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Development of Aptamer Beacons for Rapid Presumptive Detection of *Bacillus* Spores

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Abstract A library of 92 DNA aptamer sequences was developed against *Bacillus anthracis* (nonpathogenic Sterne strain) spores and anthrose sugar immobilized on magnetic beads. The selected DNA sequences were studied for similarities and potential binding pockets between the *B. anthracis* spore and anthrose aptamers. Several recurring loop structures were identified and tested for their potential to act as aptamer beacons when labeled with TYE 665 dye on their 5′ ends and Iowa Black quencher on their 3′ ends. Of these candidate sequences, two beacons designated BAS-6F and BAS-6R emerged which gave strong fluorescence responses at high spore concentrations (greater than 30,000 spores/ml). These aptamer beacons also detect *B. cereus* and *B. thuringiensis* spores with greater fluorescence intensity, but do not strongly detect vegetative cells from an array of other bacterial species. BAS-6F and 6R are also not capable of detecting pure anthrose, thereby probably ruling that epitope out as a spore surface target for these particular beacons. While not extremely sensitive, the BAS-6F and 6R aptamer beacons are potentially valuable for rapid presumptive detection of anthrax or *Bacillus* spores in suspect powders or bioterrorist activity where spore concentrations are anticipated to be high. The sequence similarities of these beacons to other published *Bacillus* spore aptamers are also discussed.

Keywords Anthrax · Anthrose · *Bacillus* · Beacon · FRET · SELEX · Spore · Terrorism

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Introduction

Rapid, presumptive detection of *B. anthracis* spores on site in buildings or envelopes and packages is important and desired by governments to avoid any casualties or expensive remediation as occurred in the aftermath of the attacks of Sept. 11, 2001. While a number of very sensitive immunoassay [1–3], PCR-based [4, 5], and laser fluorescence-aerosol particle counter technologies [6] capable of anthrax detection exist, these are generally expensive, time-consuming, or not portable. Aptamer beacons, initially produced from Sytematic Evolution of Ligands by Exponential enrichment (SELEX), have the advantages of speed (15–20 min assays) with good sensitivity in most cases [7–12] and potential portability when used with a commercially available handheld fluorometer [7–10] to enable more informed on site decisions.

The problem of anthrax detection, however, is further complicated by the very close genetic relationship of *B. anthracis* to other species of *Bacillus* such as *B. cereus* and *B. thuringiensis* [13, 14]. Even within the *B. anthracis* species, only strains carrying the pXO1 (toxin coding) and pXO2 (anti-phagocytic capsule) plasmids are pathogenic. Hence, for confirmatory or forensic analyses, PCR and other molecular biological techniques are required. But for rapid and portable detection of suspect powdery materials in air or in envelopes and packages and on fomites, a rapid presumptive one step or homogeneous assay is highly desirable to facilitate the maintenance of tight homeland security.

Therefore, we set out to determine if we could identify DNA aptamer beacons which could simply bind and detect *B. anthracis* or other *Bacillus* spore species in a “lights on” fashion in a matter of minutes. We also examined the feasibility of detecting the presence of *Bacillus* spores in a commercially available handheld fluorometer with comparison to results for the same samples obtained using a large bench-top

spectrofluorometer. Portable and rapid detection of *Bacillus* spores is advantageous for food safety testing [15] as well and detection of the natural *B. thuringiensis* (BT) pesticides or tracking and monitoring of BT spores used as biowarfare simulants in military defense field trials [16]. A number of predecessors have also developed aptamers against *Bacillus* spores [3, 16–19] and comparisons are drawn between their reported aptamer DNA sequences and those derived from the present study.

Experimental

Materials, Bacterial Cultures and Spore Preparation

Synthetic anthrose sugar [20–22] was purchased from Carbosynth, Ltd. (Berkshire, UK). Live *Bacillus anthracis* (nonpathogenic $pXO2^-$ Sterne strain) spore vaccine was purchased from the Colorado Serum Co., (Denver, CO). *B. cereus* strain 19637 was purchased from American Type Culture Collection (ATCC). *B. thuringiensis* (kurstaki strain) spores were obtained from the U.S. Air Force Research Laboratory [16]. All *Bacillus* species were streaked onto blood agar plates and cultured for 10 days at 37 °C to ensure sporulation followed by natural degradation of most of the vegetative cells. *Bacillus* culture plates were then flooded with 5 ml of sterile phosphate buffered saline (PBS, pH 7.2). Spores and remaining cells were scraped from the wetted plates and allowed to settle in tubes overnight at 4 °C. The supernate was decanted and the pellet revealed an almost pure spore population in each case which was kept cold and used for experiments. Other bacterial species were obtained from ATCC and cultured similarly on blood agar at 37 °C overnight and collected in PBS, but not settled or decanted. Approximate cell counts were obtained by hemacytometer and confirmed or adjusted by colony counts on blood agar spread plates cultured overnight at 37 °C.

DNA Aptamer Development, Cloning and Sequencing

One ml aliquots of approximately 2×10^7 *B. anthracis* (Sterne strain) spores suspended in 1X Binding Buffer (1XBB; 0.5 M NaCl, 10 mM Tris–HCl, and 1 mM $MgCl_2$, pH 7.5–7.6,) were used as targets for DNA aptamer selection. On mg of anthrose was immobilized via its hydroxyl groups by Fischer esterification onto 1 ml (2×10^9) carboxylic acid-coated M270 magnetic beads (MBs; Invitrogen Corp.) in 1 ml of sterile PBS adjusted to pH 5.0 with gentle mixing at 37 °C for 24 h followed by 3 washes in sterile 1XBB.

All DNA oligonucleotides and aptamer beacons were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Whole spore and MB-based SELEX were performed essentially as previously described [8, 16, 23] with 144 nanomoles of the degenerate SELEX template library sequence which was: 5′-ATCCGTCACACCTGCTCT-N_{36}-TGGTGTTGGCTCCCGTAT-3′, where N_{36} represents the randomized 36-base region of the DNA library. Primer sequences were: 5′-ATACGGGAGCCAACACCA-3′ (designated forward) and 5′-ATCCGTCACACCTGCTCT-3′ (designated reverse) to prime the template and nascent strands, respectively. The random library was reconstituted in 500 μl of sterile nuclease-free water and heated to 95 °C for 5 min to ensure that the DNA library was completely ss and linear. The hot template solution was added to 100 μl of anthrose-MBs (2×10^8 beads) with 600 μl of sterile 2X Binding Buffer (2XBB). The DNA libraries plus spores or anthrose-conjugated MB suspension (1.2 ml) were mixed at room temperature (RT) for 1 h.

