Chapter 2
Spore Surface Components and Protective Immunity to *Bacillus anthracis*

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2.1 Introduction

Bacillus anthracis, the etiological agent of anthrax, is a Gram-positive, rod-shaped, aerobic soil bacterium. The disease affects all mammals, including man. Like other *Bacillus* species, *B. anthracis* forms spores in response to starvation. Mature spores are metabolically inactive cells and have a highly ordered structure. This structure contributes to resistance to extreme temperatures, radiation, desiccation, harsh chemicals, and physical damage *(1)*. These properties allow the spore to survive and persist for several decades in the soil until encountering environmental conditions favorable for germination. Entry of spores into the mammalian host is the initial event of anthrax infections and spores can infect the host via intradermal inoculation, ingestion, or inhalation. Fully virulent strains of *B. anthracis* carry two large plasmids, pXO1 and pXO2, which encode the primary virulence factors: toxin production and capsule formation responsible for toxemia and septicemia, respectively *(1)*. The capsule, a polymer of gamma-*D*-glutamic acid contributes to pathogenicity by enabling the bacteria to evade the host immune defenses. Curing strains of pXO2 yields attenuated toxinogenic strains with vaccinal properties, like the Sterne strain *(2)*.

As the surface of the spore represents the first point of contact with host defenses and a target for identifying specific detection markers, there has been much interest in its structure and composition.

2.1.1 Spore Surface Structure

Spores of *B. anthracis* and its closely related species *Bacillus cereus* and *Bacillus thuringiensis* possess an exosporium, which is the outermost structure surrounding the mature spore. It consists of a paracrystalline basal layer and a hair-like outer layer (Figure 2.1A). The exosporium is composed of proteins, lipids and carbohydrates *(3)*. It contains several proteins that are synthesized concomitantly with the cortex and the coat *(4)*. A *B. anthracis* exosporium glycoprotein, BclA, which is the structural component of the hair-like filaments (Figure 2.1A) has been identified *(5)*. BclA contains a central region presenting similarity to mammalian collagen proteins. This collagen-like region consists of GXX collagen-like triplets including a large proportion of GPT triplets. The number of GXX repeats varies considerably between strains and this variation is responsible for the length variation of the filament nap covering the outer layer of the exosporium *(6)*.

Most of the monoclonal antibodies raised against spores recognize epitopes of BclA. Some of these epitopes have been shown to be specific for *B. anthracis*, and represent efficient detection tools *(7, 8)*.

Spores of *bclA* deletion mutant strains are totally devoid of filaments *(5)*. However, the structure of the exosporium crystalline basal layer is unchanged and a crystalline organization can be observed on both the inner and the outer surface of this basal layer *(5)*. Several proteins have been described to be present in or tightly associated with the exosporium. ExsFA and ExsFB are required for the localization of BclA on the spore surface and contribute to the stability of the exosporium crystalline layers (*9*, *10*). ExsY and CotY proteins are also required for the correct assembly of the exosporium and/or coat *(11, 12)*. In *B. cereus*, the *cotY* deletion mutant spores have an intact exosporium surrounding the spore; however, they are affected in the assembly of the coat *(11)*. Similar observations were made with *B. anthracis* (P. Sylvestre, unpublished results). In contrast, *exsY* deletion mutant spores of *B. cereus* and *B. anthracis* lack an intact exosporium layer *(11, 12)*. Some fragments of the exosporium, still bearing the hairy nap, form a cap-like fragment covering one end of the spore. Other fragments can be observed free in the spore preparation (Figures 2.1C and 2.1E). Spores of the double deletion mutant *exsY cotY* are more severely affected and have significant exosporium and coat defects *(11)*. Fragments of coat and of

