

# DNA Aptamers Specific for Legionella Pneumophila: Systematic Evolution of Ligands by Exponential Enrichment in Whole Bacterial Cells

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## Research Article

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# Abstract

*Legionella pneumophila* is the major causative agent of Legionnaires' disease and Pontiac fever, which pose major public health problems. Rapid detection of *L. pneumophila* is important for global control of these diseases. Aptamers, short oligonucleotides that bind to targets with high affinity and specificity, have great potential for use in pathogenic bacterium detection, diagnostics, and therapy. Here, we used a whole-cell SELEX (systematic evolution of ligands by exponential enrichment) method to isolate and characterize single-stranded DNA (ssDNA) aptamers against *L. pneumophila*. A total of 60 ssDNA sequences were identified after 17 rounds of selection. Other bacterial species (*Escherichia coli*, *Bacillus subtilis*, *Pseudomonas syringae*, *Staphylococcus aureus*, *Legionella quateirensis*, and *Legionella adelaidensis*) were used for counterselection to enhance the specificity of ssDNA aptamers against *L. pneumophila*. Four ssDNA aptamers showed strong affinity and high selectivity for *L. pneumophila*, with  $K_d$  values in the nanomolar range. Bioinformatic analysis of the most specific aptamers revealed predicted conserved secondary structures that might bind to *L. pneumophila* cell walls. In addition, the binding of these four fluorescently labeled aptamers to the surface of *L. pneumophila* was observed directly by fluorescence microscopy. This is the first study to use SELEX to target *L. pneumophila* whole cells. The aptamers identified in this study could be used in the future to develop medical diagnostic tools and public environmental detection assays for *L. pneumophila*.

## Introduction

*Legionella* species, the major causative agents of Legionnaires' disease and Pontiac fever, are commonly found in environmental samples (Xiong et al., 2016). They have been shown to induce lung infection and cause dysfunction in other organs, such as the heart and kidney, and in the central nervous system (Xiong et al., 2015). *Legionella pneumophila* specifically has been identified as the primary cause of Legionnaires' disease. Early detection of *L. pneumophila* would allow for faster recovery and a reduction in the number of fatal cases. Additionally, monitoring the *L. pneumophila* population in the environment could reduce the likelihood of Legionnaires' disease outbreaks (Xiong et al., 2017). However, traditional culturing methods used for detecting *L. pneumophila* in samples rely on enrichment and selective plating followed by biochemical, serological, and molecular identification. These methods take 2 to 3 days to obtain a result and are labor intensive and tedious (Rajendhran and Gunasekaran, 2011). Recently, highly specific detection techniques, such as ELISA, PCR, and real-time qPCR, have shortened detection time. However, these methods depend on complex and technically difficult steps and are prohibitively expensive for routine testing (Woo et al., 2008). Biosensors have demonstrated a potential for rapid bacterial detection but rely on nanomaterials or complex platforms such as functional magnetic nanoparticles, electrochemical analysis systems, and field-effect transistors (Gopinath et al., 2014). Antibodies have also been applied for more than 40 years in assays for microbial detection but have many limitations despite their widespread application. For instance, the synthesis of antibodies requires a host animal, antibodies may be unstable at room temperature, and antibodies will not be obtained when target chemicals are toxic to the host organism.

Aptamers are single-stranded DNAs (ssDNAs) or RNAs that can bind to a wide range of non-nucleic acid targets with high affinity and specificity (Keefe et al., 2010). In detection and diagnostic assays, aptamers have several advantages over antibodies (Keefe et al., 2010). For instance, aptamers are obtained through an *in vitro* selection process, which is achieved at a lower cost and with less batch variation than *in vivo* antibody production. Due to their high stability at a range of temperatures and pHs, aptamers are also relatively easy to handle. In addition, aptamers can be easily modified with dyes or other functional groups to be labeled or immobilized on a substrate (Syed and Jamil, 2018).

Aptamer sequences are usually identified through the SELEX (systematic evolution of ligands by exponential enrichment) process, which starts with incubating the ssDNA or RNA library with the target, followed by separation and exponential amplification of the binding ssDNA or RNA (Torres-Chavolla and Alocilja, 2009). By repeating this process 8–20 times before cloning and sequencing the oligonucleotides that bind to the target, the most suitable aptamers can be identified (Dua et al., 2016). Aptamers can be selected for a wide variety of targets, from small molecules to whole cells (Bruno, 2015; Cao et al., 2009; Sypabekova et al., 2017). In recent years, aptamers have been selected against cells from different bacterial species, including *Escherichia coli* K88 (Peng et al., 2014), *Salmonella typhimurium* (Duan, Wu, et al., 2013; Dwivedi et al., 2013; Moon et al., 2013), *S. enteritidis* (Kolovskaya et al., 2013; Park et al., 2014), *S. paratyphi* A (Yang et al., 2013), *Vibrio parahaemolyticus* (Duan et al., 2012; Tang et al., 2013), *Listeria monocytogenes* (Suh et al., 2014; Suh and Jaykus, 2013), *Shigella dysenteriae* (Duan, Ding, et al., 2013), *Staphylococcus aureus* (Cao et al., 2009), *Proteus mirabilis* (Savory et al., 2013), *Pseudomonas aeruginosa* (Wang et al., 2011), *Mycobacterium tuberculosis* (Chen et al., 2007), and *Campylobacter jejuni* (Dwivedi et al., 2010). However, no specific aptamers have been reported for *L. pneumophila*.

