

Processing, assembly and localization of a *Bacillus anthracis* spore protein

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All *Bacillus* spores are encased in macromolecular shells. One of these is a proteinaceous shell called the coat that, in *Bacillus subtilis*, provides critical protective functions. The *Bacillus anthracis* spore is the infectious particle for the disease anthrax. Therefore, the coat is of particular interest because it may provide essential protective functions required for the appearance of anthrax. Here, we analyse a protein component of the spore outer layers that was previously designated BxpA. Our data indicate that a significant amount of BxpA is located below the spore coat and associated with the cortex. By SDS-PAGE, BxpA migrates as a 9 kDa species when extracted from Sterne strain spores, and as 11 and 14 kDa species from Ames strain spores, even though it has predicted masses of 27 and 29 kDa, respectively, in these two strains. We investigated the possibility that BxpA is subject to post-translational processing as previously suggested. In *B. subtilis*, a subset of coat proteins is proteolysed or cross-linked by the spore proteins YabG or Tgl, respectively. To investigate the possibility that similar processing occurs in *B. anthracis*, we generated mutations in the *yabG* or *tgl* genes in the Sterne and Ames strains and analysed the consequences for BxpA assembly by SDS-PAGE. We found that in a *tgl* mutant of *B. anthracis*, the apparent mass of BxpA increased. This is consistent with the possibility that Tgl directs the cross-linking of BxpA into a form that normally does not enter the gel. Unexpectedly, the apparent mass of BxpA also increased in a *yabG* mutant, suggesting a relatively complex role for proteolysis in spore protein maturation in *B. anthracis*. These data reveal a previously unobserved event in spore protein maturation in *B. anthracis*. We speculate that proteolysis and cross-linking are ubiquitous spore assembly mechanisms throughout the genus *Bacillus*.

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INTRODUCTION

In response to nutrient depletion and, possibly, other environmental stresses, the aetiological agent of anthrax, *Bacillus anthracis*, forms highly resistant spores. The spore is the infectious particle of disease (Friedlander, 2000). The outer layers of the spore define the spore-surface topography and are responsible for important properties,

including resistance to host defences and adhesion (Bozue *et al.*, 2007a, b; Giorno *et al.*, 2007; Kang *et al.*, 2005). A better understanding of the formation of these structures may identify new targets for vaccines, detection and prophylaxis, and drive the improvement of decontamination methods.

All *Bacillus* spores are built as a set of concentric shells. The innermost spore compartment is the core, which houses the chromosome. The cortex is a thick layer of peptidoglycan that surrounds the core (Popham, 2002). The cortex, in turn, is surrounded by the coat, a proteinaceous layer with important roles in protection (Driks, 2002, 2003; Henriques & Moran, 2007; Riesenman & Nicholson, 2000; Setlow, 2006). In some species, including *B. anthracis*, there

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Abbreviations: IEM, immuno-electron microscopy; TEM, transmission electron microscopy.

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

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14. ABSTRACT All Bacillus spores are encased in macromolecular shells. One of these is a proteinacious shell called the coat that, in Bacillus subtilis, provides essential protective functions. In Bacillus anthracis, the spore is the infectious particle for disease. Therefore, the coat is of particular interest because it may provide essential protective functions required for the appearance of anthrax. In our study, we identified a protein component of the spore outer layers that was previously designated as BxpA. By SDS-PAGE, BxpA migrates as a 9-kD species in Sterne strains spores, and as 11- and 14-kD species in Ames strain spores, even though it has predicted masses of 35 kD and 38 kD, respectively. We investigated the possibility that BxpA is subject to posttranslational processing. In B. subtilis, a subset of coat proteins is proteolyzed or crosslinked by the spore proteins YabG or Tgl, respectively. To investigate the possibility that similar processing occurs in B. anthracis, we generated mutations in the yabG or tgl genes in the Sterne and Ames strains, and analyzed the effects on BxpA migration by SDS-PAGE. As expected from the functions of YabG and Tgl in B. subtilis, we found that in yabG or tgl mutants of B. anthracis, the apparent mass of BxpA increased. These data reveal a previously unobserved event in spore protein maturation in B. anthracis. We speculate that proteolysis and crosslinking are ubiquitous events in Bacillus spore assembly.			
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is an additional layer that envelopes the coat, called the exosporium. In the case of *B. anthracis* (but not, for example, *Bacillus megaterium*), a series of fine hair-like projections, also called a nap, extends from the exosporium (Aronson & Fitz-James, 1968; Gerhardt & Ribí, 1964; Gerhardt, 1967; Vary, 1994).

Spore coat assembly in *Bacillus subtilis* has been well studied as a basic model for protein assembly (Aronson & Fitz-James, 1976; Driks, 1999, 2002, 2003; Henriques & Moran, 2000). Because many coat proteins are conserved between *B. subtilis* and *B. anthracis*, including those most critical to assembly in *B. subtilis*, it is likely that there is substantial overlap in the mechanisms controlling coat assembly in the two species (Driks, 2002; Henriques & Moran, 2007). Recent studies have indicated that *B. anthracis* homologues of these critical *B. subtilis* coat proteins do play important roles in *B. anthracis* coat assembly, albeit roles that differ from those of their *B. subtilis* orthologues (Giorno *et al.*, 2007). Studies in *B. anthracis*, as well as in *Bacillus cereus*, have further shown that a number of spore proteins not present in *B. subtilis* are also important to coat and exosporium formation in those species (Boydston *et al.*, 2006; Johnson *et al.*, 2006; Steichen *et al.*, 2005; Sylvestre *et al.*, 2005).