Following interaction with the randomized DNA libraries, DNA-spore complexes were separated from unbound DNA by centrifugation at 14,000 × g for 5 min on a microcentrifuge. Similarly, anthrose-MBs with any bound DNA (round 1 aptamers) were magnetically separated from unbound DNA with a Dynal MPC-S® magnetic rack. The DNA-anthrose-MB and DNA-spore complexes were then washed three times in 400 μl of sterile 1XBB.

Following the third wash, the MB or spore pellets (about 75 μl each) were used in separate PCR reactions to amplify the bound DNA as follows. The MB pellet was split into 15 μl aliquots and added to five Easy Start™ Micro 50 tubes (Molecular BioProducts, Inc., San Diego, CA), which contained most of the nonperishable components of a PCR reaction beneath a wax seal. A total of 3 μl of 1:10 primer mix (10% forward primer plus 10% reverse primer) in nuclease-free deionized water or ~20 nanomoles of each primer per ml plus 1 μl (5 U) of Taq DNA polymerase (Fisher Scientific, Inc., Pittsburgh, PA) and 5 μl of 2 mM $MgCl_2$ were added to each of the five tubes. PCR reactions were supplemented with 0.5 μl of Perfect Match™ *E. coli* single-strand binding protein (SSBP, Stratagene Inc., La Jolla, CA) to inhibit high molecular weight concatamer formation. PCR was carried out as follows: an initial 95 °C phase for 5 min, followed by 30 cycles of 1 min at 95 °C, 1 min at 53 °C, and 1 min at 72 °C followed by a 72 °C completion stage for 7 min, and refrigeration at 4 °C. This constituted the first of five rounds of whole spore or anthrose-MB SELEX. To begin the second round and all subsequent rounds, four complete tubes of the five original PCR tubes were heated to 95 °C for 5 min to release bound DNA from whole spores or anthrose-MBs. The fifth tube was always retained and refrigerated as a back-up for that round of the SELEX process. All available DNA (25 μl per tube) was siphoned out of the hot tubes without removing the whole spores or anthrose-MBs before the tubes cooled significantly and the DNA was pooled. The 100 μl of

Table 1 DNA aptamer sequences selected against anthrose and whole *B. anthracis* spores

