Localization and assembly of proteins comprising the outer structures of the *Bacillus anthracis* spore

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Bacterial spores possess a series of concentrically arranged protective structures that contribute to dormancy, survival and, ultimately, germination. One of these structures, the coat, is present in all spores. In *Bacillus anthracis*, however, the spore is surrounded by an additional, poorly understood, morphologically complex structure called the exosporium. Here, we characterize three previously discovered exosporium proteins called ExsFA (also known as BxpB), ExsFB (a highly related paralogue of *exsFA/bxpB*) and lunH (similar to an inosine–uridine-preferring nucleoside hydrolase). We show that in the absence of ExsFA/BxpB, the exosporium protein BclA accumulates asymmetrically to the forespore pole closest to the midpoint of the sporangium (i.e. the mother-cell-proximal pole of the forespore), instead of uniformly encircling the exosporium. ExsFA/BxpB may also have a role in coat assembly, as mutant spore surfaces lack ridges seen in wild-type spores and have a bumpy appearance. ExsFA/BxpB also has a modest but readily detected effect on germination. Nonetheless, an *exsFA/bxpB* mutant strain is fully virulent in both intramuscular and aerosol challenge models in Guinea pigs. We show that the pattern of localization of ExsFA/BxpB–GFP is a ring, consistent with a location for this protein in the basal layer of the exosporium. In contrast, ExsFB–GFP fluorescence is a solid oval, suggesting a distinct subcellular location for ExsFB–GFP. We also used these fusion proteins to monitor changes in the subcellular locations of these proteins during sporulation. Early in sporulation, both fusions were present throughout the mother cell cytoplasm. As sporulation progressed, GFP fluorescence moved from the mother cell cytoplasm to the forespore surface and formed either a ring of fluorescence, in the case of ExsFA/BxpB, or a solid oval of fluorescence, in the case of ExsFB. lunH–GFP also resulted in a solid oval of fluorescence. We suggest the interpretation that at least some ExsFB–GFP and lunH–GFP resides in the region between the coat and the exosporium, called the interspace.

Received 15 August 2008
Revised 24 November 2008
Accepted 12 December 2008

INTRODUCTION

The bacilli and clostridia encompass a large number of species that produce highly resistant dormant cells, called

spores, in response to stress (Fritze, 2004; Nicholson, 2002). Prominent among these species is *Bacillus anthracis*, which has played a central historical role in our understanding of the transmission of disease and the mechanisms of toxin action, and which has reemerged as a serious threat to human life in light of its utility as a biological weapon (Alibek, 1999; Koch, 1876; Mock & Fouet, 2001). Because the *B. anthracis* spore is the infectious particle for the disease anthrax, a deeper understanding of spore composition, assembly and function could be very useful in combating this threat.

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Abbreviations: AFM, atomic force microscopy; IFM, immunofluorescence microscopy; IM, intramuscular; TEM, thin-section electron microscopy.

A supplementary table listing PCR primers used in this study is available with the online version of this paper.

Spores from diverse *Bacillus* species possess a common architecture. Surrounding the core, which houses the DNA and protective DNA-binding proteins called small acid-soluble spore proteins (SASPs) (Setlow, 2006), is a specialized peptidoglycan called the cortex (Dowd *et al.*, 2008; Popham, 2002). The cortex, in turn, is housed in a complex multi-layered protein shell called the coat (Aronson & Fitz-James, 1976; Warth *et al.*, 1963). From studies in *Bacillus subtilis*, we know that the coat protects the spore against a variety of harmful molecules and facilitates germination, the process by which spores leave the dormant state and resume vegetative growth (Moir *et al.*, 2002).

In some species, including *B. subtilis*, the coat is the outermost spore structure. In others, such as *B. anthracis*, spores possess an additional structure called the exosporium, which encases the entire spore. A thin basal layer appears to be a structural feature shared by exosporia of most or all species (Aronson & Fitz-James, 1976; Holt & Leadbetter, 1969). In some species, such as *Bacillus megaterium*, this layer is relatively thick (Aronson & Fitz-James, 1976; A. Driks, unpublished observations; Holt & Leadbetter, 1969; Vary, 1994). In others, such as *B. anthracis* and *Bacillus cereus*, a series of fine hair-like projections (also called a nap) extend a short distance from the basal layer. A striking feature of the *B. anthracis* exosporium is the significant space between the exosporium and the coat, a region we refer to as the interspace. Although no attachment points between the two structures are evident in conventional thin-section electron micrographs, such connections must exist in order for the exosporium to have been able to assemble around the spore.

The function of the exosporium is currently an area of active investigation. Efforts to identify its purpose are complicated by its presence in a taxonomically diverse subset of species that includes non-pathogenic organisms such as *B. megaterium* and *Bacillus odyseyi* (La Duc *et al.*, 2004; Vary, 1994). Nonetheless, insight into possible exosporium functions has come from recent studies that indicate that the exosporium mediates adhesion to certain eukaryotic cell types representative of host tissues, provides some degree of resistance to the oxidative burst produced by macrophages in culture, and helps moderate the innate immune response to spores (Basu *et al.*, 2007; Bozue *et al.*, 2007b; Weaver *et al.*, 2007). Additionally, the major exosporial protein BclA has been shown to mediate the phagocytosis of spores via interaction with the macrophage cell-surface integrin Mac-1 (Oliva *et al.*, 2008). These functions could provide advantages to spores during pathogenesis and in a variety of niches other than that of an infected host.

