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Selection of Aptamers against Live Bacterial Cells

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Single-stranded DNA or RNA aptamer molecules have usually been selected against purified target molecules. To eliminate the need of purifying target molecules on the cell surface, we have developed a selection technique using live bacterial cells in suspension as targets, to select for ssDNA aptamers specific to cell surface molecules. *Lactobacillus acidophilus* cells were chosen to demonstrate proof of principle based on their high abundance of surface molecules (potential targets). Aptamer pools obtained after 6−8 rounds of selection demonstrated high affinity for and selective binding with *L. acidophilus* cells when tested via flow cytometry, microscopy, and fluorescence measurements. Out of 27 aptamers that were cloned and sequenced, one sequence, hemag1P, was found to bind to *L. acidophilus* much more strongly and specifically than other cells tested. This aptamer was predicted to have a tight hairpin secondary structure. On average, an estimated 164 ± 47 aptamer molecules were bound to a target cell with an apparent K_d of 13 ± 3 nM. A likely putative molecular target of hemag1P is the S-layer protein on the cell surface.

Aptamers are single-stranded oligonucleotides that bind with high specificity and affinity to a target molecule. They are created via Systematic Evolution of Ligands via Exponential Enrichment (SELEX).[1,2] SELEX is a combinatorial chemistry technique in which a random DNA or RNA library is incubated with a target molecule and the oligonucleotides that bind the target are then separated from the nonbinders, polymerase chain reaction (PCR) amplified, and used as refined libraries in the next round of selection. This process is typically repeated 5−15 times until oligonucleotides with a suitably high affinity and specificity for the target molecule are obtained. Aptamers bind to target molecules with similar affinity and specificity as antibodies, yet possess a variety of advantages over antibodies for both therapeutic and diagnostic applications. They are easy to synthesize, inexpensive, simple to chemically modify, minimally immunogenic, have low variability between batches, do not require animals for synthesis, and have a small size, which allows for increased tissue penetration. DNA aptamers also possess increased temperature stability over antibodies.

The first SELEX procedures generated RNA aptamers against the protein bacteriophage T4 DNA polymerase[2] and organic dyes.[1] SELEX has since been applied to generate aptamers that bind to a plethora of target molecules. The most common SELEX targets are proteins. Aptamers have been developed against a variety of mammalian cell surface proteins such as the cancer markers tenascin C, MUC1, and PMSA, as well as actin, receptor tyrosine kinase, and the rat homologue of pigpen.[3−9] Other aptamer targets include amino acids, nucleosides, small organic molecules, cell membrane fragments, viruses, parasites, and spores.[10−23]

Conventional SELEX methodologies use purified target molecules affixed to a solid support such as a column. A solid support

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provides the dual benefits of immobilizing the target and easing separation of bound from unbound oligonucleotide sequences. However, a solid support does not represent the native environment in which the target is found. Hence, fixing the target to a support may alter its conformation. Recent developments in modified SELEX techniques using capillary electrophoresis (CE) separation eliminate the need to immobilize the target molecule on a solid surface. Instead, the target molecules and the DNA library sequences are incubated in solution, and the bound sequences are separated from the unbound sequences by CE. These CE-SELEX techniques require purified target molecules for each round of incubation. Purification may change the conformation of a protein from its native state and may not be possible for unknown targets such as those present on the surface of a cell. Hence, recently modified SELEX techniques use whole mammalian cells,[3,7,8,24−26] e.g., to select aptamers against target proteins overexpressed on a mammalian cell surface. Cell-based aptamer selection techniques targeting whole live cells in suspension have been carried out to create ssDNA aptamers against leukemia, liver cancer, and lung cancer cells[27,29] without prior knowledge of a specific target molecule. SELEX has rarely been used against bacterial surface molecules as targets,[21,22,30,31] and only one previous study involved live bacterial cell-based SELEX against *Mycobacterium tuberculosis.*[32] Live bacterial cells as targets have the advantageous characteristic of being easy to grow in suspension, allowing for simple separation via centrifugation.

The aim of this study is to develop a technique to create aptamers against live bacterial cells, grown in suspension to allow for separation of bound from unbound oligonucleotides via simple centrifugation. The bacterium *Lactobacillus acidophilus* ATCC 4356 was chosen as a target. It is nonpathogenic and possesses a Gram-positive cell wall with a rich variety of potential targets, and it is also a well-known probiotic bacterium.[33] Potential targets include peptidoglycan and techoic acid, transport proteins, polysaccharides, lectins, and mucus binding proteins. The cell surface is also covered by an S-layer composed of repeated subunits of the 43-kDa S-protein.[34] Whole live *L. acidophilus* cells are incubated in suspension with a ssDNA library. Separation of bound from unbound aptamers is achieved via centrifugation and washing of the cells to remove unbound and weakly bound sequences. The resultant aptamers are cloned, sequenced, and tested for their specific binding to the target cells.

EXPERIMENTAL SECTION

Bacterial Strains and Culture Media. The *L. acidophilus* strains ATCC 4355, 4356, and 4357, as well as *Streptococcus bovis, Escherichia coli* K12 and *Saccharomyces cerevisiae,* were obtained from the American Type Culture Collection (ATCC). All *L. acidophilus* strains were grown in MRS medium (BD Difco, Sparks, MD) under anaerobic conditions at 37 °C. *E. coli* and *S. bovis* were grown under aerobic conditions at 37 °C in Luria−Bertani (LB) and BBL Brain Heart Infusion media, respectively (BD Difco). *S. cerevisiae* was grown aerobically at 30 °C in Yeast Peptone Dextrose medium (BD Difco). All bacteria were harvested in their logarithmic phase of growth. *E. coli* DH5α-T1^R cells (Invitrogen, Carlsbad, CA) were used for all transformations.

