Published in final edited form as:

J Microbiol Methods. 2011 May ; 85(2): 143–148. doi:10.1016/j.mimet.2011.02.009.

Current Physical and SDS Extraction Methods Do Not Efficiently Remove Exosporium Proteins from *Bacillus anthracis* spores

Brian M. Thompson^{a,b}, Jana M. Binkley^a, and George C. Stewart^{a,*}

^aDepartment of Veterinary Pathobiology, Bond Life Sciences Center, University of Missouri

^bDepartment of Biochemistry, Bond Life Sciences Center, University of Missouri

Abstract

Biochemical studies of the outermost spore layers of the *Bacillus cereus* family are hindered by difficulties in efficient dispersal of the external spore layers and difficulties in dissociating protein complexes that comprise the exosporium layer. Detergent and physical methods have been utilized to disrupt the exosporium layer. Herein we compare commonly used SDS extraction buffers used to extract spore proteins and demonstrate the incomplete extractability of the exosporium layer by these methods. Sonication and bead beating methods for exosporium layer removal were also examined. A combination of genetic and physical methods is the most effective for isolating proteins found in the spore exosporium.

Keywords

Bacillus anthracis; spores; exosporium; extraction; anthrax

1. Introduction

Anthrax is an acute infectious disease most common in livestock caused by the Gram-positive spore-forming bacterium *Bacillus anthracis*. During unfavorable conditions an alternative development pathway, sporulation, is utilized by all members of the *Bacillus* genus (reviewed in Driks, 2009). The end result of sporulation is the formation of an endospore which is resistant to various environmental hazards such as heat, desiccation, and UV radiation. Due to these resistance properties, endospores are able to persist in soil for long periods of time. In addition, the spore is the infectious form of *B. anthracis*.

The outermost layer of the *B. anthracis* spore is the exosporium. The exosporium is composed of a basal layer and an outer nap layer which consists of hair-like projections comprised primarily of the collagen-like glycoprotein BclA (Sylvestre *et al.*, 2002; 2003). The known proteins forming this outer spore layer are found only on certain *Bacillus* species including *B. cereus*, *B. thuringiensis*, and *B. megaterium*, but absent on others including the best studied spore-former, *B. subtilis*. An exosporium is also present on *Clostridium* spores, including those of *C. botulinum* and *C. difficile* (Takumi *et al.*, 1979; Panessa-Warren *et al.*,

© 2011 Elsevier B.V. All rights reserved.

Corresponding author: George C. Stewart, Ph.D., 471e Bond Life Sciences Center, University of Missouri, 1201 Rollins St., Columbia, MO 65211 USA, 01-573-884-2866, 01-573-884-5414 (fax), stewartgc@missouri.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1997; Henriques and Moran, 2007). The function of the exosporium is not well understood. The exosporium layer is a stable structure of *B. anthracis* spores, with exosporium-deficient spores rarely, if ever, spontaneously arising in spore preparations (Thompson *et al.*, 2007). However, suggestions are creeping into the literature that the *B. anthracis* exosporium is fragile and easily removed from the spores by physical methods such as sonication (Redmond *et al.*, 2004; Kang *et al.*, 2005; Basu *et al.*, 2007), or shear stress (Faille *et al.*, 2007). Extrapolation of results that indicate that at least in one strain of *B. cereus*, exosporium-negative spores can arise spontaneously and their frequency is increased by inducing sporulation at elevated temperatures (Faille *et al.*, 2007) has led to speculation that exosporium loss may similarly occur in *B. anthracis* spore preparations (Hugh-Jones and Blackburn, 2009). Our experience with *B. anthracis*, particularly with the Sterne strain, is that the exosporium layer is an incredibly stable structure and not easily removed.

