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Identification of *Salmonella* Typhimurium-specific DNA aptamers developed using whole-cell SELEX and FACS analysis

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ABSTRACT

Conventional methods for detection of infective organisms, such as *Salmonella*, are complicated and require multiple steps, and the need for rapid detection has increased. Biosensors show great potential for rapid detection of pathogens. In turn, aptamers have great potential for biosensor assay development, given their small size, ease of synthesis and labeling, lack of immunogenicity, a lower cost of production than antibodies, and high target specificity. In this study, ssDNA aptamers specific to *Salmonella* Typhimurium were obtained by a whole bacterium-based systematic evolution of ligands by exponential enrichment (SELEX) procedure and applied to probing *S.* Typhimurium. After 10 rounds of selection with *S.* Typhimurium as the target and *Salmonella* Enteritidis, *Escherichia coli* and *Staphylococcus aureus* as counter targets, the highly enriched oligonucleic acid pool was sorted using flow cytometry. In total, 12 aptamer candidates from different families were sequenced and grouped. Fluorescent analysis demonstrated that aptamer C4 had particularly high binding affinity and selectivity; this aptamer was then further characterized.

1. Introduction

Salmonella infection causes severe illness in the elderly, infants, and those with compromised immune systems. The major symptoms are fever, abdominal pain, nausea, vomiting, and diarrhea (Moon et al., 2009; Kim et al., 2009). *Salmonella* outbreaks have been associated with raw or undercooked eggs and poultry; however, recently these outbreaks have resulted from various plant foods, like fresh-cut vegetables and fruits (Beuchat, 1998). *Salmonella* has been recognized as a major cause of food poisoning and is related to common and large-scale food-borne outbreaks (Velge et al., 2005). In Korea, *Salmonella* caused 9.1% of foodborne illnesses recorded during 2009−2011 (7.45% in 2009, 9.96% in 2010, and 9.9% in 2011) (Lee, 2012). In the USA, 40,000 cases of salmonellosis are reported annually (Lampel et al., 2012).

Methods for detection and control of these organisms are well established. Microbiological testing has played an important role in verifying the presence of *Salmonella*. However, conventional detection methods are complicated and require multiple steps, so that the need for rapid detection of *Salmonella* has increased (Joshi et al., 2009). In this context, biosensors show great potential for rapid detection of pathogens (Kim et al., 2009). Biosensors utilize various biological reactions at the surface of a physical sensor and involve immobilizing bio-ligands, such as enzymes, antibodies, cells, and nucleic acids (Torres-Chavolla and Alocilja, 2009). Although biosensors directly monitor these bio-reactions in real-time, their sensitivity and specificity depend on the affinity of the ligands for pathogen capture (Joshi et al., 2009; Kim et al., 2011). Antibodies have limited application, as it is difficult to modify the immune system, and it is hard to control the stability in different environments (Cibiel et al., 2011).

Aptamers have great potential for biosensor assay development, given their small size, ease of synthesis and labeling, lack of immunogenicity, a lower cost of production than antibodies, and high target specificity (Tombelli et al., 2005). In particular, whole-cell systematic evolution of ligands by exponential enrichment (SELEX), can be used to create aptamers that can bind to live bacteria (Mao et al., 2009). This methodology is composed of multiple steps, followed as: 1) screening random oligonucleotide bound with the target, 2) multiple separating and exponential amplifying the binding oligonucleotide, and 3) cloning and sequencing of the final specific binding molecules, identified as the best sequences. These specific nucleic acid sequences are called aptamers and can bind to targets with high affinity and specificity (Torres-Chavolla and Alocilja, 2009). These aptamers hold great potential for use as probes for the development of biosensors for *Salmonella* detection. A few reports on the use of aptamers for the detection of *Salmonella* have been published in recent years (Pan et al., 2005; Joshi et al., 2009; Hyeon et al., 2012). However, single-stranded DNA (ssDNA) aptamers that identify specific proteins extracted from *Salmonella*, rather than the whole cell, were reported (Pan et al., 2005; Joshi et al., 2009). Therefore, in order to find ligands with a higher affinity for the target than that of antibodies, the SELEX method was adopted.