DNA Library. An 80-nt oligonucleotide single-stranded DNA library consisting of a 40-nt randomized region flanked on both sides by 20-nt primer regions was used. The initial ssDNA library and the primers used to amplify it were obtained from Integrated DNA Technologies (Coralville, IA). DNA library or aptamer pools were rendered single-stranded in one of two ways: (i) via heat denaturation at 94 °C for 5 min and subsequent cooling at 0 °C for 10 min, or (ii) via purification of the forward strand using streptavidin-coated magnetic beads (Dynabeads, Invitrogen) and a biotinylated reverse primer.

PCR Amplification and Gel Electrophoresis. The primers used to amplify the ssDNA library and subsequent aptamer pools have the following sequences:

forward: 5′-AGCAGCACAGAGGTCAGATG-3′

reverse: 5′-TTCACGGTAGCACGCATAGG-3′

The PCR conditions for amplification of the DNA library and subsequent aptamer pools during SELEX were 1× PCR reaction buffer, 2 mM $MgCl_2$, 0.4 μM concentrations of each primer, 0.2 mM dNTPs, 1 EU of platinum Taq DNA polymerase, and either 10 ng of DNA library or 39.5 μL of fraction supernatant (all reagents were from Invitrogen). Thermocycling parameters were 94 °C for 5-min denaturation, followed by 20 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 20 s. A final extension step of 72 °C for 5 min was carried out following the last cycle (MJ Mini Gradient Thermocycler, Bio-Rad Laboratories, Hercules, CA). In order to amplify aptamer pools for binding assays, 1 μL of DMSO was added to the 50-μL reaction, and the annealing temperature was raised to 69 °C while all other conditions and reagents were kept the same. This change was to minimize the formation of misamplification products due to the increasing GC content of the evolving aptamer pools, as observed in Kang et al.[35]

After PCR, the reaction products were separated on 7.5% nondenaturing polyacrylamide gel electrophoresis (PAGE) in 1× TBE buffer (Bio-Rad Protean III) at 60−120 V. The gels were stained with ethidium bromide and photographed under UV light. All PCR products were purified using Qiagen MinElute PCR Purification Kit (Qiagen Inc., Valencia, CA).

Aptamer Selection. *L. acidophilus* 4356 cells were grown overnight in liquid culture, and a subculture was grown the next

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morning until an OD_{600} of 0.3 was obtained. Cells were pelleted at 5000*g* and 4 °C and then washed twice in 1× binding buffer (1× BB) (50 mM Tris-HCl (pH 7.4), 5 mM KCl, 100 mM NaCl, 1 mM $MgCl_2$) at room temperature. We initially compared the use of 100−300-pmol DNA pools and 10^5−10^9 cells in the preliminary selection experiments. We found that 100-pmol DNA pool (equivalent to 6×10^{13} molecules) and 10^8 cells were appropriate for the subsequent experiments. Thus, a total of 10^8 cells were incubated in binding buffer (600 μL for the initial round, 350 μL for subsequent rounds) for 45 min at room temperature with the ssDNA library (2 nmol initial round) or aptamer pool (100 pmol for subsequent rounds). An excess of tRNA and BSA (Invitrogen) was added to the incubation buffer (10-fold molar excess of each in the initial round, up to an 80-fold molar excess in the eighth round). Following incubation, the cells were centrifuged at 5000*g* and 4 °C for 5 min, the supernatants were removed, and the cells were washed twice in 250 μL of 1× BB with 0.05% BSA (via resuspension and centrifugation) before a final resuspension in 100 μL of 1× PCR Reaction Buffer (Invitrogen). The cells were next heated at 94 °C for 10 min and placed on ice for 10 min in order to denature and elute cell-bound aptamers. The mixture was then centrifuged as described above and the supernatant isolated and designated as the cell-bound aptamer, or CA, fraction. All fractions collected were amplified by PCR, and the PCR products of the CA fraction were used in the next round of selection. In between each incubation, washing, and elution step, the resuspended cell solution was transferred to a fresh microcentrifuge tube in order to eliminate aptamers that bind to the tube wall. A total of eight rounds of selection were performed using fresh aliquots of cells for each round. Negative controls consisting of cells incubated with all medium components but without the oligonucleotide libraries were prepared for each round of selection.

Flow Cytometric Analysis. A FACScan flow cytometer with PowerMac G4 workstation and CellQuest software (Flow Cytometry Facility, Faculty of Medicine and Dentistry, University of Alberta) was used to assess the binding of the aptamer pool and individual aptamer sequences to different types of cells (*L. acidophilus* 4355, 4356, 4357, *E. coli* K12, *S. bovis*, *S. cerevisiae*). The aptamer pools were fluorescently labeled via PCR amplification with 5′-FAM modified primers (IDT), whereas the selected final individual aptamers were purchased with the fluorescent label (5′-FAM) attached (IDT). Aptamer pools were either heat denatured or rendered single-stranded via streptavidin-coated magnetic bead purification prior to incubation with target cells. The binding assays were carried out by incubating 100 pmol of fluorescently labeled aptamer/aptamer pool with 10^7 cells for 45 min in binding buffer and then washing the cells once in 1× BB with 0.05% BSA prior to resuspension in 1× BB for immediate flow cytometric analysis. Forward scatter, side scatter, and fluorescence intensity were measured, and gated fluorescence intensity above background (cells with no aptamers added) was quantified.

