Biotechnol Lett (2013) 35:909–914
DOI 10.1007/s10529-013-1154-1

ORIGINAL RESEARCH PAPER

Selection of aptamers against inactive *Vibrio alginolyticus* and application in a qualitative detection assay

Xuemin Tang · Jiang Zheng · Qinpi Yan · Zhongbao Li · Yubao Li

Received: 28 September 2012 / Accepted: 25 January 2013 / Published online: 5 February 2013

Abstract Aptamers against inactive *Vibrio alginolyticus* were selected from an 82-nt ssDNA random library by systematic evolution of ligands by exponential enrichment. After 15 rounds of selection, the final pool of aptamers was highly specific for inactivated *V. alginolyticus* and had a dissociation constant of 27.5 ± 9.2 nM. Using these aptamers and PCR, *V. alginolyticus* could be detected at 100 cells/ml. Sequencing of the final pool of aptamers revealed that some sequences, termed high-frequency aptamers, appeared more than once; these may be of practical application. All sequences obtained were divided into nine families according to their homology tree, some conserved sequences were also found in each of the six families. One sequence was found in significant proportions of the aptamers, suggesting that this conserved sequence might be important for forming the three-dimensional aptamer structure.

Keywords Aptamer · Exponential enrichment · Systematic evolution of ligands · *Vibrio alginolyticus*

X. Tang · J. Zheng (✉) · Q. Yan · Z. Li · Y. Li
Key Laboratory of Healthy Mariculture for the East China Sea, Ministry of Agriculture, Fisheries College of Jimei University, No. 43, Yindou Road, Xiamen 361021, Fujian, China
e-mail: zhengjiang618@163.com

X. Tang
e-mail: inming@126.com

Q. Yan
e-mail: yanqp@jmu.edu.cn

Z. Li
e-mail: li2000zb@gmail.com

Y. Li
e-mail: liyubao@jmu.edu.cn

Introduction

Vibrio alginolyticus is a highly pathogenic bacterium that causes otitis and wound infections (Lin et al. 2001). It mainly lives in oceans and estuaries and usually reaches epidemic levels during summertime. People who are in frequent contact with seawater or shellfish can easily get infected by this microorganism (Oberbeckmann et al. 2011; Chen et al. 2006). Therefore, it is of the upmost importance that current measures to detect *V. alginolyticus* in water and seafood are strengthened.

Systematic evolution of ligands by exponential enrichment (SELEX) is a valuable screening technology developed in 1990 (Stoltenburg et al. 2007). A typical SELEX process starts with a chemically synthesized random RNA or ssDNA library consisting of 10^{13} to 10^{15} different sequence motifs. In the first selection round, this library of molecules is incubated directly with its target. Target-binding oligonucleotides are eluted, amplified by PCR, and used for the subsequent selection round. After several rounds of

selection, high affinity and target-specific oligonucleotides, called aptamers, are obtained (Brody and Gold 2000; Ylera et al. 2002).

In this study, we employed the SELEX method, starting with a random ssDNA library and formaldehyde-inactivated bacteria, to discover aptamers useful for the qualitative detection of *V. alginolyticus* in an aquaculture environment.

Materials and methods

Materials and reagents

The required microorganisms were provided by the Laboratory of Pathogenic Microorganisms of Jimei University; microorganisms were inactivated with a formaldehyde solution as previously described (Ellen et al. 2003). The original random ssDNA library, 5′-TCAGTCGCTTCGCCGTCTCCTTC-N35-GCACAAGAGGGAGACCCCAGAGGG-3′, was 82 nucleotides (nt) long, with 35 random oligonucleotides in the center. Three primers were used in the process of selection and affinity detection: primer P1 (5′-TCAGTCGCTTCGCCGTCTCCTTC-3′), P2 (5′-CCCTCTGGGGTCTCCCTCTTGTGC-3′), and P3 (5′-Digoxin–TCAGTCGCTTCGCCGTCTCCTTC-3′). Other materials, such as reagents and buffers, were obtained as reported in Hao et al. (2011).

