, respectively, in the top 50 sequence candidates of the reselection, suggesting that there is a more conserved, and likely more favorable base pairing. Upon further investigation, the C and G bases of the CG base pair at the same position as AT in MET4 were 68% and 46% conserved,

respectively, among the top 50 sequence candidates of the reselection, and therefore were substituted for analysis in MET10.

MET10 showed the best activity at 1 h (82%) in comparison to all the other truncated sequences. However, moving forward with the MET10 sequence for other experiments, it became apparent that the percent cleavage exhibited by MET10 was not consistent across the same 1 h timepoint. A new stock of MET10 was prepared but inconsistent cleavage continued to be observed. Given that the DNAzyme appeared not to be responsible, the CEM-LP was then investigated. An approximate 50% difference in the percent RNA-cleavage of MET10 by fresh vs frozen CEM was observed. To investigate whether this discrepancy in activity between fresh and frozen CEM was unique to MET10 or not, all the truncated MET sequences were re-assessed.

Figure 5 shows that most of the truncated sequences exhibit enhanced RNA cleavage values when incubated with fresh CEM-LP. MET7 demonstrates similar RNA-cleaving activity when incubated with fresh or frozen CEM-LP. The MET7 sequence is 101 bases in length, which approaches the length of the full length DNAzyme that is 112 bases. This suggests that although the full-length sequence can be truncated, and comparable RNA-cleavage activity to the full length can be observed, this activity is dependent on the storage of the CEM-LP and whether it is fresh or frozen. The truncated sequences appear more susceptible to CEM storage conditions.

Moving forward, these differences need to be taken into consideration when choosing which sequence to develop into a sensor. While truncated sequences are shorter, more cost effective and easy to work with, they can lack robustness when it comes to use in different sample conditions. Perhaps the differences in cleavage activity

between the truncated DNAzymes in fresh vs frozen CEM-LP are only observed in the current solution-based setup but may not be observed if the sequence is turned into an actual sensor.

4.2 Internal F&Q modified nucleotides may impact bacteria accessibility to ribonucleotide.

The high specificity of Lp1 towards CEM-LP after a 1 h incubation is likely due to the presence of the internal F&Q modified nucleotides, given that their removal results in non-specific cleavage activity seen in 4TFP and MET10, which have only an F-modified nucleotide at the 5’ end. The fluorescein (F) and dabcyl (Q) modifications are bulky molecules that could potentially influence access of the target to the ribonucleotide. This guarding is apparent when specificity is assessed, as Lp1 is highly specific whereas 4TFP and MET10 are not but is also observed when incubated with CEM-LP. The rate of Lp1 in comparison to 4TFP and MET10 is reduced approximately 10-fold, suggesting that even our desired *L. pneumophila* target has reduced accessibility to the ribonucleotide. When comparing Lp1 and Lp3, which have identical sequences differing only in the inclusion of an internal F&Q or external 3’ fluorophore, respectively, the Lp3 rate is 1000-fold greater than Lp1 towards CEM-LP. The inclusion of the F&Q modified nucleotides provides high specificity but reduced activity.

4.3 Non-specific activity exhibited by Lp3, 4TFP, and MET10 likely due to production of RNAse I by other bacteria.

The elimination of any non-specific cleavage by Lp3, 4TFP, and MET10 with the addition of RNase inhibitor suggests that the non-specific cleavage observed with these DNAzymes was likely due to RNases produced by other bacteria. The RNase inhibitor used can inhibit RNases A, B, C, I, and T1. Another RNase inhibitor was tested that inhibits only RNase A, B, C, but was unable to mitigate the non-specific cleavage activity observed (data not shown). This suggests that it is either RNase T1 or RNase I that is being inhibited in the CEM by the inhibitor. RNase T1 is fungal ribonuclease that is not expressed by bacteria, therefore RNase I is likely responsible for the observed Lp3, 4TFP, and MET10 non-specific cleavage activity. Furthermore, the RNase I protein sequences produced by the 8 species capable of inducing cleavage share high sequence identity. These species express similar RNases I that can both activate the DNAzymes and be inhibited by the addition of the RNase inhibitor.

