

Examination of *Bacillus anthracis* Spores by Multiparameter Flow Cytometry

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Abstract

The ability to rapidly differentiate *Bacillus anthracis* spores from spores belonging to other *Bacillus* spp. is potentially useful for combating the intentional release of this biothreat agent. Furthermore, not all *B. anthracis* strains are fully virulent and the ability to determine the potential virulence of the endospore is also important. In this chapter, we describe a two-color flow cytometric assay capable of simultaneously identifying *B. anthracis* spores and the presence of spore-associated protective antigen, a virulence marker for strains harboring the pXO1 plasmid.

Key words: *Bacillus anthracis* spores, Flow cytometry, Protective antigen, Antibody conjugation, Fluorescence, Identification

1. Introduction

Bacillus anthracis, a Gram-positive spore-forming bacterium, is a member of the *Bacillus cereus* group (along with *B. cereus*, *B. thuringiensis*, and *B. mycoides*), which can exist ubiquitously in nature and is genetically related. One of the only distinguishing features among this group is plasmids that encode for virulence factors. Strains of *B. anthracis* can harbor two major virulence plasmids, pXO1 and pXO2 (1). The *B. anthracis* pXO1 plasmid encodes for the three proteins protective antigen (PA), edema factor (EF), and lethal factor (LF), which interact synergistically to form edema toxin (PA and EF) and lethal toxin (PA and LF). Fully virulent isolates of *B. anthracis* also harbor the pXO2 plasmid that encodes for an antiphagocytic poly-D-glutamic acid capsule. The *B. anthracis* Ames strain possesses both these virulence

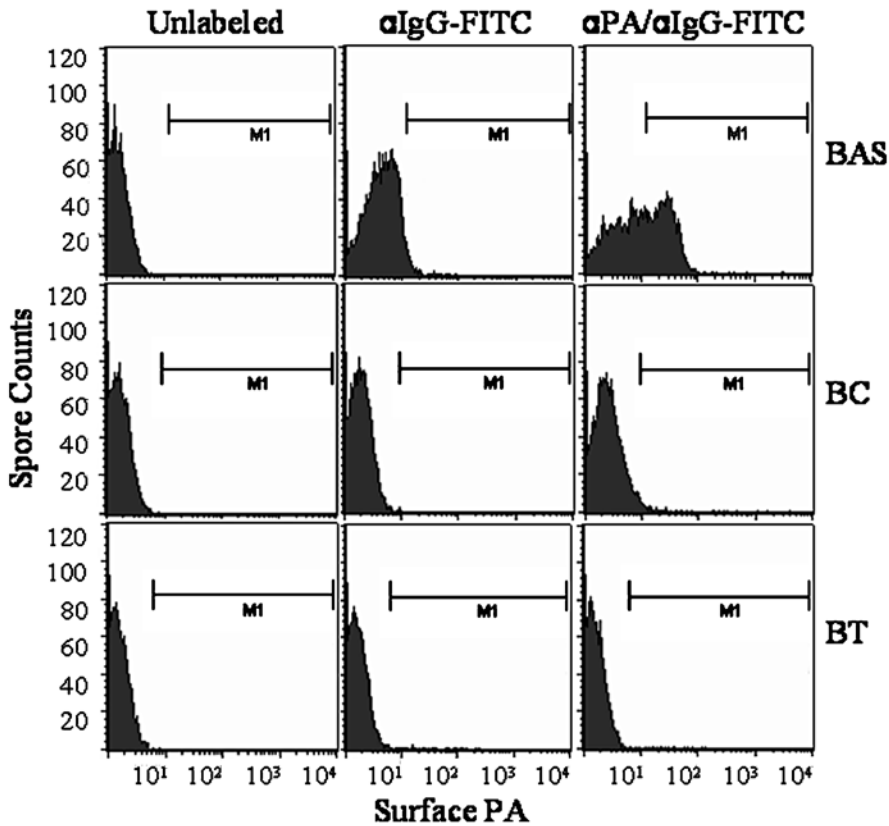


Fig. 1. Typical flow cytometry data for the surface PA assay. The monoclonal antibody against PA was found to bind tightly with *B. anthracis* Sterne spores (BAS) as compared to *B. cereus* (BC) and *B. thuringiensis* (BT) spores. Compared to the intrinsic fluorescence of unlabeled spores, IgG-FITC did nonspecifically bind to BAS. The FITC cutoff marker (M1; M1 = positive event) was adjusted to negate its contribution to the overall surface PA signal. α , anti.