In this study, we isolated four sequences for DNA aptamers specific to *L. pneumophila* using a whole bacterial cell SELEX process. The predicted secondary structures of selected aptamers were analyzed, and the binding of these aptamers to target cells was observed by fluorescence microscopy. In addition, we characterized their affinity and selectivity for *L. pneumophila* cells. The results provide an important reference for pathogen detection using aptamers in the future.

## Results

### *In vitro* selection of aptamers

To select aptamers that bind to *Legionella pneumophila*, we applied a cell-SELEX strategy using a library of ssDNA sequences containing a 45-base random region flanked by two defined primer binding regions (5'-GCAATGGTACGGTACTTCC-N45-CAAAAGTGCACGCTACTTTGCTAA-3'; Table 1). A total of 17 rounds of selection were performed until the ssDNAs binding to the target cells dominated the DNA pool (Fig. 1).

The genus *Legionella* includes more than 57 species (including subspecies) and more than 70 serogroups, not all of which are associated with human disease. The species most frequently detected in diagnosed cases is *L. pneumophila*, consisting of 16 serogroups (Casini et al., 2017); *L. pneumophila* serogroup 1, responsible for the first identified outbreak in 1976 in Philadelphia (USA), is the cause of

95% of infections in Europe and 85% of infections worldwide (Cloutman-Green et al., 2019). The phylogenetic diversity of this Philadelphia-1 strain has since expanded, now including the JR32 and Lp02 strains (Schmolders et al., 2017) commonly used for *L. pneumophila* research (Maita et al., 2016). In this study, JR32 and Lp02 were used as the target strains for aptamer selection. Live cells were used because the conformation of cell wall molecules, which represent the most likely aptamer targets, may change when cells die. Also, considering bacterial cell wall composition changes during growth in culture, we used stationary-phase bacterial cells in all experiments. Following incubation, the target-bound aptamers were eluted and enriched at each selection round by amplification using PCR. After each round of selection, the obtained ssDNAs were quantified by a spectrophotometer and analyzed by agarose gel electrophoresis (Fig. 1A).

With each SELEX round, the percentage of ssDNA binding to the target cells gradually increased. However, there were slight drops in elution yield after the second and sixth rounds, most likely due to counterselection (Fig. 1B). During SELEX, four counterselection processes were performed to increase the selectivity of aptamers to the target cells using a mixture of other bacterial species (*Escherichia coli*, *Bacillus subtilis*, *Pseudomonas syringae*, *Staphylococcus aureus*, *Legionella quateirensis*, and *Legionella adelaidensis*) at the second, sixth, ninth, and thirteenth rounds of selection. After the 17 rounds of selection, ssDNA bound to target cells dominated the DNA pool with about 90% elution yield. A total of 60 cloned transformants (*E. coli* cells harboring a vector that contained the aptamer sequences) were sequenced to identify specific aptamer candidates. From these transformants, we identified four unique aptamer sequences (S11, S25, S28, and S29) that were specific to LP02 cells and JR32 cells (Table 2).

### Sequencing and bioinformatics analysis

The sequences of the S11, S25, S28, and S29 *L. pneumophila* aptamers are listed in Table 2. The computed GC/AT ratio of all four aptamers varies from 1.63 to 0.96. Since the GC/AT ratio is one of the most basic sequence characteristics in terms of nucleotide composition, aptamer sequences can be modified and optimized in future studies by taking this ratio into account. To understand the impact of aptamer sequence structure on binding, we generated predicted secondary structures for the most efficient *L. pneumophila*-binding aptamers using the mfold algorithm. Aptamers S11 and S29 had similar predicted stem-loop region secondary structures, which contained GGGCA residues at the apical loops (Fig. 2). S25 and S29 also contained a nearly identical sequence, CAxCTGTA. In addition, S11, S25, and S28 all shared one conserved stem loop with TACTT residues, while S28 and S29 had an identical stem loop containing the sequence CAAAAGTG (Fig. 2).

