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A genetic approach for the identification of exosporium assembly determinants of *Bacillus anthracis*

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Abstract

The exosporium is the outermost layer of spores of the zoonotic pathogen *Bacillus anthracis*. The composition of the exosporium and its functions are only partly understood. Because this outer spore layer is refractive to traditional biochemical analysis, a genetic approach is needed in order to define the proteins which comprise this important spore layer and its assembly pathway. We have created a novel genetic screening system for the identification and isolation of mutants with defects in exosporium assembly during *B. anthracis* spore maturation. The system is based on the targeting sequence of the BclA exosporium nap layer glycoprotein and a fluorescent reporter. By utilizing this screening system and gene inactivation with Tn916, several novel putative exosporium-associated determinants were identified. A sampling of the mutants obtained was further characterized, confirming their exosporium defect and validating the utility of this screen to identify novel spore determinants in the genome of this pathogen.

Keywords

Bacillus anthracis; spore; exosporium; mutagenesis; transposon

1. Introduction

There are a number of endospore-forming bacteria that are of concern to public health, including *Bacillus anthracis* and *Bacillus cereus*. For these organisms, the spore is usually the infectious form. The spores from each of these pathogens contain an outer spore layer known as the exosporium, which provides the sites of initial interactions with the infected host. The exosporium is separated from the spore coat by a region known as the interspace (Beaman *et al.*, 1971; Gerhardt, 1967; Gerhardt and Ribí, 1964; Giorno *et al.*, 2007, 2009). The exosporium layer of *B. anthracis* consists of a basal layer surrounded by an external nap of hair-like projections (Hachisuka *et al.*, 1966; Gerhardt, 1967; Sylvestre *et al.*, 2002; 2003; 2005; Tan and Turnbough, 2010). The collagen-like glycoprotein BclA is the principal component of this hair-like nap, is an immunodominant antigen, and is involved in the uptake of spores by alveolar macrophages and dendritic cells through its interaction with the integrin Mac-I [CR3] (Steichen *et al.*, 2003; Boydston *et al.*, 2005; Bozue *et al.*, 2005; Brahmbhatt *et al.*, 2007; Oliva *et al.*, 2008).

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Additional protein constituents of the exosporium have been identified. These include the collagen-like proteins BclB (Waller *et al.*, 2005; Thompson *et al.*, 2007, 2012; Thompson and Stewart, 2008), and BclA (Thompson *et al.*, 2011b), as well as the basal layer-associated BxpB protein (also called ExsFA) which is needed for assembly of the collagen-like proteins (Steichen *et al.*, 2005; Sylvestre *et al.*, 2005; Thompson and Stewart, 2008; Thompson *et al.*, 2011b; 2012). CotY, ExsA, ExsB, ExsK, ExsFB, ExsM, and ExsY have also been implicated in exosporium formation (Bailey-Smith *et al.*, 2005; Sylvestre *et al.*, 2005; Boydston *et al.*, 2006; Johnson *et al.*, 2006; Steichen *et al.*, 2007; Severson *et al.*, 2009; Fazzini *et al.*, 2010; McPherson *et al.*, 2010; Thompson *et al.*, 2011c). Enzymes associated with the exosporium, including alanine racemase, arginase, and superoxide dismutase, may be involved in preventing premature germination and providing protection against macrophage killing by detoxifying superoxide free radicals (Raines *et al.*, 2006; Weaver *et al.*, 2007; Chesnokova *et al.*, 2008; Cybulski *et al.*, 2009).

The overall assembly of the exosporium layer of the spore is still not well understood. The exosporium is the final layer of the spore to be assembled, and many of its proteins are deposited around the spore in a progressive engulfment process (Ohye and Murrell, 1973; Hilbert and Piggot, 2004; Thompson and Stewart, 2008; Giorno *et al.*, 2009; Tan and Turnbough, 2010; Thompson *et al.*, 2011c). The assembly of the nap closely follows the progressive assembly of the basal layer (Thompson and Stewart, 2008; Giorno *et al.*, 2009; Thompson *et al.*, 2011c). BclA is one of the major proteins comprising the hair-like nap found on the external surface of the exosporium. BclA assembly has been well studied, from its assembly process and timing, its N-terminal cleavage events and resulting covalent attachment to BxpB, and its association into large molecular weight complexes during exosporium assembly (Boydston *et al.*, 2005; Steichen *et al.*, 2005; Sylvestre *et al.*, 2005; Thompson and Stewart, 2008; Tan and Turnbough, 2010; Tan *et al.*, 2011; Thompson *et al.*, 2011c, 2012).

Much of what we have learned about spore formation comes from genetic studies with *Bacillus subtilis* (reviewed in Stragier and Losick, 1996). Because this organism lacks a distinct exosporium layer and homologs of the known *B. cereus* family exosporium determinants, it provides no insight regarding the biosynthetic process for exosporium formation. Proteomic studies have been used to identify exosporium proteins of *B. anthracis* (Steichen *et al.*, 2003; Todd *et al.*, 2003; Liu *et al.*, 2004; Redmond *et al.*, 2004). However, these studies are limited to the identification of structural proteins present in sufficient quantities for identification. Trace components, or proteins required for the exosporium assembly process but which are not present in the mature spore, would not be identified by these approaches. To develop a genetic system to broadly identify exosporium-associated *B. anthracis* determinants, we took advantage of the BclA targeting domain and the fluorescent reporter protein DsRed, to create a method to identify mutants whose phenotype is a defect in incorporation of the exosporium surface protein BclA. Since BclA is a latecomer structural protein as far as exosporium assembly is concerned, this approach has the potential for identifying genes involved in many of the steps involved in exosporium synthesis and assembly. Mutants were generated using a Tn916 transposon mutagenesis system that has been shown to be useful for mutagenesis experiments with *B. anthracis* (Ivins *et al.*, 1988). Representative mutants obtained with this system were evaluated for exosporium defects as a validation of the genetic system.