About 20 exosporium-associated proteins and glycoproteins have been identified from analyses of *B. anthracis* and *B. cereus* (Redmond *et al.*, 2004; Steichen *et al.*, 2003; Sylvestre *et al.*, 2002; Todd *et al.*, 2003; Waller *et al.*, 2005). In addition, other, less well-characterized molecules are also present, including lipids and carbohydrates (Bozue

et al., 2005; Fox *et al.*, 2003; Matz *et al.*, 1970). The best-characterized *B. anthracis* exosporium protein is BclA, which is on the exosporium surface, is highly immunogenic, and is a major structural component of the hair-like projections (referred to below as 'projections') (Boydston *et al.*, 2005; Rety *et al.*, 2005; Steichen *et al.*, 2003, 2005; Sylvestre *et al.*, 2002, 2003, 2005). Several other exosporium proteins or candidate exosporium proteins have been characterized in *B. anthracis*. ExsFA (or BxpB) has also been shown to reside in the *B. anthracis* basal layer, and to have at least a partial role in the formation of projections and in BclA deposition (Steichen *et al.*, 2005; Sylvestre *et al.*, 2005). ExsFB, a paralogue of ExsFA/BxpB, also has a role in exosporium formation, since *exsFA/bxpB exsFB* double mutant spores have a more severe exosporium defect than *exsFA/bxpB* mutant spores (Sylvestre *et al.*, 2005). The *B. anthracis* proteins CotY and ExsY (orthologues of the *B. subtilis* coat proteins CotY and CotZ) have also been found in the exosporium (Redmond *et al.*, 2004). ExsY has a role in exosporium assembly, as *exsY* mutant spores possess only a fragment of this structure (Boydston *et al.*, 2006). The view that these proteins are together in the exosporium is supported by the finding that ExsFA/BxpB is present as part of a complex with BclA and ExsY (Redmond *et al.*, 2004). The exosporium glycoprotein BclB has also been shown to play an important role in exosporium stability, as *bclB* mutant spores assemble only partial exosporia, or lack the structure entirely (Thompson *et al.*, 2007).

Some spore proteins play roles in assembly of both the coat and exosporium. The *B. anthracis* coat protein CotE directs assembly of at least one coat protein other than itself (Giorno *et al.*, 2007). In addition, in most *cotE* mutant sporangia, an exosporium is synthesized, but accumulates in the mother cell without encircling the forespore. This and other data support the inference that CotE directs the attachment of the exosporium to the forespore surface.

Some exosporium proteins are orthologues of previously identified enzymes. For example, alanine racemase (Alr) and inosine hydrolase (IunH) are present in *B. anthracis* and *B. cereus* exosporia, consistent with a role for the exosporium in the modification of germinant molecules (Boydston *et al.*, 2006; Redmond *et al.*, 2004; Steichen *et al.*, 2007; Todd *et al.*, 2003). Arginase activity is also present in the *B. anthracis* exosporium, and plays a role in protection against oxidative bursts generated by macrophages, as already discussed (Weaver *et al.*, 2007).

In this work, we further characterize ExsFA/BxpB and show evidence to suggest that ExsFB and IunH do not entirely colocalize with ExsFA/BxpB. We show that ExsFA/BxpB affects spore surface morphology and germination, localizes to the exosporium basal layer, and plays a role in distributing BclA throughout the exosporium surface. Finally, we demonstrate that, in two animal challenge models, ExsFA/BxpB is dispensable for virulence.

METHODS

General methods. *Escherichia coli* strains were cultured in Luria-Bertani (LB) medium and antibiotics were used at standard concentrations (Sambrook *et al.*, 1989). For *B. anthracis*, erythromycin, tetracycline and kanamycin were used at concentrations of 5, 5 and 20 µg ml⁻¹, respectively. Sporangia and spores from the Sterne 34F2 strain and Sterne 34F2-derived strains of *B. anthracis* were prepared by exhaustion in Difco sporulation medium (DSM) (Lai *et al.*, 2003), unless stated otherwise. Sporulation at room temperature was performed using either 2 × SG medium (Harwood & Cutting, 1990) (for fluorescence microscopic analysis) or DSM [for immunofluorescence microscopy (IFM)], as indicated. We prepared Ames strain spores by culture in Leighton-Doi broth (Ivins *et al.*, 1990; Leighton & Doi, 1971). Time 0 (*t*₀) of sporulation was defined as the first point at which the culture leaves exponential-phase growth. We carried out further purification of Ames strain spores by centrifugation through Hypaque-76 gradients (Nycomed). All preparations possessed >95% spores. For germination and virulence studies, we diluted the spores in sterile water, heated them at 65 °C for 30 min, and then kept them on ice. Electron and atomic force microscopy (AFM) were carried out according to Catalano *et al.* (2001) and Chada *et al.* (2003), respectively.

Recombinant DNA methods. Recombinant DNA techniques were performed using standard methodologies (Sambrook *et al.*, 1989) or according to manufacturer's instructions. Strains and plasmids are referenced or described in Table 1. Primers are referenced in Supplementary Table S1. To construct plasmid pMR6, we first used PCR to amplify approximately 1 kb stretches of DNA on either side of *exsFA/bxpB*, using primers OL298 and OL299 (to amplify the upstream region), and primers OL300 and OL301 (to amplify the downstream region). The PCR products were cloned into plasmid pMR1, such that they flanked the plasmid-borne kanamycin-resistance marker. To do this, we digested the upstream PCR product and pMR1 with *SacI* and *NotI*, and ligated the products. We then digested the resulting plasmid and the downstream PCR product with *BamHI* and *SalI*, and ligated the resulting digestion products together. To generate strains bearing fusions of genes to *gfp*, we first built plasmids (pAS1 and pRG30) bearing *gfp*. To build pAS1, we PCR-amplified *gfp* from plasmid pKL147 (Lemon & Grossman, 1998) with primers OL471 and OL472, digested the PCR product and pUTE-29 with *BamHI* and *HindIII*, and ligated the resulting fragments. To build pRG30 we digested pAS1 with *HindIII* and *SmaI*, and ligated the *gfp*-bearing fragment into similarly digested pKS1. We then used pAS1 or pRG30 to build a set of plasmids bearing *gfp* fusions to different spore proteins, which were designed to integrate into the chromosome. To build plasmid pAS4, we first PCR-amplified the 3' end of *exsFA/bxpB* with primers OL504 and OL505. We digested the PCR product with *BamHI* and *EcoRV*, and pAS1 with *BamHI* and *PmeI*, and ligated these together. To build pAS2 we first amplified the 3' end of *iunH* with primers OL476 and OL498. We digested the PCR product with *BamHI* and *EcoRV*, and pAS1 with *BamHI* and *PmeI*, and ligated the digestion products. To build pRG25 we digested pAS4 with *BamHI* and *HindIII*, and ligated it to *BamHI*- and *HindIII*-digested plasmid pEO-3 (Mendelson *et al.*, 2004). To build pRG31 we first amplified the 3' end of *exsFB* with primers OL846 and OL847. We digested the PCR product with *NotI* and *EcoRV*, and pRG30 with *NotI* and *PmeI*, and ligated the digestion products.