For the aptamers S11, S25 and S28, mfold yielded four predicted secondary structures, while five different alternative conformations were predicted for S29. However, the conserved loops in different aptamers were stable in different predicted secondary structures, which suggests that these features could play an important role in the interaction of aptamers with their ligands in the bacterial cell wall.

### Assessment of selectivity

To determine that the selected aptamers were capable of binding *L. pneumophila* cells, the four *L. pneumophila*-binding DNA aptamers (S11, S25, S28, and S29) were examined using the same concentration of aptamers (250 nM) and visualized by fluorescence microscopy. Other bacterial species (*E. coli*, *B. subtilis*, *P. syringae*, *S. aureus*, *L. quateirensis*, and *L. adelaidensis*) were used as negative controls. As shown in Fig. 3A, fluorescently labeled *L. pneumophila* JR32 and Lp02 cells were observed with 6-carboxyfluorescein (FAM)-labeled S29, while the negative control cells incubated with FAM-S29 did not show any definite fluorescence signal on cells. Using Multiscan Spectrum, the intensity of fluorescence was detected. All four of the aptamers showed selective binding to *L. pneumophila* JR32 and Lp02, and they had very low binding capacity for other bacterial species, including *L. quateirensis* and *L. adelaidensis* cells (Fig. 3B). These results indicate that the four ssDNA aptamers (S11, S25, S28, and S29) showed affinity and high selectivity to *L. pneumophila* JR32 and Lp02.

### Determination of equilibrium dissociation constants

To evaluate aptamer binding in more detail, we determined the affinity of individual aptamers (S11, S25, S28, and S29) to *L. pneumophila* JR32 and Lp02. The fluorescein-labeled ssDNA aptamers (in increasing concentrations of 12.5, 31.25, 62.5, 125, 250, and 500 nM) were incubated with  $10^7$  JR32 or Lp02 cells for 60 min at 20°C with mild shaking. The fluorescence intensity of each sample was measured with a fluorospectrophotometer, and  $K_d$  values were determined by nonlinear regression analysis using Origin 9.5 software. As shown in Fig. 4, computed  $K_d$  values of all four aptamers for *L. pneumophila* JR32 and Lp02 varied from 12.98 to 35.00 nM. Aptamer S29 had the highest affinity for *L. pneumophila* JR32 cells, with a low  $K_d$  value (12.98 nM), while S28 had the lowest  $K_d$  value (22.11 nM) for *L. pneumophila* Lp02 cells.

## Discussion

*L. pneumophila* is an opportunistic pathogen comprising more than 16 serotypes, with serogroup 1 being responsible for most serious infections such as Legionnaires' disease, causing 95% of infections in Europe and 85% of infections worldwide (Casini et al., 2017; Cloutman-Green et al., 2019). In our study, the representative strains JR32 and Lp02 (Maita et al., 2016) were used as target cells to develop specific, high-affinity aptamers for pathogenic *L. pneumophila*. Notably, the binding affinity of each aptamer varied between these two strains (Fig. 3 and 4). These results may be due to experimental error during assessments of affinity and specificity for aptamers against JR32 and Lp02 cells. However, it is also possible that, although JR32 and Lp02 were isolated simultaneously, the two strains currently display distinct genomic features leading to differences in cell wall composition (Maita et al., 2016).

In this study, we determined that using fluorescein-modified primers to amplify the aptamers makes the product more visible under ultraviolet light, which aids in product recovery. In addition, to avoid primer contamination during the SELEX process we used a gel extraction kit to purify and recover products. The fluorescein group may help increase the recovery efficiency during purification and recovery steps.

Using the mfold algorithm, we generated predicted secondary structures for the most efficient *L. pneumophila*-binding aptamers (Fig. 2). We observed that in the constant region primer, S11, S25, and S28 share one conserved stem loop with TACTT residues. In-depth analysis revealed that the generations of these conserved secondary structures were due to site mutations in the constant primer regions of these aptamers during the PCR amplification process (Fig. 2). These results demonstrated that the conserved stem loop with TACTT residues could be the key secondary structures that allow these aptamers to bind the target cells. In future studies, we will test this hypothesis by truncation or site-directed mutation assays. In addition, elucidation of the specific binding targets on the cells themselves may also help us better understand this mechanism.

To our knowledge, this is the first study to report the binding of aptamers to *L. pneumophila*. Importantly, some of the aptamers described here recognize pathogenic *L. pneumophila* strains with affinity and specificity suitable for potential use in clinical diagnosis, therapeutics, and environmental pathogen detection applications.