2. Methods

2.1. Strains and growth conditions

Strains are listed in Table 1. *E. coli* was grown at 37°C with shaking (225 rpm) in Luria-Bertani broth (LB). *B. anthracis* was grown at 37°C with shaking in brain heart infusion

broth (BHIB; Difco). Solid media was prepared using 1.5% agar. When required, media were supplemented with ampicillin to 100 µg/ml ampicillin, tetracycline to 10 µg/ml, or chloramphenicol to 10 µg/ml. For relatively synchronous production of spores, *B. anthracis* was cultured in Tiger broth, a modified version of ModG medium (Bergman *et al.*, 2006; Thompson *et al.*, 2011c). The onset of sporulation (T_0) is defined as the point at which the culture deviated from exponential growth.

2.2 Production of spores

Overnight *B. anthracis* cultures in BHIB with appropriate antibiotic selection were swab inoculated onto nutrient agar (Difco) plates (with antibiotics) and incubated at 30°C. Microscopy was used to monitor progression of spore production. When sporulation exceeded 95%, the spores were harvested with sterile PBS. Spores were thrice washed in sterile PBS to remove residual vegetative cells. Purity of the spore samples were checked by phase contrast microscopy. Purified spores were stored in sterile PBS and concentration determined by counting on a hemocytometer.

2.3 Transposon mutagenesis

The recipient strain was MUS1694, ΔSterne bearing pBT3777 (Thompson and Stewart, 2008) while the Tn916 donor strain was *Enterococcus faecalis* CG110 (Gawron-Burke and Clewell, 1982). Overnight cultures (5 ml) were prepared with appropriate antibiotics (tetracycline for the selection of the donor and chloramphenicol for the recipient). The strains were combined and collected on 47 mm 0.45 µ nitrocellulose filters (Thermo Fisher cat. no. 09-740-30D) at two different ratios (2:1 or 3:1 recipient:donor). The filters were removed and placed culture side up on brain heart infusion agar plates, and incubated at 30°C for 18 hours. After incubation the filters were removed from the BHI plates, placed in 5 ml LB in a 50 ml conical tube, and the cells washed from the filter with BHIB. Serial dilutions (1:10, 1:100, 1:1000) of the harvested cells were plated on BHI plates containing 10 µg/µl chloramphenicol and 10 µg/µl tetracycline and incubated at 30°C. After 24–36 hours the colonies were replica plated onto nutrient agar plates containing 10 µg/µl chloramphenicol and 10 µg/µl tetracycline and incubated at 30°C. DsRed fluorescence was monitored daily using a Dark Reader Spot Lamp (Clare Chemical Research) which generates light output between 400–500 nm. The colonies which failed to become fluorescent, indicating that sporulation did not progress to the point of expressing the BclA NTD-DsRed fusion, were marked and not pursued further. Incubation was continued until the sporulation process was complete and the colonies reexamined for fluorescence. Those initially fluorescent colonies now exhibiting a reduction or complete loss of fluorescence were selected. A bank of 500 of these clones was stored at –80°C in BHIB + 20% glycerol.

2.4 Characterization of the Tn916 insertion sites

B. anthracis Tn916 insertion mutants displaying reduced BclA-DsRed spore-associated fluorescence were cultured in BHI broth containing tetracycline and chloramphenicol, and chromosomal DNA extracted with phenol and chloroform as described (Thompson *et al.*, 2007). Genomic DNA was digested with *Sau3a* and the DNA fragments electrophoresed on a 1% agarose gel and Southern blotted onto a nylon membrane (Magnacharge, 0.45 µm). A Tn916-specific DNA probe was prepared by PCR (primers: Tn916 probe 5p, CTAATTTACTACTTATGAATGAGC and Tn916 probe 3p, CCTTTTACCACGCTGTTTCATGCG). The resultant 996 bp PCR DNA fragment was purified using a Qiaquick PCR Clean Up kit (Qiagen) and labeled using a commercial digoxigenin labeling and detection kit (Roche). Hybridization was conducted at 68°C in 5X SSC, 0.04% SDS, 0.1% N-lauroylsarcosine, and 10% blocking solution (Roche). Hybridized probe was identified using an anti-DIG alkaline phosphatase conjugate and 2% (v/v) NBT/BCIP (Thermo-Fisher Scientific).

Characterization of the Tn916 insertion sites was performed using inverse PCR as previously described (Smidt *et al.*, 1999). Briefly, chromosomal DNA was digested with *Hind*III, purified and ligated at a concentration of 50 ng/μl. Five nanograms of self-ligated DNA was used as the template in a 50 μl PCR reaction mixture containing 37.5 ng of each primer (Tn916 62–38, TAGAATAAGGCTTTACGAGC and Tn916 12185–12190, CCTTGATAAAGTGTGATAAG); 2 mM MgCl₂; 200 μM (each) dATP, dCTP, dGTP, and dTTP; and 1.25 U of ExTaq polymerase (Takara Bio). After the mixture was preheated to 94°C for 3 min, 35 amplification cycles were performed, consisting of denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and elongation at 72°C for 3 min. A final extension of 7 min at 68°C was performed. The PCR products were identified by electrophoresis on a 1% agarose gel, purified with a Qiaquick PCR Purification kit, and the nucleotide sequence determined at the University of Missouri DNA core using the individual inverse PCR primers. The site of each Tn916 insertion was identified from the sequences.