Currently there is limited information available concerning the assembly and function of the exosporium layer. Biochemical analysis of the exosporium requires harsh detergent extraction of the spores. The two most common methods involve boiling the spores with SDS-PAGE sample buffer (Steichen *et al.* 2003, 2005, 2007; Boydston *et al.* 2005, 2006; Severson *et al.* 2009; Giorno *et al.* 2005, 2007, Brahmabhatt *et al.* 2007) or boiling the spores in the presence of SDS plus 8M urea (Sylvestre *et al.* 2002, 2003, 2005, Waller *et al.* 2003, 2005; Thompson *et al.* 2007, 2008, 2011). A comparative analysis of the two SDS-based methods was undertaken to determine the ability of these SDS-based protocols to remove the exosporium of *B. anthracis*.

2. Methods

2.1. Bacterial strains and culture conditions

We utilized ΔSterne, a plasmid-free derivative of the Sterne veterinary vaccine strain as our source of *B. anthracis* spores. Bacteria were cultured in tryptic soy broth (TSB; Difco) or on tryptic soy agar (TSA) plates at 37°C. Sporulation was induced by growth on nutrient agar (Difco) plates at 30°C, as the cells sporulate efficiently under these conditions (Thompson *et al.*, 2007). Sporulation was essentially complete (>95%) by 72 hr. The degree of sporulation was assessed by phase contrast microscopy. Spores were harvested from the plates, washed 3 times in PBS, and stored at room temperature. Washing thrice in PBS was sufficient to remove the vast majority of vegetative cell debris when monitored by light microscopy.

2.2. Spore chemical extraction methods

Buffer-washed spores (5×10^7) were subjected to boiling in SDS sample buffer (50 mM Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 2% β-mercaptoethanol, 0.02% bromophenol blue) or SDS-8M urea sample buffer (50 mM Tris-HCl, pH 10, 10% glycerol, 4% SDS, 8 M urea, 2% β-mercaptoethanol, 0.02% bromophenol blue) for 10 minutes. Spores were then pelleted by centrifugation and the supernatant was retained for western blot analysis. The spore pellet was washed in PBS buffer, and dialyzed against PBS overnight at 4°C. Spores were collected and were prepared for transmission electron microscopy (TEM) as previously described (Thompson *et al.* 2007, Thompson and Stewart, 2008). The glycoprotein stain ruthenium red was added to permit the visualization of extensively glycosylated exosporium nap layer (Waller *et al.*, 2004).

2.3. Spore physical extraction methods

Bead beating: Spores were reacted with 0.1 mm glass beads using a Bead Beater apparatus in 1 ml of cold PBS (Biospec Products). 1×10^8 PBS-washed spores were subjected to bursts of bead beating for 1, 2, 3, 5, and 60 minutes at 4°C.

Sonication: 1×10^8 PBS-washed spores were disrupted by sonication at 4°C. Spores were sonicated (Branson Sonifier 150; Branson Ultrasonics Co., Danbury, CT) with maximum power (amplitude, 12 μ m; 10 min/50 W) for either 30s or 1, 2, 10, or 60 1-min bursts, each separated by 2 min of cooling on ice, as described by Kang *et al.* (2005).

Spores (treated and control) were pelleted by centrifugation and supernatants were collected and quantified via the Bradford method (BioRad; Bradford, 1976) to determine levels of extracted proteins.

2.4. Transmission electron microscopy

To the PBS-washed spores, 1 ml of a 2% glutaraldehyde, 0.1 M sodium cacodylate solution containing 0.1% ruthenium red (Electron Microscopy Sciences, Fort Washington, PA) was added and incubated for 1 hour at 37°C. Each pellet was then washed in cacodylate buffer and fixed for 3 h at room temperature in a 1% osmium tetroxide (Electron Microscopy Sciences), 0.1 M sodium cacodylate solution containing 0.1% ruthenium red. Spores were washed in buffer and embedded in 3% agar (EM Science, Gibbstown, NJ). Dehydration involved sequential treatment with 25, 50, 75, 95, and 100% acetone. Polymerization was carried out at 60°C in Epon/araldite resin. Sections were cut at 85 nm thickness and put on 200 mesh carbon-coated copper grids and then stained with a 2% uranyl acetate solution (Electron Microscopy Sciences) for 40 min at 37°C. The sections were then treated with Sato's Triple Lead for 3 minutes, washed in ultrapure water, and stained again for 18 minutes in 5% uranyl acetate, followed by one final wash and were observed by transmission electron microscopy with a JEOL 1200EX electron microscope.