## Conclusions

We applied the whole-cell SELEX (systematic evolution of ligands by exponential enrichment) method to isolate and characterize single-stranded DNA (ssDNA) aptamers against *L. pneumophila*. Four ssDNA aptamers were obtained and showed strong affinity and high selectivity for *L. pneumophila*, with  $K_d$  values in the nanomolar range. Bioinformatic analysis of the most specific aptamers revealed predicted conserved secondary structures that might bind to *L. pneumophila* cell walls. In addition, the binding of these four fluorescently labeled aptamers to the surface of *L. pneumophila* was observed directly by fluorescence microscopy. The aptamers identified in this study could be used in the future to develop medical diagnostic tools and public environmental detection assays for *L. pneumophila*.

## Materials And Methods

### Microorganisms and culture conditions

*Escherichia coli* (DH5 $\alpha$ ), *Bacillus subtilis* (13407), *Pseudomonas syringae* (DC3000), *Legionella pneumophila* (Lp02, Philadelphia-1, *thyA*, *rpsL*, *hsdR*), *Legionella pneumophila* (JR32, Philadelphia-1, JR32), *Legionella quateirensis* (27-1), *Legionella adelaidensis* (49625), and *Staphylococcus aureus* (26111) were obtained from the American Type Culture Collection (ATCC). Cells were cultured in Luria broth (LB) or AYET medium at 37°C. Growing cultures (OD<sub>600</sub> of 3.0) were used in all experiments. Cells were washed three times with wash buffer (PBS, pH = 7.5, 0.1% Tween 20) before use.

### Aptamer selection process (SELEX)

A whole-cell SELEX approach was used to select 6-carboxyfluorescein (FAM)-labeled ssDNA aptamers with binding affinity and specificity to *L. pneumophila*. The method of Dwivedi et al. (2010) was used, with minor modifications. To initiate the selection process, an 88-mer combinatorial ssDNA library (5'-

GCAATGGTACGGTACTTCC-N45-CAAAGTGCACGCTACTTTGCTAA-3') procured from Ruibiotech (Guangzhou, PRC) was amplified using the FAM-labeled forward constant region primer (5'-FAM-GCAATGGTACGGTACTTCC-3') and reverse

constant region primer (5'-TTAGCAAAGTAGCGTGCACTTTTG-3') in 2 × Phanta Master Mix (Vazyme, Nanjing, PRC). Random library nucleotides (approximately  $10^{14}$  molecules) diluted in selection buffer (PBS, pH = 7.5, 0.05% Tween 20) were denatured for 10 min at 96°C, cooled for 10 min at 4°C, and incubated with 1 mL of  $10^8$  colony forming units (CFU)/mL of bacterial cells for 45 min at 20°C under constant agitation (30 rpm). The bacterial cells were concentrated by centrifugation (6000 g, 3 min) and washed three times with wash buffer (PBS, pH = 7.5, 0.1% Tween 20) to remove unbound and nonspecifically bound aptamers. The aptamers bound to cells were eluted by resuspending the bacteria-aptamer complex in sterile water and heating it to 95°C for 5 min. The supernatant was collected and amplified by PCR in a 100- $\mu$ L reaction, using 2× Phanta Master Mix (Vazyme, Nanjing, PRC) with 0.4  $\mu$ M FAM-labeled forward constant region primer and 0.4  $\mu$ M P-reverse constant region primer. Amplification conditions were as follows: 96°C for 2 min (denaturation and heat activation of polymerase); 12 cycles of a) 96°C for 20 s, b) 56°C for 33 s, and c) 72°C for 24 s (annealing and extension); 72°C for 5 min (final extension); and 6°C for 2 min (end of amplification). Products were resolved by 2% agarose gel. To obtain ssDNA, PCR products were obtained by digestion with 5 units of lambda exonuclease (New England Biolabs, Ipswich, MA, USA) per 50-mL reaction. Reactions were incubated for 30 min at 37°C and then heat inactivated for 5 min at 80°C. Digested products were extracted using the D205 StarPrep Gel Extraction Kit (Genestar, Beijing, PRC) and used for the next round of SELEX. Concentration of ssDNA was measured using a NanoDrop 1000 Spectrophotometer (ThermoScientific, DE, USA). For counter-SELEX, the candidate aptamer pool obtained after multiple rounds of SELEX was incubated with a cocktail of nontarget bacterial cells [*E. coli* (DH5 $\alpha$ ), *B. subtilis* (13407), *P. syringae* (DC3000), *L. quateirensis* (27-1), *L. adelaidensis* (49625), and *Staphylococcus aureus* (26111)] at a pooled concentration of  $10^8$  CFU/mL. In this case, the cell-bound aptamer molecules were discarded, and the unbound aptamer pool was recovered by centrifugal washing. After completion of all 17 rounds of SELEX and counter-SELEX processes, the final aptamer pool was enriched by PCR with forward constant region primer and reverse constant region primer. Products were cloned using the TOPO TA cloning kit (Invitrogen, USA) and transformed into *E. coli* TOP10 cells. Positive clones were sequenced, and aptamer sequences were analyzed using the Clustal and Mfold structure prediction algorithms.