2.5 Transmission electron microscopy

Spores were fixed in 1 ml 2% glutaraldehyde-100 mM sodium cacodylate solution containing 0.1% ruthenium red (Waller *et al.*, 2004; Sigma-Aldrich) for 1 hour at 37°C. Each spore pellet was then washed in 100 mM sodium cacodylate buffer, embedded in Histogel, and washed with 100 mM sodium cacodylate buffer containing 0.01 M 2-mercaptoethanol and 0.13 M sucrose (2-ME buffer). Samples underwent secondary fixation in 1% osmium tetroxide (Electron Microscopy Sciences). ΔSterne spores were used as a wild-type control and were treated identically to the mutant spores. Spores were washed in 2-ME buffer and dehydrated with sequential treatments of 20, 50, 75, 90, and 100% acetone. Polymerization occurred at 60°C in Epon/Spurrs resin after extended resin infiltration. Samples were cut into 85 nm thick sections and the sections mounted on 200 mesh nickel grids and stained with 2% uranyl acetate (Electron Microscopy Sciences) for 20 minutes at room temperature. The samples were treated with lead for 5 minutes at room temperature. Grids were washed in ultrapure water and observed by transmission electron microscopy (TEM) using a JEOL 1400 electron microscope.

2.6 Spore counts

The percentage of spores with the mutant phenotype spores were determined and compared to wild-type. A minimum of 350 spores were generally counted per strain from multiple grids, images, and different viewing fields representative of the samples. Percentages were then calculated.

2.7 Flow cytometric analyses

Wild-type and Tn916-bearing strains harboring the BclA NTD-DsRed plasmid were induced to sporulate in Tiger broth until >95% free spores (Thompson *et al.*, 2011c). 1×10⁸ PBS-washed spores were resuspended in 500 μl of 4% paraformaldehyde in PBS and incubated for 2 hours at room temperature. The spores were thrice washed in PBS and then sorted using a FACScan flow cytometer (Beckton Dickinson Biosciences) in the University of Missouri Cell and Immunobiology Core Laboratory. 75,000–100,000 spores were counted and the raw data analyzed and graphed using WinMDI 2.9 (Purdue University Cytometry Laboratories) software.

2.8 Western blot analysis

Proteins were extracted from 10⁸ spores by boiling in SDS-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 (Thompson *et al.*, 2011a). Samples were then centrifuged to remove

remaining spores, and the extracted protein was electrophoresed on 4–20% polyacrylamide gels (BioRad) and the proteins blotted to PVDF membranes. The blots were probed with rabbit polyclonal antibodies to rBclA, rBclB, or rBAS1747 respectively. Western blots were performed using goat anti rabbit immunoglobulin G conjugated to horseradish peroxidase as the secondary antibody. Immuno-reactive proteins were determined using the Pierce ECL Western Blotting Substrate kit (Thermo Scientific).

2.9 Preparation of anti-BAS1747 antiserum

The *bas1747* open reading frame was PCR amplified using primers (1747USec, **GGATCCATGTTGGTTTCATCTATTA**AAAAGATTTTAATTGG and 1747DSec, **GTCGACTTAGAAAGCTACAATTA**AAAATAATCGATGC) and cloned into the pQE30 plasmid (Qiagen) as a *Bam*HI to *Sal*I fragment. His-tagged protein was expressed in *E. coli* M15 (pREP4) by induction with 1 mM IPTG and purified using the His-Spin Protein purification kit (Zymo Research). Anti-rBAS1747 polyclonal antiserum was prepared in rabbits using Titermax Gold as adjuvant (CytRx Corp).

2.10 Immunolabeling of spores

Ten mg of paraformaldehyde-fixed spores were resuspended in 750 μ l SuperBlock Blocking buffer (Thermo Scientific) and incubated for at least 20 minutes at room temperature. The samples were vortexed every 5 minutes to ensure the spores remained suspended. The spores were harvested by centrifugation and the spore pellet was resuspended in 250 μ l SuperBlock blocking buffer with 1 μ l primary antibody (rabbit polyclonal antibodies used were against BclA [34], BclB [16], and BAS1747) and incubated at room temperature for 20 minutes. The spores were harvested by centrifugation and washed with 750 μ l of SuperBlock blocking buffer. The pellet was then resuspended in 250 μ l of SuperBlock Blocking buffer with secondary antibody conjugate (Protein A conjugated goat anti-rabbit IgG-Alexa Fluor 568; Invitrogen). The spores were incubated at room temperature for 20 minutes, pelleted and washed with 750 μ l SuperBlock blocking buffer followed by three washes with 750 μ l PBS, and finally resuspended in 250 μ l PBS. DABCO anti-fade reagent (Diazabicyclooctane, Acros Organics) at a final concentration of 0.4% was added and the spores examined by epi-fluorescence microscopy using a Nikon E1000 microscope.