A still poorly understood aspect of coat assembly in *B. subtilis* is coat protein processing. Several *B. subtilis* coat proteins are cleaved during or after assembly (Kuwana *et al.*, 2006, 2007; Takamatsu *et al.*, 2000). At least some of this proteolytic processing is under the control of YabG (Kuwana *et al.*, 2007; Ozin *et al.*, 2000; Takamatsu *et al.*, 2000). Cleavage is not the only type of coat protein modification; strong evidence for cross-linking has been provided by the observation that about 30% of the *B. subtilis* coat is in a form that cannot enter a conventional SDS-PAGE gel (Goldman & Tipper, 1978; Pandey & Aronson, 1979). More recently, it has been shown that at least some coat protein cross-linking is controlled by the coat-associated transglutaminase Tgl (Kobayashi *et al.*, 1998; Monroe & Setlow, 2006; Zilhao *et al.*, 2005).

Given the similarities between *B. anthracis* and *B. subtilis* spore function and assembly and the presence of *tgl* (ba4173) and *yabG* (ba0040) orthologues in the *B. anthracis* genome (Read *et al.*, 2003), it is highly likely that proteolysis and cross-linking are also steps in spore maturation in *B. anthracis*. In this study, we examined the assembly and processing of the *B. anthracis* spore protein BxpA, previously identified as an exosporium component (Liu *et al.*, 2004; Steichen *et al.*, 2003). Through a gene transcription study, *bxpA* has been assigned to a cluster of genes (Wave 5) that are expressed during sporulation (Bergman *et al.*, 2006). Our data indicate that a significant amount of BxpA is located below the spore coat, associated with the cortex, and is modified in a manner that depends on YabG and Tgl. These results provide support for the view that proteolysis and cross-linking of spore proteins is widespread among the bacilli.

METHODS

Growth conditions and maintenance of plasmids. *B. anthracis* and *Escherichia coli* strains and plasmids used in this study are listed in Table 1. *E. coli* and *B. anthracis* were grown in Luria-Bertani (LB) broth or on LB agar. Spores of *B. anthracis* were prepared in Difco Sporulation medium (Schaeffer *et al.*, 1965). Spores were purified by washing three times with cold sterile water, passage through a density gradient (Hypaque-76, GE Healthcare), and washing three additional times with water (Bozue *et al.*, 2005).

For maintenance of plasmids and selection in *B. anthracis*, kanamycin and erythromycin were used at 20 and 5 $\mu\text{g ml}^{-1}$, respectively. For *E. coli*, ampicillin and kanamycin were used at 100 and 50 $\mu\text{g ml}^{-1}$, respectively. Plasmid DNA was isolated from *E. coli* strains using the Promega Wizard Miniprep kit.

Mutant construction. To construct *B. anthracis* mutants, genes of interest were PCR-amplified using Phusion polymerase (NEB) from purified Ames chromosomal DNA with the primers listed in Supplementary Table S1. To construct *bxpA* mutants, a PCR fragment containing *bxpA* was cloned into the *Bacillus-E. coli* shuttle vector pEO3 (Bozue *et al.*, 2005; Mendelson *et al.*, 2004). To inactivate *bxpA* in the Ames (*bxpA*^A) and Sterne (*bxpA*^S) strains of *B. anthracis*, the Ω Kan-2 cassette (Perez-Casal *et al.*, 1991) was cloned into the *PshAI* site within a fragment of DNA containing the *bxpA* gene from the Ames strain. The *PshAI* site is located 212 bp downstream of the *bxpA* ATG start site. Transformation via electroporation of *B. anthracis* and co-integration of the *bxpA::\Omega* Kan-2 plasmid were performed as previously described after passing plasmids through *dam dcm E. coli* strain GM2163 (Bozue *et al.*, 2005). Allelic exchange mutants were selected as previously described (Cote *et al.*, 2008). Mutants were confirmed by PCR analysis using the appropriate primers (Supplementary Table S1) and *Taq* polymerase from Qiagen. The Ames strain mutant in *bxpA* was complemented by transformation with the shuttle plasmid pHT315 (Arantes & Lereclus, 1991) containing intact *bxpA* alleles along with flanking upstream and downstream sequence. The primers used for constructing the complementation plasmids are listed in Supplementary Table S1.

Mutants of *yabG* and *tgl* in *B. anthracis* were created by cloning an internal fragment of the respective genes (generated by PCR) into plasmid pEO3. Gene inactivation in *B. anthracis* took place by a Campbell-type insertion of the appropriate plasmid into the chromosome as previously described (Giorno *et al.*, 2007). Double mutants were constructed by introducing the *bxpA::\Omega* Kan-2 mutation into the appropriate backgrounds by phage transduction, using the CP51 phage as described by Thorne (1968). All mutations were confirmed by PCR analysis (Supplementary Table S1).

Protein extraction and analysis. Spore protein extracts (containing largely coat and exosporium proteins) of *B. anthracis* were prepared and fractionated as previously described (Giorno *et al.*, 2007; Little & Driks, 2001), except that 12% Bis-Tris gels (Invitrogen) were used and stained using the Gel Code Blue kit (Pierce). Where indicated, Complete Protease Inhibitor cocktail (Roche) was used.