Anthrose (Ant)	
Ant -1aF	ATACGGGAGCCAACACCACCTCCTACCCCCAACCCCGGTCCCCGTAAATGTACTAGAGCAGGTGTGACGGAT
Ant -1aR	ATCCGTCACACCTGCTCTAGTACATTTACGGGGACCGGGGTTGGGGGTAGGAGGTGGTGTTGGCTCCCGTAT
Ant -1bF	ATACGGGAGCCAACACCATGATTGAATAGGTGGAATTAGGTAAGTAACCGACCTAGAGCAGGTGTGACGGAT
Ant -1bR	ATCCGTCACACCTGCTCTAGGTCGGTTACTTACCTAATTCCACCTATTCAATCATGGTGTTGGCTCCCGTAT
Ant - 3 F	ATACGGGAGCCAACACCAGCCAAGCCCAGCCCTCCCTTCTCCTCTCTCTCCCTCAGAGCAGGTGTGACGGAT
Ant - 3 R	ATCCGTCACACCTGCTCTGAGGGAGAGAGAGGAGAAGGGAGGGCTGGGCTTGGCTGGTGTTGGCTCCCGTAT
Ant - 4 F	ATACGGGAGCCAACACCATCTAGCAAGACGTACGTAGATAAGGTTATTCCCACAAGAGCAGGTGTGACGGAT
Ant - 4 R	ATCCGTCACACCTGCTCTTGTGGGAATAACCTTATCTACGTACGTCTTGCTAGATGGTGTTGGCTCCCGTAT
Ant - 6 F	ATACGGGAGCCAACACCAGACGATATGACCGTAGTTCGGCGACACTCTATGGGGAGAGCAGGTGTGACGGAT
Ant - 6 R	ATCCGTCACACCTGCTCTCCCCATAGAGTGTCGCCGAACTACGGTCATATCGTCTGGTGTTGGCTCCCGTAT
Ant - 7 F	ATACGGGAGCCAACACCACAGACCCCCTCATCCCGCCGGCAGCCCCCTTTGCCGAGAGCAGGTGTGACGGAT
Ant - 7 R	ATCCGTCACACCTGCTCTCGGCAAAGGGGGCTGCCGGCGGGATGAGGGGGTCTGTGGTGTTGGCTCCCGTAT
Ant - 8 F	ATACGGGAGCCAACACCAACCCCACTAAGTCCCCTATCATTTAAGATGCCCTAAAGAGCAGGTGTGACGGAT
Ant - 8 R	ATCCGTCACACCTGCTCTTTAGGGCATCTTAAATGATAGGGGACTTAGTGGGGTTGGTGTTGGCTCCCGTAT
Ant - 9 F	ATACGGGAGCCAACACCAGACAACCCTTCTACCCCTCAAGTCGCCAATAACGGTAGAGCAGGTGTGACGGAT
Ant - 9 R	ATCCGTCACACCTGCTCTACCGTTATTGGCGACTTGAGGGGTAGAAGGGTTGTCTGGTGTTGGCTCCCGTAT
Ant - 10 F	ATACGGGAGCCAACACCAGGTTTAAAGATTCATTCGCAGCTCAAATATCTTTGGAGAGCAGGTGTGACGGAT
Ant - 10 R	ATCCGTCACACCTGCTCTCCAAAGATATTTGAGCTGCGAATGAATCTTTAAACCTGGTGTTGGCTCCCGTAT
Ant - 11 F	ATACGTGAGCCAACACCATAAATAGAAAGCCAAGAGAGGTTAGGGTTAATGAGTAGAGCAGGTGTGACGGAT
Ant - 11 R	ATCCGTCACACCTGCTCTACTCATTAACCCTAACCTCTCTTGGCTTTCTATTTATGGTGTTGGCTCACGTAT
Ant - 12 F	ATACGGGAGCCAACACCACCCACCTTCCCTCTTCATACGCCACCTCACACATCCAGAGCAGGTGTGACGGAT
Ant - 12 R	ATCCGTCACACCTGCTCTGGATGTGTGAGGTGGCGTATGAAGAGGGAAGGTGGGTGGTGTTGGCTCCCGTAT
Ant - 13 F	ATACGGGAGCCAACACCAAGTCCGAAAGAACTTATAACGCAATGGAATCCTGGGAGAGCAGGTGTGACGGAT
Ant - 13 R	ATCCGTCACACCTGCTCTCCCAGGATTCCATTGCGTTATAAGTTCTTTCGGACTTGGTGTTGGCTCCCGTAT
Ant - 14aF	ATACGGGAGCCAACACCAGAATTACACTCTGGTGGGGTGGGGGAAGAGGGATGTAGAGCAGGTGTGACGGAT
Ant - 14aR	ATCCGTCACACCTGCTCTACATCCCTCTTCCCCCACCCCACCAGAGTGTAATTCTGGTGTTGGCTCCCGTAT
Ant - 14bF	ATACGGGAGCCAACACCAAAACAACACAATACGCCCTATAGCGAAAGACAACGTAGAGCAGGTGTGACGGAT
Ant - 14bR	ATCCGTCACACCTGCTCTACGTTGTCTTTCGCTATAGGGCGTATTGTGTTGTTTTGGTGTTGGCTCCCGTAT
Ant - 15 F	ATACGGGAGCCAACACCATCTTTCTTAGGATACAAAGCCAAACTGAGCCCGTGCAGAGCAGGTGTGACGGAT
Ant - 15 R	ATCCGTCACACCTGCTCTGCACGGGCTCAGTTTGGCTTTGTATCCTAAGAAAGATGGTGTTGGCTCCCGTAT
Ant - 16 F	ATACGGGAGCCAACACCACGACATTATTATTGTATCATGTACGGTAGCCTTTTGAGAGCAGGTGTGACGGAT
Ant - 16 R	ATCCGTCACACCTGCTCTCAAAAGGCTACCGTACATGATACAATAATAATGTCGTGGTGTTGGCTCCCGTAT
Ant - 17 F	ATACGGGAGCCAACACCAACGTTCCAGCTCAAAGACTCTTTTCTAAAACGGTGTAGAGCAGGTGTGACGGAT
Ant - 17 R	ATCCGTCACACCTGCTCTACACCGTTTTAGAAAAGAGTCTTTGAGCTGGAACGTTGGTGTTGGCTCCCGTAT
Ant - 18 F	ATACGGGAGCCAACACCACCAAAAACCCCTACCCCACACCAGACTTCCCCCGCCAGAGCAGGTGTGACGGAT
Ant - 18 R	ATCCGTCACACCTGCTCTGGCGGGGGAAGTCTGGTGTGGGGTAGGGGTTTTTGGTGGTGTTGGCTCCCGTAT
Ant - 19 F	ATACGGGAGCCAACACCAATAATTAAAAATAAGATAGAAAGAACATGTCAGTCTAGAGCAGGTGTGACGGAT
Ant - 19 R	ATCCGTCACACCTGCTCTAGACTGACATGTTCTTTCTATCTTATTTTTAATTATTGGTGTTGGCTCCCGTAT
Ant - 20 F (69)	ATACGGGAGCCAACACCAGAGCAGGTGTGACGGAGAGCAGGTGTGACGGGTAGAGCAGGTGTGACGGAT
Ant - 20 R (69)	ATCCGTCACACCTGCTCTACCCGTCACACCTGCTCTCCGTCACACCTGCTCTGGTGTTGGCTCCCGTAT
Ant - 21 F	ATACGGGAGCCAACACCATACTGTTTAGGCGAGTTCGCTATTTTCCTCCATGATAGAGCAGGTGTGACGGAT
Ant - 21 R	ATCCGTCACACCTGCTCTATCATGGAGGAAAATAGCGAACTCGCCTAAACAGTATGGTGTTGGCTCCCGTAT
Ant - 22 F	ATACGGGAGCCAACACCACGTCCGTCTATTCCCGGTCTTCCTCCTCCCTTGACCAGAGCAGGTGTGACGGAT
Ant - 22 R	ATCCGTCACACCTGCTCTGGTCAAGGGAGGAGGAAGACCGGGAATAGACGGACGTGGTGTTGGCTCCCGTAT
Ant - 23 F	ATACGGGAGCCAACACCATGGGGGAAATGGGGATGCAGTAAATTACATGAGTGCAGAGCAGGTGTGACGGAT
Ant - 23 R	ATCCGTCACACCTGCTCTGCACTCATGTAATTTACTGCATCCCCATTTCCCCCATGGTGTTGGCTCCCGTAT
Ant - 24aF	ATACGGGAGCCAACACCATCTCTCTTAGGATACAAAGCCAAACTGAGCCCGTGCAGAGCAGGTGTGACGGAT
Ant - 24aR	ATCCGTCACACCTGCTCTGCACGGGCTCAGTTTGGCTTTGTATCCTAAGAGAGATGGTGTTGGCTCCCGTAT
Ant - 24bF	ATACGGGAGCCAACACCACGTCAACTAAGCGAGAATTCAGAAAGCTGTTTGACCAGAGCAGGTGTGACGGAT
Ant - 24bR	ATCCGTCACACCTGCTCTGGTCAAACAGCTTTCTGAATTCTCGCTTAGTTGACGTGGTGTTGGCTCCCGTAT
Ant - 25 F	ATACGGGAGCCAACACCACCCCCTCCGCTTTACTCTCCCACACTGCCCTATCACAGAGCAGGTGTGACGGAT
Ant - 25 R	ATCCGTCACACCTGCTCTGTGATAGGGCAGTGTGGGAGAGTAAAGCGGAGGGGGTGGTGTTGGCTCCCGTAT

Table 1 (continued)