## Secondary structure prediction

To predict aptamer structural folding, we used the mfold algorithm (<http://unafold.rna.albany.edu/?q=mfold/DNA-Folding-Form>). The modeling was done assuming ionic conditions of 157 mM Na<sup>+</sup> at 4°C.

## Microscopy observation of aptamer binding

To visualize aptamer binding on the surface of viable target cells, FAM-labeled ssDNA aptamers (250 nM) were incubated with the target strains ( $10^7$  cells) for 45 min at 20°C, and then cells were centrifuged and

the supernatant discarded. The cells then went through two rounds of washing and centrifugation, with the supernatants again discarded. The cells were resuspended in 40  $\mu\text{L}$  of washing buffer. Imaging of the bacteria was performed with a microscope (ZEISS, Axio Scope.A1) under excitation of 488 nm and emission in the range of 502 to 554 nm.

### **Aptamer binding analysis**

Aptamer  $K_d$  was determined by measuring the fluorescence intensity of each sample. The FAM-labeled ssDNA aptamers (in increasing concentrations of 12.5 , 31.25 , 62.5 , 125 , 250, and 500 nM) were incubated with the target strains ( $10^7$  cells) for 60 min at 20°C. Cells were washed two times to remove unbound ssDNA from cells and resuspended in 100  $\mu\text{L}$  of washing buffer. The  $K_d$  values for each aptamer were determined by nonlinear regression analysis using Origin 9.5 software.

## **Abbreviations**

FAM, 6-carboxyfluorescein; SELEX, systematic evolution of ligands by exponential enrichment; ELISA, enzyme linked immunosorbent assay; CFU, colony forming units.

## **Declarations**

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### **Availability of data and materials**

All data generated or analyzed during this study are included in this published article, and are available from the corresponding author (sunyunhaoscope@163.com) on reasonable request.

### **Competing interests**

The authors declare that they have no competing interests.

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### Authors' contributions

Lina Xiong, Mingchen Xia and Yunhao Sun conceived and designed the study. Lina Xiong, Qinglin Wang, Zhen Meng, Jie Zhang, Guohui Yu and Zhangyong Dong performed the experiments. Lina Xiong, Yongjun Lu and Yunhao Sun wrote the paper. Guohui Yu and Zhangyong Dong reviewed and edited the manuscript. All authors read and approved the manuscript.

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## Tables

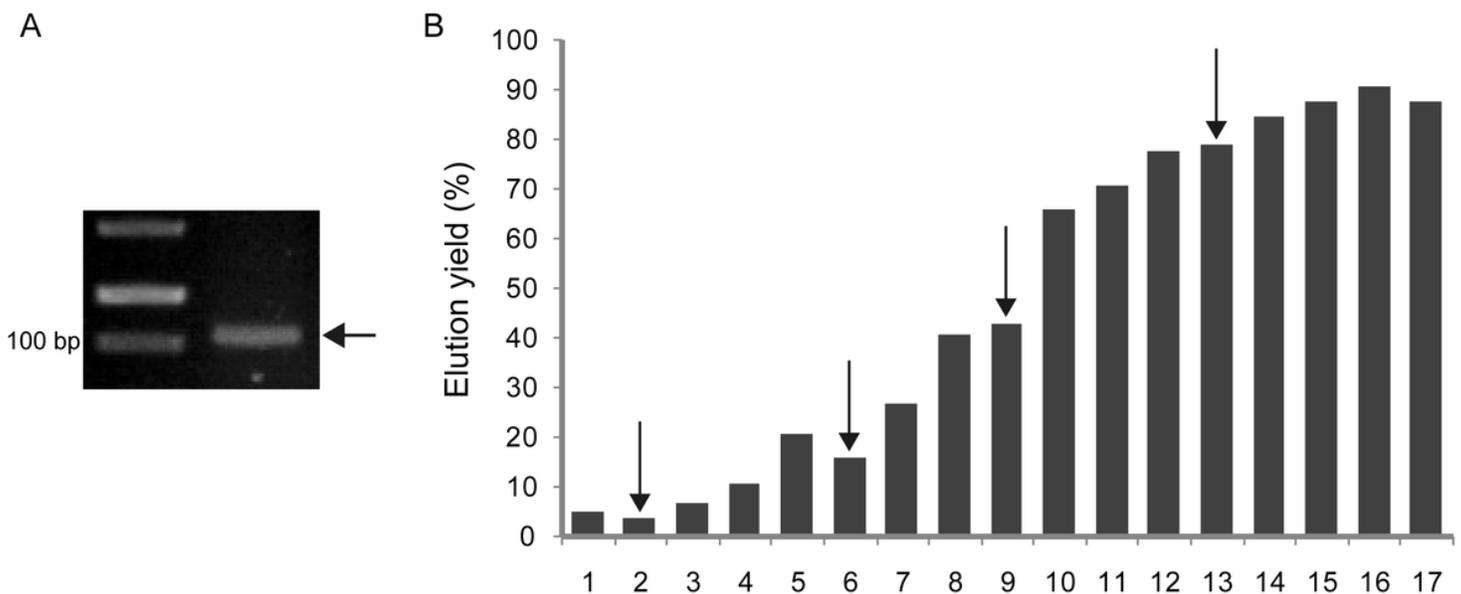
Table 1. Oligonucleotides used in the selection and characterization of aptamers with binding affinity to *Legionella pneumophila*.