Vegetative cell extracts were prepared by removing 10 ml samples from mid-exponential phase cultures of wild-type Ames and Sterne strain spores, and isogenic *bxpA* mutants. The cultures were centrifuged, the supernatant fluids discarded, and the pellets resuspended in 1 ml ice-cold water. The pellets were homogenized in Blue matrix tubes (MP Bio) with a FastPrep (MP Bio) instrument. The cleared cultures were fractionated and stained as described above.

For Western analysis, fractionated proteins were transferred onto a PVDF membrane overnight at 4 °C in 1× NuPAGE transfer buffer (Invitrogen), 0.35% SDS and 20% methanol. After transfer, the membranes were blocked with 10% skimmed milk in Tris-buffered

Table 1. Strains and plasmids

Plasmid or strain	Genotype or description	Reference or source
Plasmids		
pEO3	Erm ^r /Amp ^r , temperature-sensitive shuttle vector	Mendelson <i>et al.</i> (2004)
pJRS102.0	Source of Ω Kan-2 cassette	Perez-Casal <i>et al.</i> (1991)
pEO3- <i>bxpA</i> :: Ω kan	<i>bxpA</i> :: Ω kan	This study
pHT315	Erm ^r /Amp ^r , shuttle vector	Arantes & Lereclus (1991)
pEO3- <i>yabG</i>	pEO3 containing an internal fragment of <i>yabG</i>	This study
pEO3- <i>tgl</i>	pEO3 containing an internal fragment of <i>tgl</i>	This study
pET23A	Protein expression vector	EMD Biosciences
pET23A-BxpA ^A	Expression vector for <i>bxpA</i> ^A	This study
pKH-KSM4	pHT315 Δ <i>lacZ</i>	This study
pKSM5	pKH-KSM4 + MCS of pET23A	This study
pKSM6	pKSM5 + <i>bxpA</i> region	This study
pKSM7	pKSM6 with 133R→A	This study
<i>E. coli</i> strains		
GM2163	<i>dam</i> ⁻ <i>dcm</i> ⁻	NEB
NEB 10-Beta	Cloning	NEB
BL21 DE3	Expressing rBxpA ^A	EMD Biosciences
<i>B. anthracis</i> strains		
Ames	pXO1 ⁺ pXO2 ⁺	Little & Knudson (1986)
Ames <i>bxpA</i>	<i>bxpA</i> :: Ω kan	This study
Ames <i>yabG</i>	<i>yabG</i> ::pEO3- <i>yabG</i>	This study
Ames <i>bxpA yabG</i>	<i>bxpA</i> :: Ω kan <i>yabG</i> ::pEO3- <i>yabG</i>	This study
Ames <i>bxpA</i> + pKSM5	<i>bxpA</i> + shuttle vector	This study
Ames <i>bxpA</i> + pKSM6	<i>bxpA</i> complemented with functional <i>bxpA</i>	This study
Ames <i>bxpA</i> + pKSM7	<i>bxpA</i> complemented with <i>bxpA</i> 133R→A	This study
Ames <i>tgl</i>	<i>tgl</i> ::pEO3- <i>tgl</i>	This study
Ames <i>bxpA tgl</i>	<i>bxpA</i> :: Ω kan <i>tgl</i> ::pEO3- <i>tgl</i>	This study
Sterne	34F2 background	A. Driks
Sterne <i>bxpA</i>	<i>bxpA</i> :: Ω kan	This study

saline. Primary polyclonal rabbit antibodies against BxpA (described below) were used at a dilution of 1 : 1000 and secondary goat anti-rabbit horseradish peroxidase was used at a dilution of 1 : 2500. Bands were visualized using 4-chloronaphthol/3,3'-diaminobenzidine (Pierce).

MS. Gel pieces were excised, placed in 1.5 ml polypropylene tubes, and then processed according to the procedure of the University of Wisconsin Biotechnology Center (<http://biotech.wisc.edu/ServicesResearch/MassSpec/ingel.htm>). Briefly, gel pieces were washed, destained, dehydrated, then dried slightly in a centrifugal evaporator. Cysteine residues were reduced and alkylated, and excess reagents removed with water washes after reaction. Gel pieces were dehydrated and dried briefly in a centrifugal evaporator to facilitate the uptake of trypsin (Promega) in ammonium bicarbonate. After digesting for 18 h at 37 °C, peptides were extracted from the gel pieces using 0.1 % trifluoroacetic acid and two extractions with 0.3 % trifluoroacetic acid in 70 % acetonitrile. Extracts were combined, evaporated to dryness in a centrifugal evaporator, and reconstituted for MS analysis in 0.1 % formic acid. MS-MS data were acquired and processed as described previously (Hines *et al.*, 2005). Proteins were identified from the peptide data using the Mascot Database search engine (http://www.matrixscience.com/search_form_select.html).