plasmids, whereas minimally virulent Sterne and Pasteur strains lack pXO2 or pXO1, respectively.

Flow cytometric methods for examining *Bacillus* spp. have increasingly appeared in the literature because they work rapidly and can detect both physical and chemical features within a spore population (2–4). The technique is based on the interaction of light with spores contained in a hydrodynamically focused liquid stream. Light scattered off the spores is indicative of size and granularity, while emitted light, intrinsic or extrinsic, can reveal information about structure and function. Typical analysis speeds exceed 1,000 spores per second (5), making flow cytometry faster than the 1–2 days required for most standard microbiological tests (6). And unlike polymerase chain reaction (PCR) methods, which require the extraction of endospore DNA (7), flow cytometric methods can rely on simple labeling assays that detect surface antigens on intact spores. However, until recently most spore-based labeling assays were not selective among members of the *B. cereus* group (8, 9).

In 2003, short peptide fragments that exhibited tight binding to *Bacillus* spores were discovered (10), and numerous studies have since reported on their effectiveness (11, 12). One such peptide, ATYPLPIRGGGC (ATYP), was conjugated to R-phycoerythrin (RPE) and found by flow cytometry to bind species specifically to *B. anthracis* spores. Unfortunately, the ATYP–RPE conjugate could not differentiate spores from different strains of *B. anthracis* (10).

Recently, the PA protein expressed by pXO1-harboring strains of *B. anthracis* was implicated as a spore surface antigen (13). PA is present during the sporulation process and thought to be non-covalently entrapped in the spore coat (SC) and exosporium (EX). Since the pXO1 plasmid encodes for several virulence factors, surface PA can be considered a virulence marker for *B. anthracis* spores. The protocol that follows describes a two-color flow cytometry assay which couples a fluorescein isothiocyanate (FITC)-conjugated antibody-based PA assay (see Fig. 1) to the *B. anthracis* spore-specific ATYP–RPE assay. We demonstrate the potential of this multiparameter assay by differentiating PA-positive (+PA) *B. anthracis* spores from PA-negative (–PA) *B. cereus* and *B. thuringiensis* spores.

2. Materials

2.1. Bacterial Stocks

1. *B. anthracis* Sterne 34F2 (pXO1⁺/pXO2⁻, seed from the live-spore veterinary vaccine; Colorado Serum Company, Denver, CO), *B. anthracis* Ames (pXO1⁺/pXO2⁺), *B. cereus* (pXO1⁻/pXO2⁻), and *B. thuringiensis* (pXO1⁻/pXO2⁻) seed lots.
2. Leighton Doi medium (LDM): 15 g bactopectone, 6.9 g glucose, 3 g yeast extract, 3 g NaCl, 1.88 g KCl, 0.294 g CaCl₂·2H₂O, 0.0246 g MgSO₄·6H₂O, 0.0028 g FeSO₄·6H₂O, and 0.00169 g MnCl₂. Adjust volume to 1 L with double-distilled water (ddH₂O) and autoclave.
3. Modified G medium (MGM): 2 g yeast extract, 2 g (NH₄)₂SO₄, 0.5 g K₂HPO₄, 0.2 g MgSO₄·6H₂O, 0.05 g MnSO₄·4H₂O, 0.025 g CaCl₂·2H₂O, 0.005 g ZnSO₄·2H₂O, 0.005 g CuSO₄·5H₂O, and 0.0005 g FeSO₄·6H₂O. Adjust volume to 1 L with ddH₂O and autoclave.
4. Renografin 76 sedimentation gradient (see Subheading 3.2, items 2 and 3).
5. Sterile H₂O, Gram's stain, blood agar plates, sterile loops, sterile laboratory glassware, Pasteur pipettes, and sterile centrifuge tubes.
6. Equipment: autoclave, box shaker, incubation chamber, centrifuge, hemocytometer, and optical microscope.