2.5. Epi-fluorescence microscopy

5×10^7 of spores were resuspended in StartingBlock (Thermo Scientific) and incubated at room temperature for 45 minutes with occasional mixing. The spores were then pelleted and resuspended in StartingBlock. Rabbit polyclonal antiserum (1:250 dilution) against BclA was added and incubated at room temperature for 45 minutes with occasional mixing. The spores were then thrice washed in StartingBlock and incubated with FITC-Protein A conjugate (Sigma Chemical Co.) and incubated for 45 minutes at room temperature with occasional mixing. The spores were washed 3X with StartingBlock, resuspended in PBS, and examined by epi-fluorescence microscopy using a Nikon E600 microscope.

2.6. Western blot analyses

Extracted spore proteins were size fractionated on 4-20% gradient Tris-HCl polyacrylamide gels (Bio-Rad) in Tris-glycine-SDS buffer. Proteins were transferred to nitrocellulose filters. Anti-BclA and anti-BclB antisera were prepared in rabbits using Ribi adjuvant (Corixa). Western blots were conducted using goat anti-rabbit IgG-alkaline phosphatase (Pierce) as the secondary antibody and the immunoreactive proteins were identified using SuperSignal Detection Substrate (Pierce). Quantitative analyses of western blot data were obtained using the Multi-Gauge analytical software (Fujifilm). The presence of 8M urea in the sample buffer results in decreased mobility under SDS-PAGE conditions and the slight variation in the apparent size of BclA complexes visualized by western blot analysis.

2.7. Flow cytometry

Spores were reacted with antibody and labeled with FITC-protein A as described above for epi-fluorescence microscopy. The spores were washed 3X with StartingBlock, followed by 2X with PBS and processed on a FACScan flow cytometer using a 488 nm argon laser (Beckton Dickinson Biosciences). Data were analyzed using Cell Quest analysis software (Beckton Dickinson).

3. Results

3.1. Examination of extracted spores by transmission electron microscopy

Untreated, SDS-extracted, and SDS+urea-extracted spores were examined by TEM (Fig. 1). Untreated spores had an intact exosporium layer without any observable defects in the exosporium or spore coat layers (Fig. 1, A and D). SDS-extracted spores displayed a damaged exosporium layer, but the exosporium remained as a distinct feature of the spores (Fig. 1, B and E). The spores extracted with both SDS and urea had a more complete disruption of the exosporium hair-like nap layer and exhibited a collapse of the so-called “interspace” layer between the exosporium and the spore coat (Fig. 1, panels C and F; Giorno *et al.*, 2009). Closer analysis of the exosporium nap (arrowheads) stained with glycoprotein stain ruthenium red demonstrated the different extraction efficiencies on the exosporium layers (Fig. 1, D-F). In the spores extracted with SDS there was a partial loss of filaments in the hair-like nap layer and a partial disruption of the basal layer. The spores that were extracted with both SDS and urea an increased destruction of the basal layer and a greater loss of the nap layer (Fig. 1, E-F).

3.2. Extraction of exosporium proteins by SDS versus SDS + urea

In addition to the modest loss of exosporium structure observed by TEM analysis, the proteins extracted by each method were subjected to western blot analysis for the detection of the exosporium proteins BclA and BclB using rabbit polyclonal anti-BclA and anti-BclB antibodies (1:10,000, Thompson *et al.* 2007).

As shown in Fig. 2, the addition of 8M urea to the SDS extraction buffer greatly enhanced the yield of BclA recovered from the spores (Fig. 2A). Besides extracting more BclA, the mass of the protein complexes extracted differed in the presence of 8M urea versus SDS extraction in the absence of urea. Certain exosporium components, such as the surface-exposed BclB glycoprotein, were only efficiently extracted from the exosporium in the presence of 8M urea (Fig. 2B). In addition, the yield of the exosporium basal layer protein BxpB was increased in the presence of 8M urea (data not shown).