Expression and purification of recombinant BxpA, and antibody development. To express BxpA, the Ames *bxpA* gene was PCR-amplified from genomic DNA with Phusion polymerase, using the appropriate primers (listed in Supplementary Table S1). The PCR product was cloned into plasmid pET23A (EMD Biosciences), the resulting plasmids were introduced into *E. coli* strain NEB 10-Beta

[New England Biolabs (NEB)], and then into *E. coli* BL21 DE3 (EMD Biosciences). The recombinant protein was expressed in *E. coli* and purified using HisBind columns (EMD Biosciences) according to the manufacturer's instructions (Fig. 3b shows an SDS-PAGE gel of the purified recombinant protein).

Rabbit antisera against rBxpA^A were made by Covance. IgG was purified from the antisera as well as from preimmune sera as described previously (Welkos *et al.*, 2004).

Electron microscopy. For transmission electron microscopy (TEM), wild-type Ames and *bxpA* mutant spores were fixed in 4 % formaldehyde and 1 % glutaraldehyde in Millonig's phosphate buffer for several days and washed. The samples were then fixed in 1 % osmium in Millonig's phosphate buffer for 1 h and washed. Further staining and preparation for TEM were performed as previously described (Bozue *et al.*, 2007b). Immuno-electron microscopy (IEM) was performed on wild-type and *bxpA* mutant spores, which were fixed in 4 % formaldehyde in Millonig's phosphate buffer as previously described (Cote *et al.*, 2008) using IgG purified from polyclonal rabbit antisera directed against BxpA^A. The specificity of the immune sera for BxpA^A was demonstrated by blocking the immune sera with rBxpA^A prior to incubation with the thin-section samples.

Complementation of *bxpA*. The *lacZ* gene of pHT315 was deleted by inverse PCR using the primers listed in Supplementary Table S1 and Phusion DNA polymerase (NEB). The PCR product was treated with End-It (Epicentre) and self-ligated, creating pKH-KSM4. The region between the T7 terminator and T7 promoter of pET23A (EMD

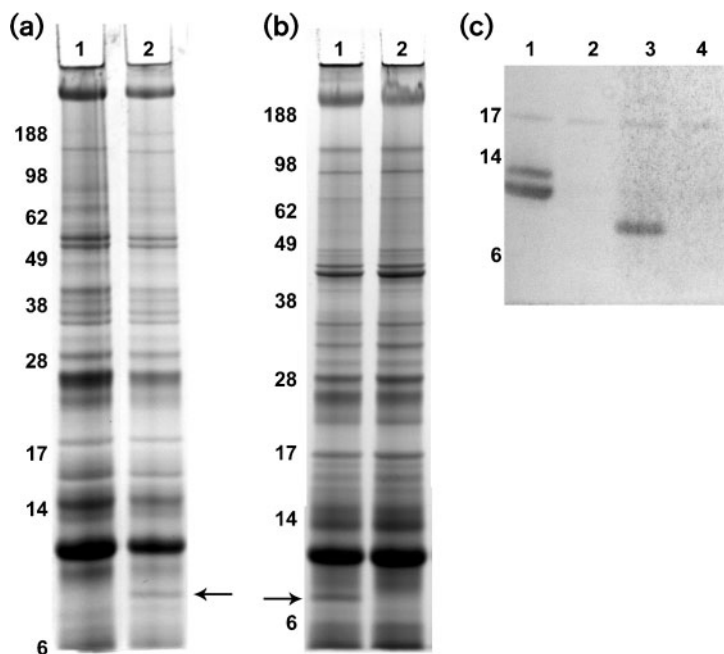


Fig. 1. SDS-PAGE analysis of spore proteins from *B. anthracis* wild-type and *bxpA* mutant spores. Spore proteins were purified and fractionated on a 12% Bis-Tris gel. (a) Wild-type Ames (lane 1) and Sterne (lane 2). (b) Wild-type Sterne strain (lane 1) and *bxpA* mutant (lane 2) spore proteins were subjected to Gel Code Blue staining. The arrows indicate the 9 kDa band which contains a portion of the BxpA protein. (c) Western blot analysis of spore proteins from Ames and Sterne strain spores. Lanes: 1, Ames; 2, Ames *bxpA*; 3, Sterne; 4, Sterne *bxpA* spores. The BxpA bands are present in the wild-type strains and missing in the mutants. Molecular masses are indicated on the left in kDa.

Biosciences) was PCR amplified and cloned into pKH-KSM4 to form shuttle vector pKSM5.

The *bxpA^A* gene and its promoter region were amplified and cloned into appropriately digested pET23A. Site-directed mutagenesis was performed using Phusion DNA polymerase and the appropriate primers to convert the arginine (R) at nucleotide 133 (relative to the *bxpA^A* ATG start site) to an alanine (A). The mutation in *bxpA* was confirmed by sequencing. Both the wild-type *bxpA* gene and the mutant allele (133R→A) were separately subcloned as *NheI/XhoI* fragments into plasmid pKSM5 to create plasmids pKSM6 and pKSM7, respectively. These plasmids were then transformed into the *bxpA* Ames mutant strain. Spore proteins from these strains were analysed as described above.

Germination assays. The degree of germination of spores was measured by monitoring changes in optical density via refractility, as previously described (Bozue *et al.*, 2007a; Ragkousi *et al.*, 2003). Briefly, 10^7 spores ml^{-1} was heat-activated at 65 °C for 30 min and added to AAC medium (Welkos *et al.*, 2004) (at a 1 : 10 dilution). Germination was measured by dividing the optical density, measured at various times after addition of spores to AAC medium [OD_{600} (t)], by the initial optical density recorded before addition to AAC medium [OD_{600} (init.)].

