

Identification and Characterization of *Bacillus anthracis* Spores by Multiparameter Flow Cytometry[∇]

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In response to the need for methods that can rapidly detect potentially virulent *Bacillus anthracis* spores, we developed 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.

Assays that can rapidly and accurately detect *Bacillus anthracis*, the etiologic agent of anthrax, are important tools for combating bioterrorism (5, 13, 16). This gram-positive spore-forming bacterium is a member of the *Bacillus cereus* group (along with *B. cereus*, *B. thuringiensis*, and *B. mycooides*) whose members exist ubiquitously in nature and are genetically related (7). One of the few distinguishing features among this group is plasmids that encode virulence factors (5). Fully virulent strains of *B. anthracis* harbor two virulence plasmids, pXO1 (1) and pXO2 (6), whereas minimally virulent strains lack one or both.

Common detection methods for *B. anthracis* include real-time PCR (11) and standard microbiological tests (14), but such assays require either the extraction of genetic material (PCR) or lengthy analysis times (microbiological tests). Comparatively, spore-based methods that analyze intact spores can be rapid because surface antigens are detected directly by using simple labeling procedures (5); however, many of these strategies are not selective for specific members of the *B. cereus* group (9, 17). Through a phage display screening process, short peptide fragments that exhibited species-specific binding to *Bacillus* spores were discovered (19, 21), and several investigators have used them successfully (2, 15). One such peptide, ATYPLPIRGGGC (abbreviated ATYP), was conjugated to *R*-phycoerythrin (RPE) and found by flow cytometry (FCM) to bind only to *B. anthracis* spores; however, the ATYP-RPE conjugate cannot differentiate spores from different strains of *B. anthracis* (21).

Recently, the protective antigen (PA) protein expressed by pXO1-harboring strains of *B. anthracis* has been identified as a spore surface antigen (4, 20). PA is present during the sporulation process and thought to be noncovalently entrapped in the spore coat and exosporium (4). Since the pXO1 plasmid encodes several virulence factors, surface PA can be considered a virulence marker for *B. anthracis* spores. Here, we report on a two-color FCM assay which couples a fluorescein isothiocyanate (FITC)-conjugated antibody-based PA assay 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.

Bacterial stocks. The following *Bacillus* spp. were used in this study: *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⁻). Bacterial strains were grown, sporulated, and purified according to previously described methods (10, 12, 18). Subsequent labeling assays were carried out in phosphate-buffered saline, pH 7.2.

Surface PA assay. An amount of 80 µg/ml of a mouse monoclonal antibody against *B. anthracis* PA (BAP0101; Abcam) was mixed with approximately 10⁶ spores and incubated for 1 h at 37°C. Bound antibody was detected by incubation with 25 µl of a 1:100 dilution of FITC-conjugated goat anti-mouse immunoglobulin G (IgG) (heavy plus light chains) (IgG-FITC; Jackson ImmunoResearch) for an additional hour at 37°C. Wash/centrifuge steps were used before and after the addition of IgG-FITC to remove unbound reagents. Under these conditions, PA was detected on the surface of *B. anthracis* Sterne spores by using FCM (FAC-SCalibur instrument/CellQuest Pro software; BD Biosciences) (Fig. 1). As expected, PA was not detected on the surface of *B. cereus* or *B. thuringiensis* spores since they do not harbor the pXO1 virulence plasmid.

Two-color assay. The ATYP peptide (GenScript Corporation) was conjugated to RPE (Invitrogen) through the heterobifunctional cross-linker sulfosuccinimidyl-4-(*N*-maleimido-methyl)cyclohexane-1-carboxylate (Sulfo-SMCC; Pierce) (8). PA antibody-treated spores (10⁶) were prepared and labeled stepwise with 25 µl of 1:100 IgG-FITC and then 25 µl of 2 µM ATYP-RPE. Each reagent was incubated for 1 h at 37°C, and wash/centrifuge steps were used before and after the addition of ATYP-RPE. The FCM data showed that for one lot of *B. anthracis* Sterne spores (BAS7), the two-color assay double labeled 30% of the population (Table 1). An additional 26% of the spores were positive for ATYP-RPE and negative for surface PA, which reflects the heterogeneity of surface PA con-