Name	Oligonucleotides
DNA aptamer library	5'-GCAATGGTACGGTACTTCC-N45- CAAAAGTGCACGCTACTTTGCTAA-3'(88-mer)
Forward constant region primer	5'-GCAATGGTACGGTACTTCC-3'
Reverse constant region primer	5'-TTAGCAAAGTAGCGTGCACTTTTG-3'
FAM-forward constant region primer	5'-FAM-GCAATGGTACGGTACTTCC-3'
P-reverse constant region primer	5'-phosphate-TTAGCAAAGTAGCGTGCACTTTTG-3'

Table 2. List of four identified *Legionella pneumophila* binding ssDNA aptamers.

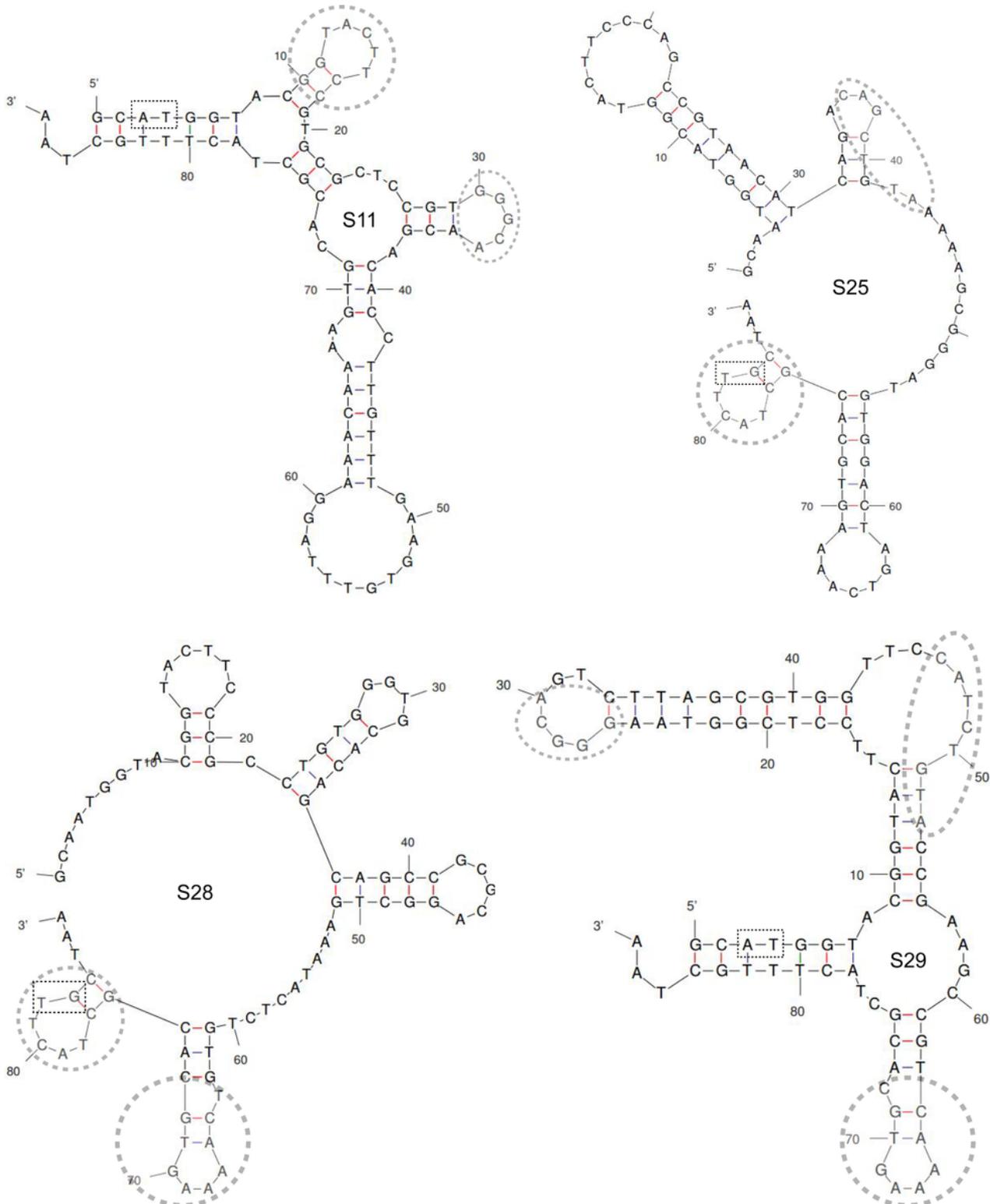
Aptamer	Sequence of N45 random region (5' to 3')	%GC
S11	GTGCGCTCCGTGGGCAACGACACCTTGTGTTGAAGTGTGTTAGGAAA	51
S25	CAGCCGTAACATCAGACAGCTGTAAAAAGCGGGATGTGGACTAGT	49
S28	CGCCTGTGGGTGCACAGCAGCCGCGCAGGCTGAAATACTCTGTGT	62
S29	TCGGTAAGGGCAGTCTTAGCGTGTTCCATCTGTACCGAAGCCGT	56