SELEX process

The SELEX procedure included six sequential steps: binding, separating, washing, heating, collecting, and amplification. Initially, the ssDNA library (30 µl, 10 µM) was diluted to 100 µl with 2× binding buffer. The ssDNA library was denatured at 95 °C for 5 min, incubated in an ice bath for 10 min, and mixed with 100 µl inactive *V. alginolyticus* (2×10^9 cells/ml) on a rotary shaker for 30 min at 30 °C to allow formation of the binding complex. The mixture was centrifuged for 5 min at 6,000×*g* for isolation of the ssDNA/bacteria complexes; unbound ssDNA in the supernatant was discarded. The accumulated complexes were washed (resuspended in 1× binding buffer and subsequently re-isolated) three times to remove weakly-bound ssDNA. After 100 µl binding buffer was added, the complex was heated to 95 °C for 5 min and centrifuged for 10 min at 15,000×*g*. Finally, the supernatant was collected and used as a template for asymmetric PCR amplification (P1:P2 = 25:1). The denaturation step of the asymmetric PCR was performed at 94 °C for 4 min. A total of 40 cycles of denaturation (30 s, 94 °C), annealing (30 s, 58 °C), and extension (20 s, 72 °C) were performed, and this was followed by a final extension for 7 min at 72 °C. After amplification, the PCR products were analyzed by agarose gel electrophoresis. PCR product, 100 µl, was used as the ssDNA library for the next round of selection.

Negative selection was carried out every three selection rounds to improve the specificity of the pool of aptamers against *V. alginolyticus*. In the negative selection step, the target bacterium (inactive *V. alginolyticus*) was replaced with the following inactivated microorganisms: *Edwardsiella tarda, Aeromonas hydrophila,* and *Vibrio harveyi*. Negative selection did not include the heating step because the ssDNA bound to these three microorganisms was to be discarded, while the unbound ssDNA in the supernatant was to be collected. The other steps for negative selection were the same as those for positive selection.

The SELEX process described above were repeated until the affinity of the pool of enriched aptamers for the bacterium increased significantly.

Affinity analyses

Horseradish peroxidase (HRP)-conjugated antibody against digoxin was used to determine the affinity between the pool of enriched aptamers and inactive *V. alginolyticus*. First, the enriched pool of ssDNA aptamers was amplified by a modified version of the asymmetric PCR described previously, where primers P2 and P3 (P2:P3 = 1:25) were used to label ssDNA with digoxin. After denaturation at 95 °C for 5 min and cooling in an ice bath for 10 min, the PCR products were bound, separated, and washed according to the SELEX method. Subsequently, a rabbit anti-digoxin IgG/HRP conjugate (1:1,000) was added to the solution and incubated for 10 min. The reaction products were centrifuged for 5 min at 6,000×*g* and the supernatant, which included unbound ssDNA, was discarded. The bacterial precipitate was washed three times with binding buffer, then incubated with 200 µl freshly prepared substrate (1 mg TMB/ml: substrate buffer: 30 % v/v H_2O_2 = 100: 900: 1) in the dark for 10 min. Finally, 200 µl 2 M H_2SO_4 was added to stop

the color reaction. The absorbance of the final solution was measured at 450 nm using an ELISA reader. This measurement can be interpreted as the affinity of the pool of enriched aptamers for the bacterium (Bruno and Kiel 1999; Hao et al. 2011).

Validation of specificity

Four species of bacteria (*V. alginolyticus*, *E. tarda*, *A. hydrophila*, *V. harveyi*) were inactivated as described above, and resuspended in binding buffer at 2.5×10^9 cells/ml. The affinity of the last pool of enriched aptamers (the PCR products of the 15th round of selection) was tested against each of these samples using the affinity analyzes method described above.

Determination of affinity constant (Kd) of last pool of enriched aptamers

The pool of ssDNA aptamers were enriched after the last round of selection, and amplified with asymmetric PCR according to the affinity analyses method described above. The PCR products were serially diluted with binding buffer, and ssDNA concentration was determined with a micro-spectrophotometer. Samples containing different concentrations of ssDNA were bound, separately, with an excess of inactivated *V. alginolyticus*, and subsequently, the affinity was determined by affinity analyses. Plotting the affinity versus the concentration of ssDNA, we obtained a saturation curve for the binding of aptamers to the *V. alginolyticus* bacteria. The affinity constant (Kd) was calculated with nonlinear fitting using Origin 8 software.