Immunolabeling of SDS- or SDS+urea-extracted and untreated control spores with anti-BclA polyclonal antiserum revealed that the amount of BclA exposed on the spores did not change appreciably (Fig. 3). Extracted spores, particularly SDS + urea-treated spores, displayed an increased tendency to clump.

3.3. Effect of bead beating on the exosporium

It has been suggested that physical extraction methods can strip the exosporium layer from spores. To determine whether a commonly used bacterial cell disrupting method, bead beating, would suffice to dislodge the exosporium layer of *B. anthracis* spores, we subjected 1×10^8 PBS-buffer washed purified spores to cycles of bead beating for 1, 2, 3, and 5, and 60 minutes (Fig. 4). Proteins released from treated and control spores were quantified (Fig. 4A). No significant removal of spore proteins (Fig. 4A) or the major exosporium protein BclA (assayed by western blotting, Fig. 4B) occurred by bead beating. The amount of BclA that could be extracted from the spores following bead beating was comparable to that of control spores not subjected to bead beating (Fig. 4C). Increasing the bead beating time to 60 minutes only resulted in a minor release of protein into the supernatant. To assay for any variation in the BclA content of the treated spores, we immunolabeled spores following increasing treatment time to search for differences in the reactivity of the spores to anti-BclA antibodies. No change in the overall antibody reactivity to BclA on the surface of the spores was found despite the bead-beating treatment (Fig. 4D). If substantial amounts of exosporium had been removed by bead beating, the amplitude of the peaks would have

diminished. This was not observed, consistent with the exosporium layers remaining largely intact despite the sonication treatment.

3.4. Effect of sonication on the exosporium

It has been reported that sonication is sufficient to remove the exosporium from *B. anthracis* spores (Redmond *et al.*, 2004; Basu *et al.*, 2005; Kang *et al.*, 2007). To test this, 1×10^8 of PBS-washed purified spores were exposed to increasing times of sonication followed by assessment of the damage to the exosporium layer of *B. anthracis* spores (Fig. 5). No significant removal of total spore proteins (Fig. 5A) or specifically BclA (Fig. 5B) occurred with sonication. Only with 10 minutes or 60 minutes of sonication was a barely detectable level of spore protein released from the spores (Fig. 5A). The minor increase in the 10 minute sample was insufficient for BclA detection by western blot analysis (Fig. 5B). The overall concentration of BclA on the sonicated spores remained the same following all sonication treatments (Fig. 5C).

3.5. Effect of sonication on the *cotE*-null spore exosporium

Deletion of the *cotE* determinant leads to the production of spores that contain broken exosporium pieces loosely attached to the spores and strips of exosporium that are not spore-associated in the mother cell (Giorno *et al.*, 2007). CotE-negative spore preparations contain substantial amounts of free exosporium strips, as shown by TEM analysis (Fig. 6A and B). The loose exosporium fragments tend to be lost with repeated washing of the spores in PBS. We took PBS-washed Δ Sterne and *cotE*-null mutant spores and subjected them to sonication (10 1-minute bursts). Spores were pelleted by microfugation, and the supernatants were filtered through 0.45 μ m filters and concentrated by vacuum centrifugation. Concentrated supernatant samples were either boiled or unheated in the presence of SDS + urea and resolved by SDS-PAGE (Fig. 6C-E). Supernatants from the wild-type Δ Sterne spores did not have any detectable exosporium as assayed by BclA reactivity (Fig. 6C). Exosporium proteins were found in the *cotE*-null spore supernatants treated in this fashion (Fig. 6D). Upon boiling in the presence of SDS, some BclA was released from the high MW complexes into its ~70 kDa monomeric form (Fig 6D, arrows).