Slide-Binding Assays and Electron Microscopy. Streptavidin-coated microscope slides (Xenopore Corp., Hawthorne, NJ) were incubated with denatured biotinylated aptamer pools. A 3 μg/mL biotinylated aptamer pool solution in 1× SSC buffer was denatured at 94 °C for 10 min before being applied onto a 2-cm^2 surface area of each slide, saturating the streptavidin sites on the slide (maximum binding is 5 pmol of biotin binding sites/cm^2 of slide). Slides were incubated for 10 min at 37 °C and high humidity and then left to air-dry at room temperature. These slides, having aptamers immobilized on the surface, were stored at 4 °C when not in use. A negative control slide incubated with buffer alone was also prepared for each experiment.

To test the binding of cells to the immobilized aptamers on the slides, ~10^6 *L. acidophilus* 4356 cells were suspended in 300 μL of 1× BB and incubated over the 2-cm^2 surface area for 45 min at room temperature. The slides were each then washed 6 times in 1× BB, prior to fixation with 75% methanol and staining with crystal violet and Gram's iodine (Fisher Scientific Gram stain kit, Fairlawn, NJ). Slides were then visualized at room temperature under 1000× magnification on a light microscope (Leica DMRXA Upright Microscope with Optronics MacroFire Digital Camera, Advanced Microscopy Facility, Department of Biological Sciences, University of Alberta). For each slide, a photograph was taken of five random fields of view, and the number of cells in five random fields was counted. The aptamer pools from each round of SELEX were tested using the same approach on duplicate slides and with the same number of cells. The total number of cells in each field was counted, and totals from the 10 random fields were obtained for each aptamer pool.

Scanning electron microscopy (SEM) was used to confirm the *L. acidophilus* 4356 cells bound to the aptamer-immobilized slides (Advanced Microscopy Facility, Biological Sciences, University of Alberta). The cells were prepared for SEM via fixation with a fresh mixture of 1 part 3% glutaraldehyde in 0.1 M phosphate buffer and 2 parts 2% (w/v) aqueous osmium tetraoxide. The cells were then washed in 0.1 M phosphate buffer, desiccated, dehydrated with ethanol, and then treated with increasing amounts of HMDS (25−100%) prior to drying on a polycarbonate filter and gold-coating. The gold-coated samples were visualized the next day and photographed at 25000× on a Phillips XL SEM (Advanced Microscopy Facility, Biological Sciences, University of Alberta).

Flow Injection Coupled with Fluorescence Detection. To further confirm the specific binding of aptamers to their targets, the fluorescently labeled aptamer (hemag1) was incubated with *L. acidophilus* 4356 cells in 1× BB, and the fluorescence was measured before and after the incubation and removal of the cells. From 10^5 to 10^9 cells were incubated with 0.25 or 0.5 pmol (0.5 or 1.0 nM) of aptamer for 45 min in 1× BB. Incubation times ranging from 10 to 120 min were also varied, using 5 pmol of aptamer. The mixture was then either centrifuged at 5000*g* or filtered through a 0.22-μm membrane to remove the cells. A 50-μL aliquot of the supernatant or filtrate was injected into an Agilent 1100 HPLC system coupled to an Agilent 1100 series fluorescence detector to measure the remaining fluorescent aptamer. Fluorescence was detected at 520 nm with excitation at 495 nm. Peak areas were integrated, and the concentrations of the remaining aptamers in the solution were measured against a standard calibration of 0−1 nM fluorescently labeled aptamer. The decrease of aptamer in the solution after incubation with the cells was a measure of the bound aptamer to the cells (which were removed by centrifugation or filtration). Negative control incubations of 10^5−10^9 cells in the absence of DNA were carried out, the cells were removed, and the fluorescence intensities of the negative control incubation supernatants were subtracted from the incubation supernatants containing fluorescently labeled aptamers.

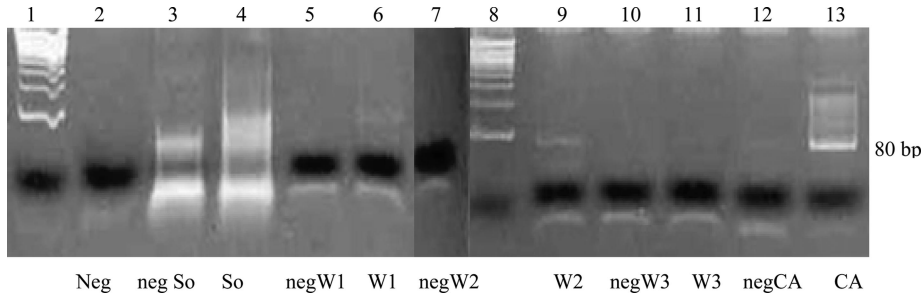

Figure 1. Native PAGE of PCR-amplified oligonucleotide fractions after the first round of SELEX. A randomized, single-stranded DNA library was incubated with *L. acidophilus* cells in the presence of tRNA and BSA. Following incubation, the cells were centrifuged to remove the incubation supernatant (So) and repeatedly washed and centrifuged to remove DNA sequences that are nonspecifically or weakly bound (W1, W2, W3). The cells were next heated at 94 °C, the suspension was centrifuged, and the supernatant was collected containing the heat-eluted cell aptamer fraction (CA). A SELEX negative control was carried out in which cells were incubated in the absence of DNA library (negSo), washed (negW1, negW2, negW3), and heat-eluted (negCA). The different fractions collected during SELEX (So, W1−W3, CA) and the parallel negative control were PCR-amplified and analyzed via PAGE. Lanes 1 and 8 on the gel contain the DNA ladder (100−2072 bp) and lane 2 contains the PCR negative control.