Qualitative detection of the target bacterium

Inactive *V. alginolyticus* (at 10, 10^2, and 10^3 cells/ml), a mixture of three other inactivated bacteria (*E. tarda, A. hydrophila,* and *V. harveyi*) at $>10^3$ cells/ml, and a blank solution (H_2O) were each prepared and bound to the last pool of enriched aptamers. The procedures were same as the SELEX method described above, except that the binding complex was washed six times in this case. Following these steps, the supernatants were collected and used as the template for normal PCR (P1:P2 = 1:1). The denaturation step of the PCR amplification was performed at 94 °C for 4 min. A total of 35 cycles of denaturation (30 s, 94 °C), annealing (30 s, 58 °C), and extension (20 s, 72 °C) were performed, and this was followed by a final extension for 7 min at 72 °C. After amplification, the PCR products were analyzed by agarose gel electrophoresis. Theoretically, the samples of *V. alginolyticus* would have bright bands in the electrophoretogram, whereas the other samples would have no bands, which could be useful for the SELEX process.

Cloning and sequencing

The aptamers derived from the last round of selection were amplified by asymmetric PCR as described previously with primers P1 and P2. The PCR products were cloned and sequenced by Shanghai Majorbio Biopharm Technology Co., Ltd. The sequence analysis and alignments were performed using the software DNAMAN.

Statistical analysis

The data were analyzed for statistical significance using a *t* test. Differences with a p value of <0.05 were considered to be statistically significant.

Results and discussion

Using SELEX to select aptamers targeting *V. alginolyticus*

As shown in Fig. 1, the affinity of aptamer enriched pool was 0.025 after the first selection round; however, the affinity was 0.452 after 15 rounds of selection thereby, indicating that the affinity between the aptamers and *V. alginolyticus* increased significantly with additional selection rounds ($p < 0.05$). However, there were no significant differences ($p > 0.05$) between the affinities of the 11th, 13th, and 15th rounds of selection, suggesting that the binding sites of *V. alginolyticus* might be fully occupied by the aptamers beginning with the 11th selection round.

The final enriched aptamers exhibited the highest affinity for *V. alginolyticus* with little or no affinity for the other three bacteria tested (Fig. 2). Statistical analysis also revealed that the affinity between the aptamer pool and *V. alginolyticus* was significantly higher than those measured with the other three

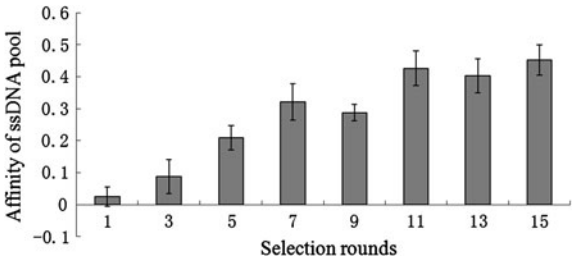

Fig. 1 Changes in affinity following rounds of selection. Affinity was measured as the absorbance at 450 nm resulting from oxidation of TMB by horseradish peroxidase-conjugated antibody bound to digoxin-tagged aptamers contained within aptamer-target complexes. Results represent the mean of three experiments; *error bars* represent the standard deviation

bacterial species ($p < 0.01$), suggesting that the aptamers of the last enriched pool have good specificity for *V. alginolyticus*.

Kd is a very important parameter used to characterize the stability of aptamer-bacteria complexes, and usually ranges from 1 to 100 nM (Wärnmark et al. 2005). As shown in Fig. 3, the affinity gradually increased as the concentration of ssDNA increased, resulting in a typical saturation curve. Kd was determined to be 27.5 ± 9.2 nM, demonstrating that the enriched aptamers have a good affinity for the target bacterium *V. alginolyticus*.

Qualitative detection of target bacterium

As seen in Fig. 4, the pool of enriched aptamers had good specificity for *V. alginolyticus*, and could detect the presence of 10^2 and 10^3 cells/ml of *V. alginolyticus*. Lane H of Fig. 4, representing inactivated *V. harveyi*, also had a band of weak brightness, which may be due to the commonality of binding sites on *V. alginolyticus* and *V. harveyi*. The absence of signal in the lane