This study aimed to design and validate the whole-cell SELEX method targeting *Salmonella* Typhimurium, including estimation of its selectivity

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and sensitivity when preceded by counter-SELEX, flow cytometry, and fluoro-photometric analysis.

2. Methods and materials

2.1. Bacterial strains

Stock cultures of *S.* Typhimurium were obtained from the Korean Collection of Type Cultures (KCTC, South Korea). Cells were grown overnight at 35 °C in brain heart infusion medium (Difco, NJ, USA). Cells were harvested by centrifugation, washed 3 times in phosphate buffered saline (PBS), and finally suspended in 100 μl of PBS prior to use in experiments.

2.2. Preparation of DNA library

The DNA template was synthesized as a single-stranded DNA containing 40 random nucleotides 5′-CGGATGCGAATTCCCTAATACGACT CACTATAGGGCGT-N_{40}-GGTGGATCCATATTCCTACTCG-3′. The initial DNA library was prepared by PCR amplification with Pyrobest polymerase (Takara, Japan).

2.3. SELEX procedures

Aptamers were screened using a modification of Liu's methods (Liu et al., 2012). In the initial selection rounds, 1.0 μg DNA library dissolved in 50 μl PBS (pH 7.4, Sigma, MO, USA) was denatured by heating at 95 °C for 10 min and then placed on ice for 10 min. The denatured ssDNA library was incubated with 100 μl of 1.0×10^9 cfu/ml *S.* Typhimurium, with shaking (500 rpm) at room temperature for 45 min. Unbound ssDNA was removed by centrifugation at 5,000 × *g* for 10 min. Then, the bound ssDNA was eluted by heating bacteria–aptamer complexes at 95 °C for 10 min in 500 μl of sterile ddH_2O. After centrifugation, the supernatant was used as an aptamer candidate template for amplification by PCR (30 cycles each of 30 s at 98 °C, 30 s at 68 °C, 15 s at 72 °C, followed by 5 min at 72 °C). Electrophoresis on 2.0% agarose gel was used to confirm the purity and verify the size of the PCR products. All PCR products were purified using Ultracel (Millipore, MA, USA). After verification of the band on the gel, the target band was carefully cut out and extracted using a MinElute gel extraction kit (Qiagen, Germany). These extraction methods were also used in the subsequent rounds of selection.

After 5 selection-rounds, the selected ssDNA pool was mixed with 100 μl of 1.0×10^8 cfu/ml *S.* Typhimurium to allow binding. To acquire aptamers with high affinity and specificity, we used 6-rounds of counter-SELEX against *Escherichia coli*, *Salmonella* Enteritidis, and *Staphylococcus aureus*.

2.4. Flow cytometric analysis (FACS; fluorescence-activated cell sorting)

To monitor the enrichment of aptamers in the selected ssDNA pool, *S.* Typhimurium was prepared with 1.0×10^9 cfu/ml in 100 μl of PBS. This analysis was used to assess the binding of the individual selected ssDNA to *Salmonella* cells. The selected DNA was fluorescently labeled via PCR amplification with 5′-FAM (fluorescein)- and 3′-FAM-modified primers. The dsDNA was denatured to ssDNA by heat and ice shock. Binding assays were then carried out by incubating 1.0 μg of fluorescently labeled aptamer candidates with 10^8 cells for 45 min in PBS and then washing the cells once in PBS buffer. Thereafter, cells were resuspended in 100 μl of PBS for flow cytometry analysis. This method has been called to fluorescence-activated cell sorting (FACS) analysis for measuring cell-assorted fluorescence (Herzenberg et al., 2006). Forward scatter, side scatter, and fluorescence intensity were measured, and gated fluorescence intensity above background was quantified. Aptamer candidates bound to *S.* Typhimurium were sorted by FACS and were then amplified with primers. These amplified aptamer candidates were cloned and sequenced at Macrogen (Seoul, Korea). The aptamer sequences were analyzed using a commercial genetic analysis program (CLC Workbench 6, Aarhus, Denmark).