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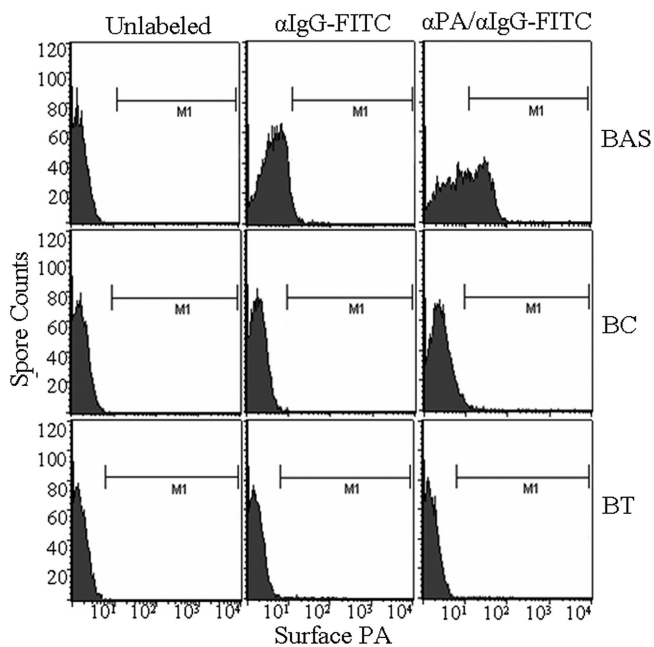


FIG. 1. Typical FCM data for the surface PA assay. The monoclonal antibody against PA was sensitive and selective for *B. anthracis* Sterne spores (BAS) as compared to *B. cereus* (BC) and *B. thuringiensis* spores (BT). In comparison to the intrinsic fluorescence of unlabeled spores, we did observe some nonspecific binding of IgG-FITC to Sterne spores. As such, the FITC cutoff marker (M1; M1 = positive event) was adjusted to minimize the contribution from nonspecifically bound IgG-FITC to the authentic surface PA signal. All FCM data are presented as the average of 10,000 events. α, anti.

tent. We also verified that double labeling occurred on individual Sterne spores by using confocal microscopy (TCS SP2 AOBS instrument; Leica Microsystems) (Fig. 2). Lower surface PA values were obtained for Sterne spores when the detection reagents were applied simultaneously or in opposite order (ATYP-RPE followed by IgG-FITC) (data not shown). This discrepancy may indicate that surface PA exists close to the ATYP peptide receptor(s) on the exosporium basal layer (3, 4) and can be blocked by binding of ATYP-RPE.

TABLE 1. Characterization of different spore lots by using the FCM two-color assay

Organism	Lot	DOP ^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 ^e	09/2003	12	72	8.6
	BAS2 ^e	09/2003	11	75	6.8
	BAS3 ^e	06/2005	58	36	36
	BAS4 ^c	08/2005	58	33	33
	BAS5 ^c	06/2006	51	23	23
	BAS6 ^d	05/2007	67	32	32
	BAS7 ^c	05/2007	56	30	30
	BAS8 ^e	06/2007	71	43	43

^a DOP, date (month/year) of spore lot preparation.
^b % DP, percentage of spores that are double positive.
^c Leighton Doi medium/modified G medium/Renografin 76.
^d Leighton Doi medium/modified G medium/no Renografin 76.
^e Leighton Doi medium/nutrient agar plate/Renografin 76.

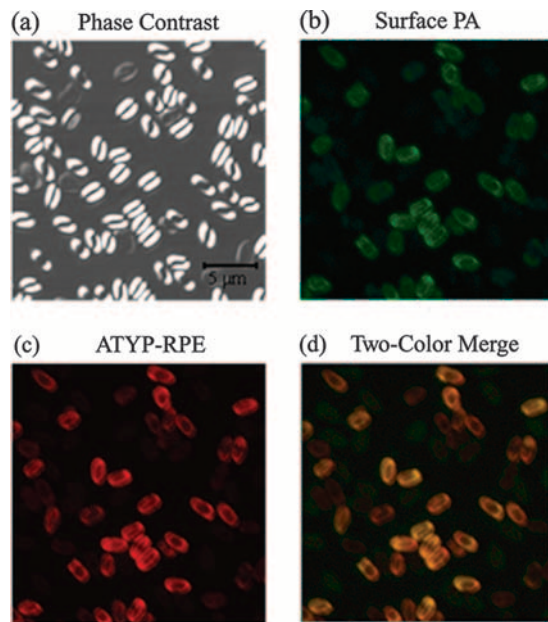


FIG. 2. Phase-contrast (a) and fluorescence (b and c) images of double-labeled *B. anthracis* Sterne spores. The surface PA fluorescence image (b) and the ATYP-RPE fluorescence image (c) were used to produce the merged image (d).