Cloning, Sequencing, and Structural Analysis of Aptamers. The highest affinity aptamer pools were chosen for sequencing analysis. Aptamer pools were cloned using a TOPO TA Cloning Kit for Sequencing (Invitrogen), transformed into *E. coli* DH5α-T1^R cells (Invitrogen), and colonies containing the vector were selected via overnight incubation at 37 °C on LB plates containing 50 μg/mL kanamycin. From each aptamer pool, 25 colonies were chosen for screening. The plasmid DNA was purified (Qiaex II gel extraction kit, Qiagen, Mississauga, ON, Canada) and analyzed for the presence of an 80-bp insert via digestion with 1 U of *Eco*R1 at 37 °C for 30 min, followed by 7.5% native PAGE. A total of 30 inserts were then sequenced (Molecular Biology Services Unit, Department of Biological Sciences, University of Alberta), yielding a total of 27 usable sequences. The secondary structure of each sequence was predicted using Oligoanalyzer 3.0 (IDT), with input conditions of room temperature (21 °C) and 1 mM $MgCl_2$.

Capillary Electrophoresis. Capillary electrophoresis with laser-induced fluorescence (CE-LIF) was carried out on mixtures of a S-layer protein preparation and fluorescently labeled hemag1P. The S-layer protein was extracted from the surface of *L. acidophilus* 4356 using the procedure of Lortal et al.[36] For each mixture, the concentration of hemag1P was kept constant at 5 nM and the concentration of the S-layer protein preparation was varied from 0 to 5 μM. The mixtures were incubated in 1× BB without NaCl for 45 min. Fluorescein was included as an internal standard at a concentration of 7.5 nM. Fluorescence intensity was measured at 515 nm with excitation by an argon ion laser at 488 nm.

RESULTS AND DISCUSSION

Selection of DNA Aptamers against Live *L. acidophilus*. Our technique for the selection of aptamers against live bacterial cells involves five main steps (Supporting Information Figure S1): (1) incubating DNA library with live bacterial cells, (2) separating the bound from the unbound DNA sequences via centrifugation (and washing the cells), (3) releasing the bound DNA from the cell surface, (4) amplifying the incubation supernatant, wash fractions, and bound DNA sequences, (5) characterizing the selected (bound) DNA for binding affinity (as new library for the subsequent round of selection) and for their sequences and structures (at the end of the SELEX cycles), prior to cloning and sequencing the selected DNA aptamers. *L. acidophilus* 4356 was chosen as a target due to the high abundance of molecules on its surface available for binding to DNA in the library. This nonpathogenic bacterium is easy to maintain and robust to handle. Two sets of SELEX, of eight rounds each, were performed. Prior to incubation with the target cells, the double-stranded DNA aptamer pool was rendered single-stranded via either (i) heat denaturation in the first set of SELEX or (ii) streptavidin−bead purification in the second set of SELEX. Following the incubation of ssDNA library with the *L. acidophilus* cells, most of the DNA library/aptamer pool remained unbound in the supernatant as illustrated by the large number of PCR amplification products around 70−100 bp (Figure 1, lane 4). Only a small amount of DNA was present in the first two washes of the centrifuged cells (lanes 6 and 9). No DNA was amplified from the third wash fraction from any of the SELEX rounds. The amplification products of the heat-eluted cell aptamer (CA) fraction (lane 13) represented the DNA sequences strongly bound to the cells. No DNA was amplified from the wash or CA fractions of the negative control, which consisted of cells treated to the incubation, wash and heat elution procedures without the addition of library or aptamer pool DNA. The observation of a single 80-bp band on the gel after each round of selection and PCR amplification of the CA fraction suggests that the cells are able to bind to a pool of aptamer sequences. The PCR products of the CA fraction were used in the next round of selection following purification.

Binding Affinity and Specificity of Aptamer Pools Following Each Round of Selection. After each round of selection, the aptamer pools were assessed for binding affinity and specificity to the target *L. acidophilus* 4356 cells using both flow cytometry (Supporting InformationFigure S2) and microscopy.

Flow cytometric analyses of incubation mixtures containing fluorescently labeled aptamer pools and the target cells show that, with increasing rounds of selection, the percent of cells with fluorescence above background increased to a maximum average of 67% at round 7 for the heat-denatured aptamer pools and 49% at round 6 for the streptavidin-purified aptamer pools (Figure 2a). This increase in the number of fluorescent cells is due to the increased binding of the fluorescent aptamers to the target cells.

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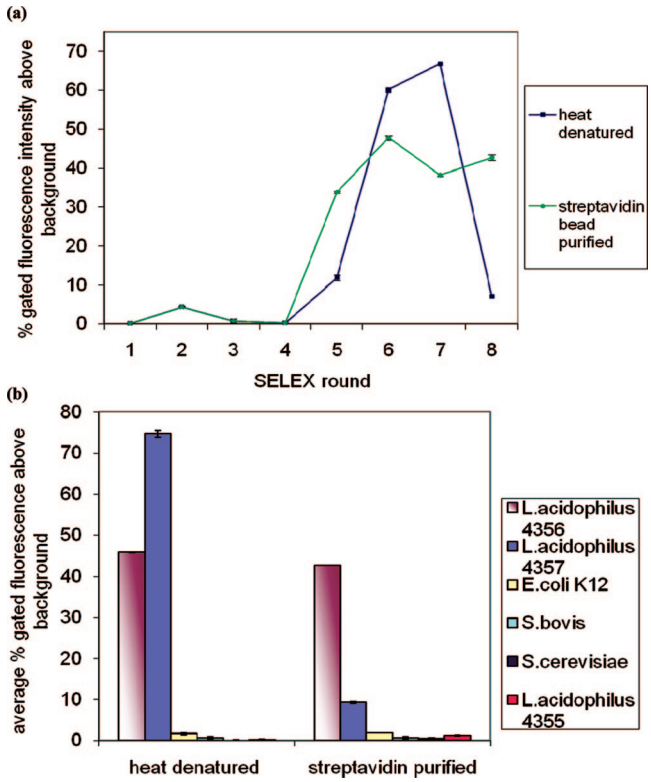