## Figures



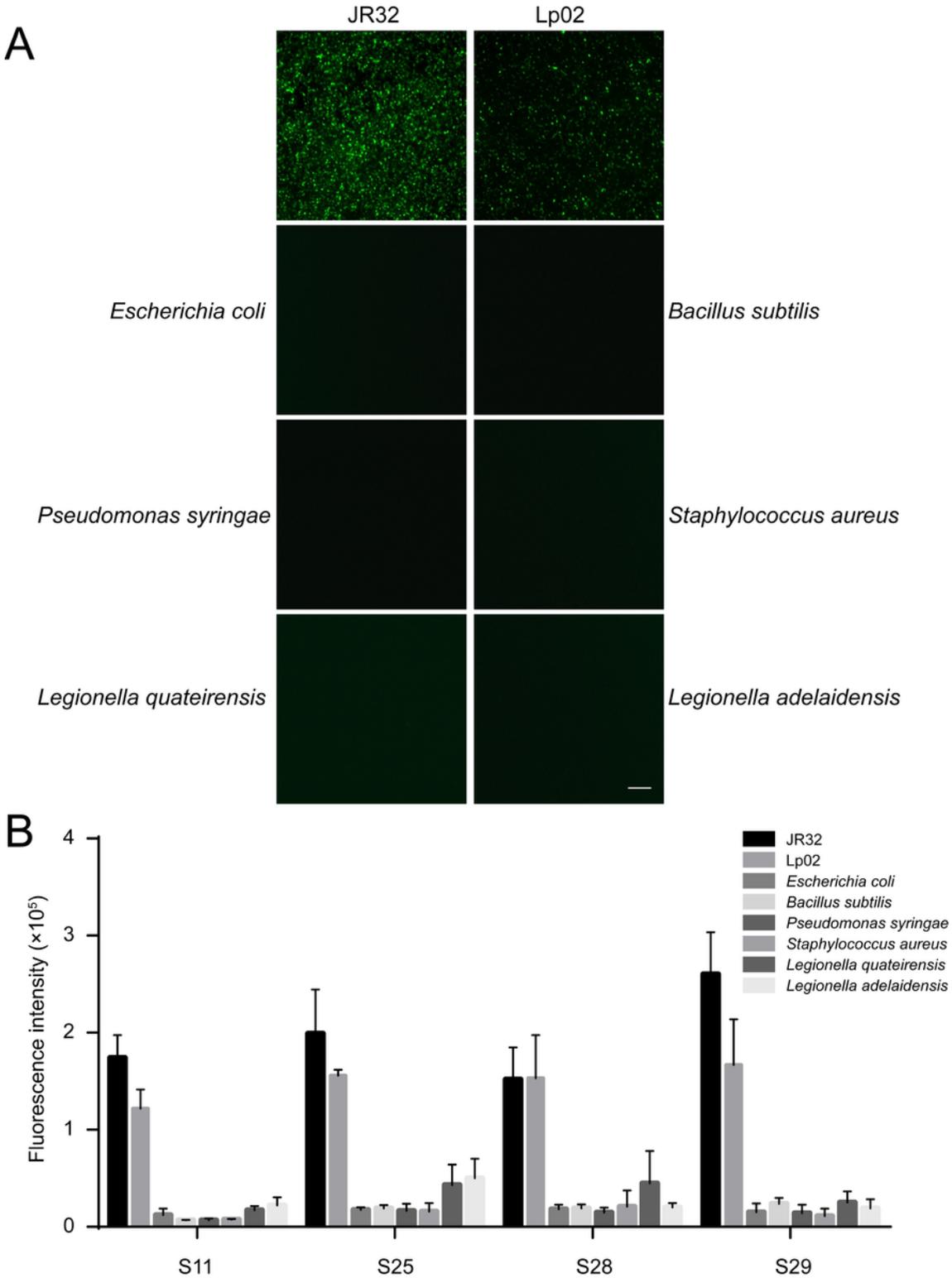
# Figure 1

In vitro selection of aptamers. A. The PCR products of the 9th round of selection were analyzed by agarose gel electrophoresis. Lane 1, DNA ladder; lane 2, amplified DNA products. Black arrow indicates the PCR product band. B. Progression of the selection process based on the portion of ssDNA bound to the target cells in the DNA pool. Numbers 1–17 represent the seventeen rounds of selection. Black arrows indicate the rounds of counter selection.



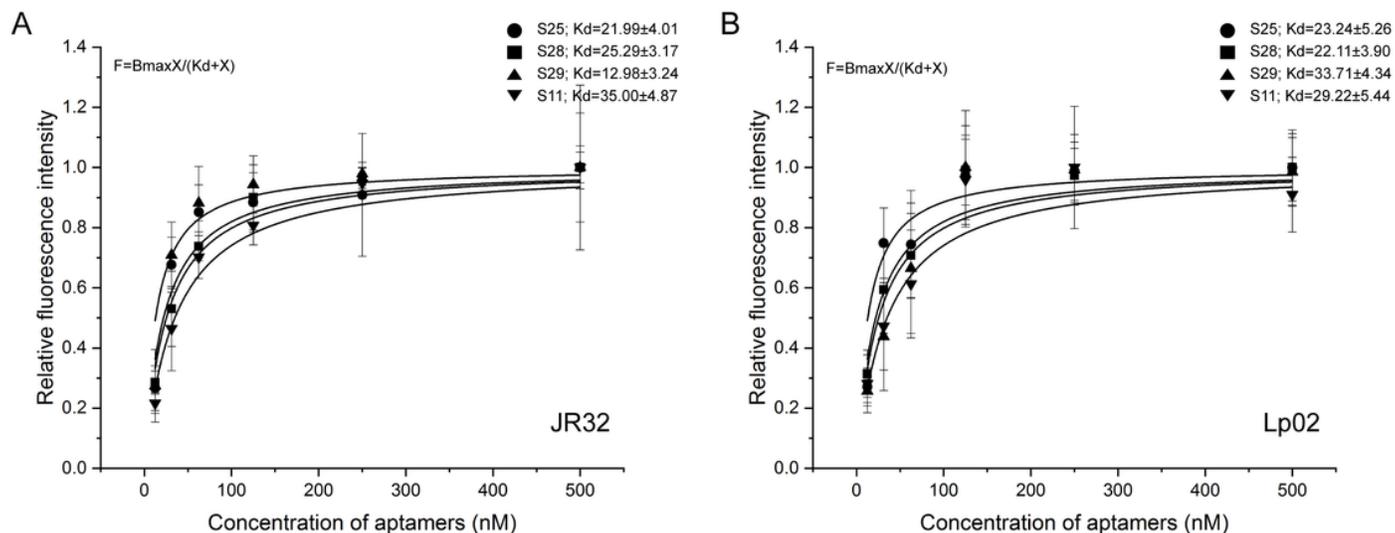
**Figure 2**

Predicted secondary structures of four obtained aptamers (S11, S25, S28, and S29). The DNA folding platform from the mfold web server based on a free energy minimization algorithm was used. The conserved stem loops are depicted in dotted circles.



**Figure 3**

Detection and specificity of aptamers for *Legionella pneumophila*. A. Fluorescein-labeled ssDNA aptamers (250 nM in binding buffer; representative pictures of aptamer S29 are shown) were incubated with  $10^7$  bacteria at 20°C for 45 min (scale bar, 10  $\mu$ m). B. Selectivity of aptamers (S11, S25, S28, and S29) for *Legionella pneumophila*, *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas syringae*, *Staphylococcus aureus*, *Legionella quateirensis*, and *Legionella adelaidensis* cells.



**Figure 4**

Binding affinity of four selected aptamers for *Legionella pneumophila*.

The 6-carboxyfluorescein-labeled ssDNA aptamers (in increasing concentrations of 12.5, 31.25, 62.5, 125, 250, and 500 nM) were incubated with the target strains JR32 (A) ( $10^7$  cells) and Lp02 (B) ( $10^7$  cells) for 60 min at 20°C. The  $K_d$  values for each aptamer were determined by nonlinear regression analysis using Origin 9.5 software. Data represent the mean SD of three independent experiments.