***Bacillus anthracis* Spores (BAS)**	
BAS - 2/11 F	ATACGGGAGCCAACACCATACCTGACCCCCCGCCGCAATCCTAGTCTACCTCCGAGAGCAGGTGTGACGGAT
BAS - 2/11 R	ATCCGTCACACCTGCTCTCGGAGGTAGACTAGGATTGCGGCGGGGGGTCAGGTATGGTGTTGGCTCCCGTAT
BAS - 3 F	ATACGGGAGCCAACACCA***CCCGTGGCCTTCACCCAGCCAGGG***A***CCCCGTCTCTG***AGAGCAGGTGTGACGGAT
BAS - 3 R	ATCCGTCACACCTGCTCT***CAGAGACGGGGG***T***CCCTGGCTGGGTGAAGGCCACGGG***TGGTGTTGGCTCCCGTAT
BAS - 5 F	ATACGGGAGCCAACACCACACACAGAGCGCCATGGACTCAGTCAGATGTGATGTAGAGCAGGTGTGACGGAT
BAS - 5 R	ATCCGTCACACCTGCTCTACATCACATCTGACTGAGTCCATGGCGCTCTGTGTGTGGTGTTGGCTCCCGTAT
BAS - 6 F	ATACGGGAGCCAACACCATCCCTCTTAGGATACAAAGCCAAACTGAGCCCGTGCAGAGCAGGTGTGACGGAT
BAS - 6 R	ATCCGTCACACCTGCTCTGCACGGGCTCAGTTTGGCTTTGTATCCTAAGAGGGATGGTGTTGGCTCCCGTAT
BAS - 7 F	ATACGGGAGCCAACACCATCAGCAGCTCTGCTTACAGGCGCCCGTAATCCGGACAGAGCAGGTGTGACGGAT
BAS - 7 R	ATCCGTCACACCTGCTCTGTCCGGATTACGGGCGCCTGTAAGCAGAGCTGCTGATGGTGTTGGCTCCCGTAT
BAS - 8 F	ATACGGGAGCCAACACCATGTGCCAGGTAGAGCCCGATAATCCTTACAGTACGCTAGAGCAGGTGTGACGGAT
BAS - 8 R	ATCCGTCACACCTGCTCTAGCGTACTGTAAGGATTATCGGGCTCTACCTGGCACATGGTGTTGGCTCCCGTAT
BAS - 9 F	ATACGGGAGCCAACACCACGTACATACCCTCCATCTAACCGCCGTCCCCAGTCAAGAGCAGGTGTGACGGAT
BAS - 9 R	ATCCGTCACACCTGCTCTTGACTGGGGACGGCGGTTAGATGGAGGGTATGTACGTGGTGTTGGCTCCCGTAT
BAS - 12 F	ATACGGGAGCCAACACCACGAGGACCTAGACTTGTCCGACATCACAGTGTGCGAGAGCAGGTGTGACGGAT
BAS - 12 R	ATCCGTCACACCTGCTCTCGCACACTGTGATGTCGGACAAGTCTAGGTCCTCGTGGTGTTGGCTCCCGTAT
BAS - 13 F	ATACGGGAGCCAACACCACGTATTGCGCGATCCGTTGCCAACTCTTATCGGGCCAGAGCAGGTGTGACGGAT
BAS - 13 R	ATCCGTCACACCTGCTCTGGCCCGATAAGAGTTGGCAACGGATCGCGCAATACGTGGTGTTGGCTCCCGTAT
BAS - 14 F	ATACGGGAGCCAACACCACCCGCACCCTGATCCCTTCCACCCGTCCCCTCCCGCAGAGCAGGTGTGACGGAT
BAS - 14 R	ATCCGTCACACCTGCTCTGCGGGAGGGGACGGGTGGAAGGGATCAGGGTGCGGGTGGTGTTGGCTCCCGTAT
BAS - 15 F	ATACGGGAGCCAACACCATGAAATGGTCCGATGCTGTATTATCCCTCCGCAGCGAGAGCAGGTGTGACGGAT
BAS - 15 R	ATCCGTCACACCTGCTCTCGCTGCGGAGGGATAATACAGCATCGGACCATTTCATGGTGTTGGCTCCCGTAT
BAS - 17aF	ATACGGGAGCCAACACCATACGGGATAGCGGGTAGGATCCCGTGCCATCACGTCAGAGCAGGTGTGACGGAT
BAS - 17aR	ATCCGTCACACCTGCTCTGACGTGATGGCACGGGATCCTACCCGCTATCCCGTATGGTGTTGGCTCCCGTAT
BAS - 17bF	ATACGGGAGCCAACACCAGGTTGACATCTGTAAAGTTCCGTACTGGTGGCTAGGAGAGCAGGTGTGACGGAT
BAS - 17bR	ATCCGTCACACCTGCTCTCCTAGCCACCAGTACGGAACTTTACAGATGTCAACCTGGTGTTGGCTCCCGTAT
BAS - 18 F	ATACGGGAGCCAACACCACCCCCATACCGTACACTGTCCATCCCGCCTTTCTCCAGAGCAGGTGTGACGGAT
BAS - 18 R	ATCCGTCACACCTGCTCTGGAGAAAGGCGGGATGGACAGTGTACGGTATGGGGGTGGTGTTGGCTCCCGTAT
BAS - 19 F	ATACGGGAGCCAACACCACGGAGTGCCCAGTGCAAGTTTACGCCAGATGTGCGCAGAGCAGGTGTGACGGAT
BAS - 19 R	ATCCGTCACACCTGCTCTGCGCACATCTGGCGTAAACTTGCACTGGGCACTCCGTGGTGTTGGCTCCCGTAT
BAS - 20 F	ATACGGGAGCCAACACCACCTGTCATCCATGCTCCGCTTCTCCCGGTACCGTCCAGAGCAGGTGTGACGGAT
BAS - 20 R	ATCCGTCACACCTGCTCTGGACGGTACCGGGAGAAGCGGAGCATGGATGACAGGTGGTGTTGGCTCCCGTAT
BAS - 21/25 F	ATACGGGAGCCAACACCA***CCCGTGGCCTTCACCCAGCCAGGG***G***CCCCGTCTCTG***AGAGCAGGTGTGACGGAT
BAS - 21/25 R	ATCCGTCACACCTGCTCT***CAGAGACGGGGG***C***CCCTGGCTGGGTGAAGGCCACGGG***TGGTGTTGGCTCCCGTAT
BAS - 22 F	ATACGGGAGCCAACACCAGGAGCGTCCCCCGTCCCCCGCACCCAACGGCGAGCCAGAGCAGGTGTGACGGAT
BAS - 22 R	ATCCGTCACACCTGCTCTGGCTCGCCGTTGGGTGCGGGGGACGGGGGACGCTCCTGGTGTTGGCTCCCGTAT
BAS - 23 F	ATACGGGAGCCAACACCAATAGAGCGTCTACCCGTATCCCGCCTTGGATTCCACAGAGCAGGTGTGACGGAT
BAS - 23 R	ATCCGTCACACCTGCTCTGTGGAATCCAAGGCGGGATACGGGTAGACGCTCTATTGGTGTTGGCTCCCGTAT
BAS - 24 F	ATACGGGAGCCAACACCACTGTCTACGTGGGTCTCACAGTCCGTGCTATTTGCCAGAGCAGGTGTGACGGAT
BAS - 24 R	ATCCGTCACACCTGCTCTGGCAAATAGCACGGACTGTGAGACCCACGTAGACAGTGGTGTTGGCTCCCGTAT