To build plasmid pEO-3-*exsFA/BxpB*, we PCR-amplified an internal fragment of *exsFA/bxpB* corresponding to nucleotides 105–390, using primers *ExsF-KpnI* and *ExsF-HindIII*. We digested this product with *KpnI* and *HindIII*, and ligated it into similarly digested plasmid pEO-3.

Construction of *B. anthracis* mutant strains. We transformed the Sterne strain by electroporation (Koehler *et al.*, 1994), using one or

another of the plasmids described above. To build RG124 and RG130, we isolated strains bearing a chromosomally integrated copy of the plasmid by culturing transformants bearing plasmid pMR6 or pAS2 in LB medium for several days, diluting the culture 1:10 twice a day, and plating on LB agar supplemented with either kanamycin (to isolate the *exsFA/bxpB* single-integration mutant) or tetracycline (to isolate the *iunH-gfp*-bearing mutant, strain RG130). We then screened individual colonies for plasmid integration by PCR. To generate the *exsFA/bxpB* deletion strain, an additional reverse reciprocal excision had to take place. Therefore, we cultured a kanamycin- and tetracycline-resistant colony in LB medium over the course of several days, as described. We screened samples of the culture until we were able to isolate a kanamycin-resistant and tetracycline-sensitive colony, which became strain RG124. To build RG151, we introduced pRG25 into the Sterne strain and selected for chromosomally integrated plasmids by plating on solid medium with erythromycin, at the nonpermissive temperature as described previously (Mendelson *et al.*, 2004). To build RG155, we introduced pRG31 into the Sterne strain and selected for chromosomally integrated plasmids as described elsewhere (Shatalin & Neyfakh, 2005). To build a version of the Sterne strain in which *bclA* is inactivated (Sterne-JAB-13), we followed the approach of Bozue *et al.* (2007b). Each strain was confirmed by PCR analysis.

Phage transduction. To generate strains RG157, RG158 and RG169 we used the CP51ts generalized transducing phage (Thorne, 1968). Donor strains where grown overnight at 37 °C in LB medium and 100 µl aliquots of cell culture were infected with 100 µl of 10-fold serially diluted solutions of phage. Infected cells were then plated onto solid PA medium (Thorne, 1968) using the soft-agar overlay technique. After incubation at 30 °C in a humidity chamber for up to 2 days, plaques were collected and the cell debris was removed by centrifugation. The phage-containing supernatant was collected and filtered through either a 0.2 or a 0.45 µm pore-size filter and serially diluted as before. The recipient strain was grown overnight in brain-heart infusion (BHI) broth supplemented with 0.5% (v/v) glycerol, transferred into fresh medium, and grown for 6–7 h. Of this culture, 1 ml was infected with 100 µl of each phage dilution, incubated at room temperature without shaking for 30 min, and then in a roller drum for a further 2 h. Finally, 0.1–1.0 ml of infected cells was plated onto LB plates supplemented with tetracycline for strains RG157 and RG158, or kanamycin for strain RG169, and incubated at 37 °C (to cure the phage and allow for marker expression).

Fluorescence and IFM. We analysed Sterne strain spores and sporangia according to Pogliano *et al.* (1995) using a Leica DM IRB fluorescence microscope equipped with a MagnaFire cryo-cooled charge-coupled device (CCD) camera. Spores were water-washed prior to adherence to slides, and sporangia were treated with methanol at –20 °C for 5 min after adherence to access interior epitopes. Images were processed with Adobe Photoshop 7.0 software. To detect GFP fusion proteins, rabbit anti-GFP antibodies (Promega) were used at a 1:2000 dilution, and Alexa Fluor 568-conjugated goat anti-rabbit antibodies (Molecular Probes) were used at a 1:300 dilution. To detect BclA, we used the anti-BclA mAb BA-MAB 5 (Critical Reagents Program, Department of Defense) at a 1:80 000 dilution and Alexa Fluor 568-conjugated goat anti-mouse antibodies (Molecular Probes) at a 1:300 dilution. Chromosomes were visualized using the DNA-specific probe Hoechst 33342 (Sigma) as previously described (Catalano *et al.*, 2001). Data presented are representative of more than 150 spores observed.

Germination assays. The spectrofluorometric germination assay was done according to Welkos *et al.* (2004). The tetrazolium overlay assay was done according to Giorno *et al.* (2007). We monitored germination [using spores from three independent sporulations

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
Sterne strain and derivatives		
RG1	Sterne wild-type 34F2, attenuated	P. J. Jackson*
RG124	<i>exsFA/bxpB::km</i>	This study
RG130	<i>iunH</i> ΩpAS2	This study
RG151	<i>exsFA/bxpB</i> ΩpRG25	This study
RG155	<i>exsFB</i> ΩpRG31	This study
RG157	<i>cotE::km exsFA/bxpB</i> ΩpRG25	This study
RG158	<i>cotE::km exsFB</i> ΩpRG31	This study
RG169	<i>bclA::km exsFB</i> ΩpRG31	This study
Sterne-JAB-13	<i>bclA::km</i>	This study
Ames strain and derivatives		
Ames strain	Wild-type, fully virulent	Little & Knudson (1986)
Ames-JAB-5	<i>exsFA/bxpB</i> ΩpEO3- <i>exsFA/bxpB</i>	This study
<i>E. coli</i> strains		
DH5α	Cloning host	Laboratory collection
GM1684	<i>dam</i> used for transformation of Sterne strain	T. Koehler
GM2163	<i>dam dcm</i> used for transformation of Ames strain	New England Biolabs
Plasmids		
Bifunctional plasmids		
pAS1	Ap ^r in <i>E. coli</i> , Tc ^r in <i>B. anthracis</i> for construction of 3' <i>gfp</i> fusions	This study
pAS2	<i>iunH</i> - <i>gfp</i> fusion vector	This study
pAS4	<i>exsFA/bxpB</i> - <i>gfp</i> fusion vector	This study
pEO3	<i>E. coli</i> / <i>Bacillus</i> shuttle vector	Mendelson <i>et al.</i> (2004)
pEO3- <i>bxpB</i> / <i>exsF</i>	<i>exsFA/bxpB</i> inactivation vector	This study
pJRS182	Source of Ω <i>km</i> -2	Perez-Casal <i>et al.</i> (1991)
pKL147	Source of <i>gfp mut2</i>	Lemon & Grossman (1998)
pKS1	Temperature-sensitive vector in <i>B. anthracis</i>	Shatalin & Neyfakh (2005)
pMR1	Derivative of pUTE-29 containing Ω <i>km</i> -2 from pJRS182	Giorno <i>et al.</i> (2007)
pMR6	<i>exsFA/bxpB</i> deletion vector	This study
pRG25	<i>exsFA/bxpB</i> - <i>gfp</i> fusion vector	This study
pRG30	Derivative of pKS1 bearing <i>gfp</i>	This study
pRG31	<i>exsFB</i> - <i>gfp</i> fusion vector	This study
pUTE-29	Ap ^r in <i>E. coli</i> , Tc ^r in <i>B. anthracis</i>	Koehler <i>et al.</i> (1994)