4. Discussion

Multiple methodologies have been described to remove the exosporium from isolated *Bacillus* spores. We have demonstrated that the simple use of SDS sample buffer is insufficient to efficiently extract exosporium proteins from *B. anthracis* spores. Addition of 8M urea permits greater disruption of exosporium components and increases the yield of exosporium proteins. We chose BclA, a well-studied and highly abundant exosporium nap protein to evaluate extraction of the *B. anthracis* exosporium. In addition, BclB was chosen as representative of spore proteins which require SDS + urea extraction for efficient extraction from the exosporium of *B. anthracis* (Thompson *et al.*, 2007). We suggest that future research into known and unknown exosporium proteins use the addition of 8M urea in their SDS sample buffer to expand both the spectrum and yields of recovered exosporium proteins.

Physical methods have also been employed for the removal of the exosporium. We demonstrate that the use of mechanical disruption by bead beating was not efficient in the removal of detectable levels of total protein from the spore and BclA in particular nor did it result in significant loss of the exosporium layer from the spores as assessed by transmission electron microscopy (data not shown). Sonication was similarly inefficient in extracting spore proteins or removing of the exosporium layer from the spores. This result differs from those reported with the Ames strain of *B. anthracis* by Redmond *et al.* (2004). The different

results may be due to strain differences or how the spores were prepared or maintained prior to sonication.

One approach to more efficiently extract exosporium proteins is the use of mutant strains of *B. anthracis*, such as *cotE*-negative, that exhibit defects in exosporium assembly or attachment to the spore coat layer. CotE-deficient spores have loosely associated exosporium layers that can be released from the spores by sonication. A combined genetic and physical / detergent extraction approach should greatly facilitate exosporium protein isolation.

5. Conclusions

Treatment of *B. anthracis* spores with SDS sample buffer alone does not greatly affect the overall appearance of the exosporium nor lead to high yields of exosporium proteins. The addition of 8M urea to the SDS extraction buffer greatly enhances both the extraction of the exosporium and the yield obtained via western blot analyses. Importantly, both SDS chemical extraction methods still do not greatly alter the overall structure of the spore or exosporium, indicative of the robust interactions among exosporium proteins. Physical methods utilized for the removal of the exosporium, such as sonication and bead beating, were not able to release spore proteins from the spores and do not alter the exosporium content of the spores.

Acknowledgments

B. Thompson was supported in part by a Veterinary Postdoctoral Fellowship through the Midwest Regional Center of Excellence for Biodefense and Emerging Infectious Disease Research. This work was supported in part by NIH grant AI074589 to G.C.S.

References

- Basu S, Kang TJ, Chen WH, Fenton MJ, Baillie L, Hibbs S, Cross AS. Role of *Bacillus anthracis* spore structures in macrophage cytokine responses. *Infect Immun*. 2007; 75(5):2351–2358. [PubMed: 17339355]
- Boydston JA, Chen P, Steichen CT, Turnbough CL Jr. Orientation within the exosporium and structural stability of the collagen-like glycoprotein BclA of *Bacillus anthracis*. *J Bacteriol*. 2005; 187(15):5310–5317. [PubMed: 16030225]
- Boydston JA, Yue L, Kearney JF, Turnbough CL Jr. The ExsY protein is required for complete formation of the exosporium of *Bacillus anthracis*. *J Bacteriol*. 2006; 188(21):7440–7448. [PubMed: 16936017]
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976; 72:248–254. [PubMed: 942051]
- Brahmbhatt TN, Janes BK, Stibitz ES, Darnell SC, Sanz P, Rasmussen SB, O'Brien AD. *Bacillus anthracis* exosporium protein BclA affects spore germination, interaction with extracellular matrix proteins, and hydrophobicity. *Infect Immun*. 2007; 75(11):5233–5239. [PubMed: 17709408]
- Driks A. The *Bacillus anthracis* spore. *Mol Aspects Med*. 2009; 30(6):368–373. [PubMed: 19683018]
- Faille C, Tauveron G, Le Gentil-Lelièvre C, Slomianny C. Occurrence of *Bacillus cereus* spores with a damaged exosporium: consequences on the spore adhesion on surfaces of food processing lines. *J Food Prot*. 2007; 70(10):2346–2353. [PubMed: 17969617]
- Giorno R, Bozue J, Cote C, Wenzel T, Moody KS, Mallozzi M, Ryan M, Wang R, Zielke R, Maddock JR, Friedlander A, Welkos S, Driks A. Morphogenesis of the *Bacillus anthracis* spore. *J Bacteriol*. 2007; 189(3):691–705. [PubMed: 17114257]
- Giorno R, Mallozzi M, Bozue J, Moody K, Slack A, Qui D, Wang R, Friedlander A, Welkos S, Driks A. Localization and assembly of proteins comprising the outer structures of the *Bacillus anthracis* spore. *Microbiology*. 2009; 155(4):1133–1145. [PubMed: 19332815]