All sequences are presented 5′ to 3′ from left to right. The 18 bases on each end represent the constant primer regions. Shaded or highlighted sequences are identical or highly similar. Abbreviations: Ant; anthrose sugar and BAS; *Bacillus anthracis* spore aptamers. Most aptamers were full-length 72 base oligonucleotides, but numbers in parentheses indicate the lengths of truncated aptamers

hot DNA was added to 100 µl of fresh OMP-MBs in 200 µl of 2XBB and allowed to mix for 1 h at RT. Thereafter, the selection and amplification process was repeated for four more rounds of whole spore and anthrose- MB SELEX with verification of 72 bp aptamer PCR products by ethidium bromide-stained 2% agarose electrophoresis after each round. Following round 5, aptamers were cloned into chemically competent *E. coli* using a Lucigen GC cloning kit (Middleton, WI) and clones were sent to Sequetech, Inc. (Mountain View, CA) for proprietary GC-rich DNA sequencing. Beacons were 5′ labeled with TYE 665 dye and 3′ labeled with Iowa Black quencher and HPLC-purified at Intergrated DNA Technologies, Inc.

Secondary Structure Determinations

Secondary structures of aptamers were determined using free internet-based Vienna RNA software (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) using DNA parameters at 25 °C [24–26].

Fluorescence Microscopy

Bacillus spore populations at ~1×10^7 spores/ml were gently mixed with 1 μg/ml of the BAS-6F or 6R aptamer beacons in PBS for 20–30 min at room temperature and then pelleted at 14,000 × g for 5 min. The supernates were removed and wet mounts of spore pellets were prepared on microscope slides beneath coverslips. Specimens were then imaged on an Olympus BH-2 fluorescence microscope using bright-field and epifluorescence microscopy using a standard rhodamine filter cube and a 2 Megapixel Motic 2000 color CCD camera (Motic Corp., China) and Motic Image Plus version 2.0 image analysis software.

Spectrofluorometer and Handheld Fluorometer Measurements

Serial ten-fold, two-fold, or other dilutions of *Bacillus* spores or other types of bacteria as indicated in each figure were made in 1 ml of PBS in polystyrene cuvettes. One ml of either the BAS-6F or 6R aptamer beacon was added to each 1 ml of bacterial or spore dilution at 2 μg/ml in PBS per cuvette to give a final aptamer beacon concentration of 1 μg/ml and cuvettes were gently mixed for 20 min at room temperature. Fluorescence was then assessed with a Varian Cary Eclipse spectrofluorometer using a 645 nm excitation and scanning emissions from 650 nm to 720 nm using a PMT setting of 1,000 V. Aptamer beacon-stained bacteria and spores were pipetted up and down several times immediately prior to fluorescence scanning to ensure a uniform cell suspension for fluorescence assessment. In many cases, the same cuvette samples were also assessed with a Turner Biosystems (now Promega Corp.) Picofluor™ or Quantifluor™-P handheld fluorometer specially modified to excite at 650 nm and read fluorescence from 660 nm to 720 nm. The handheld fluorometer was set to maximal sensitivity (i.e., STD VAL=999.0) and again spores and vegetative bacteria were resuspended just prior to fluorescence readings.

Results

Table 1 catalogues all 92 of the aptamer sequences derived during the course of this study against anthrose sugar and

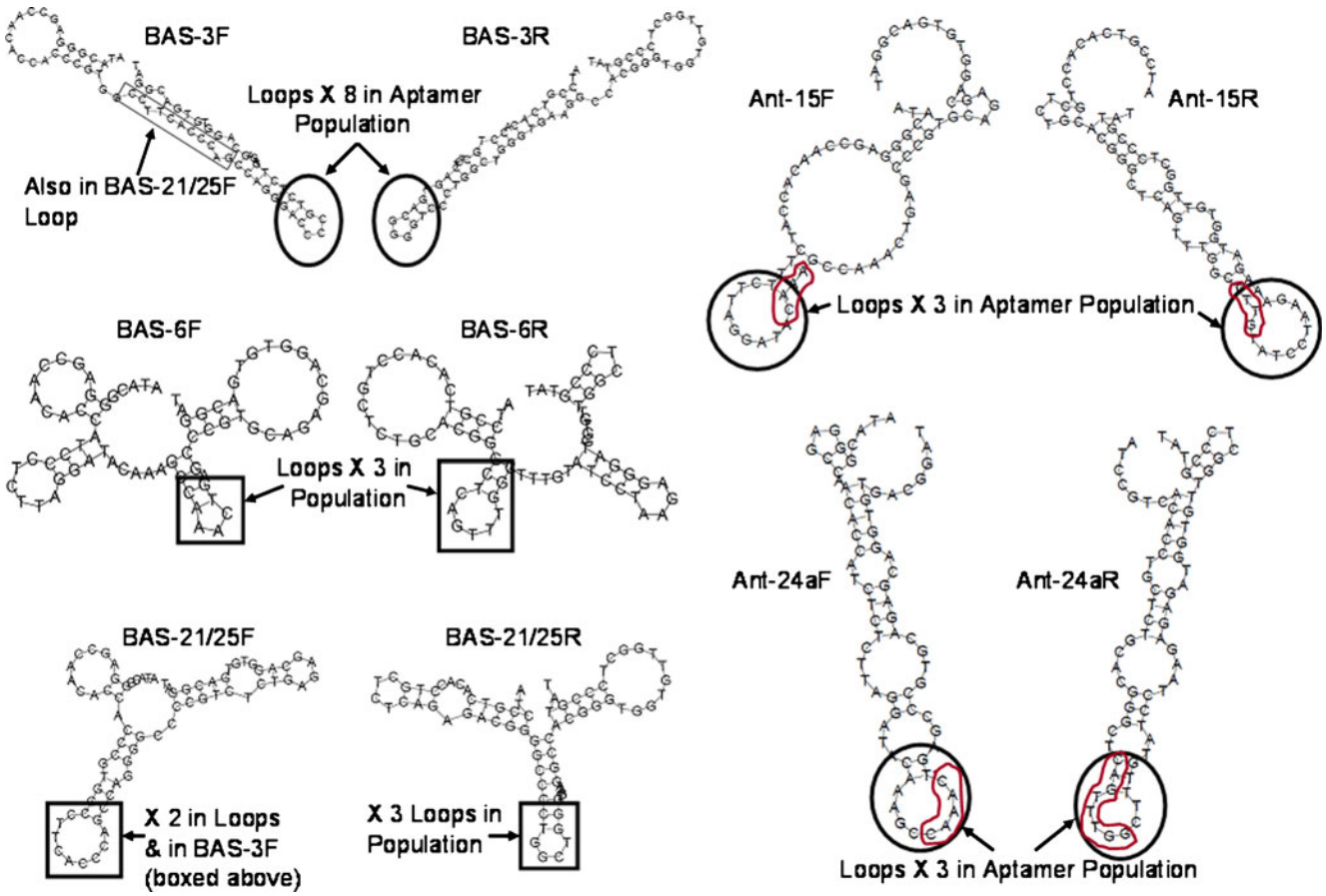

Fig. 1 Secondary stem-loop structures of some of the key whole *B. anthracis* spore (BAS) and anthrose (Ant) aptamers as calculated by Vienna RNA software using DNA parameters and 25 °C. Boxed and circled regions define common sequences or loop structures which formed the basis for aptamer beacon development