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(prepared by culture in DSM)] via the loss of optical density assay according to Harwood & Cutting (1990), except that to initiate germination, spores were resuspended at OD₅₈₀ 0.1 at room temperature in RPMI–BHI medium (Steichen *et al.*, 2005).

Animal challenges. For intramuscular (IM) challenges, female Hartley Guinea pigs (350–400 g) (Charles River Laboratories) (*n*=10) were given intramuscular injections of 1000 spores, representing the equivalent of 10 Ames LD₅₀ (Ivins *et al.*, 1994). We performed whole-body aerosol challenges as described previously (Cown *et al.*, 1957; Friedlander *et al.*, 1993; May, 1973) with an inhaled dose representing approximately 5 Ames LD₅₀ and measured survival rates.

Statistical analysis. Survival rates and mean times to death were compared between each treatment group and control group by Fisher's exact test and Student's *t* test, respectively, with permutation adjustment for multiple comparisons. All analyses were conducted using SAS version 8.2 (SAS Institute, SAS OnlineDoc, version 8). Data collected from the loss of optical density germination assay were analysed by the Mann–Whitney U test (Mann & Whitney, 1947).

RESULTS

Characterization of *exsFA/bxpB* mutant spores

In the course of a proteomic analysis designed to discover spore coat proteins, we identified several previously described exosporium proteins (Lai *et al.*, 2003; Redmond *et al.*, 2004; Steichen *et al.*, 2003; Todd *et al.*, 2003). To better understand the role of one of these, ExsFA/BxpB, in spore function, we generated and characterized versions of the Ames and Sterne strains of *B. anthracis* in which *exsFA/bxpB* was inactivated (strains Ames-JAB-5 and RG124, respectively). Both strains grew and sporulated similarly to the corresponding wild-type strains. By thin-section electron microscopy (TEM), however, we found that the projections, or nap, extending from the exosporium were missing from spores of both mutant strains (data not shown), consistent with the

findings of Steichen *et al.* (2005). However, Sylvestre *et al.* (2005) found that *exsFA/bxpB* mutant spores had fewer projections.

We also characterized the mutant spore surface by AFM. This is a non-optical imaging technology that can produce high-resolution images of cell surfaces without any fixation or other preparation (Fotiadis *et al.*, 2002; Shao *et al.*, 1996; Wang *et al.*, 2007). We allowed Sterne strain or *exsFA/bxpB* mutant spores to air-dry and imaged at least 150 spores for each sample in the tapping mode (Chada *et al.*, 2003). Using AFM, the coat surface can be distinguished from the exosporium surface by distinctive ridges that run along the coat surface (Chada *et al.*, 2003; Plomp *et al.*, 2004). In *B. anthracis*, the ridges usually appear in closely apposed pairs that are relatively narrow, rounded and of about equal width (Fig. 1a) (Chada *et al.*, 2003; Giorno *et al.*, 2007). The surface of *exsFA/bxpB* mutant spores (from strain RG124) was distinct from that of wild-type, in that they lacked ridges and had a bumpy appearance (Fig. 1b, c). These results suggest that ExsFA/BxpB has a role in directing coat surface topography. Because we rarely observed exosporia on *exsFA/bxpB* mutant spores from strain RG124 we were unable to obtain good quality images of the mutant spore exosporium surface. Our inability to obtain images of the exosporium surface may be related to an earlier observation that the exosporium basal layer is unstable in *exsFA/bxpB* spores (Sylvestre *et al.*, 2005).

Earlier reports have shown that at least some BclA is spore-associated in *exsFA/bxpB* mutant spores (Steichen *et al.*,

2005; Sylvestre *et al.*, 2005). Therefore, BclA deposition can be divided into two phases: ExsFA/BxpB-dependent and ExsFA/BxpB-independent. To analyse the subcellular location of the ExsFA/BxpB-independent BclA during sporulation, we used IFM, with the anti-BclA mAb BA-MAB-5, to monitor BclA localization in wild-type and *exsFA/bxpB* mutant cells that had completed forespore engulfment (known as sporangia) in the Sterne strain background. To visualize the orientation of the forespore and mother cell compartments, we analysed fixed and Hoechst 33342-stained cells by bright-field and fluorescence microscopy simultaneously. In these experiments, the forespore appears as an oval-shaped structure and the mother cell chromosome as a bright focus (Fig. 2a, BF/DNA). We first analysed wild-type sporangia to determine when BclA would initially be detected by IFM. In this and subsequent IFM experiments, we analysed a minimum of 150 cells. After 4 h of sporulation, a time at which BclA is known to be expressed (Sylvestre *et al.*, 2002), we did not detect BclA in sporangia (data not shown). After 7 h, BclA was clearly localized around the forespore in wild-type sporangia (Fig. 2a). The same fluorescence pattern was seen in free spores (Fig. 2b). To confirm the specificity of the mAb, we also examined a *bclA* mutant strain (Sterne-JAB-13). Anti-BclA fluorescence was not detected in *bclA* mutant sporangia or spores (data not shown).

In *exsFA/bxpB* mutant sporangia after 7 h of sporulation, the BclA localization pattern suggested that at least some of the protein was located in the mother cell (Fig. 2a). After 8 h, BclA was localized exclusively around the forespore, in both wild-type and mutant strains. However, in the *exsFA/*

Fig. 1. Atomic force micrographs of Sterne strain spores of *B. anthracis*. (a) Wild-type Sterne and (b, c) RG124 (*exsFA/bxpB*) spores were analysed in the tapping mode. Bars, 1 μ m. In panels (a ii), (b ii) and (c ii), the white dotted lines indicate spore outlines and the white dashed lines indicate surface ridges.