3. Results

3.1 Creation of a mutagenesis system to identify novel exosporium determinants

To develop a method to screen for mutants defective in exosporium synthesis, we took advantage of the mechanism by which the collagen-like BclA glycoprotein is incorporated onto the exosporium surface as a component of the outer hair-like nap layer. Inclusion of an N-terminal domain (NTD) from BclA, C-terminally fused to the fluorescent reporter DsRed, results in covalent attachment of the DsRed reporter onto the spore surface (Thompson and Stewart, 2008). The BclA NTD-DsRed fusion is strongly expressed from the *bclA* promoter and appears in the cytoplasm of the cells approximately three hours into the sporulation process (Thompson and Stewart, 2008; Thompson *et al.*, 2011c). The NTD sequence results in the localization of the reporter protein around the developing spore and subsequent covalent attachment of the fusion protein to the exosporium of the spore. When the sporulation process is complete, the mother cells lyse releasing the mature spore. Colonies of sporulating cells become fluorescent when the reporter is expressed in the mother cell and remain fluorescent when the sporulation process is complete because the BclA NTD-DsRed is abundantly and stably anchored to the spore surface. Mutants that are unable to incorporate the BclA surface protein reporter lose fluorescence when the mother cells lyse and the unincorporated BclA NTD-DsRed diffuses away from the colony or becomes inactive through protein turnover. Bound BclA NTD-DsRed is quite stable (Thompson and

Stewart, 2008). Thus loss of colony fluorescence can be used to identify mutants potentially defective in exosporium maturation resulting in defective BclA fusion incorporation. To eliminate early sporulation gene mutants which fail to sporulate, colonies can be screened early in the sporulation process and any which fail to express the BclA NTD-DsRed reporter (blocked prior to BclA expression and do not develop fluorescence) can be identified and eliminated from consideration.

We utilized a Tn916 (Gawron-Burke and Clewell, 1982; Ivins *et al.*, 1988; Flannagan *et al.*, 1994) conjugative transposon mutagenesis system. The donor strain was *Enterococcus faecalis* CG110 and the recipient strain was MUS1694, Δ Sterne bearing the *bclA* NTD-DsRed reporter plasmid pBT3777 (Thompson *et al.*, 2011c, 2012). The transconjugant *B. anthracis* cells bearing Tn916 were identified by plating on media containing tetracycline (to select for Tn916 and chloramphenicol (to select for pBT3777). Plates were incubated at 30°C and examined using a Dark Reader lamp for fluorescence. Colonies which failed to express the BclA NTD-DsRed reporter were identified, marked, and not considered further. Colonies that expressed the reporter but which exhibited reduced fluorescence once sporulation was complete were identified and selected for further study (Fig. 1).

DNA was isolated from cells of representative colonies that exhibited reduced fluorescence at the end of the sporulation process. The genomic DNA was cleaved with *Sau3a*, electrophoresed in an agarose gel, Southern blotted onto a charged nylon membrane, and probed with a DIG-labeled Tn916 probe. Rare strains possessing more than one copy of Tn916 were not further characterized. Inverse PCR was performed and the PCR products sequenced to identify the insertion site of the Tn916 transposon. Representative putative exosporium development mutants identified are shown in Table 2. Most of the determinants are not annotated as genes expected to be associated with assembly of the outer spore layers. Therefore, studies were conducted to validate these determinants as being spore assembly-associated.

To confirm that the mutants were defective in BclA-NTD-DsRed incorporation onto the spore surface, the mutant spores were compared to the wild-type parent by flow cytometry. Representative examples (Tn916 inactivated *bas0389*, *bas0605*, *bas1726*, and *bas1747*) are shown in Fig. 2. Each mutant exhibited a reduced level of DsRed fluorescence compared to wild-type levels. The results confirmed the fluorescence colony screen results that the mutant spore population exhibited a reduced BclA-NTD-DsRed expression on the spore surface.

Three novel exosporium determinants were selected for further study. Two of these, BAS0389 and BAS1747, are of unknown function. BAS0605 was annotated as a sodium/alanine symporter family protein.

3.2 *bas0389*

The *bas0389* determinant harbored the Tn916 element 501 bp into the open reading frame. This determinant is the penultimate determinant in an apparent seven gene operon (*bas0384-bas0390*). These genes are annotated as a ROK family protein (BAS0384), tellurium resistance protein, putative (BAS0385), tellurium resistance protein (BAS0386), tellurium resistance protein (BAS0387), tellurium resistance protein, putative (BAS0388), conserved hypothetical protein (BAS0389), and tellurite resistance protein, putative (BAS0390) respectively. The Sterne strain is not, however, resistant to tellurite (Spreng and Stewart, unpublished).

Flow cytometry analysis of spore fluorescence revealed that the *bas0389*:Tn916 mutant exhibited an intermediate level of fluorescence when compared to the positive control (Fig.

2B). To examine the native BclA content of the *bas0389::Tn916* spores, a derivative of MUS1754 lacking the reporter plasmid was identified and designated MUS1834. Proteins were extracted from MUS1834 spores by boiling in SDS and urea, the resulting proteins and complexes were resolved by SDS-PAGE, transferred to PVDF, and probed with rabbit polyclonal antibodies to rBclA. The amount of extract loaded into each lane corresponded to an equivalent number of spores. The western blot results showed a modest reduction in the amount of BclA extracted from the *bas0389::Tn916* mutant spores relative to that from the wild-type Sterne strain (Fig. 3A). In addition, the mutant strain exhibited a more prominent lower molecular weight BclA-containing complex (slightly below the position of the 220 kDa molecular weight standard) than the wild-type spores. The wild-type spores gave the expected heterogeneous high molecular weight BclA-containing complexes.

Because *bas0389* is the penultimate gene in a possible multigene operon, the phenotype resulting from the *Tn916* insertion may have been due to a polar effect on *bas0390*. To determine if the defect in BclA incorporation into the spores of the *bas0389::Tn916* mutant was due to the inactivation of the *bas0389* determinant, a complementation study was conducted. Spores were produced from the *bas0389::Tn916* mutant strain bearing the *bas0389*-complementing plasmid, pKS4395. The spores from the wild-type, mutant, and complemented strains were harvested, purified, and then examined via western blot with rabbit polyclonal antiserum against BclA (Fig. 3B). Spores from cells harboring the complementation plasmid regained both wild-type levels of BclA and the wild-type pattern of BclA-containing complexes which are stable to boiling in the presence of SDS and urea. Thus the decrease in BclA incorporation in the mutant spores was the result of the inactivation of the *bas0389* determinant by *Tn916* and not due to expression defects on the downstream determinant. This experiment also indicated the defect was not due to a spontaneous mutation elsewhere in the *B. anthracis* genome.