2.2. Two-Color Assay

1. Mouse monoclonal antibody against *B. anthracis* PA (anti-PA, 2 mg/mL, Abcam, Cambridge, MA).
2. FITC-conjugated goat anti-mouse immunoglobulin G (IgG), H+L (IgG-FITC, Jackson ImmunoResearch, West Grove, PA).
3. Synthetic peptide with amino acid sequence ATYPLPIRG GGC (GenScript, Piscataway, NJ).
4. RPE (4 mg/mL, Invitrogen, Carlsbad, CA).
5. Sulfo-succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC, Pierce, Rockford, IL).
6. Staining buffer: Dulbecco's calcium-/magnesium-free phosphate-buffered saline (PBS), pH 7.2, 1% fetal bovine serum.
7. Wash buffer: Dulbecco's PBS, pH 7.2.
8. 2% Paraformaldehyde in wash buffer.
9. Microcentrifuge tubes, Slide-A-Lyzer 10K MWCO dialysis cassettes, and sterile 96-well plates (conical bottom).
10. Equipment: Vortex mixer, micropipettors, microcentrifuge, incubation chamber, flow cytometer, and laser-scanning confocal microscope.

3. Methods
3.1. Preparation of Bacterial Stocks

1. Streak blood agar plate with a sample of the bacterial seed lot using a sterile loop and incubate for 12–18 h at 37°C.
2. Using a sterile loop, remove a single colony off the cultured plate and add to 15–20 mL LDM; incubate with shaking for 12–18 h at 37°C.
3. Transfer the 15–20 mL culture into a 200 mL volume of LDM and incubate with shaking for 10 h at 37°C.
4. Centrifuge cultured bacteria for 20 min at 4,000 × *g* and 4°C, then discard supernatant and transfer pellet into 250 mL MGM; cover container with aluminum foil and shake gently for 3–5 days at 20–25°C.
5. Mix an aliquot of the MGM (containing spores) with Gram's stain in a 1:1 ratio (v/v) and evaluate the extent of sporulation with a hemocytometer or a Petroff-Houser counter. If sporulation is complete and there is less than 10–15% vegetative cells, then proceed to purification; if there is greater than 15% vegetative cells, continue the incubation at room temperature for an additional 24 h.

3.2. Purification of Bacterial Stocks

1. Centrifuge the MGM (containing spores) for 20 min at 4,000 × *g* and 4°C, discarding the supernatant.

2. Prepare a stock solution of Renografin 76 by mixing 16 mL of filter sterilized meglumine diatrizoate (833 mg/mL) with 2 mL of sodium diatrizoate in ddH₂O. Adjust the density of the Renografin 76 stock solution to 1.25 g/mL using additional ddH₂O.
3. Divide the stock Renografin 76 solution into two equal volumes (stock A and B); stock A = bottom phase of gradient (do not modify). Adjust the density of stock B to 1.12 g/mL using additional ddH₂O.
4. Pipette 10 mL stock B into sterile centrifuge tube containing the crude spore pellet, then vortex. Transfer the entire volume into a sterile Oak Ridge centrifuge tube, and then carefully pipette 10 mL of stock A to the bottom of the centrifuge tube, being sure not to disturb the density interface.
5. Centrifuge for 1 h at 8,000 × *g*. The pellet contains mature *B. anthracis* spores. Remove the Renografin density gradient and any bands which contain immature spores and vegetative cells, leaving the pellet. Wash the pellet three times with ddH₂O, then reconstitute in 3 mL sterile H₂O, and store at 4°C.
6. Using the serial dilution method, determine the colony forming units per milliliter (CFU/mL) of the purified spore preparation.