Resistance assays. Ames wild-type and *bxpA* mutant spores were heat-activated and tested for resistance in the presence of 1 M HCl, 250 mg lysozyme ml^{-1} or 0.5 M NaOH. In each case, spores were incubated for 30 min at 37 °C, and dilutions were plated on LB agar plates to assay viable cells.

In vivo challenges. Female Hartley guinea pigs (350–400 g) were obtained from Charles River Laboratories. Guinea pigs were challenged intramuscularly (Fellows *et al.*, 2001) by injection of 200 μl of heat-activated spores suspended in water. The animals were monitored several times each day, and morbidity and mortality rates were recorded.

Pathogen-free female BALB/c mice were obtained from the National Cancer Institute, Fort Detrick. For intranasal challenge experiments,

mice were anaesthetized with 100 μl ketamine, acepromazine or xylazine injected intramuscularly. The mice were challenged by intranasal instillation (Lyons *et al.*, 2004) of either *B. anthracis* Ames spores or *bxpA* mutant spores. The total volume of inoculum instilled was 50 μl . Mice were monitored several times each day and mortality rates recorded.

RESULTS

Identification of BxpA in *B. anthracis*

Ames and Sterne strain spores have similar coat protein composition, as judged by analysis of the proteins readily extracted and fractionated on 16% Tris-glycine gels (Giorno *et al.*, 2007). However, fractionation of spore proteins by a method which extracts both coat and exosporium proteins (see Methods) on 12% Bis-Tris gels revealed the presence of an additional 9 kDa species in the Sterne strain that was not observed in Ames spores (Fig. 1a). To determine the composition of this band, it was excised and subjected to MS. Although the band contained several proteins, we focused on one of these, corresponding to the protein annotated as either BA2162 (Ames) or BAS2008 (Sterne), because the predicted protein sequence from the Sterne strain genome lacks 14 amino acids that are present in the Ames protein sequence (Steichen *et al.*, 2003). This protein has been identified in the Sterne strain as an exosporium component protein named BxpA (Steichen *et al.*, 2003).

To demonstrate that *bxpA* encodes the 9 kDa band in Sterne strain spore extracts, we inactivated the *bxpA* gene by allelic replacement in both the Ames and Sterne strain backgrounds. As expected, *bxpA^S* mutant spores lacked the 9 kDa band (Fig. 1b). SDS-PAGE analysis of the extractable proteins from Ames wild-type and *bxpA^A* mutant spores

did not reveal any observable differences in the electrophoretic patterns (results not shown). However, we readily detected BxpA in both the Ames and Sterne wild-type strains by Western blot analysis using polyclonal rabbit anti-BxpA serum (Fig. 1c). Interestingly, two bands, corresponding to 11 and 14 kDa bands, were detected by Western blotting in Ames strain spore extracts (Fig. 1c). These bands are most likely obscured by the bands present in these regions of the SDS-PAGE gel of Ames spore extracts when staining proteins (Fig. 1a).

BxpA is produced during sporulation

Assembly of BxpA into the spore most likely occurs in one of two ways: it is either (1) synthesized during sporulation, under the control of the mother cell sigma factor σ^K (Steichen *et al.*, 2003) and assembled into the spore before mother cell lysis; or (2) produced during vegetative cell growth and associated with the spore surface after mother cell release, when vegetatively produced proteins can adhere to the spore surface (Cote *et al.*, 2005; Williams & Turnbough, 2004). To distinguish between these possibilities, we analysed extracts of vegetative cells and spores by Western blotting using BxpA antiserum. BxpA was not found in Ames vegetative cell extracts but was found on washed density gradient-purified spores, as expected (data not shown). Therefore, BxpA was very likely synthesized and assembled on the spore during sporulation, rather than adhering to the spore after mother cell lysis.

Localization of BxpA

A previous study identified the C-terminal domain of BxpA as a component of purified exosporium because BxpA is present in extracts that specifically isolate this structure (Steichen *et al.*, 2003). To further localize BxpA within the spore, we performed IEM on wild-type Ames spores using anti-BxpA polyclonal antibodies and gold-conjugated secondary antibodies. We detected gold grains primarily over the cortex (12.3 ± 6.15 grains per spore) and to a lesser extent the core (3.50 ± 3.13 grains per spore), but not the exosporium, interspace or spore coat of Ames strain spores (Fig. 2, Table 2). We observed significantly reduced labelling when we blocked the anti-BxpA polyclonal antibodies with rBxpA prior to staining sections of wild-type Ames strain spores or when we applied anti-BxpA antibodies to sections of *bxpA* mutant spores (Fig. 2, Table 2), indicating that our antisera were specific for BxpA. Our results suggest that most of the BxpA is in the cortex. Taken together with earlier results (Steichen *et al.*, 2003), we interpret these results as indicating that most of the BxpA is in the cortex and that perhaps a smaller amount is in the exosporium.

YabG-dependent maturation of BxpA

BxpA migrates as a smaller protein than is predicted from its sequence for the Ames and Sterne strains, 27 and 29 kDa,

respectively (Steichen *et al.*, 2003). It was possible that the discrepancies between the predicted and measured masses of BxpA^A and BxpA^S were due to the actions of proteases liberated during protein extraction. To address this possibility, we performed Western blot analysis using the anti-BxpA polyclonal antibodies to analyse spore extracts with and without a protease inhibitor (see Methods). Regardless of the extraction conditions, the same-sized BxpA bands were observed by Western analysis (results not shown).