Characterization of *Bacillus* spores by two-color FCM. We next examined several spore lots to see if differences in preparation, purification, and storage conditions affected the outcome of the two-color assay (details presented in Table 1). Lots

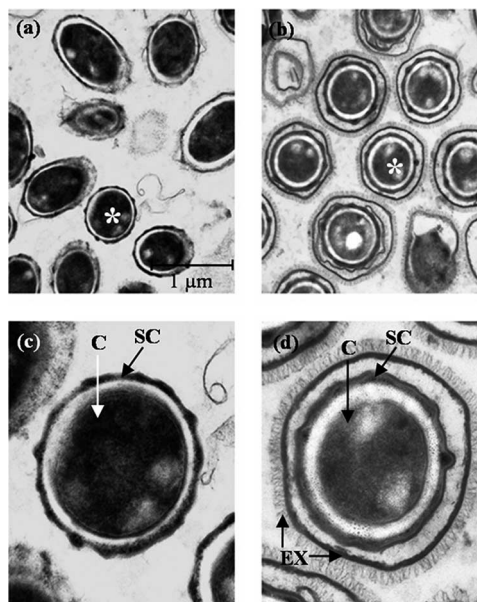


FIG. 3. Transmission electron micrographs of spores from lots BAS1 (a and c) and BAS8 (b and d). Asterisks are placed on spores shown in the enlarged images. Arrowheads point to the exosporium (EX), spore coat (SC), and core (C). Image magnifications are ×21,300 (a and b) and ×60,000 (c and d).

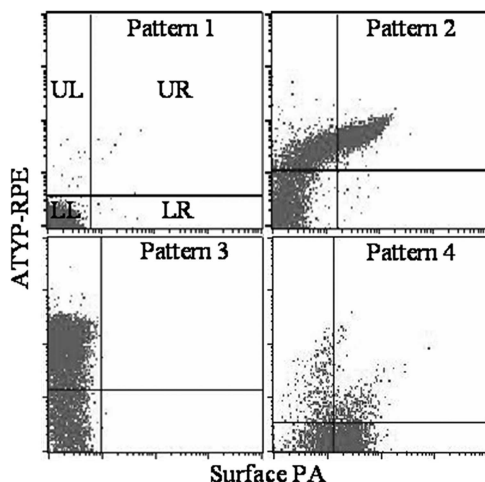


FIG. 4. Prototypical FCM dot-plot patterns for identification and characterization of unknown samples of bacterial spores. *Bacillus* species and potential virulence can be predicted by using one of the following patterns: pattern 1, ATYP-RPE⁻/PA⁻; pattern 2, ATYP-RPE⁺/PA⁺; pattern 3, ATYP-RPE⁺/PA⁻; and pattern 4, ATYP-RPE⁺/PA⁺ (no exosporium). Quadrant abbreviations are as follows: LL, lower left; UL, upper left; UR, upper right; and LR, lower right.

BAS3 through BAS8 were labeled similarly by ATYP-RPE (51 to 71% positive) and had comparable surface PA values (23 to 43% positive). Only lots BAS1 and BAS2 demonstrated lower ATYP-RPE binding (11 to 12% positive) and higher surface PA values (72 to 75% positive). Lots BAS1 and BAS2 were stored in double-distilled water at 4°C for a longer period of time (4 years) than any other lot used in this study. The examination of spores from lots BAS1 and BAS8 by using transmission electron microscopy revealed that a majority of lot BAS1 spores were missing the outermost exosporium (Fig. 3a and c). Though the reason is still unclear, we believe the structural damage to lot BAS1 spores was caused either by repeated handling during the prolonged storage or unknown protease activity (possibly through contamination). From the electron micrographs in Fig. 3 and data in Table 1, we concluded the following about the two-color assay: (i) *B. anthracis* spores only bind ATYP-RPE if they possess an exosporium and (ii) the detection of subexosporium PA (i.e., spore coat PA) is possible in the absence of an exosporium. As expected, *B. cereus* and *B. thuringiensis* spores produced double-negative results by the two-color assay.

Based on the two-color results, we have developed a set of prototypical FCM dot-plot patterns for predicting the species of spores belonging to the *Bacillus* genus and their potential virulence (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* spores. 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 spores, 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 spores, that are likely to be minimally virulent. Like pattern 2, pattern 4 (events occur in lower-left, upper-left, upper-right, and lower-right quadrants) also occurs with

ATYP-RPE⁺/PA⁺ *B. anthracis* spores that are potentially virulent; however, the 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⁺ strains of *B. anthracis* from pXO1⁻ strains in only a few hours. One drawback of this assay is that it cannot resolve *B. anthracis* Sterne spores from *B. anthracis* Ames spores since they both harbor the pXO1 plasmid. Preliminary data indicated that these two strains were labeled similarly by the two-color assay (data not shown). Work to identify another spore surface antigen capable of making that distinction is ongoing. Once discovered, we envision its use in a three-color assay. Nonetheless, this two-parameter detection assay marks an important step toward rapid and complete characterization of the dangerous pathogen known as *B. anthracis*.

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