3.3. Preparation of ATYP–RPE Conjugate

1. Centrifuge 50 µL RPE for 5 min at 9,000 × *g* (at 20–25°C), then discard the supernatant, and reconstitute the pellet in 25 µL wash buffer.
2. Dialyze RPE against several changes of wash buffer to remove residual ammonium sulfate (total volume after 4 h of dialysis at 20–25°C ≈ 30 µL).
3. Dissolve 2 mg of sulfo-SMCC into 450 µL wash buffer (10.2 mM sulfo-SMCC).
4. Mix 30 µL RPE with 15 µL sulfo-SMCC stock solution (molar ratio ≈ 1 RPE: 180 sulfo-SMCC) and incubate for 1 h at 30°C (see Note 1). Remove unbound sulfo-SMCC via dialysis into wash buffer (total volume after 6 h of dialysis at 4°C ≈ 50 µL).
5. Dissolve 2 mg of ATYP peptide into 200 µL wash buffer (8.3 mM ATYP peptide).
6. Mix 50 µL sulfo-SMCC-activated RPE with 100 µL ATYP peptide stock solution (molar ratio ≈ 1 RPE: 1,000 ATYP peptide) and incubate for 18 h at 30°C. Remove unbound ATYP peptide via dialysis into wash buffer (total volume after 18 h of dialysis at 4°C ≈ 200 µL). This corresponds to approximately 4 µM of purified ATYP–RPE conjugate.

3.4. Two-Color Assay

1. Pipette a volume containing approximately 10^6 spores into a sterile 96-well plate, add staining buffer and centrifuge at $700 \times g$ and 4°C , discarding the supernatant. To the spore pellet, add 25 μL of a 1:12.5 dilution of the anti-PA antibody (in staining buffer), then incubate for 1 h at 37°C . Remove unbound anti-PA antibody via repeated wash/centrifuge steps (at $700 \times g$ and 4°C) with 200 μL aliquots of wash buffer.
2. Mix spores with 25 μL of a 1:100 dilution (in wash buffer) of IgG-FITC for 1 h at 37°C . Remove unbound anti-IgG-FITC via repeated wash/centrifuge steps (at $700 \times g$ and 4°C) with 200 μL aliquots of wash buffer.
3. Mix spores with 25 μL of 2 μM ATYP-RPE and incubate for 1 h at 37°C . Remove unbound conjugate via repeated wash/centrifuge steps (at $700 \times g$ and 4°C) with wash buffer (see Note 2).
4. Fix spores in 2% PFA prior to analysis by flow cytometry (see Note 3) and laser-scanning confocal microscopy (see Note 4).

Confocal microscopy was used to visually confirm that double-labeling occurred on Sterne spores and not single-color labeling of different populations (see Fig. 2). We next examined several spore lots by flow cytometry to see if differences in preparation, purification, and storage conditions affected the outcome of the assay (see Table 1). Lots BAS 3 through BAS8 were labeled similarly by ATYP-RPE (51–71% positive) and had comparable surface PA values (23–43% positive). Only lots BAS1 and BAS2 demonstrated lower ATYP-RPE binding (11–12% positive) and higher surface PA values (72–75% positive). Lots BAS1 and BAS2 were stored in ddH_2O at 4°C for a longer period of time (4 years) than any other lot used in this study (see Note 5 and Fig. 3). As expected, *B. cereus* and *B. thuringiensis* spores produced double-negative results by the two-color assay.