A possible explanation for the migration of BxpA is controlled cleavage by a sporulation-specific protease, as occurs for some coat proteins in *B. subtilis* (Cutting *et al.*, 1991; Takamatsu *et al.*, 2000). Therefore, in the Ames and Sterne strains we inactivated the *yabG* gene, which encodes the homologue of the *B. subtilis* coat protein protease, and analysed the mutant spore extracts by Western blot analysis using anti-BxpA antibodies. Consistent with a role for YabG in the maturation of BxpA in both strains, in *yabG* mutant spores, the lower-molecular-mass bands were reduced in intensity or absent, and a series of higher-molecular-mass bands appeared (Fig. 3a).

To determine whether any of the BxpA-containing bands detected by Western blot analysis of *yabG* mutant extracts could plausibly represent full-length BxpA, we analysed the electrophoretic behaviour of a full-length, histidine-tagged and T7-tagged version of BxpA overproduced in *E. coli*. The resulting proteins migrated as a ~40 kDa protein (Fig. 3b). The added tags are unlikely to account for the difference between this mass and the predicted mass of 29 kDa. Western blot analysis using anti-6 × histidine and anti-T7 tag antibodies demonstrated that the N and C termini were intact (results not shown). From these results, we infer that the ~38 kDa species detected in Fig. 3(a) likely represents the unprocessed form of BxpA.

In *B. subtilis*, YabG processes several coat proteins by a trypsin-like protease activity (Takamatsu *et al.*, 2000). If BxpA is cleaved by YabG directly, it is likely that this depends on an arginine (R) residue in BxpA, as would be the case for cleavage by trypsin and as suggested for this protein (Steichen *et al.*, 2003). To test this prediction, we introduced a plasmid bearing either wild-type *bxpA* (pKSM6) or *bxpA* 133R→alanine (A) (pKSM7) into *bxpA*^A mutant cells. Western blot analysis of spore extracts bearing the wild-type *bxpA*-containing plasmid resulted in the expected electrophoretic pattern (Fig. 4). However, in extracts of spores bearing the *bxpA* 133R→A-containing plasmid, bands corresponding to the mature form of BxpA were significantly reduced in intensity and multiple higher-molecular-mass bands appeared (Fig. 4). These results are consistent with the possibility that YabG cleaves BxpA directly.

BxpA^A maturation requires Tgl

We found that in *yabG* mutant spores, BxpA is present in multiple forms, some of which are larger than predicted for the full-length protein (Fig. 3). The presence of these larger