To determine if inactivation of the *bas0389* determinant resulted in alterations in the morphology of the *B. anthracis* spore, MUS1834 and wild-type parent spores were examined by transmission electron microscopy. The MUS1834 spores exhibited an intact exosporium and possessed a distinct hair-like nap layer, similar to that of wild-type spores (Fig. 4A, B). A structural abnormality, however, was evident in the mutant spores. With the MUS1834 mutant spores, the predominant distinction from wild-type spores was the presence of putative spore coat or exosporium fragments in the interspace (Fig. 4B, C) and what appears to be nap-containing shed exosporium debris in the sample (Fig. 4C). The nature of the interspace-localized fragments has not yet been defined. A small subpopulation of spores also presented with a structural defect in the exosporium with an apparent loss of structural integrity (Fig. 4B, C). These defects were rarely observed in TEM images of wild-type spores. Enumeration of the defects observed is presented in Table 3.

3.3 *bas0390*

BAS0389 is encoded in an apparent operon. It is possible, therefore, that other genes in the operon may encode exosporium-associated proteins. To test this, the *bas0390* open reading frame was fused to the eGFP reporter gene. The fusion construct was then subcloned into the pMK4 shuttle plasmid (Sullivan *et al.*, 1984) to produce pKS1824 and introduced to the Δ Sterne strain of *B. anthracis* by electroporation. Expression of the fusion protein was monitored in relatively synchronous cells cultured in Tiger broth. At T₃ (3 hours after onset of the sporulation process) the BAS0390-eGFP fusion protein is present in the mother cell cytoplasm (Fig. 5A). At T₅, the fusion protein localized to the developing spore (Fig. 5B). The localization pattern of fluorescence was not uniform; there was a higher concentration at one pole of the developing spore, similar to that seen with BclA fusions (Thompson and Stewart, 2008). At T₇, a decrease in fluorescence was seen in the mother cell cytoplasm as the fusion protein had localized to the developing spore, which was uniform around two-

thirds of the spore. Lesser incorporation of the BAS0390-eGFP fusion protein was observed at one spore pole in a subpopulation of the spores (Fig. 5C, D). Upon release from the mother cells, the free spores retained fluorescence. However, the fluorescence of the released spores was reduced relative to that observed prior to mother cell lysis. The fusion protein studies indicate that BAS0390 is a spore surface-associated protein, and the timing of its incorporation into the spore is consistent with it being an exosporium protein of *B. anthracis*.

3.4 *bas1747*

Reduced BclA-DsRed incorporation into the exosporium was identified in a strain that harbored a Tn916 insertion in the *bas1747* 365 codon open reading frame. The insertion site was located 41 nucleotides into the ORF. BAS1747 is encoded by an apparent two gene operon, and is annotated as a hypothetical protein, as is BAS1748. Both show sequence similarity to amino acid transporters. Analysis of spore fluorescence by flow cytometry revealed the *bas1747::Tn916* mutant spores presented with a reduced level of BclA NTD-DsRed fluorescence (Fig. 2B). SDS + urea extracts of the plasmid-cured *bas01747::Tn916* mutant spores revealed reduced levels of BclA present (Fig. 6A) relative to that obtained from the wild-type spores.

Because BAS1747 appears to be encoded in a bicistronic operon, the phenotype resulting from the Tn916 insertion may have resulted from polar effects on *bas1748*. To prove that the defect in BclA incorporation into the spores of the *bas1747::Tn916* mutant was due to the inactivation of the *bas1747* determinant, complementation studies were conducted. Spores were prepared from the *bas1747::Tn916* mutant strain bearing pKS4427, a *bas1747* complementing plasmid which carries the *bas1747* determinant expressed from its native promoter. Spores from cells harboring the complementation plasmid regained wild-type levels of BclA (Fig. 6A), indicating the reduction of BclA seen in the western blot was due to inactivation of *bas1747*.

An additional characterized exosporium collagen-like protein is BclB (Thompson *et al.*, 2007, 2012). Loss of BclB contributes to decreased structural integrity of the exosporium and an alteration in exosporium construction (Thompson *et al.*, 2007, 2012). Western blots were performed with rabbit polyclonal antibodies against rBclB to determine if BclB incorporation was affected by inactivation of *bas1747*. Extracts from the *bas1747::Tn916* mutant spores displayed increased levels of BclB (Fig. 6B). In addition, a broader size range of BclB-containing complexes was observed.

To gain a better insight into the BAS1747 content of spores, a rabbit polyclonal antiserum was raised against rBAS1747. Western blot studies using this serum showed that BAS1747 is not present in detectable amounts in SDS + urea extracts from wild-type Δ Sterne spores. However, proteins extracted from *bclA*-null spores yielded three reactive species, which migrated at positions of 20, 38, and 55 kDa (Fig. 7). Proteins extracted from the exosporium basal layer protein BxpB-deficient spores failed to react with the r1747 antibodies. Because these spores possess greatly reduced levels of BclA (Steichen *et al.*, 2005; Sylvestre *et al.*, 2005; Thompson *et al.*, 2011c), the elevated BAS1747 content of BclA-negative spores is not solely the consequence of a reduction in BclA spore content. BAS1747 may be directly or indirectly dependent upon BxpB for its incorporation into the spore. Dramatically increased levels of BAS1747 were evident in extracts of *bclB*-null spores, indicating an inverse relationship between BclB and BAS1747 protein levels in the spore. Three distinct species of approximately 30 kDa, 40 kDa, and 70 kDa, were identified with the anti-rBAS1747 serum in the BclB-negative extracts (Fig. 7). The predicted molecular mass of BAS1747 is 38.2 kDa and thus BAS1747 is likely present in spores in the form of SDS + urea-resistant complexes. The 20 and 30 kDa species detected with the anti-BAS1747 serum

may represent processed forms of the protein. In addition, the content of BAS1747 in CotE-deficient spores was examined.