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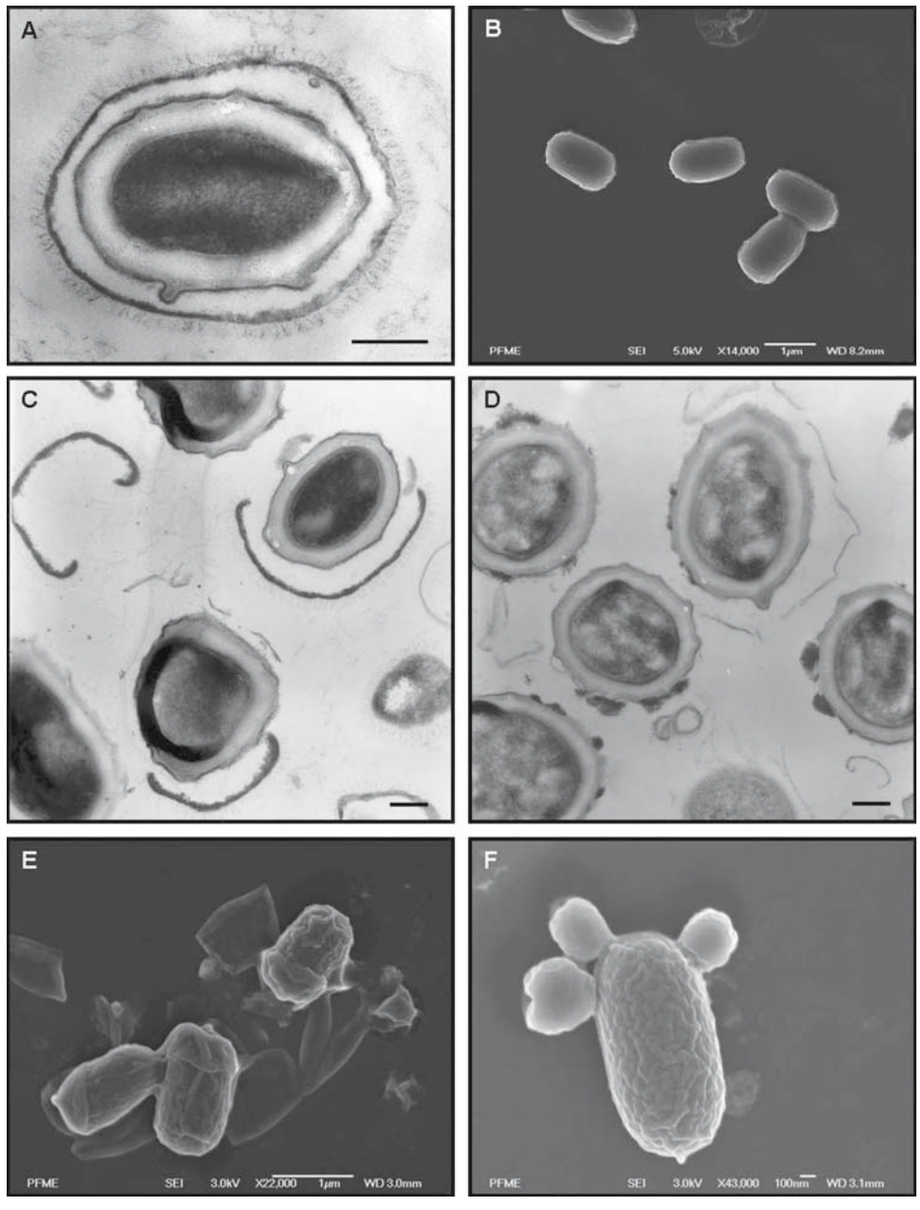

FIGURE 2.1. Electron micrographs of mutant spores of Sterne (A and B), Sterne Δ*exsY* (C and E), and Sterne Δ*cotY* Δ*exsY* (D and F) strains. Spores were analyzed by thin-section transmission electron microscopy (A, C and D) or scanning electron microscopy (B, E, and F). Scale bars in A, C, and D represent 200 nm.

exosporium-like layer, devoid of the BclA filaments, are found either associated to spores or free (Figures 2.1D and 2.1F).

2.2 Spores and Host Interaction

Because BclA is exposed on the spore surface, it is the first structure of the pathogen to interact with the host. This protein may play a role in the interaction of spores with host's cells and in the early steps of infection.

Spores of the Sterne 7702, 9602, and 4229 strains with a filament length of 60, 30, and 14 nm, respectively *(6)*, were analyzed for their adhesion properties on macrophages. As shown in Figure 2.2, similar results were obtained with the three strains (two to three spores/cell). Thus, the filament length does not influence spore adhesion to macrophages. In contrast, when spores of the Sterne *bclA* mutant strain were used, the absence of filaments led to an increase of spore adhesion to cells (16–23 spores/cell; Figure 2.2). The phenotype of the parental strain was restored when the mutant strain