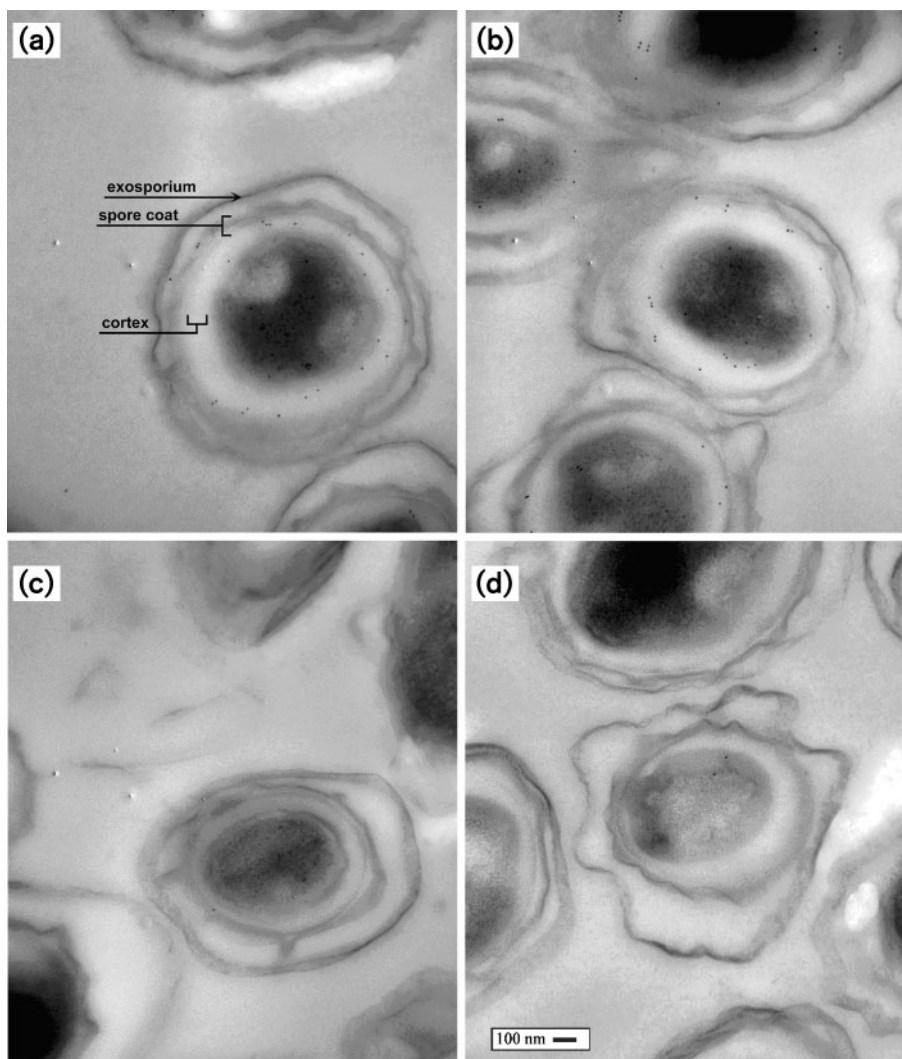


Fig. 2. Localization by IEM of the *B. anthracis* BxpA protein in spores of the Ames strain. (a, b) Polyclonal anti-BxpA^A antibodies were applied to thin sections of Ames wild-type spores. Gold grains indicate locations of antibody staining. (c) The same experiment was performed as in (a) and (b), except that BxpA, overproduced in *E. coli*, was applied to the thin sections prior to addition of the primary antibody. (d) The same experiment was performed as in (a) and (b), except that *bxpA* mutant spores were analysed. Bar, 100 nm.

forms could be explained if BxpA was cross-linked. To explore this possibility, we inactivated the Ames strain homologue of *tgl* and determined the consequences for BxpA mass by Western blot analysis. We predicted that the

absence of a BxpA cross-linking activity would result in either (1) the absence of the higher-molecular-mass bands or (2) the appearance of previously unseen bands (because proteins that are usually cross-linked into a form too large

Table 2. Number of gold grains associated with the structure per spore

The values represent the mean \pm SD of the number of gold grains associated with each spore.

Sample	Exosporium	Interspace	Spore coat	Cortex	Core	<i>n</i>
α BxpA ^A + Ames	0.08 \pm 0.31	0.03 \pm 0.22	0.47 \pm 1.21	12.3 \pm 6.15	3.50 \pm 3.13	220
rBxpA + α BxpA ^A + Ames	0.13 \pm 0.43	0.05 \pm 0.26	0.15 \pm 0.55	0.10 \pm 0.40	0.33 \pm 0.80	237
α BxpA ^A + <i>bxpA</i>	0.01 \pm 0.06	0 \pm 0	0.02 \pm 0.16	0.06 \pm 0.28	0.05 \pm 0.25	173

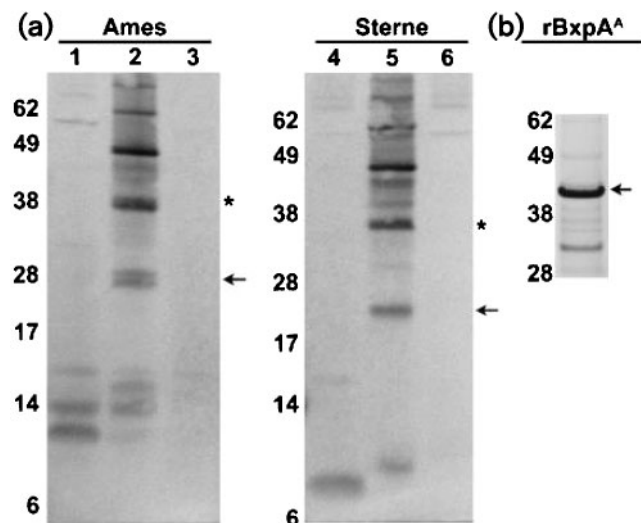


Fig. 3. Western blot analysis of the effect of YabG on BxpA. (a) Spore proteins were extracted from spores of Ames (lane 1), Ames *yabG* (lane 2), Ames *yabG bxpA* (lane 3), Sterne (lane 4), Sterne *yabG* (lane 5) and Sterne *yabG bxpA* (lane 6) strains, fractionated on a 12% Bis-Tris gel and subjected to Western analysis using anti-BxpA^A antibodies. The likely full-length forms of BxpA^A and BxpA^S are indicated by asterisks. The bands marked by the arrows are alternative cleavage products. (b) BxpA^A was expressed in *E. coli*, and the purified product fractionated on a 12% Bis-Tris gel and stained with GelCode Blue. The band marked by the arrow is full-length rBxpA^A. Molecular masses are indicated on the left in kDa.

to enter the gel are now able to do so), or both. In *tgl* mutant spores, we detected the 11 and 14 kDa bands corresponding to mature BxpA, as well as new bands at 35 and 40 kDa (Fig. 5). Therefore, Tgl affects BxpA maturation. While this experiment does not conclusively demonstrate that Tgl cross-links BxpA, it is consistent with the possibility that Tgl directs the cross-linking of BxpA into a form that does not enter the gel.

The role of BxpA in spore functions

We did not detect an effect of mutation of *bxpA* on spore morphology (based on TEM), germination, or resistance to NaOH, HCl or lysozyme (a measure of coat integrity) (results not shown). Consistent with the lack of an obvious major role in morphology or function, we did not detect a role for BxpA in virulence, using either a mouse intranasal model (Lyons *et al.*, 2004), following challenge with approximately 4×10^5 spores, or a guinea pig intramuscular model of infection (Bozue *et al.*, 2005; Fellows *et al.*, 2001), following challenge with approximately 1100 spores (data not shown).