In the absence of CotE, the exosporium is made, but it fails to attach to the developing spores and consequently is found in long strips in sporulating cells and only small fragments of exosporium are spore-associated (Giorno *et al.*, 2007, 2009). A single faintly reactive species at about 38 kDa was observed (Fig. 7). The increased reactivity observed with CotE-deficient spores, relative to wild-type spores, suggests this BAS1747 species may be present in the outer spore coat or interspace regions of the spore and thus more efficiently extracted when the exosporium layer is largely absent.

To determine if inactivation of the *bas1747* determinant results in phenotypic changes in the appearance of the *B. anthracis* spore, *bas1747::Tn916* and Δ Sterne parent spores were examined by transmission electron microscopy. The *bas1747::Tn916* spores possessed an intact exosporium and a distinct hair-like nap layer (Fig. 8). The appearance of the BclA-containing exosporium nap layer was altered in the *bas1747::Tn916* mutant as the nap appeared less dense (Fig. 8B and D) relative to that of the wild-type spore nap but the individual fibrils appear in a more extended conformation (Fig. 8A and C). A small subpopulation of mutant spores, approximately 6%, had an exosporium wrapped twice or thrice around the spore (Fig. 9 and Table 4). These extra layers appeared to be exosporium layers as the nap layer was evident on both layers. Debris was also apparent in the *bas1747::Tn916* mutant spore samples, which also had the appearance of exosporium fragments (Fig. 8F) as compared with a representative field of Δ Sterne wild-type (Fig. 8E). Spores lacking an intact exosporium and several shed exosporia were present. Twelve per cent of the mutant spores lacked an exosporium and an additional 19% of spores appeared to have structural defects in the exosporium layer (Table 4).

4. Discussion

Much of what we know regarding the process of sporulation in the genus *Bacillus* comes from genetic studies with *Bacillus subtilis*, an organism whose spores lack a distinct exosporium layer. Although *B. subtilis* spore surfaces possess certain similarities to the surface of exosporium-containing spores (Sousa *et al.*, 1976; Waller *et al.*, 2004), they are not analogous structures. Known *B. anthracis* exosporium genes often have homologs present in the exosporium-containing *B. cereus* family of bacteria, but determinants with significant sequence similarity are usually absent in spore forming bacteria lacking a visible exosporium, including *B. subtilis*.

Our genetic system to identify genes involved in exosporium identification makes use of our understanding of the BclA nap protein on the surface of the exosporium. The system possesses sufficient sensitivity to identify mutants with quantitative defects in BclA incorporation onto the spore surface. An advantage of this genetic approach is that it can identify genes whose products affect exosporium development and which are not necessarily present in the spore (biosynthetic rather than structural roles). This genetic system can additionally be modified to use reporter fusions to other identified exosporium determinants to identify genes affecting the incorporation of these proteins, and thus identify exosporium biosynthetic pathways which may not show up in a BclA-based reporter system. Although we were successful in identifying mutants using the *Tn916* transposon, the system can be improved using the more promiscuous mariner transposon element which has been adapted for use with *B. anthracis* (Tam *et al.*, 2006; Wilson *et al.*, 2007).

Most of the genes identified to date using this system have gene annotations that are not helpful in assigning an exosporium biosynthetic role. Thus it was important to validate their involvement in the sporulation process. The genes characterized were found to be important

for incorporation of BclA onto the exosporium at wild-type levels. Based on the microarray studies by Hanna and coworkers (Liu *et al.*, 2004; Bergman *et al.*, 2006), the genes identified in Table 2 show some degree of expression at time points consistent with exosporium maturation. However, many do not show peak expression at late sporulation times. For example, peak expression of the *bas0389/bas0390* determinants occurs broadly over the logarithmic and stationary growth phases and yet the BAS0390 protein is incorporated into the spore five hours into the sporulation process.

Spore formation is a highly ordered process. Loss of one protein, whether it be a structural or a nonstructural protein involved in the assembly process, potentially impacts incorporation of downstream proteins. Assembly of the exosporium is likely to involve the same degree of hierarchical protein interdependency. Evidence for this was seen with BAS1747 and the inverse relationship with BclB. Loss of BAS1747 resulted in elevated spore levels of BclB. Similarly, lack of BclB resulted in elevated expression of BAS1747 in the spores. The presence of BAS1747 in different molecular weight complexes that are stable to boiling in SDS and urea, provides a means to biochemically identify the interactive partner or partners of this spore protein.

Inactivation of the *bas1747* determinant produced a subpopulation of spores exhibiting an exosporium assembly defect with multiple exosporium layers present. Assembly defects resulting in a double exosporium layer have been noted in *exsM* mutants of both *B. cereus* and *B. anthracis* (Fazzini *et al.*, 2010). The multiple exosporium layers in both the *exsM* and *bas1747* mutant spores retain the BclA nap. Thus the exosporium maturation process leading to nap formation occurs in an apparently normal fashion in these mutants. The similarity in phenotypes raises the possibility that ExsM and BAS1747 may function at the same stage in the exosporium assembly process.