whole *B. anthracis* spores. From this data, we deduced several identical or very similar sequences either between the whole spore aptamers (e.g., BAS 2/11 or 21/25F and R each came from two different clones raised against whole spores) or common across the anthrose and whole spore aptamer families. In particular, the anthrose (Ant) 15 and 24 clones were of special interest due to their high degree of similarity with the eventual best aptamer beacons (BAS-6F and 6R).

Figure 1 gives the secondary stem-loop structures of all the candidate aptamers chosen for occurring more than once in Table 1 or having common loop sequences that occurred multiple times in the population of 92 aptamer sequences (denoted in Fig. 1). Candidate aptamer beacons were derived from these loop structures by synthesizing truncated aptamers demarcated by the boundaries of boxes or circles over loop structures in Fig. 1. Each candidate aptamer beacon was synthesized with 5′-TYE 665 dye and 3′-Iowa Black end labels. As Fig. 1 illustrates, the secondary loop structures of the Ant 15 and 24 clones shared some similarity with the BAS-6 clones (circled or boxed in Fig. 1) except that the loops of the BAS-6 group were generally smaller (consisted of fewer bases) than those of the Ant 15 or 24 clones. In addition, some of the potentially key bases which are identical between Ant and BAS-6 aptamers (bolded and underlined in Table 2) are generally more involved in double-stranded stem regions of the anthrose aptamers and may therefore require induced fit and greater energy to open for a "lights on" fluorescence response. Table 2 also shows some other bolded or underlined sequence segment similarities between the aptamer beacon candidates derived in this study and those of Fan et al. [17], Ikanovic et al. [16] and a U.S. patent by Vivekananda and Kiel [18]. In each case some similarities can be found suggesting that these may represent points of physical contact between the spores and the aptamers.

It became clear through a great deal of empirical testing (not shown for brevity), that the only beacon candidates capable of detecting nonpathogenic Sterne strain *B. anthracis* spores were BAS-6F and 6R. It is interesting that these two aptamer candidates derived from complementary forward and reverse-primed aptamers from the same clone. As Fig. 2 illustrates BAS-6F was generally a stronger fluorescing aptamer candidate, but both aptamer beacons responded to varied, but high levels of *B. anthracis* spores as illustrated using a spectrofluorometer (Figs. 2a and b) and the handheld Picofluor™ flourometer (Fig. 2c). We also determined from multiple spread plate colony counts of the lowest detectable fluorescence above background that BAS-6F and 6R only had a limit of detection (LOD) of approximately 30,000 to 60,000 *B. anthracis* spores per ml.

In cross-reactivity studies presented in Fig. 3, the BAS-6F and 6R aptamer beacons detected BT spores somewhat better than *B. anthracis* spores, but did not detect other bacterial species (Gram positive or Gram negative) nearly

Table 2 Similarities between DNA sequences of aptamer beacon candidates used in this study and other published or patented *Bacillus* spore aptamers

Anthrose-15F Beacon	CTTTCTTAGGATA**CAAA**G
Anthrose-15R Beacon	C***TTTG***TATCCTAAGAAAG
Anthrose-24aF Beacon	**CAAA**GC**CAAACT**G
Anthrose-24aR Beacon	***CAGTTTG***GC**TTTG**
BAS-6F Beacon	**CAAACTG**
BAS-6R Beacon	**CTCA*GTTTGG***
BAS-13F Beacon	GACCCCGTC
BAS-13R Beacon	GACGGGGTC
BAS-21-25F Beacon	GCCTTCACCCAGC
BAS-21-25R Beacon	CTGGCTGG
Fan et al., 2008	
(+) Strand	ACCCCTGCATCC***TTTG***CTGGAGAGGAATGT-ATAAGGATGTTCCGGGCGTGTGGGTAAGTC-AGTCTAGAGGGCCCCCAGAAT
(-) Strand	ATTCTGGGGGCCCTCTAGACTGACTTACCC-ACACGCCCGGAACATCCTTATACATTCCTCT-CCAG**CAAA**GGATGCAGGGGT
Ikanovic et al., 2007	CATCCGTCACACCTGCTCTGGCCACTAACA-TGGGGACCAGGTGGT***GTTGG***CTCCCGTATC
U.S. Patent 6,569,630	
SEQ ID NO. 1	CCCCTGCAGGTGAT***TTTG***CTCAAGT
SEQ ID NO. 6	ACCC***GGTT***AATTCGTAGTAGAGGAGGGTC ***GTTTGG***AGTCA