- Henriques AO, Moran CP Jr. Structure, assembly, and function of the spore surface layers. *Ann Rev Microbiol.* 2007; 61:555–588. [PubMed: 18035610]
- Hugh-Jones M, Blackburn J. The ecology of *Bacillus anthracis*. *Molec Aspects Med.* 2009; 30(6): 356–367. [PubMed: 19720074]
- Kang TJ, Fenton MJ, Weiner MA, Hibbs S, Basu S, Baillie L, Cross AS. Murine macrophages kill the vegetative form of *Bacillus anthracis*. *Infect Immun.* 2005; 73(11):7495–7501. [PubMed: 16239551]
- Panessa-Warren BJ, Tortora GT, Warren JB. Exosporial membrane plasticity of *Clostridium sporogenes* and *Clostridium difficile*. *Tissue Cell.* 1997; 29(4):449–461. [PubMed: 9281845]
- Redmond C, Baillie LW, Hibbs S, Moir AJ, Moir A. Identification of proteins in the exosporium of *Bacillus anthracis*. *Microbiology.* 2004; 150(2):355–363. [PubMed: 14766913]
- Severson KM, Mallozzi M, Bozue J, Welkos SL, Cote CK, Knight KL, Driks A. Roles of the *Bacillus anthracis* spore protein ExsK in exosporium maturation and germination. *J Bacteriol.* 2009; 191(24):7587–7596. [PubMed: 19837802]
- Steichen C, Chen P, Kearney JF, Turnbough CL Jr. Identification of the immunodominant protein and other proteins of the *Bacillus anthracis* exosporium. *J Bacteriol.* 2003; 185(6):1903–1910. [PubMed: 12618454]
- Steichen CT, Kearney JF, Turnbough CL Jr. Characterization of the exosporium basal layer protein BxpB of *Bacillus anthracis*. *J Bacteriol.* 2005; 187(17):5868–5876. [PubMed: 16109927]
- Steichen C, Kearney JF, Turnbough CL Jr. Non-uniform assembly of the *Bacillus anthracis* exosporium and a bottle cap model for spore germination and outgrowth. *Mol Microbiol.* 2007; 64(2):359–367. [PubMed: 17493122]
- Sylvestre P, Couture-Tosi E, Mock M. A collagen-like surface glycoprotein is a structural component of the *Bacillus anthracis* exosporium. *Mol Microbiol.* 2002; 45(1):169–178. [PubMed: 12100557]
- Sylvestre P, Couture-Tosi E, Mock M. Polymorphism in the collagen-like region of the *Bacillus anthracis* BclA protein leads to variation in exosporium filament length. *J Bacteriol.* 2003; 185(5): 1555–1563. [PubMed: 12591872]
- Sylvestre P, Couture-Tosi E, Mock M. Contribution of ExsFA and ExsFB proteins to the localization of BclA on the spore surface and to the stability of the *Bacillus anthracis* exosporium. *J Bacteriol.* 2005; 187(15):5122–5128. [PubMed: 16030205]
- Takumi K, Kinouchi T, Kawata T. Isolation and partial characterization of exosporium from spores of a highly sporogenic mutant of *Clostridium botulinum* type A. *Microbiol Immunol.* 1979; 23(6): 443–454. [PubMed: 386051]
- Thompson BM, Hsieh HY, Spreng K, Stewart GC. Co-dependency of BxpB/ExsFA and BclA for proper incorporation into the exosporium of *Bacillus anthracis*. *Mol Microbiol.* 2011; 79(3):799–813. [PubMed: 21255119]
- Thompson BM, Stewart GC. Targeting of the BclA and BclB proteins to the *Bacillus anthracis* spore surface. *Mol Microbiol.* 2008; 70(2):421–434. [PubMed: 18761690]
- Thompson BM, Waller LN, Fox KF, Fox A, Stewart GC. The BclB glycoprotein of *Bacillus anthracis* is involved in exosporium integrity. *J Bacteriol.* 2007; 189(18):6704–6713. [PubMed: 17644587]
- Waller L, Fox N, Fox KF, Fox A, Price RL. Ruthenium red staining for ultrastructural visualization of a glycoprotein layer surrounding the spore of *Bacillus anthracis* and *Bacillus subtilis*. *J Microbiol Meth.* 2004; 58(1):23–30.
- Waller LN, Stump MJ, Fox KF, Harley WM, Fox A, Stewart GC, Shahgholi M. Identification of a second collagen-like glycoprotein produced by *Bacillus anthracis* and demonstration of associated spore-specific sugars. *J Bacteriol.* 2005; 187(13):4592–4597. [PubMed: 15968070]