Based on the two-color results, we developed a set of prototypical flow cytometry dot-plot patterns for predicting the species of spores belonging to the *Bacillus* genus and their potential virulence (see Fig. 4). Pattern 1 (events occur in lower-left quadrant) corresponds to ATYP-RPE⁻/PA⁻ non-*B. anthracis* spores, such as *B. cereus* or *B. thuringiensis*. Pattern 2 (events occur in lower-left, upper-left, and upper-right quadrants) is indicative of ATYP-RPE⁺/PA⁺ spores, such as *B. anthracis* Sterne or Ames, that are potentially virulent. Pattern 3 (events occur in lower-left and upper-left quadrants) is indicative of ATYP-RPE⁺/PA⁻ spores, such as *B. anthracis* Pasteur, that are likely to be minimally virulent. Like pattern 2, pattern 4 (events occur in all four quadrants) also results from ATYP-RPE⁺/PA⁺ spores that are potentially

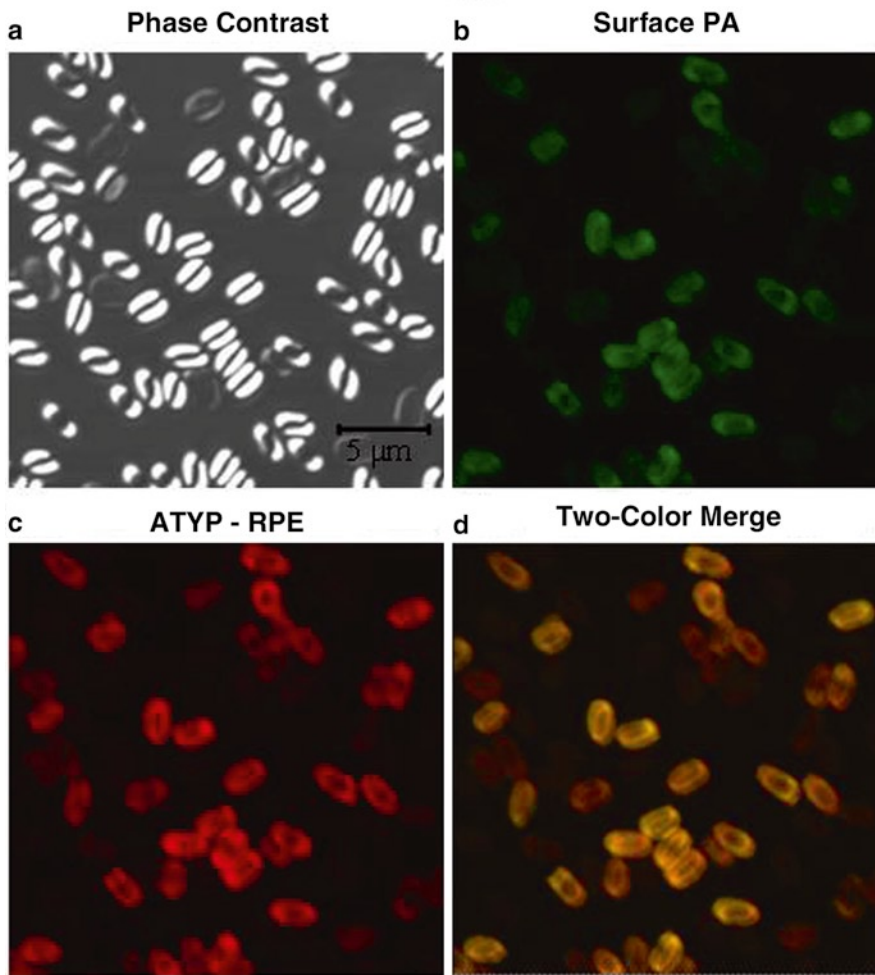


Fig. 2. Phase contrast (a) and fluorescence (b and c) images of double-labeled *B. anthracis* Sterne spores. The fluorescence observed in image (b) was attributed to IgG-FITC binding with anti-PA/surface PA complexes. The fluorescence in image (c) was caused by surface-bound ATYP-RPE. Fluorescence images (b and c) were used to produce the merged image (d), which confirmed that double-labeling occurred on most Sterne spores.

virulent; however, the large population in the lower right quadrant indicates that a significant fraction of the spores containing PA are nonreactive with the ATYP-RPE conjugate.

In conclusion, this two-color assay successfully differentiated pXO1⁺ (+PA) strains of *B. anthracis* from pXO1⁻ (-PA) strains in only a few hours. One drawback of this assay is that it cannot resolve *B. anthracis* Sterne spores (BAS) from *B. anthracis* Ames spores, since they both harbor the pXO1 plasmid. Nonetheless, this two-parameter detection assay marks an important step toward rapid and complete characterization of this dangerous pathogen.