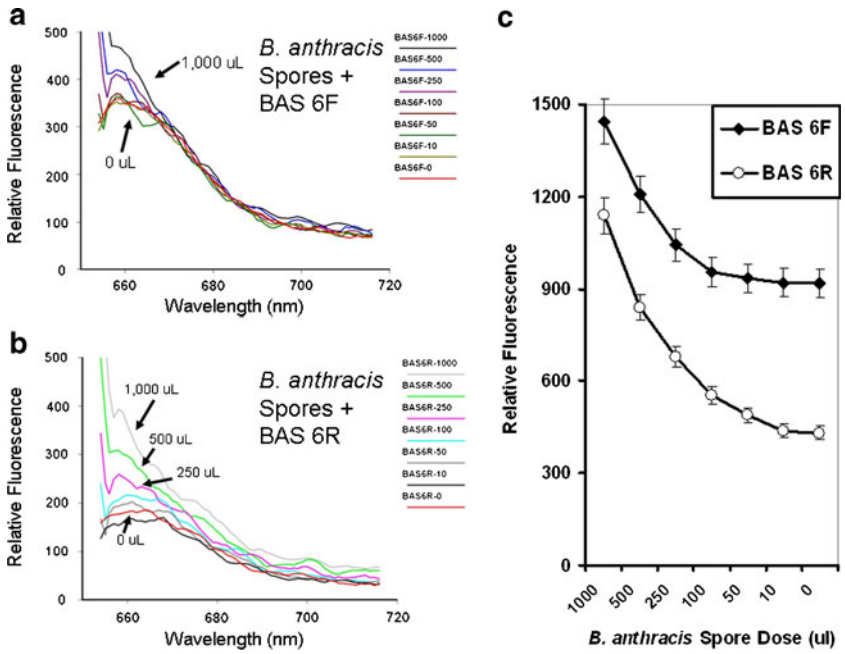

Fig. 2 A and B represent fluorescence spectra for the BAS-6F and 6R aptamer beacons at 1 μg/ml in PBS (pH 7.2) in the presence of 0 to 1,000 μl of stock *B. anthracis* Sterne strain spores (~3×10^7 spores per ml). Spectrofluorometer excitation was at 645 nm and the PMT setting was 1,000 V. Panel C shows the corresponding handheld Picofluor™ fluorometer mean values with 2× standard deviation error bars (N=5 readings) for the same cuvettes analyzed in panels **a** and **b**

as well. We further showed in Fig. 4 that the BAS-6F and 6R aptamer beacons detected *B. cereus* spores most intensely of the three *Bacillus* spore species, but in all cases the LOD was consistently only 30,000 to 60,000 spores per ml. This lack of sensitivity may be due to a paucity of the unknown epitope on the surface of the spore. We determined that BAS-6F and 6R aptamer beacons could not detect pure anthrose sugar in the nanogram to microgram ranges (data not shown). Unfortunately too, as aforementioned, none of the anthrose aptamer beacon candidates could detect whole *Bacillus* spores very well, if at all. Therefore, despite some similarities in primary and secondary structures of the candidate aptamers and their derived candidate beacons, anthrose itself can probably be eliminated as a potential epitope target for BAS-6F or 6R. In addition, the fluorescence spectrum at the bottom of Fig. 4b is characteristic of the very low autofluorescence of any of the three *Bacillus* species spore populations examined at their highest concentrations (i.e., autofluorescence was insignificant in the red region when bacteria or spores were excited at 645 nm).

We also considered the possibility that a nuclease from the *Bacillus* spores or vegetative cell remnants was responsible for the fluorescence as a function of spore concentration by cleaving off the 5′-TYE 665 somewhere in the beacon structure. However, the red surface fluorescence on various species of *Bacillus* spores shown in Figs. 5c and d suggests that real aptamer beacon binding to the spore's surface and "lights on" fluorescence due to separation of the TYE 665

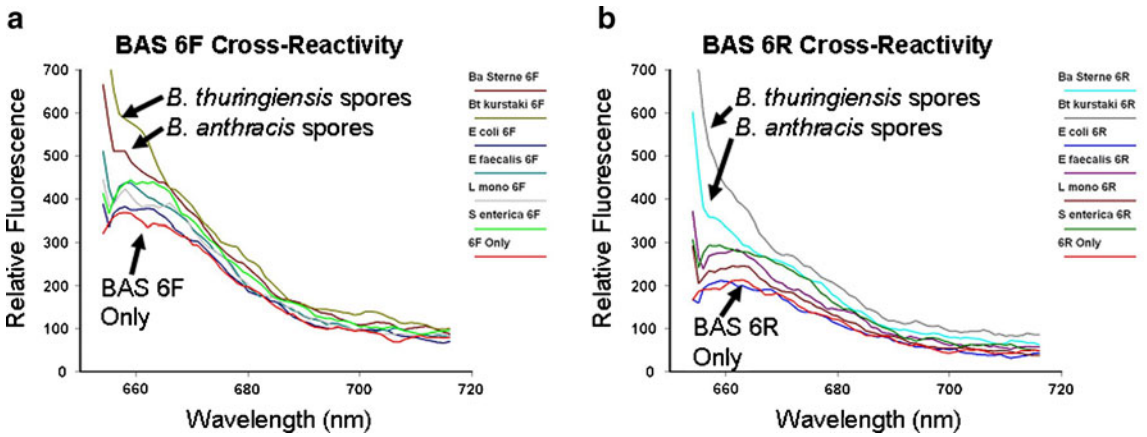

Fig. 3 Cross-reactivity study of the BAS-6F and 6R aptamer beacons (1 μg/ml in PBS) versus 30 million spores or vegetative cells per ml from each of the species shown: *B. anthracis* Sterne strain, *B. thuringiensis* kurstaki strain, *Escherichia coli* ATCC 8739 strain, *Enterococcus faecalis*, *Listeria monocytogenes*, and *Salmonella enterica*. Excitation was at 645 nm and the PMT setting was 1,000 V

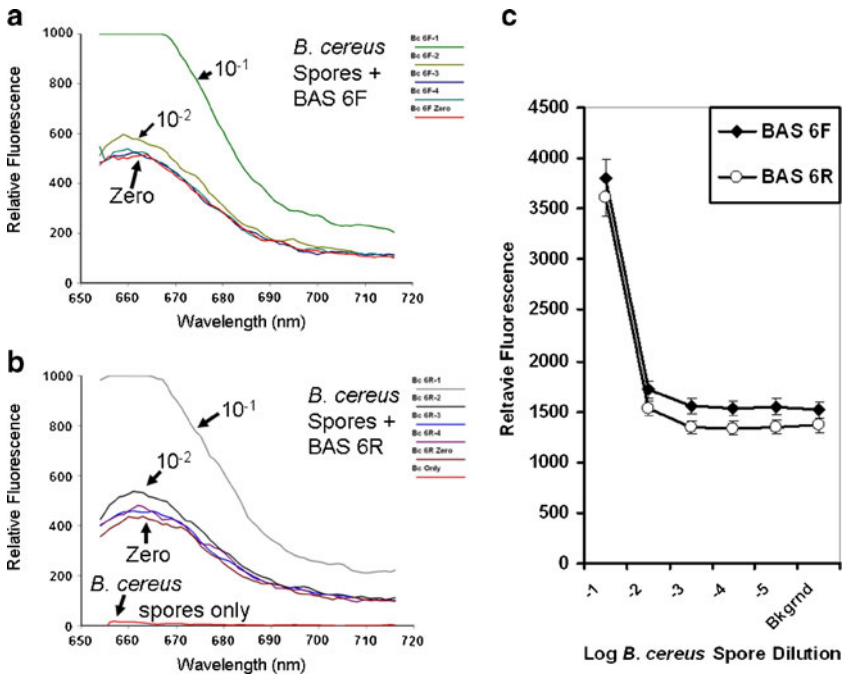

Fig. 4 **a** and **b** represent fluorescence spectra for the BAS-6F and 6R aptamer beacons at 1 μg/ml in PBS (pH 7.2) in the presence of serial ten-fold dilutions of stock *B. cereus* spores (stock~3×10^7 spores per ml). Excitation was at 645 nm and the PMT setting was 1,000 V. Panel **c** shows the corresponding handheld Picofluor™ fluorometer mean values with 2× standard deviation error bars (N=5 readings) for the same cuvettes analyzed in panels **a** and **b**

fluorophore and quencher beyond the Förster distance accounts for the fluorescence as a function of spore concentration.

