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Selection and characterization of DNA aptamers specific for *Listeria* species

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ABSTRACT

Single-stranded (ss) DNA aptamers with binding affinity to *Listeria* spp. were selected using a whole-cell SELEX (Systematic Evolution of Ligands by EXponential enrichment) method. *Listeria monocytogenes* cells were grown at 37 °C and harvested at mid-log phase or early stationary phase to serve as the targets in SELEX. A total of 10 unique aptamer sequences were identified, six associated with log phase cells and four with stationary phase cells. Binding affinity of the aptamers was determined using flow cytometry and ranged from 10% to 44%. Four candidates having high binding affinity were further studied and found to show genus-specific binding affinity when screened against five different species within the *Listeria* genus. Using sequential binding assays combined with flow cytometry, it was determined that three of the aptamers (LM6-2, LM12-6, and LM12-13) bound to one apparent cell surface moiety, while a fourth aptamer (LM6-116) appeared to bind to a different cell surface region. This is the first study in which SELEX targeted bacterial cells at different growth phases. When used together, aptamers that bind to different cell surface moieties could increase the analytical sensitivity of future capture and detection assays.

Members of the genus *Listeria* are gram-positive, rod-shaped, facultative anaerobic bacteria. The genus comprises eight non-pathogenic species (*L. marthii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. grayi*, *L. rocourtiae*, *L. fleischmannii*, and *L. weihenstephanensis*) and two pathogenic species (*L. monocytogenes* and *L. ivanovii*) which can infect humans and animals [1]. These bacteria can be found in a variety of environments including soil, water, and sewage, as well as foods such as vegetables, meat, dairy, and ready-to-eat products [2]. Although rare, listeriosis is a serious illness that predominantly affects sensitive populations, i.e., pregnant women, the elderly, and immunocompromised persons. The ability to grow at low temperatures and grow/persist in many different environments, along with the significant morbidity and mortality associated with *L. monocytogenes*, makes this pathogen of substantial concern for food safety [3].

Traditional cultural methods for detecting *L. monocytogenes* in food and environmental samples rely on cultural enrichment and selective/differential plating followed by biochemical, serological, and/or molecular identification. These methods are labor intensive and tedious, taking 2–3 days to confirm a negative sample and up to a week or more for a positive sample. Newer, highly specific detection techniques (i.e., ELISA, PCR, and real-time qPCR)[2] have shortened time to detection, bypassing the selective/differential plating steps. However, the need for prior cultural enrichment remains and this is the rate-limiting factor for truly rapid or "real-time" detection. It has been suggested that detection could be accelerated if the target pathogens were separated, concentrated, and purified from the sample matrix before detection [4].

Antibodies are currently the most commonly used ligand for the capture and detection of bacterial pathogens. Emerging ligands such as peptide and nucleic acid aptamers are promising alternatives because of their ease of production and modification, stability, low cost, and unique chemical properties [5]. Nucleic acid aptamers are single-stranded oligonucleotides having three-dimensional structures that impart binding affinity for specific target molecule(s). They are selected using an iterative process called SELEX (Systematic Evolution of Ligands by EXponential enrichment) [6]. Aptamer technologies have been widely applied in the field of diagnostics for the detection of microbial agents

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[2] *Abbreviations used:* ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; qPCR, quantitative PCR; SELEX, systematic evolution of ligands by exponential enrichment.

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including spores, vegetative cells, and metabolic byproducts (e.g., mycotoxins) [5].

Different growth temperatures and growth phases can cause variation in the expression of surface and other functional proteins of *Listeria* [7,8]. In this study, we produced single-stranded (ss) DNA aptamers specific for *L. monocytogenes* using cells in different growth phases as targets. The aptamers were selected using a whole-cell SELEX approach and further characterized for binding inclusivity and exclusivity, as well as to determine if they bound to different cell surface moieties. The ability to bind to different cell surface sites would provide the potential to use aptamers in combination, potentially increasing the analytical sensitivity of future capture and detection assays.

Materials and methods

Bacterial strains, culture conditions, and preparation of cells

The bacterial strain used as the target for aptamer selection was *Listeria monocytogenes* ATCC19115, obtained from the American Type Culture Collection (Manassas, VA). Other *Listeria* spp. used in the inclusivity studies included *L. innocua* ATCC33091, *L. ivanovii* ATCC19119, and *L. grayi* ATCC25401, in addition to well-characterized naturally occurring *L. welshimeri* and *L. seeligeri* strains isolated from foods (courtesy of Dr. Sophia Kathariou, North Carolina State University, Raleigh, NC). The non-*Listeria* strains used for the counter-SELEX and exclusivity studies included *E. coli* O157:H7 ATCC43895, *Bacillus cereus* ATCC49063, *Salmonella* Enteritidis ATCC13076, *Staphylococcus aureus* ATCC23235, *Pseudomonas aeruginosa* ATCC23993, *Shigella flexneri* ATCC12022, *Brochothrix thermosphacta* ATCC11509, and *Lactococcus lactis* MG1363 (provided by Dr. Todd R. Klaenhammer, North Carolina State University, Raleigh, NC). The target *L. monocytogenes* strain used in positive SELEX was cultured in Tryptic Soy Broth (TSB, Becton-Dickinson, Sparks, MD) at 37 °C and standard growth curves were constructed as per Buchanan et al. [9]. Fresh cultures were prepared and subsamples were taken at mid-exponential phase (6 h) and the beginning of stationary phase (12 h). Each subsample was processed by its own SELEX procedure, allowing for selection of aptamers targeting cells in different growth phases. The other *Listeria* spp. and non-*Listeria* spp. were grown in TSB and Brain Heart Infusion (BHI) broth (Becton-Dickinson), respectively, by overnight incubation at 37 °C, except for *B. thermosphacta*, which was grown at 26 °C. The enumeration of cells was done by serial dilution and plating on TSB or BHI agar plates, as appropriate.