Figure 2. (a) Increase in percent gated fluorescence intensity of *L. acidophilus* 4356 cells incubated with fluorescently labeled aptamer pools after increasing SELEX rounds and (b) binding of the highest binding aptamer pools (rounds 7HE and 8MAG) to different types of cells. Aptamer pools obtained after each round of selection were fluorescently labeled at the 5′ end with a FAM-labeled primer. The aptamers were then rendered single-stranded via heat denaturation and incubated with the target cells. A total of 100 pmol of labeled aptamer pool and 10^7 cells were used. Following incubation the cells were washed, pelleted by centrifugation, and resuspended in incubation buffer to a final volume of 450 μL and subjected to flow cytometry. Error bars represent standard deviation from triplicate incubations.

It thus appears that, during SELEX, DNA rendered single-stranded via heat denaturation results in the generation of aptamers that bind more efficiently to the target cells than DNA rendered single-stranded via streptavidin-coated magnetic bead purification. This may be due to the fact that, in the streptavidin-purified pools, only the forward strand of the DNA is retained for incubation whereas in the heat-denatured pools both forward and reverse strands are retained, effectively doubling the number of sequences available for selection. It is also possible that the streptavidin-coated magnetic bead purification procedure resulted in preferential retention of certain sequences and loss of others or in nonspecific loss of sequences due to the additional purification steps present when using magnetic beads versus heat. Increase in aptamer binding with SELEX round appears to level off after round 6 for both the heat-denatured and the streptavidin-purified aptamer pools (Figure 2a). However, for the heat-denatured pools, the eighth round of SELEX results in the loss of affinity aptamers for the bacterial cells. This deselection may be due to a decrease in the aptamer pool complexity, inefficient partitioning of bound from unbound sequences during the eighth round selection process, or a combination of both. As a result, subsequent cloning of aptamer sequences was carried out from the round 7 heat-denatured aptamer pool and round 8 streptavidin-purified aptamer pools.

Controls carried out using fluorescent random DNA library with *L. acidophilus* 4356 cells, fluorescent aptamer pools alone, and buffer with BSA and tRNA alone did not yield any increase in gated fluorescence above background levels (data not shown). Increasing the amount of time after incubation and washing at which the cell−aptamer solutions were analyzed via flow cytometry resulted in a maximum 9.7% decrease in maximum gated fluorescence above background. This decrease was constant up to 30-min postincubation for the aptamer pool, providing ample time for sample analysis without loss of aptamer pool binding or cell death (data not shown).

Different cells were used to test the specificity of the aptamer pools. The increase in gated fluorescence intensity was less than 2% when the fluorescent aptamer pools were tested against *S. bovis, E. coli* K12, and *S. cerevisea* (Figure 2b). This indicates that the binding observed between the aptamers and *L. acidophilus* is target specific. Two additional strains of *L. acidophilus* were also tested, ATCC 4355 and 4357, which are rat and human isolates, respectively (4356 is a human isolate). Minimal binding of the aptamer pool to the rat isolate strain 4355 was seen, but the aptamer pool bound strongly to the 4357 cells. Both strains 4356 and 4357 are human isolates, are known to be highly genetically related, and are covered in an S-layer composed of similar S-proteins.[37,38]

To further confirm the binding of the aptamer pool to the target cells, we developed a novel slide-binding assay using streptavidin-coated microscope slides and biotinylated aptamer pools. This idea is reminiscent of the previous work using aptamers to capture osteoblasts for the purpose of seeding bone growth after transplantation.[39] *L. acidophilus* 4356 cells were incubated on the slides for 45 min, washed thoroughly, fixed with methanol, stained with crystal violet, and viewed under a light microscope. Further SEM analyses of the cells captured on the slides show their morphological similarity to those of the *Lactobacillus* species (data not shown). A series of photographs is shown in Figure 3 for slides coated with heat-denatured aptamer pools from SELEX rounds 1−8. As the round of SELEX after which the aptamer pool was isolated was increased, an increasing number of cells were found to bind to the slides for both heat-denatured (Figure 3 and Supporting InformationFigure S4) and streptavidin-purified aptamer pools (Figure S4), indicative of increased binding affinity of the aptamers for the *L. acidophilus* cells. The ratio of cells bound to slides coated with heat-denatured round 7 aptamers versus uncoated slides is 61 and is 9 for round 7 heat-denatured aptamer pools versus round 1 heat-denatured aptamer pools. These results confirm those of flow cytometry, supporting the specific binding of the aptamer to the target cells. The key advantages of this slide-binding assay are that it allows visualization of bacterial cells and is rapid and inexpensive.

Cloning and Sequence Analysis of Aptamer Pools. Upon confirmation that aptamer pool binding was increasing with SELEX rounds, the heat-denatured (HE) and streptavidin-coated, magnetic bead-purified (MAG) aptamer pools were cloned and sequenced after the seventh and eighth rounds, respectively. These pools displayed

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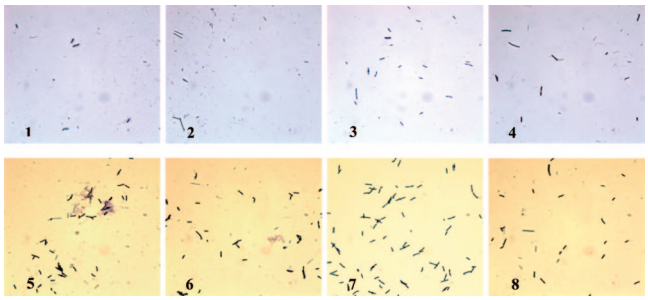