Fig. 2. Fluorescence microscopic analysis of the role of ExsFA/BxpB in BclA deposition. (a) Sporangia from the Sterne strain (wild-type; WT) and strain RG124 (*exsFA/bxpB*) were harvested after 7 and 8 h of sporulation (*t*₇ and *t*₈, respectively), fixed and treated with the anti-BclA mAb BA-MAB5 (α-BclA). Bright-field microscopy and fluorescence microscopy (BF/DNA) were performed simultaneously so that the forespore and mother cell DNA (stained with Hoechst 33342 dye) could be visualized in a single image. The locations of individual cells are indicated with brackets. Mother cell chromosomes are indicated with arrowheads. The cell indicated by a dashed bracket (wild-type at *t*₈) did not sporulate, as indicated by the chromosome morphology. (b) Spores from the wild-type or RG124 (*exsFA/bxpB*) strains were harvested 24 h after the onset of sporulation and stained with the anti-BclA antibody BA-MAB-5. Corresponding bright-field (BF) images are shown for each immunohistograph (α-BclA). Data shown are representative of a minimum of 150 cells observed for each sample and time point. We judged the immunostaining to be positive if at least 90 % of the cells showed fluorescence. All negative control experiments showed fluorescence in fewer than 10 % of cells.

bxpB mutant sporangia, BclA decoration was more intense at the edge of the forespore closest to the midpoint of the sporangium (i.e. the mother-cell pole of the forespore) (Fig. 2a). We found that BclA also localized in a polar fashion in free spores from the *exsFA/bxpB* mutant (Fig. 2b). This region of BclA decoration appears to coincide with the portion of the exosporium that forms first in sporulation, called the cap (Giorno *et al.*, 2007; Ohye & Murrell, 1973; Steichen *et al.*, 2007). These unexpected results indicate a previously unrecognized polarity to BclA localization.

Our TEM analysis indicated that released *exsFA/bxpB* mutant spores do not possess projections (or nap) (data not shown), consistent with earlier results (Steichen *et al.*, 2005). Nonetheless, as just discussed, at least some BclA is

forespore-localized in *exsFA/bxpB* mutant sporangia, as shown by IFM (Fig. 2a). The presence of forespore-associated BclA in mutant cells raised the possibility that projections were also present (presumably, if this were true, the projections are lost during or just before spore release from the mother cell). To test this, we used TEM to determine whether projections could be detected in *exsFA/bxpB* mutant sporangia after 6 and 8 h of sporulation, when the projections are readily visible in wild-type cells (Giorno *et al.*, 2007; Steichen *et al.*, 2007). We did not detect projections in *exsFA/bxpB* mutant cells (data not shown). Therefore either *exsFA/bxpB* mutant cells do not assemble projections, even transiently, or they are unstable. Overall, these data are consistent with the view that, while some BclA localizes in an ExsFA/BxpB-independent manner, this deposition is insufficient to maintain either a uniform shell of BclA or the formation of recognizable projections.

Role of ExsFA/BxpB in spore functions

The exosporium could have a role in germination or outgrowth (Redmond *et al.*, 2004; Steichen *et al.*, 2005). Therefore, we analysed the ability of *exsFA/bxpB* mutant spores to germinate and resume metabolism (known as outgrowth). We analysed early events in germination by measuring the rate and level of incorporation of the fluorescent dye Syto-9 (Welkos *et al.*, 2004). We found that the initial dye uptake kinetics of the *exsFA/bxpB* spores from either the Sterne or the Ames background were identical to those of wild-type spores (Fig. 3a). However, after 4 min, the rate of dye incorporation from the *exsFA/bxpB* spores was reduced, and after 60 min, the *exsFA/bxpB* spores had not incorporated as much dye as the wild-type spores (Fig. 3a). This effect appeared to be more severe in the Ames background. We do not know the significance of this difference between the two strain backgrounds. The effect of the *exsFA/bxpB* mutation on dye uptake is not due solely to the reduction in spore-associated BclA, since Ames spores entirely missing BclA (due to a *bclA* mutation) take up dye as well as wild-type spores (Bozue *et al.*, 2007a). In previous work, germination of *exsFA/bxpB* mutant spores was measured using RPMI–BHI medium as a germinant (Steichen *et al.*, 2005). Therefore, we also monitored germination by measuring the loss of optical density (Harwood & Cutting, 1990) using this germinant. After 5 min, the percentage loss of optical density of by *exsFA/bxpB* mutant spores (in the Sterne background, strain RG124) was lower than in wild-type, and remained so throughout the experiment (Fig. 3b). Although modest, the difference between wild-type and mutant spores in this assay was significant as determined by the Mann–Whitney U test ($P < 0.01$). Taken together, these results suggest that *exsFA/bxpB* spores have a defect in an early step of germination.

To determine whether an *exsFA/bxpB* mutation affects late events in germination and/or the resumption of metabol-

Fig. 3. Germination of wild-type (WT) and mutant spores. (a) Spores from wild-type Ames (■), wild-type Sterne (▲), Ames-JAB-5 (□) and RG124 (△) strains were analysed by the fluorescence dye uptake assay. The percentage increase in fluorescence is the difference between the fluorescence in relative fluorescence units (RFU) at a given time in germination medium and the RFU at t_0 , expressed as a percentage of the latter. (b) Spores from wild-type Sterne (▲) and RG124 (△) were monitored over time for the loss of refractility by measuring the reduction in optical density. Both assays were done in triplicate. The data shown in (a) are representative of three independent experiments, and SEM values are shown in (b).

ism (outgrowth), we used a modified version of the tetrazolium overlay assay (Harwood & Cutting, 1990; Giorno *et al.*, 2007). Spores from *exsFA/bxpB* mutant strains, in either the Sterne or the Ames background (in RG124 and Ames-JAB-5, respectively), showed wild-type levels of outgrowth by this assay (data not shown).