Abbreviations

TEM	Transmission electron microscopy
SDS	sodium dodecyl sulfate

Figure 1.

TEM of spores untreated (Panels A and D) or treated with SDS (Panels B and E) or SDS and urea buffer (Panels C and F). Arrows denote the interspace region of the spores. Arrowheads denote the hair-like nap layer. All panels stained with ruthenium red to visual the heavily glycosylated exosporium layer.

Figure 2.

Extraction efficiencies with and without 8M urea. **A)** Western blot using anti-BclA antisera. Lane 1, Δ Sterne spores boiled in SDS sample buffer, Lane 2, protein marker, Lane 3, Δ Sterne spores boiled in SDS + urea buffer. **B)** Western blot using anti-BclB antisera. Lane 1, protein marker, Lane 2, Δ Sterne spores boiled in SDS sample buffer, Lane 3, Δ Sterne spores boiled in SDS + urea buffer.

Figure 3. Immunolabeling of treated and untreated spores with anti-BclA antibodies followed by FITC-Protein A. Panels show epi-fluorescence micrographs taken at 400 $\times$ magnification. Note the clumping effect seen with SDS treatment and especially the SDS + urea treatment.

Figure 4.

The lack of exosporium disruption via bead beating. A) Total protein levels released from spores as assayed via the Bradford protein assay. B) Supernatant samples from disrupted spores western blotted for the presence of BclA. C) Samples of spores collected following bead beating and western blotted for the presence of BclA. D) Overlaid flow cytometry results for surface labeling with anti-BclA antibodies after bead beating for 0 (red), 1 (yellow), 2 (teal), 3 (purple) and 5 minutes (blue). The last histogram is a control with no secondary antibody added to untreated spores. ND = Not detectable. The lower limit for protein quantification was 0.66 ng/ μl .

Figure 5.

The lack of exosporium disruption via sonication. A) Total protein levels released from spores as assayed via the Bradford protein assay. B) Supernatant samples from disrupted spores western blotted for the presence of BclA. C) Samples of spores collected following sonication and western blotted for the presence of BclA. ND = Not detectable. The lower limit for protein quantification was 0.66 ng/ μl .

Figure 6.

The effect of sonication on *cotE*-null spores. A and B) TEM micrographs of *cotE*-null spores before treatment. Note the appearance of free exosporia and loosely associated exosporia (denoted by arrowheads). C) Concentrated protein preparations of sonicated Δ *Sterne* spores assayed for BclA reactivity as a marker of exosporium proteins. D) Concentrated protein preparations of sonicated *cotE*-null spores assayed for BclA reactivity as a marker of exosporium proteins.