Table 1
Characterization of different spore lots by using the flow cytometry two-color assay

Organism	Lot	D.O.P. ^a	%ATYP–RPE	%PA ⁺	%DP ^b
<i>B. cereus</i>	BC1 ^c	04/2007	0.070	1.5	0.03
<i>B. thuringiensis</i>	BT1 ^c	04/2007	0.26	0.16	0.08
<i>B. anthracis</i> Sterne	BAS1 ^d	09/2003	12	72	8.6
	BAS2 ^d	09/2003	11	75	6.8
	BAS3 ^d	06/2005	58	36	36
	BAS4 ^c	08/2005	58	33	33
	BAS5 ^c	06/2006	51	23	23
	BAS6 ^c	05/2007	67	32	32
	BAS7 ^c	05/2007	56	30	30
	BAS8 ^d	06/2007	71	43	43

^aD.O.P. = date (month/year) of spore lot preparation

^b%DP = percentage of spores that are double positive

^cLeighton Doi medium/modified G medium/Renografin 76

^dLeighton Doi medium/nutrient agar plate/Renografin 76

^eLeighton Doi medium/modified G medium/no Renografin 76

4. Notes

1. Sulfo-SMCC (MW = 436.37 g/mol) is a heterobifunctional cross-linker containing an NHS ester group (amine-reactive at pH 7–9) and a maleimide group (sulfhydryl-reactive at pH 6.5–7.5). Sulfo-SMCC is soluble in water at concentrations up to ≈ 10 mM. Sulfo-SMCC-activated materials should be used immediately after purification to minimize maleimide decomposition caused by neutral pH.
2. Lower surface PA values were obtained for Sterne spores when ATYP–RPE was incubated before, or simultaneous to, IgG–FITC (data not shown). This finding may indicate that surface PA exists close to the ATYP-peptide receptor(s) on the exosporium basal layer and can be blocked by binding of ATYP–RPE.
3. Flow cytometry: Samples were analyzed using an FACSCalibur instrument and CellQuest Pro software (Becton Dickinson Biosciences). All samples were excited at 488 nm with an