To detect a role for ExsFA/BxpB in infection, we measured the ability of *exsFA/bxpB* mutant spores (from Ames-JAB-5) to infect Guinea pigs by both IM and inhalational challenges. The mutant strain was fully virulent in both experiments (Fig. 4). There were no significant differences in mean time to death between strains by either the IM route ($P=0.2878$) or the aerosol route ($P=0.5062$) as measured by Student's *t* test, nor were there any significant differences in survival rates between strains after infection by the aerosol route ($P=0.2105$), as measured by Fisher's

Fig. 4. Effect of *exsFA/bxpB* on virulence. Spores from the wild-type (WT) Ames strain (■) or Ames-JAB-5 (□) were used in (a) Guinea pig IM or (b) Guinea pig inhalational challenge assays. Numbers of surviving animals are plotted.

exact test. There were no survivors in the IM-exposed group. These results are consistent with previous results (Bozue *et al.*, 2007a; Sylvestre *et al.*, 2002). Our results indicate that neither the germination defect nor the changes in spore composition and morphology of the *exsFA/bxpB* Ames mutant detectably reduce disease using these two routes of infection.

Subcellular localization and assembly of ExsFA/BxpB and ExsFB

To better understand the roles of ExsFA/BxpB and ExsFB in spore assembly and function, we examined their locations in living cells during sporulation. To do this, we built strains (in the Sterne background) bearing fusions of each protein to GFP, and examined them by fluorescence microscopy. Cells from strains bearing ExsFA/BxpB-GFP (RG151) and ExsFB-GFP (RG155) grew and sporulated normally, as judged by light microscopy (data not shown). We noted, however, that TEM showed that *exsFA/bxpB-gfp*-bearing spores lacked projections, suggesting a role for the ExsFA/BxpB C terminus in projection assembly (data not shown). Since *exsFB* null mutant spores have not been shown to have strong phenotypes (Sylvestre *et al.*, 2005), we did not further characterize ExsFB-GFP-bearing cells by TEM.

We did not detect fluorescence in cells from strain RG151 (bearing the ExsFA/BxpB-GFP fusion) at 3 h after the onset of sporulation (Fig. 5a). At 5 h, we found that fluorescence was dispersed throughout the mother cell and excluded from the forespore (data not shown). After 6 h, we detected fluorescence in both a ring around the forespore as well as dispersed throughout the mother cell (Fig. 5a). Released spores bearing ExsFA/BxpB-GFP (from strain RG151) showed rings of fluorescence that surrounded the spores and were more intense at the caps (Fig. 5a, bottom). In about 60 % of spores, we saw that one cap was brighter than the other.

To detect fluorescence due to ExsFB-GFP, we sporulated cells bearing this fusion (strain RG155) at room temperature using $2\times$ SG medium, as we did not detect fluorescence during culture at 37 °C in DSM (data not shown). Culturing cells at a decreased temperature has been shown to facilitate detection of certain fusions in both in *B. subtilis* and *B. anthracis* (Kim *et al.*, 2006; Mallozzi *et al.*, 2008; van Ooij *et al.*, 2004). Similarly to the case of ExsFA/BxpB-GFP, we first detected fluorescence in ExsFB-GFP-bearing cells (RG155) as a dispersed signal in the mother cell, beginning at stage IV of sporulation (where the stage of sporulation was determined by visualization of

Fig. 5. Fluorescence microscopic analysis of ExsFA/BxpB-GFP, ExsFB-GFP and IunH-GFP. (a) Fluorescence microscopic localization of fusion proteins during sporulation. Cells from strains RG1 (wild-type; WT), RG151 (*exsFA/bxpB-gfp*) and RG130 (*iunH-gfp*) were cultured in DSM broth at 37 °C and harvested at 0 (t_0), 3 (t_3), 6 (t_6) and 24 h (Spores) after the onset of sporulation. Cells from strain RG155 (*exsFB-gfp*) were cultured in $2\times$ SG broth at 25 °C, harvested at various times and staged by analysis of chromosome morphology. DNA staining (DNA) and GFP epifluorescence (GFP) are shown for each time point, and DNA staining was used to determine whether cells had transitioned to sporulation [t_0 or stage 0 (0)] or progressed to stage III (III) or stage IV (IV). Brackets indicate individual sporangia. GFP epifluorescence (GFP) and bright-field (BF) images of released spores are also shown for all strains. (b) Larger fields showing fluorescence microscopic localization of ExsFB-GFP and IunH-GFP. Additional examples of the solid oval fluorescence pattern of these two fusion proteins, without corresponding bright-field images, for spores of strain RG155 (*exsFB-gfp*) and RG130 (*iunH-gfp*) are shown. (c) Immunofluorescence detection of fusion proteins in released spores from strains RG151 (*exsFA/bxpB-gfp*), RG155 (*exsFB-gfp*), RG157 (*cotE exsFA/bxpB-gfp*) and RG169 (*bclA exsFB-gfp*). Data shown are representative of a minimum of 150 cells observed for each sample and time point. We judged the immunostaining to be positive if at least 90 % of the cells showed fluorescence. All negative control experiments showed fluorescence in less than 10 % of cells. Spores from strains RG155 and RG169 were cultured in DSM at 25 °C. Bright-field (BF) and anti-GFP immunofluorescence (α -GFP) are shown for each. Images were not acquired (ND) for RG130 (*iunH-gfp*) after 3 h of sporulation.

chromosome morphology using a DNA stain, and by phase-contrast microscopy) (Fig. 5a and data not shown). We noted that fluorescence was excluded from the forespore during stage IV of sporulation, which is the time when the forespore is no longer metabolically active (Losick *et al.*, 1986). In contrast, released spores bearing the ExsFB-GFP fusion (from strain RG155) had either a solid oval of fluorescence or, occasionally, rings (in 17 of 380 spores observed) of fluorescence that surrounded the spores (Fig. 5a, b, and data not shown). This is in striking contrast to what we and others have seen with fusions of coat protein genes to GFP, in both *B. anthracis* and *B. subtilis*, where the patterns of localization are usually rings, and sometimes caps or other structures that suggest incomplete rings, but never solid ovals (Bauer *et al.*, 1999; Eichenberger *et al.*, 2003; Kim *et al.*, 2006; Mallozzi *et al.*, 2008; McPherson *et al.*, 2005; van Ooij *et al.*, 2004). We note that the timing of the appearance of ExsFB-GFP fluorescence, just described, is inconsistent with the possibility that ExsFB-GFP is synthesized in the spore core. The view that ExsFB is synthesized in the mother cell is further supported by the presence of a candidate σ^K -driven promoter upstream of *exsFB* (Steichen *et al.*, 2005). Therefore, although ExsFB-GFP fluorescence appears as a solid oval, it is highly unlikely that this fluorescence emanates from the spore interior.