Discussion

In this preliminary study, we have taken significant strides towards identifying some potentially useful aptamer beacons that can detect a variety of closely related *Bacillus* spore species in the far red region of the spectrum. This is potentially quite important for the rapid, homogenous, and portable, albeit presumptive, detection of a key bioterrorist or biowarfare agent (namely anthrax) or related foodborne *Bacillus* pathogens [15, 27]. Other fluorescence resonance energy transfer (FRET) systems for *Bacillus* spore detection have been devised with antibodies [27], but these systems involved

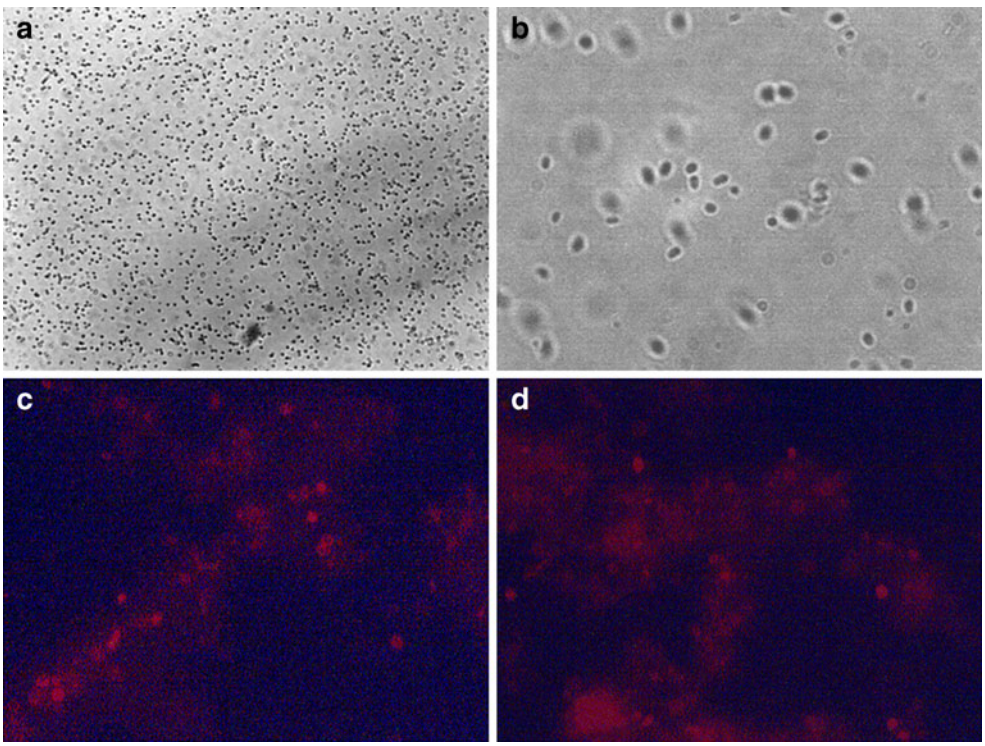

Fig. 5 Brightfield images of *B. anthracis* Sterne spores (**a**; 200X magnification) and *B. cereus* spores (**b**; 1,000X). Panels **c** and **d** show 1,000X oil immersion objective fluorescence microscopic images of *B. anthracis* and *B. thuringiensis* spores after 20–30 min at room temperature in the presence of aptamer beacons BAS-6F and 6R respectively

competitive displacement and were even less sensitive than the present aptamer beacon system.

The present work also compares aptamer DNA sequences from past efforts to develop *Bacillus* spore aptamers [16–18] to those developed for this study and identifies some interesting short sequences which are either identical or similar. These regions of sequence similarity could represent points of physical contact with the *Bacillus* spore epitopes, since they have emerged in several studies. Despite some similarities between the anthrose (sugar known to exist on *Bacillus* spores [20–22]) and the whole spore-derived aptamers, the best spore aptamer beacons (BAS-6F and 6R) demonstrated no fluorescence as a function of anthrose concentration. Therefore, while the BAS-6F and 6R aptamer beacons appear quite specific for high levels of *Bacillus* spores (e.g., Fig. 3), their actual epitope remains unknown and constitutes an area for future research.

We also questioned the basic mechanism of fluorescence, since in the presence of live bacteria or their remaining spores and aptamer beacons, one cannot automatically rule out nuclease activity as the source of fluorescence. However, three facts argue against the nuclease hypothesis. The first is that spores were washed by centrifugation and resuspended in fresh PBS immediately prior to experiments, thereby by removing most of any nucleases in the supernate. The second observation to counter the nuclease cleavage hypothesis is that two different sequences (BAS-6F and 6R) gave fluorescence in the presence of *Bacillus* spores. Although possible for an endonuclease to be involved, it seems unlikely that two sequences with very different "cut sites" would be recognized and cleaved. Similarly, a 5′ exonuclease would likely be inhibited by the 5′-TYE 665 dye. The third and strongest line of evidence against nucleases, however, comes from simple fluorescence microscopy in which unlabeled spores of all three species (Figs. 5a and b) begin to fluoresce red over time suggesting surface binding and opening of aptamer beacons beyond the Förster distance to produce the red glow seen in Figs. 5c and d. Figures 5a and b also demonstrate that fairly pure populations of spores resulted from gravitational sedimentation of old *Bacillus* cultures followed by decanting of vegetative cell remnants in the supernates.

While the present work is preliminary and improvements are needed in both sensitivity and specificity of spore detection, it is encouraging. We plan to attempt improvements in both sensitivity and specificity of the pilot assays, by manipulating the locations of the fluorophore and quencher in larger aptamer structures to perhaps enable more points of contact for enhanced molecular recognition and discrimination of the spore's surface. We are also working on identification of the actual epitopes being recognized by some of these aptamers, so that we might engage in rational design of aptamer beacons or other forms of FRET-aptamers [10, 28] for the improved detection of bacterial spores. Such endeavors may eventually enhance homeland security and food safety.

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