Figure 3. Slide-binding assay showing binding of *L. acidophilus* 4356 cells to microscope slides coated with heat-denatured aptamer pools after each round of SELEX (round number labeled on each picture). Aptamer pools obtained after each round of selection were biotinylated and attached to duplicate streptavidin-coated microscope slides. The slides were then incubated with *L. acidophilus* 4356 cells in binding buffer. Cells were prepared via centrifugation at 5000*g* and 4 °C, washing twice in binding buffer. For each slide, ~10^6 cells were suspended in 300 μL of binding buffer and incubated over the 2-cm^2 surface area for 45 min at room temperature. The slides were each then washed six times in binding buffer, prior to fixation with 75% methanol and staining with crystal violet and Gram's iodine. Slides were then stored at room temperature until visualization under a light microscope at 1000× magnification. For each slide, the number of cells in five random fields was counted.

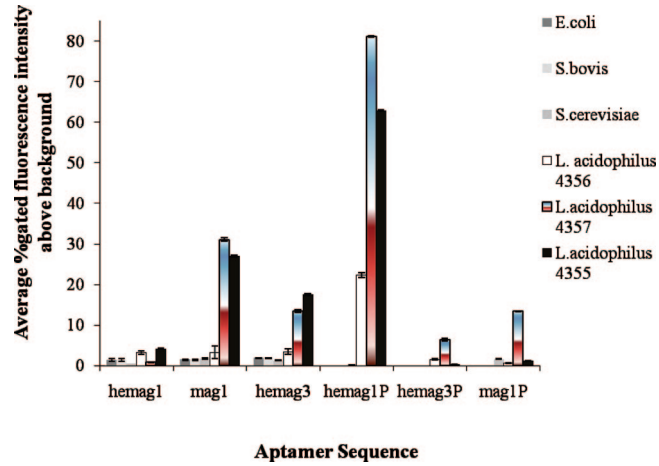

Figure 4. Sequences derived via cloning of aptamer pools, which show increased binding affinity to *L. acidophilus* 4356, 4357, and 4355 cells and minimal binding to other cells when screened via flow cytometry. All aptamers were fluorescently labeled with 5′-FAM. Incubations and centrifugation were carried out as in Figure 2. Each mixture contained 100 pmol of aptamer and 10^7 cells.

the highest affinity for the target cells when screened via flow cytometry. A total of 27 sequences from both the streptavidin-magnetic bead-purified and heat-denatured aptamer pools were obtained. Within this collection of sequences, there was one sequence that was repeated six times, three times in each pool. This sequence was named hemag1P. There were several other sequences and sequence motifs that were repeated both within and between aptamer pools. Also, many of the sequences contain a high percentage of C and G bases, especially at their 5′ and 3′ ends. This suggests the presence of secondary structural motifs.

Binding of Individual Aptamer Sequences to Target Cells. Sequences representing a variety of secondary structures, as well as those sequences that were repeated within and between the aptamer pools, were selected for further screening (Figure 4). The aptamers with and without primer sequences were included (Table 1), e.g., hemag1P and hemag1. Each aptamer sequence was fluorescently labeled and tested via flow cytometry for binding to three separate strains of *L. acidophilus*: 4355, 4356, and 4357. Higher binding to strain 4357 than to 4356 and 4355 was seen with many of the sequences. This trend is similar to that seen with the aptamer pools (Figure 2b). Binding of all the sequences was specific for *L. acidophilus* in that minimal or no binding was seen to *E. coli, S. bovis,* or *S. cerevisiae* (Figure 4). The most frequently repeated sequence with primers, hemag1P, exhibited by far the highest binding for both 4356 and 4357 cells. The secondary structure of each sequence, both with and without primers, was predicted at room temperature and 1 mM $MgCl_2$ (Supporting Information Figure S3). The aptamer sequences can be classified into three broad categories of secondary structure: (i) tight hairpins, (ii) branched hairpins, and (iii) few or no hairpins.

The predicted most energetically favorable secondary structure of aptamer hemag1P at room temperature and 1 mM $MgCl_2$ is shown in Figure 5. Inclusion of the primer sequences in hemag1P allows it to form a tight hairpin secondary structure whereas the shorter sequence hemag1 forms a more open structure (Supporting Information Figure S3). The hairpin secondary structure perhaps accounts for the superior binding of hemag1P to the target cells. Other sequences that demonstrated affinity for *L. acidophilus* (Figure 4) also have predicted hairpin or branched structures (Supporting Information Figure S3), whereas those sequences with no or minimal affinity tend to have minimal predicted secondary structures.

Binding of Aptamer hemag1P to *L. acidophilus*. Binding of hemag1P to target 4356 cells was further examined via flow cytometry and flow injection coupled to fluorescence detection. The binding curve from flow cytometric analysis of fluorescently labeled hemag1P and *L. acidophilus 4356* cells (10^7cells) is shown in Figure 6. At first, aptamer concentrations in the incubation supernatant from 0 to 500 nM were tested, with the amount of aptamer binding found to level off at less than 100 nM (data not shown). Subsequently, a lower concentration range (0−120 nM) was then examined more closely (Figure 6). The average gated fluorescence intensity above background was found to reach a maximum of 31 ± 0.8% at a hemag1P concentration of 80 nM. Heating the single-stranded aptamers at 94 °C and then cooling at 0 °C prior to room-temperature incubation had negligible effect on binding (data not shown). The K_d of hemag1P was determined to be ~13 ± 3 nM based on the fit of the nonlinear regression curve (GraphPad Prism 5).