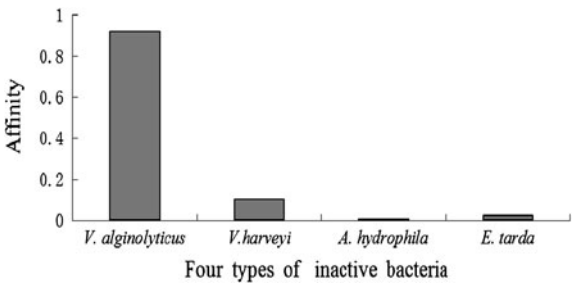

Fig. 2 Comparison of affinities of the last pool of enriched aptamers for four bacterial species. Affinities were determined as described in Fig. 1 and the "Materials and Methods". Each value represents a single determination

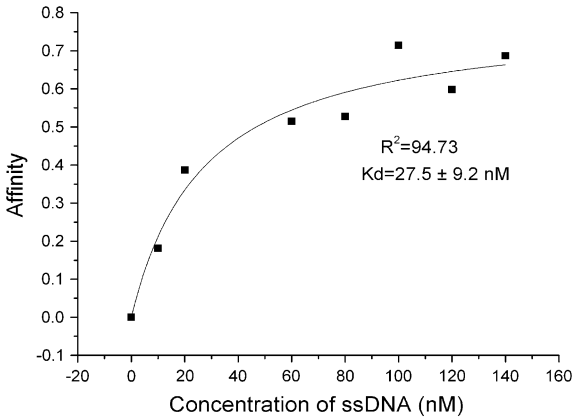

Fig. 3 Saturation *curve* and affinity constant (Kd) of the last pool of enriched apatamers. Affinities were determined using varying concentrations of oligomers and a fixed, excess concentration of inactivated *V. alginolyticus.* Each value represents a single determination. Data were fit to a single binding isotherm

lacking PCR template (lane B) demonstrates that the PCR reaction system was not contaminated and the results obtained with this system were reliable. These data demonstrate that the pool of selected aptamers, combined with PCR amplification, enabled successful qualitative detection of *V. alginolyticus*.

Analysis and alignment of aptamer sequences

To further characterize the final aptamer pool, we cloned individual aptamers for sequence analysis.

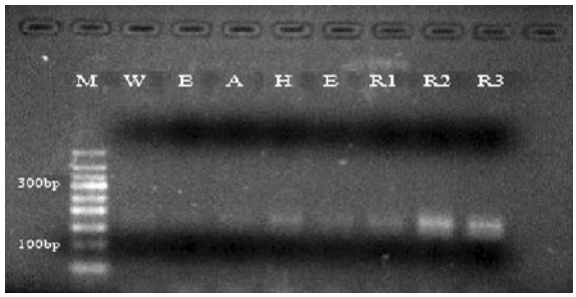

Fig. 4 Electrophoresis image of the PCR products of the qualitative detection assay. *M* marker; *W* ddH_2O instead of bacteria in the qualitative detection; *B* PCR blank, ddH_2O instead of template in PCR; *A* 1.7×10^4 cells/ml of inactive *A. hydrophila*; *H* 6.6×10^3 cells/ml of inactive *V. harveyi*; *E* 1.3×10^4 cells/ml of inactive *E. tarda*; *R1* 10^1 cells/ml of inactive *V. alginolyticus*; *R2* 10^2 cells/ml of inactive *V. alginolyticus*; *R3* 10^3 cells/ml of inactive *V. alginolyticus*