The timing of expression, pattern of assembly, and effects on BclA all suggest that the products of the genes identified in this study are involved in exosporium assembly. The known exosporium proteins have expression patterns similar to BclA, driven off of σ^K promoter elements. Expansion of this genetic system should permit the identification of additional spore surface proteins which could serve as vaccine or diagnostic targets for exosporium-containing pathogens, as well as enhance the general understanding of the construction of this novel spore layer.

5. Conclusions

We have utilized the exosporium protein BclA as a target for a genetic screen to identify mutants of *B. anthracis* which exhibit defects in production of this outer spore nap layer. The system identifies mutants which affect the sporulation process after the expression of the *bclA* determinant and as such is enriched for mutants affecting maturation of the exosporium. Although many of the genes identified were not annotated as genes likely to be spore-associated, the mutated determinants characterized were found to affect BclA incorporation into the spore. In addition, the BAS1747 and BAS0390 proteins were found to be present in *B. anthracis* spores.

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Highlights

- We describe a novel screen for identification of exosporium gene mutants of *Bacillus anthracis*
- Mutants were generated by transposon mutagenesis
- Mutants in specific genes were identified
- Mutants were characterized to confirm their exosporium defect

Figure 1.

The exosporium gene identification system. A) A BHIA plate containing tetracycline and chloramphenicol inoculated with the *E. faecalis* + *B. anthracis* conjugation mixture and following incubation at 30°C for 1 week to permit sporulation. Colonies were exposed to a Dark Reader lamp. White arrows denote locations of colonies exhibiting reduced fluorescence. B) Cells from putative reduced fluorescence colonies from the initial plating were toothpick inoculated onto a BHIA plate containing tetracycline and chloramphenicol incubated at 30°C for 1 week to permit sporulation. A control culture exhibiting wild-type BclA-NTD-DsRed fluorescence is positioned at the right side of the plate.

Figure 2.

Flow cytometry quantification of overall fluorescence levels of BclA-NTD-DsRed in spores. Red line – wild-type (MUS1694) spores as a positive control, gray area – Δ Sterne spores (as a negative control). Panel **A**) MUS1751 (*bas1747::Tn916*) mutant spores (blue); **B**) MUS1754 (*bas0389::Tn916*) mutant spores (green); **C**) MUS1761 (*bas0605::Tn916*) mutant spores (purple); and **D**) MUS1753 (*bas1726::Tn916*) mutant spores (black).

Figure 3.

Western blot probing for BclA content in spore extracts. **A)** 1×10^8 spores were extracted with SDS + urea and the extract resolved on 4–20% SDS-polyacrylamide gradient gels. Proteins were transferred to PVDF membranes and probed for BclA using rabbit polyclonal anti-rBclA serum. Lane 1, Invitrogen MagicMark XP western size standard (220, 120, 100, 80, 60, 50, 40, 30 and 20 kDa); 2, Sterne; 3, MUS1776 (*bclA* null mutant); and 4, MUS1834 (*bas0389::Tn916*). **B)** Western blot with the anti-rBclA antiserum and reduced amounts of spore extract to better show differences in BclA levels. Lane 1, Δ Sterne; 2, MUS1776 (*bclA* null mutant); 3, MUS1834 (*bas0389::Tn916*); and 4, MUS8046 (*bas0389::Tn916* complemented strain).

Figure 4.

Transmission electron microscopy of *bas0389::Tn916* spores. **A, B)** Higher magnification and **C)** lower magnification TEM micrographs of spores from MUS1834. Bars represent 1 μm . Green arrows denote positions of the exosporium or spore coat fragments and the black arrows indicate sites of apparent loss of exosporium integrity. The presence of what appears to be free exosporia is evident in the background debris in panel C.

Figure 5. Spore incorporation of BAS0390-eGFP. **A–D**) Fluorescence micrographs of sporulating cells and spores of *B. anthracis* strain MUS1824. Samples were harvested at T₃ (panel **A**), T₅ (**B**), T₇ (**C**), and released spores (**D**). Yellow arrows denote the spore pole exhibiting decreased BAS0390-eGFP fluorescence.

Figure 6.

Western blot probing for BclA and BclB content in spore extracts. Purified spores were extracted with SDS + urea and the extract resolved on 4–20% SDS-polyacrylamide gradient gels. Proteins were transferred to PVDF membranes and probed for BclA (panel A) or BclB (panel B) using rabbit polyclonal sera. **A)** Lane 1, ΔSterne extract; 2, MUS1776, *bclA* null mutant extract; 3, MUS1836 (*bas1747::Tn916*); and 4, MUS8047 (*bas1747* complemented strain). **B)** Lane 1, ΔSterne extract; 2, MUS1691, *bclB* null mutant; 3, MUS1836 (*bas1747::Tn916*).

Figure 7.

Western blot showing reactivity to polyclonal r1747 antibodies. Proteins were extracted from different spore types and electrophoresed on a 4–20% polyacrylamide gel. Proteins were transferred to PVDF membranes and probed for BAS1747. Lane 1, Invitrogen MagicMark XP western standard (220, 120, 100, 80, 60, 50, 40, 30 and 20 kDa); 2, Δ Sterne extract; 3, MUS1776, *bclA* null mutant extract; 4, RG124, *bxpB* null mutant extract; 5, MUS1691, *bclB* null mutant extract; 6, RG56, *cotE* null mutant extract; 7, *bas1747::Tn916* spore protein extract.

Figure 8.

Transmission electron micrographs of Δ Sterne and *1747::Tn916* mutant spores. **A)** Δ Sterne wild-type spore with the BclA-nap layer. **B)** *1747::Tn916* mutant spore. The nap layer has an altered appearance relative to that of the wild-type spore, and the individual fibrils appear in a more extended form. **C)** Higher magnification of a Δ Sterne wild-type spore nap layer; **D)** Higher magnification of a *1747::Tn916* mutant spore nap layer. The red arrows denote the nap layers. **E)** Representative field of Δ Sterne wild-type spores. **F)** Representative field of *bas1747::Tn916* mutant spores. The black arrows denote spores lacking an exosporium and the blue arrows indicate some of the exosporium appearing debris.