Analyses of fluorescent aptamers in supernatant before and after incubation with *L. acidophilus* 4356 indicate increased removal of hemag1P by increasing numbers of target cells. Separate experiments were carried out in which the supernatant was and was not passed through a 0.22 μM filter prior to analysis. The purpose of the filtration step was to remove any cells in the supernatant that may have dislodged from the pellet and, hence, may interfere with the fluorescence measurement. The supernatants were then measured with a fluorescence detector, where the amount of fluorescence is proportional to the aptamer concentration of the sample. Increased aptamer removal by the target cells is represented by a decrease in detectable supernatant fluorescence with increasing cell number, for both the filtered

Table 1. Screened Aptamer Sequences with (hemag1P, mag2P, hemag3P) and without (hemag1, mag1, hemag3) Primers[a]

name	sequence
hemag1	5′FAM/TAGCCCTTCAACATAGTAATATCTCTGCATTCTGTGATG-3′
mag1	5′FAM/TGAGCCCCACTAAAGTTGCAATCATGTCGTCAGCTTTGGG-3′
hemag3	5′FAM/CGTCGCGGC ATATTTCCAGTGGAACGGTTACGATATGTTG-3′
hemag1P	5′FAM/AGCAGCACAGAGGTCAGATGTAGCCCTTCAACATAGTAATATCTCTGCATTCTGTGTGCCTATGCGTGCTACCGTGAA-3′
mag1P	5′FAM/AGCAGCACAGAGGTCAGATGTGAGCCCCAGTAAAGTTGCAATCATGTCGTCAGCTTTGGGCCTATGCGTGCTACCGTGAA-3′
hemag3P	5′FAM/AGCAGCACAGAGGTCAGATGTCGTCGCGGCATATTTCCAGTGGAACGGTTACGATATGTTGCCTATGCGTGCTACCGTGAA-3′

[a] Shown here are the aptamers of high affinity for *L. acidophilus* 4356 cells, selected from a total of 27 aptamers that were sequenced. The fluorescent label FAM is shown at the 5′ end. The underlined sequences are the primers.

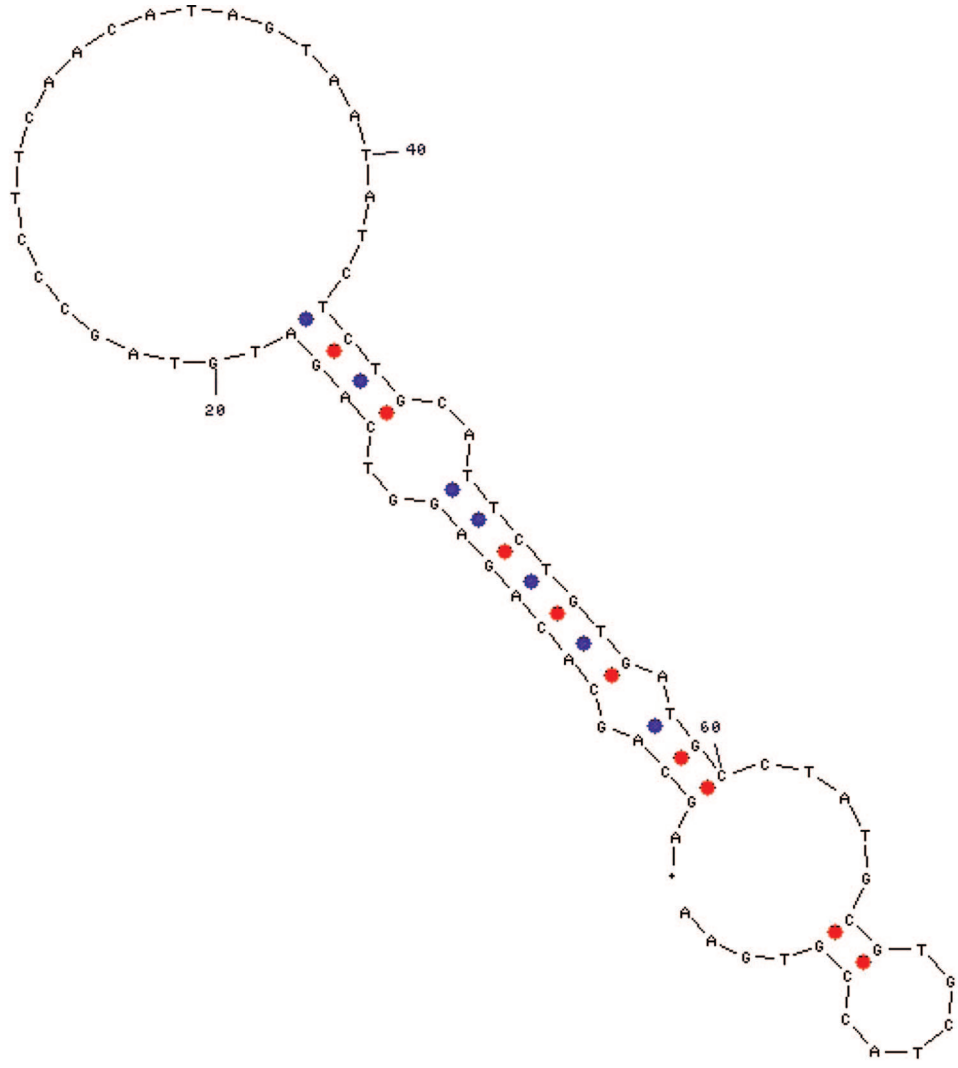

Figure 5. Predicted secondary structure of aptamer hemag1P. Structure was predicted at 21 °C and 1 mM $MgCl_2$ using Oligoanalyzer 3.0 (IDT). Base-pairing interactions are represented by blue and red dots.

and unfiltered supernatants. An average of approximately 100% of 0.5 nM aptamer or 72% of the starting 1.0 nM aptamer was removed from the unfiltered supernatants by 10^9 cells. This corresponds to the binding of 150 ± 1 and 217 ± 22 aptamers/cell, respectively. Analysis of the filtered supernatant revealed that 84% of the 0.5 nM aptamer fluorescence was removed by 10^9 cells, corresponding to 126 ± 25 aptamer molecules/cell. These results complement the flow cytometry analysis, showing the binding of the fluorescently labeled hemag1P aptamer to the *L. acidophilus* 4356 cells. These results also provide an estimate of the number of aptamer molecules binding to each cell (on average ~164 ± 47 aptamer molecules/cell).