4.4 CIM of *L. pneumophila* can be successfully prepared using liquid nitrogen

Typically, when preparing CIM, heat is used to lyse the pellet. However, after using heat to lyse the pellet to prepare CIM from *L. pneumophila*, no cleavage activity was observed by the DNAzyme (data not shown). Previous tests had revealed that the target produced by *L. pneumophila* that the DNAzyme is not heat stable. After 1 min incubation of *L. pneumophila* CEM at 90°C, no cleavage activity was observed by the DNAzyme (data not shown). Consequently, it is likely that employing heat to lyse the pellet for CIM destroyed the target and another method of lysing the pellet is required to

successfully isolate *L. pneumophila* CIM. Lysing methods that involved chemicals such as: β-Mercaptoethanol, lysozyme, and LDAO detergent, were investigated, however, again no cleavage activity was observed by the DNAzyme (data not shown). This suggests that neither heat nor chemical lysis of the pellet are good methods for preparing CIM. Therefore, mechanical lysis methods were evaluated next. Multiple freeze thaw cycles in and out of the -80°C freezer, and freeze thaw using liquid nitrogen could be used to the lyse the pellet without destroying the target in the process. Successful CIM preparation provides confirmation that the target is found both intra- and extracellularly. This knowledge is important for future applications because at low concentrations of *L. pneumophila* there will likely be low amounts of target excreted, however, lysing the bacteria to release the target that is intracellular could increase the amount of target available enabling more sensitive detection by the DNAzyme.

4.5 Cooling tower water tests suggest DNAzyme activity is maintained.

All characterization experiments were done in deionized water which is not representative of a real-world environmental sample. Therefore, it was important to determine if the DNAzyme could function in cooling tower water, given that this is the proposed application of the DNAzyme for *L. pneumophila* detection. Contrary to deionized water, which is pure, cooling tower water can contain a variety of other bacteria and chemical reagents used for water treatment[37–41]. Over 50 different cooling tower water samples from Canada and the USA were tested. Each cooling tower water sample was subjected to two tests, a positive test where the water was spiked with CEM-LP, and a negative test were the DNAzyme could incubate in the cooling tower

water without CEM-LP. For the positive test, it was expected that the DNAzyme would cleave given that *L. pneumophila* was present. Each cooling tower water sample was sent for bacterial culturing to determine if it contained *L. pneumophila* and a report of the reagents added/within the cooling tower was collected. At the time of writing, one sample was positive for *L. pneumophila* at a concentration of 580 cfu/mL, however it was not detected by the DNAzyme. As shown in Figure 10, the DNAzyme can only detect high concentrations of CEM-LP (10,000 cfu/mL) by gel based methods, and requires an extended incubation time (72 h). Ultimately, detection of *L. pneumophila* in cooling tower water will not be done via test tube incubations and gel-based methods. Therefore, the purpose of these experiments was not to detect *L. pneumophila* endogenously in cooling tower water using the DNAzyme, but rather to determine the stability of the DNAzyme and its ability to function in cooling tower water. For most of the incubations, the DNAzyme did cleave when CEM-LP was added and did not cleave when incubated with just cooling tower water. There were however some exceptions.

Firstly, there was one cooling tower that had 3 different water samples collected at different time points as shown in Figure 13 and there are bands in the negative lanes of the 10 h and 11.15 h samples. No CEM-LP was added to the negative incubations, therefore no cleavage of the DNAzyme was expected. However, a large dose of oxidizing biocide, specifically hypochlorous acid, was added just before the first sample was taken. The bottles used to collect the cooling tower water contain sodium thiosulfate to inactivate such reagents, however the amount of thiosulfate in the sample bottles was likely not enough to inactivate such a high dose. Consequently, the acid could have still been active in the water sample used for testing and caused non-

specific cleavage of the DNAzyme. By the time the third sample was taken it is likely that enough of the biocide had reacted with biological material, or had been dissipated, to no longer see any cleavage of the DNAzyme. To test this hypothesis the pH of the water samples was checked. At the time of testing, no significant difference in pH was observed, all three time point samples had a pH around 8. However, it is important to note that the pH of the samples was tested 20 days after the water was initially collected, and the actual incubation of the DNAzyme with the cooling tower water was done the day after it was collected. It is possible that the cooling tower water samples had a different pH when the cleavage reactions were carried out than when the pH was tested weeks later, depending how long the buffering system, in this case the sodium thiosulfate coating in the bottles, took to reach equilibrium. Consequently, it cannot be stated that the large dose of hypochlorous acid was responsible for the non-specific cleavage activity of the DNAzyme given that the pH at the time of sampling was not taken. In the same respect, if the pH 8 value of the water samples measured recently is a true representation of the pH at the time of testing, the basicity of the water could also explain the background cleavage. A base could deprotonate the 2'OH of the embedded ribonucleotide resulting in a nucleophilic attack on the adjacent phosphorous, and ultimately cleavage[42,43]. However, another possibility is that the DNAzyme was being cleaved by a DNase from the 3' end, given that the fluorescein molecule is present on the 5' end and there is the appearance of multiple bands. After the biocide could circulate in the cooling tower for some time, whatever was producing the DNase was eliminated, as seen by the absence of bands at the third timepoint (13 h). Future experiments could involve running various DNAzyme truncation sequences in the same

gel to deduce the size of the bands. This information can be compared against the predicted secondary structure to determine if it is areas of single stranded DNA or double stranded DNA that is being cleaved.