2.5. Aptamer binding assay

All binding assays using FAM-labeled aptamers were analyzed using an Infinite M1000 spectrophotometer (TECAN, Switzerland). The binding and selective affinity of aptamer groups (1.0 μmole) for *S.* Typhimurium among different bacterial populations (*S.* Typhimurium, *E. coli* and *S. aureus*; 5×10^7 cfu cells each) were assessed. Aptamer candidates labeled with FAM were incubated with different bacteria in 500 μl PBS for 45 min at room temperature. After incubation, bacteria–aptamer complexes were washed twice with 500 μl PBS. Finally, the pellet of FAM-ssDNA bound to bacteria was resuspended in 100 μl PBS and transferred to 96-well black microplates (Corning, USA). Fluorescence was measured at 494 nm excitation, and emission from fluorescently labeled analytes was monitored at 510 nm. Optimal binding affinity of different concentrations (0.1, 0.5, 1.0, 5.0, and 10.0 μmole) of a high-affinity FAM-labeled aptamer C4 for *S.* Typhimurium (1×10^7 cfu) was also assessed.

3. Results and discussions

3.1. Whole-cell SELEX for evolution of S. Typhimurium-specific aptamers

Our study of the use of SELEX-based aptamers against live *Salmonella* cells was characterized by 5 important steps. First, the ssDNA library was incubated with live *Salmonella* cells at room temperature to allow binding between aptamers and live cells. Second, only ssDNA bound to cells was separated by centrifugation, allowing separation from non-bound ssDNA. Third, ssDNA released from cell surfaces was amplified by incubating of fractions eluted after washing. In this process, ssDNA was concentrated by PCR-Ultracel, because the concentration of the ssDNA is too low to allow amplification for the next selection. Finally, ssDNAs were sorted, cloned, sequenced, and then individually characterized according to their binding affinity for *Salmonella* cells by flow cytometry analysis. In total, 12 aptamer candidates were sequenced after 10 rounds of selection and 6 rounds of counter-selection (Table 1).

This study was based on a whole cell-SELEX approach, in which live cells are used to select aptamers (Chen et al., 2007; Cibiel et al., 2011). Generally, the success rate of whole cell-SELEX is almost 50% (Mayer et al., 2010). Because the cell surface carries a net negative charge, DNA polyanion is bind to cells with difficult, due to charge repulsion (Sefah et al., 2010). To solve this problem, the selection process in our study included two important processes. One was the PCR amplification process after elution of ssDNA from cells. During the 1st round of selection, the target band (100 bp) could not be detected visually; however, it could be detected after the 10× concentration by Ultracel. Therefore,

Table 1
Screened aptamer sequences.

Name	Sequence
C1	5′-GCGTGCGTCGGAGCCAGGATGCGAGGTCTGTAGGTCTGCGGGCGG-3′
C2	5′-ACGGCGGGCGAGTTGACGGCGTAATCGTGCTGCCGCGTG-3′
C3	5′-GCGGGCGTGGCTCAGAGTGGGGGTCGGTCAGTCTTGTGCGCG-3′
C4	5′-ACGGGCGTGGGGGCAATGCCTGCTTGTAGGCTTCCCCTGTGCGCG-3′
C5	5′-GCGTGCGGACGCCTGCGTGGCTGAGGCTTCGGTCTGCGCCG-3′
C6	5′-CGTGCGGCGGGCAGGATGGGATGCTGTAGGTCTGCGGGCGCGCG-3′
C7	5′-CGTGCGGAGCCAGGATGGGAGGTCTGTAGGTCTGCGGGGCGCG-3′
C8	5′-GCGTGGGACGGGTACCGGGCGTGTGGCGTGCCTGCCGCG-3′
C9	5′-ACGTGCGGGCCGGATGGGAGCTCTGTAGGTTGCGGGCGCGG-3′
CA1	5′-GCGTGCGGGGGCCAGGATGCGA-3′
CA2	5′-GGTCTGTAGGTCTGCGGCGCGCG-3′
CA3	5′-GCGTGCGGGGGCCAGGATGCGAGGTCTGTAGGTCTGCGGCGCGCG-3′