Figure 9.

Transmission electron micrographs of *1747::Tn916* mutant spores with additional exosporium layers. Two examples of a small subpopulation of *bas1747::Tn916* mutant spores that exhibited multiple exosporium layers are shown. The arrows denote the additional layers of the exosporium, complete with their associated nap, wrapped twice (left panel) or thrice (right panel) around the spore.

Table 1

Strains and plasmids used in this study

Strain or Plasmid	Relevant Characteristics ^a	Source
Strains		
<i>Bacillus anthracis</i>		
Sterne	pXO1 positive, pXO2 negative	Lab stock
ΔSterne	Plasmid-free Sterne strain	Lab stock
MUS1691	ΔSterne <i>bclB</i> null; Kan ^r	(Thompson <i>et al.</i> , 2007)
MUS1694	ΔSterne pBT3777	(Thompson and Stewart, 2008)
MUS1751	ΔSterne <i>bas1747::Tn916</i> ; pBT3777	This study
MUS1753	ΔSterne <i>bas1726::Tn916</i> ; pBT3777	This study
MUS1754	ΔSterne <i>bas0389::Tn916</i> ; pBT3777	This study
MUS1761	ΔSterne <i>bas0605::Tn916</i> ; pBT3777	This study
MUS1776	ΔSterne <i>bclA</i> null; Kan ^r	(Thompson and Stewart, 2008)
MUS1824	ΔSterne pKS1824	This study
MUS1834	ΔSterne <i>bas0389::Tn916</i>	This study
MUS1835	ΔSterne <i>bas0605::Tn916</i>	This study
MUS1836	ΔSterne <i>bas1747::Tn916</i>	This study
MUS8046	MUS1834; pKS4395	This study
MUS8047	MUS1836; pKS4426	This study
RG56	Sterne <i>cotE</i> null; Kan ^r	(Giorno <i>et al.</i> , 2007)
RG124	Sterne <i>bxpB</i> null; Kan ^r	(Giorno <i>et al.</i> , 2007)
<i>Enterococcus faecalis</i>		
CG110	Tn916Tet ^r	(Gawron-Burke and Clewell, 1982)
<i>Escherichia coli</i>		
DH5	<i>endA1 hsdR17 (r_K⁻m_K⁺) glnV44 thi-1</i> <i>recA1 gyrA (NaF) relA1</i> <i>Δ(lacIZYA argF)U169</i> <i>deoR (φ80dlacΔ(lacZ)M15)</i>	Lab stock
GM48	<i>thr leu thi lacY galK galT ara fhuA tsx</i> <i>dam dcm glnV44</i>	Lab stock
SCS110	<i>rpsL thr leu endA thi-1 lacY galK galT ara</i> <i>tonA tsx dam dcm supE44 Δ(lac-proAB)</i>	Stratagene
M15	<i>lacZΔM15</i>	Qiagen
MUS3845	M15 pRep4 + pBT3845; Amp ^r , Kan ^r	This study
Plasmids		
pBT3777	pMK4 + <i>bclA-NTD-DsRed</i> ; Amp ^r , Cm ^r	(Thompson and Stewart, 2008)
pKS1824	pMK4 <i>bas0390-eGFP</i> ; Cm ^r	This study
pKS4168	pHP13 <i>bas0390</i> ; Cm ^r , Em ^r	This study
pKS4395	pHP13 <i>bas0389</i> ; Cm ^r , Em ^r	This study
pKS4426	pHP13 <i>bas1747</i> ; Cm ^r , Em ^r	This study

Strain or Plasmid	Relevant Characteristics ^a	Source
pMK4	Shuttle plasmid; Amp ^r , Cm ^r	(Sullivan <i>et al.</i> , 1984)
pQE30	His-tag expression vector; Amp ^r	Qiagen

^aCm^r, Tet^r, Kan^r, Em^r, Lm^r, and Amp^r denote resistance to chloramphenicol, tetracycline, kanamycin, 0.5 µg erythromycin ml⁻¹, 12.5 µg lincomycin ml⁻¹, and ampicillin, respectively.

Table 2

Sites of Tn916 Insertion

Sterne gene number	Gene annotation
BAS0047	SpoVG
BAS0389	Conserved hypothetical protein
BAS0483	Hypothetical protein
BAS0547	Citrate cation symporter family protein
BAS0605	Sodium/alanine symporter family protein
BAS1291	Hypothetical protein
BAS1726	Acetyl CoA hydrolase/transferase family protein
BAS1747	Hypothetical protein
BAS1820	Aminoglycoside 6-adenylytransferase, putative
BAS3594	Sensory box/GGDEF family protein

Table 3

Morphology of BAS0389-deficient spores

Strain	Number of spores with normal spore coat/exosporium	Number of spores with interspace fragments evident
Δ Sterne (wild-type)	342 (99.1%)	3 (0.9%)
MUS1834	233 (87.9%)	32 (12.1%)

Table 4

Percentage of the *basI747* mutant spores with a loss of structural integrity

Strain	Number of spores	Normal appearing exosporium	Exosporium- damaged spores	Spores without exosporium	Multiple exosporium layers
Δ Sterne	587	570 (97.1%)	6 (1%)	11 (1.9%)	0
MUS1836	590	382 (64.7%)	104 (17.6%)	69 (11.8%)	35 (5.9%)