Secondly, when Lp5 was incubated with cooling tower water from Chemco Products Company (California, USA) only a 40% average cleavage activity was observed with the addition of CEM-LP (positive test). Lp5 demonstrated 80-98% cleavage in the remaining cooling tower water samples following a 1 h incubation, suggesting there is something from the California sample that may be inhibiting the DNAzyme. Unfortunately, that was the only sample to come from California so it cannot be determined whether the reduced activity of the DNAzyme is unique to the specific cooling tower the water came from, or if the reduced activity of the DNAzyme is conserved across Chemco California cooling towers. When looking at the data sheet for that specific cooling tower, two biocides are reported: isothiazolinone and stabilized bromine, however these biocides have been reported in many of the other cooling tower water samples received and no reduced activity by Lp5 was observed. Benzotriazole is reported as the corrosion inhibitor, whereas other data sheets from different cooling towers report scale inhibitor. It is therefore difficult to determine if there is a specific reagent responsible for the observed reduction in cleavage activity. More samples from Chemco California would facilitate this investigation.

4.6 DNAzyme target likely specific for *L. pneumophila*

The specificity of the DNAzyme was assayed against other species (Figure 7) but not against other *Legionella* species. A collaboration with Dr. Alex Ensminger from U of

T facilitated this investigation. Firstly, species that were phylogenetically distant from *L. pneumophila* were selected and included: *Legionella micdadei, Legionella dumofii, Legionella pneumophila (*Philly -1 CDC*),* and *Legionella longbeachae*[44]*.* Given that very little was known about the target it was important to first start broadly then narrow down. After determining that Lp5 was only cleaved by *L. pneumophila* and none of the other *Legionella* species investigated, the next step was to determine if the DNAzyme can recognize multiple strains of *L. pnuemophila*. Again, a variety of strains were selected, ensuring they were phylogenetically distant from Philly which is seen as the more 'traditional' strain[45]. As shown in Figure 15, Lp5 was cleaved by all the strains investigated, exhibiting the best RNA-cleaving activity in the presence of Philly. The *L. pneumophila* str. Philly was investigated at two different OD_{600} values to see if different growth resulted in different RNA-cleavage activity. The Philly strain grown to a higher OD_{600} of 2.6 exhibited a slightly higher percent cleavage than the strain grown to OD_{600} of 1, however at the higher OD_{600} there is the appearance of a second band directly below the expected cleavage band. It is possible given the proximity of the two bands that they are the same cleavage product but in one cleavage fragment the terminal phosphate is in the cyclic conformation and in the other fragment it is in the open/linear conformation. *Legionella* species that are phylogenetically distant from *L. pneumophila* were chosen for initial investigations, but *Legionella* species that are phylogenetically close to *L. pneumophila* such as *L. waltersii* and *L. shakespearei* have not yet been investigated. It is possible that these close relatives to *L. pneumophila* may share the same target.

5 Conclusion and Future Directions

A DNAzyme that is specifically activated by the CEM of *L. pneumophila* was isolated after 11 rounds of *in vitro* selection. Investigations into the characteristics of the DNAzyme sequence revealed that the removal of the internal fluorophore and quencher modified nucleotides enhances the activity of the DNAzyme towards the CEM of *L. pneumophila* but consequently results in non-specific cleavage activity by the DNAzyme. This non-specific cleavage activity can be mitigated via the addition of RNase inhibitor. The DNAzyme sequence was truncated from 112 bases to 55 bases, however the RNA-cleaving activity of the truncated sequence is susceptible to storage conditions. Using dPAGE the Lp3 DNAzyme can detect on the order of magnitude 10 cfu/µL after a 72 h incubation period with the CEM of *L. pneumophila.* A method to successfully isolate the CIM of *L. pneumophila* was established using liquid nitrogen and demonstrated comparable activity to the CEM and liquid culture of *L. pneumophila*, suggesting the target is also found intracellularly. Investigations into the RNA-cleaving activity of the DNAzyme in cooling tower water suggests that the DNAzyme remains capable of detecting *L. pneumophila* in the environment of its intended application, with few exceptions that will require further assessment. Preliminary analysis of the target revealed that it is expressed by all strains of *L. pneumophila* investigated, but not present in the phylogenetically distant *Legionella* species investigated. More tests with closely related species to *L. pneumophila* are required to narrow down the specificity of the target. In the future the DNAzyme will be incorporated into an assay for the development of an on-site test than can provide early-stage detection of *L. pneumophila* in water as a means of mitigating Legionnaires' disease outbreaks.

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