DISCUSSION

There are three major results from this work: (1) localizing the majority of the *B. anthracis* protein BxpA to the spore

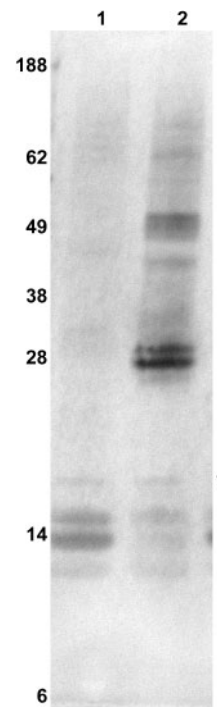


Fig. 4. Complementation of the *bxpA* mutant with a *bxpA* point mutation. The Ames *bxpA* mutant was transformed with the shuttle vector pKSM6 (bearing wild-type *bxpA*) or pKSM7 (bearing *bxpA* 133R→A). Spore extracts were fractionated on a 12% Bis-Tris gel and subjected to Western analysis using anti-BxpA^A antibodies. Lanes: 1, Ames *bxpA* + pKSM6; 2, Ames *bxpA* + pKSM7. The versions of BxpA used in this experiment bear a histidine fusion tag and therefore differ in size from wild-type BxpA. A cross-reacting band is marked by an asterisk. Molecular masses are indicated on the left in kDa.

cortex; (2) demonstrating a difference in spore protein composition between the Ames and Sterne strains; and (3) demonstrating roles for YabG and Tgl in *B. anthracis* spore protein maturation.

BxpA protein has been previously identified as an exosporium component in spores of the Sterne strain of *B. anthracis* in two studies (Liu *et al.*, 2004; Steichen *et al.*, 2003). However, a similar study of the components of purified exosporia from spores of the Ames strain did not detect this protein (Redmond *et al.*, 2004). Using IEM with polyclonal antisera generated against BxpA and spores from the Ames strain, we found that BxpA was localized primarily to the cortex region of the spore and not to the exosporium (Fig. 2, Table 2). A simple explanation for these differing results is that BxpA is present both in the exosporium and associated with the cortex, but the abundance of exosporium-associated BxpA is below the limit of detection of IEM. We note that the presence of a protein in multiple locations within the spore is not unprecedented (Cote *et al.*, 2008; Severson *et al.*, 2009; Todd *et al.*, 2003).

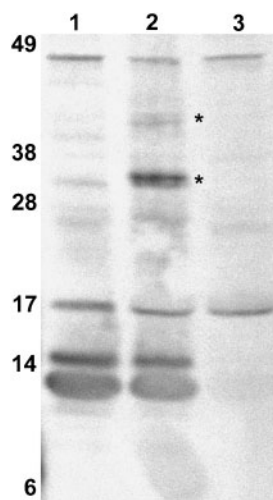


Fig. 5. Western blot analysis of the effect of Tgl on BxpA. Spore proteins were extracted from spores of the Ames strain (lane 1), Ames *tgl* mutant (lane 2) and Ames *tgl bxpA* mutant (lane 3). The extracted proteins were run on a 12% Bis-Tris gel and subjected to Western blotting with anti-BxpA^A antibodies. The bands at ~35 and ~40 kDa (indicated by asterisks) are full-length and, most likely, partially cleaved forms, respectively, of BxpA. Molecular masses are indicated on the left in kDa.

Identifying molecular or physiological differences between *B. anthracis* strains is important for at least two reasons. First, because the species is highly monophyletic (Keim *et al.*, 2000; Read *et al.*, 2003; Sylvestre *et al.*, 2003), variation in spore characteristics could be especially useful in revealing adaptations to varying niches. Second, these differences could be used to rapidly distinguish among strains. To date, very few practically useful differences have been found. Among them are the variation in collagen-like repeats in the exosporium protein BclA, which leads to variation in the length of the hair-like projections (Sylvestre *et al.*, 2003), and variation in the presence of the spore coat protein Cot β (Mallozzi *et al.*, 2008).

Perhaps the most important result is the finding that the maturation of BxpA depends on YabG and Tgl. The simplest interpretation of our data is that YabG proteolyzes and Tgl cross-links BxpA. The discovery of roles for YabG and Tgl in BxpA maturation is consistent with the possibility that, as in *B. subtilis*, YabG cleaves (Kuwana *et al.*, 2007; Takamatsu *et al.*, 2000) and Tgl cross-links coat proteins (Kobayashi *et al.*, 1998; Monroe & Setlow, 2006; Takamatsu *et al.*, 2009; Zilhao *et al.*, 2005). If this is true, then it further supports the contention that at least some of the known critical events in *B. subtilis* coat assembly are also part of the assembly in *B. anthracis*. Furthermore, this would raise the strong possibility that proteolysis and cross-linking are ubiquitous in bacterial spore formation.

The purpose of the modifications in BxpA is unclear. In principle, however, it is reasonable to suggest that cross-

linking provides spores with important mechanical and physical properties, including flexibility, porosity and resistance (Driks, 2003). The purpose of spore protein proteolysis is more difficult to rationalize. However, Kuwana and colleagues have hypothesized that the cleavage of GerQ by YabG in *B. subtilis* reveals functional groups recognized by Tgl (Kuwana *et al.*, 2006). Possibly, BxpA cross-linking similarly depends on prior cleavage. Although we do not know how these events contribute to spore function, the conservation of YabG and Tgl across divergent species suggests an important function for these proteins.

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