To ensure that the fluorescence patterns of ExsFA/BxpB-GFP and ExsFB-GFP are truly distinct, we reexamined the more intense ExsFA/BxpB-GFP signal with the camera sensitivity reduced. The signal remained a ring at all intensities. Likewise, increasing the camera sensitivity did not result in rings of ExsFB-GFP fluorescence. Therefore, the fluorescence patterns of ExsFA/BxpB-GFP and ExsFB-GFP are distinct.

To directly compare the timing of assembly of the two fusion proteins, in a separate experiment we also carried out sporulation of the ExsFA/BxpB-GFP-bearing strain (RG151) and the ExsFB-GFP-bearing strain (RG155) at room temperature (in $2 \times$ SG medium). Sporulation of RG151 cells at room temperature did not detectably alter the fluorescence pattern of ExsFA/BxpB-GFP, which formed a ring, as it did during sporulation at 37 °C. From this analysis, we found that fluorescence due to ExsFB-GFP appeared several hours later than fluorescence due to ExsFA/BxpB-GFP (data not shown). Given that sporulation is relatively slow at this temperature, the biological significance of this difference in timing is unclear.

To learn whether the unusual solid oval localization pattern of ExsFB-GFP might be a property of other exosporium-associated proteins, we also analysed the localization of a fusion of GFP to the putative nucleoside hydrolase, IunH (encoded by a gene currently annotated as BA2888), which has also been shown to fractionate with the exosporium (Redmond *et al.*, 2004; Todd *et al.*, 2003) and which we also identified in a previous proteomic study as a spore-associated protein (Lai *et al.*, 2003). We found that the pattern of fluorescence in spores bearing *iunH-gfp* (strain RG130) was very similar to that of the *exsFB-gfp*-bearing spores from strain RG155 (Fig. 5a, b), although in about 3 % of spores the fluorescence of the solid oval was much brighter than the rest (6 out of 200 spores observed) (Fig. 5a, b, and data not shown).

To interpret the solid oval patterns of ExsFB-GFP and IunH-GFP fluorescence in terms of subcellular localization of the respective proteins, we first considered the possibility that the ring-like appearance of typical spore protein-GFP fusions could be ascribed to the geometry of the microscope set-up. Because the 2D image (a ring) captured by the camera is actually due to a projection of a 3D image (a shell), the edges of the image show a greater amount of image intensity, per pixel, than does the interior. Therefore, a possible explanation for the disk of fluorescence would be that, rather than being confined to a thin shell, ExsFB-GFP and IunH-GFP form a very thick layer, as they might if they resided in the interspace. If this were true, then a significant fraction (if not all) of these fusion proteins would be hidden underneath the exosporium. Further support for this interpretation comes from the observation that GFP fluorescence is excluded from the forespore in stage IV *exsFB-gfp*-bearing cells (Fig. 5a). These data indicate that ExsFB-GFP is not in the forespore prior to

stage IV, when forespore metabolism has already ceased (Losick *et al.*, 1986). Therefore, it is extremely unlikely that the forespore-associated GFP in these cells is due to ExsFB-GFP in the core. Much more likely, ExsFB-GFP surrounds the core.

To clarify the locations of ExsFA/BxpB-GFP and ExsFB-GFP, we first determined whether they could be detected on the spore surface, using IFM. We mixed anti-GFP antibodies with spores from either strain and visualized the resulting complexes using fluorescence microscopy. ExsFA/BxpB-GFP-bearing spores reacted with anti-GFP antibodies, as expected from the known basal-layer location of ExsFA/BxpB (Steichen *et al.*, 2005) and the lack of projections, due to the *exsFA/bxpB-gfp* allele, see above (Fig. 5c) (Steichen *et al.*, 2005). In contrast, anti-GFP antibodies did not react with ExsFB-GFP-bearing spores (from strain RG155, Fig. 5c).

To investigate the locations of these proteins further, we applied anti-GFP antibodies to spores bearing either fusion, but in which the exosporium was also incompletely assembled, due to a mutation in *cotE* (from strains RG157 and RG158). As in all our IFM analyses, we examined a minimum of 150 cells. In separate work, we have shown that deletion of *cotE* results in spores that lack the exosporium (Giorno *et al.*, 2007). However, those experiments do not reveal whether CotE controls interspace formation. Spores bearing ExsFA/BxpB-GFP and a mutation in *cotE* (from strain RG157) did not react with anti-GFP antibodies (and did not show fluorescence due to GFP) (Fig. 5c), as we expected. Interestingly, we also found that we did not detect ExsFB-GFP in spores of a strain also bearing the *cotE* mutation (in strain RG158), by application of either anti-GFP antibodies or, more importantly, GFP fluorescence (data not shown). Therefore, detection of ExsFB-GFP is CotE-dependent, consistent with a location for the fusion in the interspace, the exosporium, or both.

These data above do not exclude the possibility that ExsFB also is present within the exosporium basal layer. If it were, localization by IFM might be inhibited by the presence of the BclA projections. To address this, we analysed *bclA exsFB-gfp* spores (from strain RG169) by IFM. We detected fluorescence around the spore (Fig. 5c). The pattern of immunofluorescence from strain RG169 spores was a discontinuous ring, in contrast to the continuous ring of ExsFA-GFP immunofluorescence (in strain RG151). We interpret these data to suggest that ExsFB is present in both the basal layer and the interspace.