Putative Target Molecule. The SELEX technique that has been presented herein most likely favors the selection of aptamers that bind to abundant cell surface molecules. The high affinity of hemag1P for the target cells (nM K_d) supports this assertion, since aptamers selected against a single purified target typically possess lower K_ds than those selected against complex targets in which multiple target molecules are present. The estimated K_d of 13 ± 3 nM is comparable

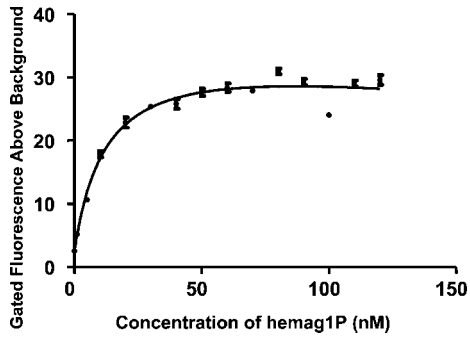

Figure 6. Binding characteristics of aptamer hemag1P as determined via flow cytometry. Various concentrations of aptamer hemag1P were incubated with 10^7 *L. acidophilus* 4356 cells in binding buffer for 45 min and analyzed via flow cytometry under the same conditions as in Figure 2. Error bars are standard error from duplicate analyses.

to K_ds of aptamers derived from purified protein targets.[2,16,40−42] The most abundant surface structure on *L. acidophilus* 4356 is the evenly distributed S-layer composed of 43-kDa S-proteins.[35] The flow cytometry results (Figure 4) support this idea, since hemag1P binds only to cells with S-layers composed of similar S-proteins (*L. acidophilus* 4355, 4356, and 4357) and not to other cells (*E. coli, S. bovis,* and *S. cerevisiae)*. Further flow cytometric screening of hemag1P against *Lactobacillus* species both with (*L. acidophilus* 4356) and without (*L. casei, Lactobacillus gasseri, Lactobacillus johnsonii*) S-layers indicates that hemag1P does not bind to cells without an S-layer (data not shown). Further evidence is provided by preliminary CE-LIF analysis of incubation mixtures of a crude S-layer protein preparation and fluorescently labeled hemag1P. A decrease in the unbound hemag1P peak intensity was seen with increasing concentration of S-layer protein preparation, along with the corresponding formation of aptamer−protein complex peaks. These preliminary results suggest that S-protein is a likely target of hemag1P. However, procedures to obtain the S-layer protein from the cell surface may alter protein conformation and hence aptamer−protein binding. Purified S-layer proteins are not available commercially. Further studies will need to optimize S-layer protein purification procedures, including affinity purification with hemag1P as an affinity tag, and to assess the strength of the interaction between the aptamers and the purified protein target.

CONCLUSIONS

By developing a modified cell-based SELEX method, we have generated several ssDNA aptamers capable of binding to the bacterium *L. acidophilus*, without use of the purified target molecule during selection. Briefly, whole live bacterial cells in solution were used as a target matrix, and bound aptamers were separated from unbound via centrifugation and heat elution. Of the resultant aptamers, hemag1P displayed the highest and most specific binding with an estimated K_d of 13 ± 3 nM.

A similar method has been employed against mammalian cancer cells.[27] Aptamers have also previously been created against purified bacterial components, including autoclaved anthrax spores, bacterial toxins, and purified *F. tularensis* antigens[22,30,31] as well as *M. tuberculosis.*[32]

Developing a SELEX technique against whole, live bacterial cells versus purified components presents several key advantages. Foremost is the increased efficiency of this technique; since a specific target molecule does not need to be identified prior to selection, many troublesome purification steps are avoided. This technique could lead to discovery of novel cell surface molecules as well as the selection of aptamers against new disease and pathogenicity markers without the necessity of purifying them. Another advantage of these aptamers is that they are selected against the target in its native conformation, a particularly important consideration for protein targets. A study by Pestourie et al. found that using mammalian cells as a matrix for an overexpressed target membrane protein, as opposed to selection against the protein on magnetic beads, was essential to evolving aptamers able to recognize the target protein in its native environment.[7]

Aptamers that bind to bacterial cell surface molecules could be used in bacterial surveillance and typing, diagnosis of bacterial infections, and therapeutics. A variety of assays already developed for protein detection using aptamers could be extended toward bacterial detection, particularly aptamer-based ELISAs and microarrays.[43,44] Similarly, aptamers could replace antibodies in bacterial typing. In addition to detecting cells expressing target molecules on their surface, aptamers could function as antimicrobials. In binding to a specific molecular target, aptamers could inhibit the growth or pathogenesis of cells expressing that target molecule. Aptamers against mammalian cell surface molecules have been found to block cellular transformation and inhibit tumor growth; aptamer-siRNA chimeras show promise in targeting tumor cells for apoptosis through the RNA interference pathway.[45,46] Aptamers have also been used as escorts to target gelonin to cells overexpressing PSMA, a prostate cancer marker.[5]

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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