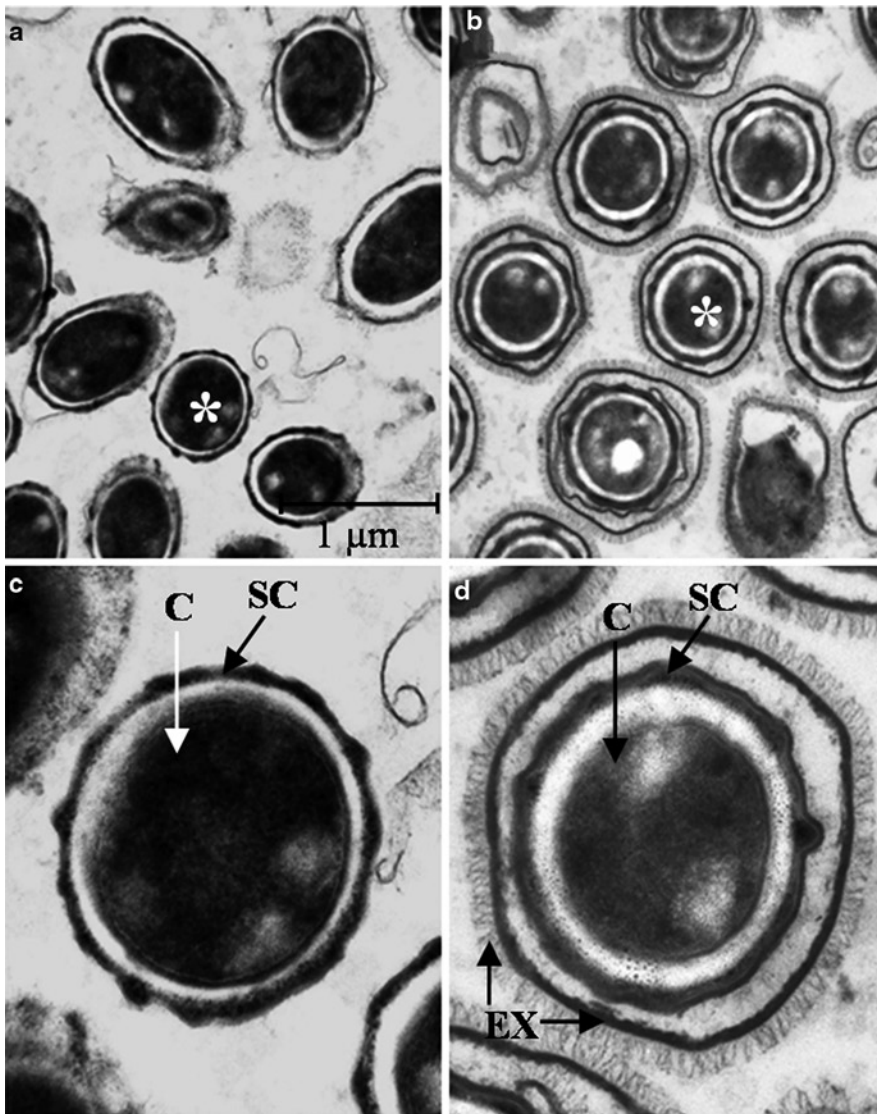


Fig. 3. Transmission electron micrographs (see Note 6) of spores from lots BAS1 (**a** and **c**) and BAS8 (**b** and **d**). Asterisks are placed on spores shown in the enlarged images. Arrowheads point to the exosporium (EX), spore coat (SC), and core (C). Image magnifications are $\times 21,300$ (**a** and **b**) and $\times 60,000$ (**c** and **d**).

argon-ion laser and detected through FL1 (FITC; 530 ± 15 nm) and FL2 (PE; 585 ± 21 nm) bandpass filters. Unlabeled spores and negative controls were used to empirically determine voltages, gains, and cutoff values for positive samples. Representative instrument settings were as follows: FL1 voltage/gain = 545/1.00, FL2 voltage/gain = 543/1.00, FITC cutoff marker = $< 2.0\%$, and RPE cutoff marker = $< 0.50\%$. Typical compensation values for two-color assays were