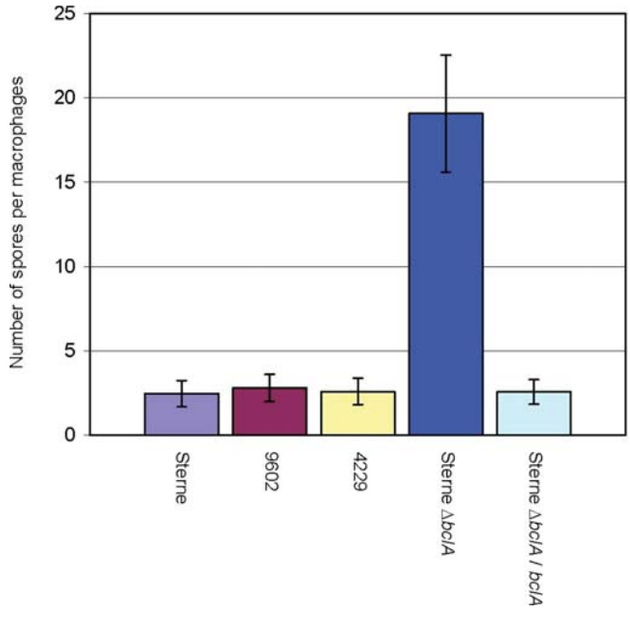

FIGURE 2.2. Spore adherence to macrophages. RAW264.7 macrophages were used to compare the adherence of spores of the parental strains Sterne 7702, 9602, and 4229, and the deletion mutant 7702 Δ*bclA* and the PF31 complemented deletion mutant *(6)*. The RAW264.7 cells were cultured on glass coverslips placed in 24-well tissue culture plates and were grown to semi-confluency (10^6 cells/well), as described previously *(22)*. Spores were prepared as previously described *(5)*, suspended in PBS and inoculated at a multiplicity of infection (MOI) of 10 spores per macrophage. The tissue culture plates were centrifuged for four minutes at 700 xg to promote contact between spores and macrophages and were incubated for 30 minutes at 4°C. The wells containing the glass cover slips were washed five times with PBS to remove the non adherent spores and the macrophage cells were fixed with PBS-2% paraformaldehyde and stained with May-Grünwald-Giemsa stain. The number of cell-associated spores was determined by counting 30 random macrophage cells in five different fields. The adherence assays were performed in triplicate, and the results were expressed as the mean number of spores per macrophage +/- standard error of the mean (*See Color Plates*).

was complemented for *bclA* (Figure 2.2). Therefore, BclA is involved in spore adhesion properties, and exposure of the exosporium basal layer components in the BclA-deficient mutant modifies the interaction of spore surface with cells.

The contribution of BclA to the virulence of *B. anthracis* was further investigated. In a previous work, no differences in virulence were observed between the attenuated toxinogenic Sterne strain and the Sterne *bclA* mutant, in a mouse subcutaneous model of infection *(5)*. Mutant strains deficient for BclA were constructed as previously described *(5)* in a fully virulent strain and in its capsulated toxin-inactivated derivative *(13)*; the virulence of this latter strain relies entirely on its multiplication properties leading to a rapid fatal septicemia in mice. No differences in virulence were observed when mice were challenged subcutaneously with either spores of the parental strains or spores of the *bclA* mutants (LD_{50} = 30 spores per mouse). Similar results were obtained when the experiments were performed using an intra-nasal route of infection, in which the LD_{50} of the strains was 5.10^5 spores per mouse. In conclusion, the differences in the adhesion properties of spores devoid of BclA did not modify the virulence of these strains, and BclA does not appear to contribute to the virulence of *B. anthracis*. These results are in agreement with those reported recently with a bclA mutant of the Ames strain tested in mice and guinea pigs *(14)*.

2.3 Spores and Protective Immunity

BclA is an immunodominant protein on the spore surface. Mice immunized with spores of the *B. anthracis* Sterne strain, either alive or inactivated, develop a strong response against BclA, which appears as the major band recognized by polyclonal antibodies on Western blots *(5)*. Other less immunogenic proteins, including ExsY, CotY, and ExsF proteins, can also be detected (*5*, *9*, *10*, *15*).

In animal studies, live spore vaccines are more protective than protective antigen (PA) alone, the cell binding component of the toxins *(16)*. Therefore, adaptive immunity to anthrax does not depend solely on control of toxemia through PA neutralizing antibodies. The addition of formaldehyde-inactivated spores (FIS) to PA has been shown to confer full protection against highly virulent strains of *B. anthracis* in mouse and guinea pig models of infection *(13)*. The contribution of specific spore antigens to immunoprotection, and mechanisms of the immune response to spores have been investigated *(17–19)*.