Table 1 Classification of the 32 different sequences based on the homology tree

Family number and homology in each family (%)	Sequences of random region of aptamers (5′–3′direction)	Frequency of aptamer
Family 1	GGGGGCGCGGTGAGGGGCT	1
100	GGGGGCGCGGTGAGGGGCT*GCACAAGAGGGAG*	2
Family 2	GGACGAGATGGCGGGAAACGAG	1
80–100	GGACG**G**GATGGGCGGGAAACGAG	1
	GGACGAG**C**TGGGCGGGAAACGAG*GCACAAGAGGGA*	1
	GGACGAGATGGGCGGGAAACGAG*GCACAAGAGGGA*	3
	GGACGAGATGGGCGGGAAACGAG*GCACAAGTGGGA*	1
Family 3	GTAGGAGGTAGTCGGAGAGGCGAATGAGAGGGGAA	1
80–100	GTAGGAGGTAGTCGGAGAGGCGAATGAGAGGGGAA*GCACAAGAGGGA*	2
	GTAGGAGGTAGTCGGAGAGGCGAATGAGAGGGG**G**AGT*ACAAGAGGGA*	1
Family 4	AGCCGGGGTGGTCAGTAGGAGCA	8
80–100	AGCCGGGGTGGTCAGTAGGAGCA*GCACAAGAGGGA*	5
	AGCCGGGGTGGTCAGTAGGAGCA*GCACAAGAGGGA*G	1
	AGCCGGGGTGGTCAGTAGGAGCA*GCACAAGAGGGA***GCACAAGAGGGA**	1
	AGCCGGGGTGGTCAGTAGGAGCA*GCACAAGAGGGA***GCACAAGAGGGA**G *GCACAAGAGGGA*	1
Family 5	TGCAGGGCCAGAACAGGGGGAAG	1
80–100	TGCAGGGCCAGAACAGGGGGAAG*GCACAAGAGGGA*	7
	TGCAGG**T**CCAGAACAGGGGGAAG*GCACAAGAGGGA*	1
	TGCAGGGCCAGAACAGGGGGGAGGTACAAGAGGGA	1
	TGCAGGGCCAGAACAGGGGGAAG*GCACAAGAGGGA***GCACAAGAGGGA**	1
	TGCAGGGCCAGAACAGGGGGAAG*GCACAAGAGGGA***GCACAAGAGGGA**G	1
Family 6	GGGTGGAGGAGGACGAAGTGAGA*GCACAAGAGGGA***GCACAAGAGGGA**	1
80–100	GGGTGGAGGAGGACGAAGTGAGA*GCACAAGAGGGA***GCACAAGATGGA**	1
	GGGTGGAGGAGGACGAAGTGAGA*GCACAAGAGGGA***GCACAAGAGGGA** *GCACAAGAGGG*G	1
Family 7	AGCACAAGAGGGA*GCACAAGA*G*GGA*G**GCACAAGAGGGA**G	1
40–60	GGGCGTGAGGTACCAAGCATGTTGTGGGAAGGGCT*GCACAAGAGGGA*	1
Family 8	GGCCGGCCATCGGTACCAGGGGT	1
40–60	AGCCGAGTGTACACGGGGAACGACACACCGTTGCA	1
Family 9	GGTCGAATTGAAGGAGTCCGCTACGCGCGTAGAAT	1
20–40	AGTCTCTGCGTCAAATAGGAGCTAGAGATCGGTAG	1
	TAATTTACAGAATTACTCGACATTCCACTTCTAAA	1
	GAGGCGTCAGGACCTGGATGGGCAAATTTTCGGAT	1

Conserved sequences of each family are shown underlined. One conserved sequence, 5′-GCACAAGAGGGA-3′ is marked by bold and italics. "Frequency of aptamer" refers to the number of times the aptamer sequence appeared in the sequencing results

Fifty-three sequences were obtained from 55 randomly picked clones, but only 32 unique sequences were found. Based on homology analysis of these 32 sequences, they could be divided into nine distinct families (Table 1). The first six families, which represent 24 of the 32 sequences, share >80 % homology and contain conserved sequences. One conserved sequence in particular, 5′-GCACAAGAGGGA-3′, was found in seven of the nine families (families 1–7), and appeared more than once in some aptamers, suggesting that these aptamers might have a common structure. The finding of high homologous aptamers

and conserved internal sequences, as well as the appearance of certain aptamers with a high-frequency, suggests that the pool of enriched single-stranded nucleotides underwent convergent evolution from the original random ssDNA library in our SELEX procedure.

Conclusions

SELEX technology was used to produce a pool of aptamers that bind selectively to formaldehyde-inactivated *V. alginolyticus* with a Kd of 27.5 ± 9.2 nM. Employing the final aptamer pool and PCR, we demonstrated qualitative detection of the inactive microorganism at concentrations as low as 100 cells/ml. Sequencing revealed that the aptamers in the final pool shared strong sequence homology to one another, and identified conserved sequences within them, suggesting convergent evolution during the SELEX process. The successful identification of aptamers that can be used to selectively detect *V. alginolyticus*, may be more useful in detection of pathogenic microorganisms in the aquaculture environment.

Acknowledgments This study was supported by the Scientific and Technological Project from the Education Department of Fujian Province (JB10096), the Science Foundation of Jimei University (ZQ2011002), the Foundation for Innovative Research Team of Jimei University (2010A004) and the Innovative Experimental Project for Students in Universities of Fujian Province.

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