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. monocytogenes* in the mid-exponential (6 h) and initial stationary (12 h) growth phases. The method of Dwivedi et al. [10] was used, with minor modifications. To initiate the selection process, an 81-mer combinatorial ssDNA library (5′-AGTATAC GTATTACCTGCAGC-N_{40}-CGATATCTCGGAGATCTTGC-3′) procured from Integrated DNA Technologies (IDT, Coralville, IA, USA) was amplified using the FAM-labeled forward constant primer (5′-/56-FAM/-AGTATACGTATTACCTGCAGC-3′) and biotinylated reverse constant primer (5′-/5Biosg/-GCAAGATCTCCGAGATATCG-3′) in 50 µl PCR containing 1× GoTaq buffer (Promega Corp., Madison, WI), 0.2 mM GeneAmp dNTPs Mix (Applied Biosystems, Foster City, CA), 5 U Go Taq DNA Polymerase (Promega), 500 nM forward and reverse primers and 1 µM template (ssDNA library). The PCR was performed in a DNA Engine (PTC-200) Peltier Thermal Cycler-200 (MJ Research/ Bio-Rad Laboratories, Hercules, CA) using a three-step thermal protocol of initial denaturation at 95 °C for 5 min followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min. To isolate the 5′ FAM-labeled forward strand from its complimentary 5′ biotinylated strand, the double-stranded PCR product was coupled to Streptavidin MagneSphere Paramagnetic Particles (SA-PMPs) (Promega) as per the manufacturer instructions. The FAM-labeled ssDNA molecules were separated from the biotin-labeled ssDNA bound to the magnetic particles using alkaline denaturation in 0.15 M NaOH, and concentrated using the Vivaspin500 DNA purification/concentration system (Satorius, Piscataway, NJ) as per the manufacturer instructions.

To perform the selection process, approximately 500 pmol (3.0×10^{14} sequences) of ssDNA library was denatured by heating at 90 °C for 5 min and renatured by flash cooling on ice for 10 min. This was incubated with the *L. monocytogenes* cells ($10^{8\text{-}9}$ CFU/ml) harvested at different growth phases in a total volume of 1 ml for 1 h with gentle rotation at room temperature. The bacterial cells were concentrated by centrifugation and washed sequentially to remove unbound and nonspecifically bound aptamers. The aptamers bound to cells were further enriched by PCR amplification (as per the protocol described above), the product of which was used in the next round of SELEX. The annealing temperature used in candidate aptamer amplification was gradually increased (up to 65 °C) as selection rounds were increased and the diversity of the aptamer pool decreased. For counter-SELEX, the candidate aptamer pool obtained after multiple rounds of SELEX was incubated with a cocktail of nontarget bacterial cells (*Escherichia coli* O157:H7, *B. cereus*, *S.* Enteritidis, *S. aureus*, *P. aeruginosa*, *S. flexneri*, *B. thermosphacta*, and *L. lactis*) at a pooled concentration of 10^8 CFU/ml. In this case, the cell-bound aptamer molecules were discarded and the unbound aptamer pool recovered by centrifugal washing. After completion of the entire SELEX and counter-SELEX processes, the final aptamer pool was washed (MERmaid Spin kit, MPbio, La Jolla, CA), cloned (TOPO TA Cloning kit, Invitrogen, Carlsbad, CA), and sequenced (Genewiz Inc., South Plainfield, NJ) to identify unique aptamer candidates.

Analysis of aptamer binding affinity using flow cytometry

Screening for the binding affinity (binding assays) of select aptamer candidates to *L. monocytogenes* and other related and nonrelated bacteria was done by flow cytometry. Specifically, 1 µM aptamer molecules was mixed with an overnight culture (10^8–10^9) of the target cell population in a 1 ml final volume followed by incubation for 45 min with gentle rotation at room temperature. Aptamer-bound cells were centrifuged at 5000 *g* for 10 min (Eppendorf, Hamburg, Germany) and the pellet was washed with 1 ml of 1× phosphate-buffered saline (PBS) three times to separate the unbound aptamers from the pelleted cell-bound aptamers. The pelleted cells were resuspended in 200 µl PBS and analyzed using flow cytometry (n = 200,000) (FACSCalibur flow cytometer, BD Biosciences, San Jose, CA) to record mean fluorescence intensity and percentage fluorescent cells. Similar studies were performed to evaluate the binding affinity of select aptamers for other *Listeria* species (*L. innocua*, *L. ivanovii*, *L. grayi*, *L. welshimeri*, and *L. seeligeri*), and non-*Listeria* spp. (*E. coli* O157:H7, *S.* Enteritidis, *B. cereus*, and *B. thermosphacta*). All flow cytometry data were analyzed using BD CellQuest Pro software (Becton-Dickinson Biosciences). Data were expressed as mean ± standard deviation of three replicate experiments. The aptamer binding data were analyzed by one-way analysis of variance (ANOVA) followed by Duncan's multiple-range test using statistical analysis software (SAS ver. 9.2, Cary, NC) ($P < 0.05$). For the inclusivity and exclusivity test, comparisons between groups were performed using Student's t tests ($P < 0.05$).