FIS vaccination induces the production of anti-spore antibodies *(13)*. Sera from mice immunized with FIS or with spores of the bclA mutant derivative (FISΔ*bclA)* were used in immunoblots against spore extracts to characterize the spore antigens recognized by the immune humoral response. The anti-FIS serum probed against the parental Sterne spore extract (Figure 2.3, lane 1) revealed a single strongly immunoreactive band at the top of the gel. This material has been characterized in previous studies as a complex of the immunodominant protein BclA and other spore components, including ExsY, CotY, and ExsF proteins (*5*, *9*, *10*, *15*). The serum reacted with other components of the high-molecular-weight complex when it was probed against an extract of spores deficient for BclA (Figure 2.3, lane 2), thus indicating that this complex can exist independently of BclA. Furthermore, two protein bands, at about 130 kDa and 60 to 70 kDa, could be detected in this extract. This suggests that the lack of BclA facilitates the extraction of additional antigens, which are, however, accessible to the immune system during immunization with FIS. The anti-FISΔ*bclA* serum was probed against the same spore extracts (Figure 2.3, lanes 3 and 4). Interestingly, this serum recognized an additional protein band at about 25 kDa present in both spore extracts. Otherwise the recognition pattern did not vary significantly from that obtained with the anti-FIS serum. Therefore, the 25 kDa antigen, which remains to

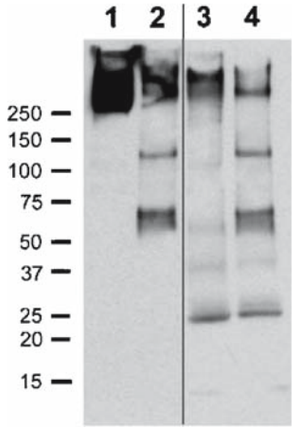

FIGURE 2.3. Characterization of the humoral immune response. Anti-FIS (1 and 2) or anti-FIS Δ*bclA* (3 and 4) sera were analyzed by immunoblotting against spore extract proteins of Sterne (1 and 3) and Sterne Δ*bclA* (2 et 4) strains separated by SDS-PAGE 4 to 15%.

TABLE 2.1. Protective immunity induced in mice after immunization with FIS or FIS*ΔbclA* +/- PA.

	Protection[b] (%) against challenge with :			
	Fully virulent strain (tox+/cap+)		Capsulated strain (tox-/cap+)	
Immunization[a]		Δ*bclA*		Δ*bclA*
FIS	0	0	60–80	60–80
FIS*ΔbclA*	0	0	60–80	60–80
FIS + PA	100	100	100	ND
FIS*ΔbclA* + PA	100	100	100	ND

[a]Mice (groups of 10 or 12) were immunized as previously described (13) with 10^8 FIS or 10^8 FIS*ΔbclA* +/– 10μg PA and were challenged subcutaneously with 10 LD_{50}s

[b]Number of surviving/number challenged is expressed as percent. ND, not determined

be identified, becomes accessible to the immune system, only when spores used for immunization are devoid of BclA.

To determine if BclA contributes to protective immunity, the efficacies of FIS and of FIS*ΔbclA* were compared in protection assays after immunization of mice. As shown in Table 2.1, mice immunized with FIS or FIS*ΔbclA* were similarly partially (60–80%) protected against a challenge with the non-toxinogenic capsulated strain or with its Δ*bclA* derivative. All the animals immunized with the combinations PA plus FIS or PA plus FIS*ΔbclA* were totally protected against a challenge with the fully virulent parental strain or its Δ*bclA* derivative. These data indicate that, as previously described, spores are essential for protection against infection with *B. anthracis*; however, the immunodominant BclA protein does not contribute to their immunoprotective properties.

To elucidate the mechanisms of the adaptive immune protection induced by immunization with FIS that functions independently of toxin neutralization, the resistance of mice to the capsulated toxin-inactivated bacteria was further studied.