DISCUSSION

An important question in *B. anthracis* spore assembly is the timing and control of BclA deposition. Earlier investigations have shown that while most BclA assembly requires ExsFA/BxpB, a significant fraction of BclA assembles in an ExsFA/BxpB-independent manner, and that ExsFA/BxpB-dependent BclA assembly is needed for formation of the

projections (Steichen *et al.*, 2005; Sylvestre *et al.*, 2005). We also found this and, additionally, we showed that BclA accumulates at the mother cell pole in *exsFA/bxpB* forespores. Therefore, the fraction of BclA that localizes independently of ExsFA/BxpB appears on the mother-cell-proximal portion of the exosporium that forms early in sporulation, and under the direction of CotE (Giorno *et al.*, 2007; Ohye & Murrell, 1973). This is likely the first portion of the exosporium to be assembled (Giorno *et al.*, 2007). Interestingly, this region of the exosporium is biochemically distinct from the rest of the exosporium, as it lacks alanine racemase and its assembly is ExsY-independent (Steichen *et al.*, 2007). Taken together, these data appear to suggest that exosporium assembly is a two-step process. In the first step, CotE directs deposition of a biochemically specialized patch of the basal layer onto the forespore pole closest to the mother cell midpoint. In the second step, ExsY directs the remainder of the polymerization of the sheet of exosporium, an event that terminates when the exosporium shell closes on itself (Steichen *et al.*, 2007). The factor(s) directing assembly of ExsFA/BxpB-independent BclA remains unknown. However, ExsFB is a plausible candidate, since *exsFA/bxpB exsFB* double mutant spores assemble less BclA than *exsFA/bxpB* mutant spores (Sylvestre *et al.*, 2005).

We detected a modest germination defect in *exsFA/bxpB* mutant spores by multiple assays, indicating at least one role for this protein beyond mediating BclA deposition. The finding of a germination defect is in contrast to an earlier finding showing that both germination and outgrowth are accelerated in mutant spores (Steichen *et al.*, 2005). We do not know the basis for this discrepancy, but it is plausibly due to differences in the physical set-up of our experimental systems. We note that while similar media were used to measure germination in one case, Steichen *et al.* (2005) used a flow chamber system, while we used assays in which germinant was not replenished during the experiment (Harwood & Cutting, 1990). Possibly, the constant flow of nutrient has a marked effect on germination in the *exsFA/bxpB* mutant that cannot be observed when the germinant is given in a single dose.

We found that ExsFA/BxpB is not needed for the typical presentation of *B. anthracis* infection in two models. This is consistent with previous findings demonstrating that even without BclA, spores from either the attenuated Sterne or the virulent Ames backgrounds can still cause infection (Bozue *et al.*, 2007a; Brahmabhatt *et al.*, 2007; Sylvestre *et al.*, 2002). Importantly, it extends those results by demonstrating the dispensability of the projections (or nap) in an entirely separate manner, and using an additional route of infection (the Guinea pig inhalational challenge). These data also are consistent with our previous finding that spores lacking the exosporium, due to a mutation in the coat protein gene *cotE*, are fully virulent in two animal models (Giorno *et al.*, 2007). These experiments raise an important caution; while defences and therapeutics targeting the exosporium will be very useful,

we should not rely on them exclusively, since this structure may be dispensable (Giorno *et al.*, 2007). Rather, we must prepare for events using spores both with and without projections.

Although we did not detect an effect of ExsFA/BxpB on disease, the exosporium may still have a role in interactions with the host. For example, it has been shown recently that the exosporium provides a degree of protection against macrophages (Weaver *et al.*, 2007). Also, the presence of BclA may be necessary for targeting spores to macrophages, as it decreases spore adherence to epithelial, fibroblast and endothelial cells (Bozue *et al.*, 2007b). Therefore, at least under certain circumstances, the exosporium could affect the natural disease process in ways not detected in our assays. Exosporium proteins or the genes encoding these proteins may also be useful as subunits in an improved anthrax vaccine, as suggested by several studies (Brossier *et al.*, 2002; Cybulski *et al.*, 2008; Enkhtuya *et al.*, 2006; Hahn *et al.*, 2006). Therefore, interactions between the host and exosporium proteins are likely to be important to future therapeutics.

If the projections are not always needed for disease, then what is their function? It is plausible that the projections have roles in resistance or attachment, in the soil or some other ecological niche. *B. anthracis* maintains a highly complex series of spore protective structures, apparently largely dedicated to survival outside the host, in spite of its adaptation to animal hosts. It may be challenging to identify these additional roles. An example of an organism that likely uses BclA for a purpose other than disease is the non-pathogenic organism *Bacillus licheniformis*, a close relative of *B. subtilis* that does not have an exosporium but which possesses an orthologue of BclA (M. Mallozzi & A. Driks, unpublished results). Preliminary phylogenetic analysis suggests that BclA was, in this case, acquired by horizontal gene transfer (H. Qin & A. Driks, unpublished results).

Most coat and exosporium genes do not have paralogues. *exsFA/bxpB* and *exsFB* are exceptions. Making this set of genes even more unusual, some *B. cereus*-group strains possess a third paralogue that differs from *exsFA/bxpB* and *exsFB* at least as much as those two genes differ from each other. *B. anthracis* has only two paralogues, as do 19 other strains, three strains have only one paralogue, and *B. cereus* E33L contains three paralogues. Interestingly, in *B. cereus* E33L two of the paralogues are on the chromosome and one is plasmid-encoded. Possibly, these variations result in adaptive changes in exosporium structure and function.

Our data argue that ExsFB-GFP and IunH-GFP do not precisely colocalize with the exosporium basal layer. We suggest the possibility that ExsFB and IunH reside in the interspace. At present, this localization is not definitive. Future experiments to support these data and to uncover additional possible interspace proteins may help clarify the function of this poorly understood spore compartment.

ACKNOWLEDGEMENTS

The authors thank Kathy Kuehl [Pathology Department, United States Army Medical Research Institute of Infectious Diseases (USAMRIID)] for preparation of electron microscope samples and Sarah Norris (USAMRIID) for statistical analysis. We thank Terri Koehler (University of Texas, Houston) for the kind gift of CP-51, plasmids and strains, and for valuable advice. We thank Peter McKenney and Patrick Eichenberger for important input into this study. Funding was provided by the NIH (AI53365, A.D.) and the US Army Medical Research and Materiel Command under Projects 02-4-5C-018 (J.B.), 02-4-5C-023 (S.W.) and 04-0-IL-002 (S.W.) and an In-House Laboratory Innovative Research Award from the Department of the Army under Project 92489 (J.B.). Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources *et al.*, 1996). The facility in which this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the US Army.

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Edited by: M. Hecker