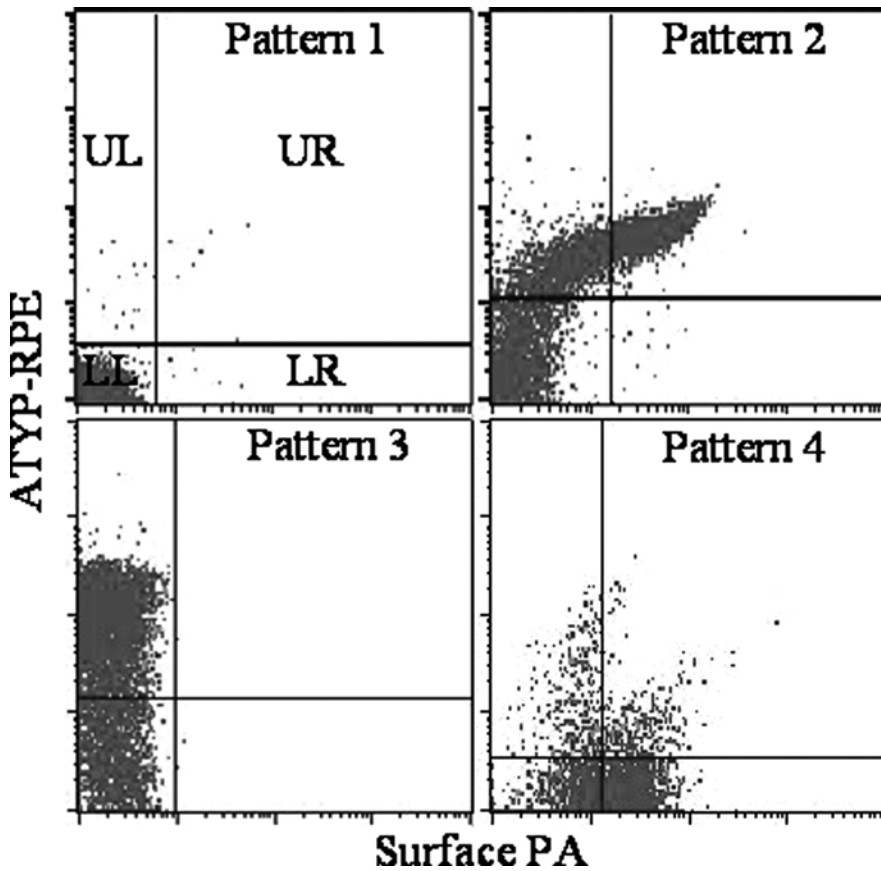


Fig. 4. Prototypical flow cytometry dot-plot patterns for identification and characterization of samples suspected to contain *Bacillus* spores. Species and potential virulence can be predicted by using one of the following patterns: pattern 1, ATYP-RPE⁻/PA⁻; pattern 2, ATYP-RPE⁺/PA⁺; pattern 3, ATYP-RPE⁺/PA⁻; and pattern 4, ATYP-RPE⁺/PA⁺ (partially nonreactive with ATYP-RPE). Quadrant abbreviations are as follows: *LL* lower left, *UL* upper left, *UR* upper right, and *LR* lower right. The following *Bacillus* samples were used to generate the dot-plot patterns: pattern 1, *B. thuringiensis*; pattern 2, *B. anthracis* Sterne; pattern 3, *B. anthracis* Sterne labeled with ATYP-RPE only (the *B. anthracis* Pasteur strain was not available for this study); and pattern 4, *B. anthracis* Sterne, lot BAS1 (no exosporium).

FL1 – 1.9% FL2, and FL2 – 17.6% FL1. All flow cytometry data was presented as an average of 10,000 events.

4. Confocal microscopy: Samples were analyzed using a Leica TCS SP2 AOBS confocal laser scanning microscope. An aliquot (10 μ L) of spore suspension was deposited on a clean glass microscope slide and dried to immobilize the spores. Dried samples were mounted with 90% glycerol, then coverslipped and imaged immediately. All samples were excited at 488 nm with an argon-ion laser and detected using FL1 (FITC; 520 ± 10 nm) and FL2 (PE; 575 ± 10 nm). Unlabeled spores and negative controls were used to empirically determine voltages for positive samples, which were typically 450 and 600 for FL1 and FL2, respectively. Using these optimized

settings, we did not observe any spectral bleed-through into either PMT. All images were collected using a 40× oil objective lens and presented as an average of ten scans.

5. Transmission electron microscopic (TEM) examination of spores from lots BAS1 and BAS8 revealed that a majority of lot BAS1 spores were missing the outermost exosporium. We believe the structural damage to lot BAS1 spores was caused by either repeated handling during the prolonged storage or unknown protease activity (possibly through contamination).
6. TEM: Unlabeled spores were prefixed with 5% glutaraldehyde in 20 mM sodium phosphate buffer, pH 7.2 for 3 days at 20–25°C. Postfixation was carried out with 1% osmium tetroxide in 50 mM sodium phosphate buffer, pH 7.2 for 24 h at 20–25°C. Serial alcohol dehydration was then performed using ethanol (15, 35, 50, 75, 90, and 100%, 3×) and then acetone (100%, 3×). Next, the samples were embedded with epon resin and polymerized at 75°C for 24 h. Ultrathin sections were obtained using an LKB Ultramicrotome (Sweden). The sections were double-stained with 1% uranyl acetate and lead citrate. EM observation was made by a Phillips EM 300 transmission electron microscope at an accelerating voltage of 60 kV. The images were recorded on Kodak electron image films.

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