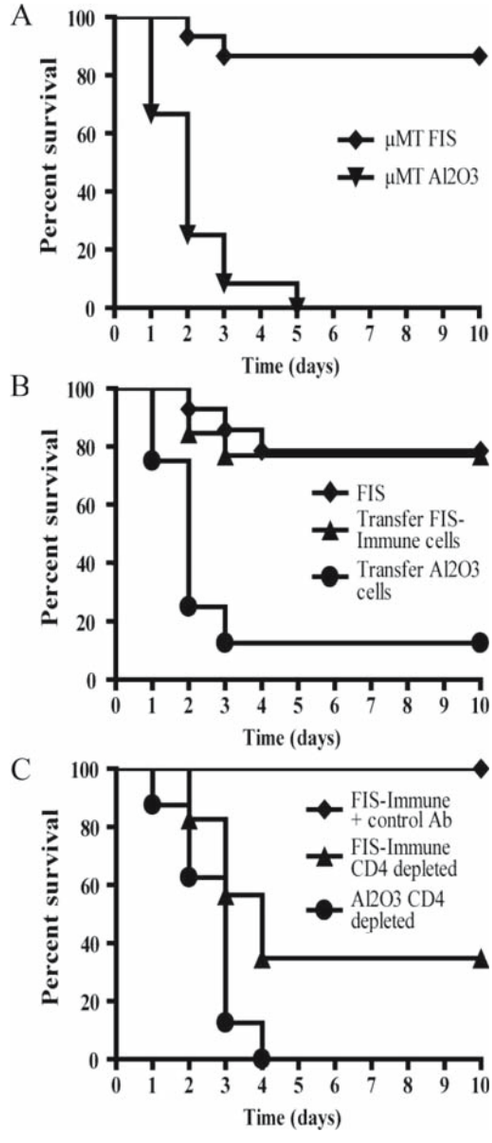

FIGURE 2.4. FIS-immune CD4 T lymphocytes are protective. (A) FIS-immunized μMT -/- (μMT FIS) or adjuvant-treated μMT (μMT Al2O3) were challenged subcutaneously with 200 LD_{50}s of a capsulated non-toxinogenic strain. (B) Splenocytes from FIS-immunized mice (Transfer FIS-Immune cells) or adjuvant treated (Transfer Al2O3 cells) mice were transferred to naïve mice and challenged as in A. A FIS-immune (FIS) control was included. (C) FIS-immune mice were treated *in vivo* with anti-CD4 antibodies (FIS-Immune CD4 depleted) or an irrelevant antibody control (FIS-Immune + control Ab), and then challenged as in A. Anti-CD4 treatment completely eliminated CD4 T lymphocytes. Adjuvant-treated mice were also treated with anti-CD4 antibody as controls (Al2O3 CD4 depleted).

Because FIS vaccination induces the production of anti-spore antibodies, we thus questioned whether humoral immunity was the mediator of FIS-induced immunity. Transfer of FIS-immune serum did not protect naïve mice from infection *(20)*. Additionally, as shown in Figure 2.4A, μMT -/- mice, which

do not produce antibodies *(21)*, were protected by FIS immunization. Together these data suggest that humoral immunity is not sufficient to protect FIS-immunized mice from infection by capsulated non-toxinogenic *B. anthracis.* We therefore hypothesized that cellular immunity functioned as the mediator of FIS-induced protection. Splenocytes were isolated from FIS-immunized mice and transferred to naïve mice, prior challenge. FIS-immunized splenocytes significantly protected naïve mice from infection, whereas splenocytes from adjuvant-only treated mice did not (Figure 2.4B). *In vivo* depletion of CD4 T lymphocytes significantly reduced the ability of FIS-immune mice to resist *B. anthracis* infection (Figure 2.4C). Furthermore, the transfer of purified FIS-immune CD4 T lymphocytes protected naïve mice from *B. anthracis* challenge, while CD4-depleted splenocytes did not *(20)*. These FIS-immune CD4 T lymphocytes reacted to FIS in an MHC-restricted manner with the production of IFN-γ. Furthermore, *in vivo* depletion of IFN-γ eliminated most of the protection induced by FIS-immunization *(20)*. The protective spore-associated antigens recognized by CD4 T lymphocytes remain to be determined, yet because the spore is a highly complex structure; it is likely that no single antigen will be solely responsible for the protective response.

These results are the first evidence that a protective immunity provided by spore immunization relies on cell mediated immunity involving CD4 T lymphocytes secreting gamma interferon as an absolute requisite for effective protection *(20)*. Thus, combination of a spore-reactive cellular immunity with a toxin-neutralizing humoral immunity will result in enhanced protection against *B. anthracis* infection.

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