Contribution of ExsFA and ExsFB Proteins to the Localization of BclA on the Spore Surface and to the Stability of the *Bacillus anthracis* Exosporium

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Received 15 February 2005/Accepted 27 April 2005

Spores of *Bacillus anthracis*, the etiological agent of anthrax, and the 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. To date, the structural contribution of only one exosporium component, the collagen-like glycoprotein BclA, has been described. It is the structural component of the hair-like filaments. Here, we describe two other proteins, ExsFA and ExsFB, which are probably organized in multimeric complexes with other exosporium components, including BclA. Single and double *exsF* deletion mutants were constructed and analyzed. We found that inactivation of *exsF* genes affects the BclA content of spores. BclA is produced by all mutants. However, it is partially and totally released after mother cell lysis of the $\Delta exsFA$ and $\Delta exsFA \Delta exsFB$ mutant strains, respectively. Electron microscopy revealed that the *exsF* mutant spores have defective exosporia. The $\Delta exsFA$ and $\Delta exsFA \Delta exsFB$ spore surfaces are partially and totally devoid of filaments, respectively. Moreover, for all mutants, the crystalline basal layer appeared unstable. This instability revealed the presence of two distinct crystalline arrays that are sloughed off from the spore surface. These results indicate that ExsF proteins are required for the proper localization of BclA on the spore surface and for the stability of the exosporium crystalline layers.

Bacillus anthracis, the etiological agent of anthrax, is a gram-positive, rod-shaped, aerobic soil bacterium. 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, 9, 35). These properties allow the spore to survive and persist for several decades in the soil until it encounters 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 (8).

In the genus *Bacillus*, the sporulation process begins by an asymmetric septation in the vegetative cell that produces two genome-containing compartments, one large compartment called the mother cell and one small compartment called the forespore. The mother cell then engulfs the forespore, surrounding it with two cell membranes in opposite orientation. Over the course of several hours, several protective structures assemble inside and around the forespore. The forespore is surrounded with a thick layer of modified peptidoglycan, called the cortex, and a multilayered protein shell, called the coat, that covers the cortex. The cortex and the coat contribute to spore protection and to germination (9–11). After a final step of spore maturation, the surrounding mother cell lyses and liberates the mature spore.

In some bacillus species, including *Bacillus subtilis*, the coat is the outermost layer of the spore. In others, including *B.*

anthracis and the closely related species *Bacillus cereus* and *Bacillus thuringiensis*, there is an additional outer structure, called the exosporium. Electron microscopy has revealed that the exosporium is composed of a paracrystalline basal layer with hexagonal periodicity and a hair-like outer layer (2, 16, 18, 20). The basal layer contains two or more crystalline sublayers (2, 12, 16, 43) and the hair-like nap differs in length between strains and between species (16, 20, 38). There is still little known about the structural components of the exosporium crystalline array, whereas the exterior components of the vegetative cell of *B. anthracis* have been well characterized (13). In rich medium in vitro, *B. anthracis* synthesizes successively one of two S-layers, one composed of SAP protein and the other composed of EA1 protein. These two S-layer proteins are organized in two-dimensional crystalline arrays (5, 26). Furthermore, during infection in vivo, a poly- γ -D-glutamate capsule completely covers an S-layer, which is exclusively composed of EA1 protein (27).

The exosporium is composed of proteins, lipids, and carbohydrates (2, 19, 24, 34). It contains several proteins that are synthesized concomitantly with the cortex and the coat (2, 7, 30). Exosporium-specific glycoproteins are synthesized by *B. thuringiensis* and by *B. cereus* (4, 15, 39). Recently, we identified a *B. anthracis* exosporium glycoprotein (BclA), which is the structural component of the hair-like filaments (37). Spores of $\Delta bclA$ strains are totally devoid of filaments (37, 38). 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 (37).

BclA contains a central region presenting similarity to mammalian collagen proteins. This collagen region consists of GXX collagen-like triplets, including a large proportion of GPT triplets. The number of GXX repeats varies considerably between

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TABLE 1. *Bacillus anthracis* strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or nucleotide	Genotype or description	Marker ^a	Source or reference
Strains			
7702	Sterne strain (pXO1 ⁺)		Laboratory stock
PF50	7702 Δ <i>exsFA</i>	Er ^r	This work
PF51	7702 Δ <i>exsFA</i>	Sp ^r	This work
PF55	7702 Δ <i>exsFB</i>	Er ^r	This work
PF56	7702 Δ <i>exsFA</i> Δ <i>exsFB</i>	Er ^r Sp ^r	This work
PF60	7702 Δ <i>exsFA</i> <i>eag::exsFA</i>	Er ^r Sp ^r	This work
PF65	7702 Δ <i>exsFB</i> <i>eag::exsFB</i>	Er ^r Sp ^r	This work
SM17	7702 Δ <i>sap</i> Δ <i>eag</i>	Sp ^r	Given by Mesnage (laboratory stock)
PF08	7702 Δ <i>sap</i> Δ <i>eag</i> Δ <i>bclA</i>	Er ^r Sp ^r	This work
Plasmids			
pUC19	Cloning vector	Ap ^r	44
pUC1318Spc	Spectinomycin resistance cassette harboring plasmid	Ap ^r Sp ^r	29
pUC1318Erm	Spectinomycin resistance cassette harboring plasmid	Ap ^r Er ^r	41
pGEM-T-Easy	Cloning vector	Ap ^r	Promega
pGEM- <i>exsFA</i>	pGEM-T-Easy carrying the <i>exsFA</i> gene	Ap ^r	This work
pQE30	<i>E. coli</i> overexpression IPTG-inducible vector	Ap ^r	Qiagen
pQE30- <i>exsFA</i>	pQE30 carrying the ExsFA-encoding sequence	Ap ^r	This work
pAT113	Conjugative suicide plasmide	Kn ^r Er ^r	42
pFX113	Conjugative suicide plasmide	Kn ^r Sp ^r	3
pSAL322	Conjugative suicide plasmide carrying deleted <i>eag</i> gene	Kn ^r Er ^r Sp ^r	25
pXP01	pUC19 carrying the <i>exsFA</i> gene	Ap ^r	This work
pXP02	pUC19 carrying the <i>exsFB</i> gene	Ap ^r	This work
pXP03	pAT113 carrying the <i>exsFA</i> gene	Kn ^r Er ^r	This work
pXP04	pFX113 carrying the <i>exsFA</i> gene	Kn ^r Sp ^r	This work
pXP05	pFX113 carrying the <i>exsFB</i> gene	Kn ^r Sp ^r	This work
pXP06	pAT113 carrying the disrupted <i>exsFA</i> gene	Kn ^r Er ^r Sp ^r	This work
pXP07	pFX113 carrying the disrupted <i>exsFA</i> gene	Kn ^r Sp ^r Er ^r	This work
pXP08	pFX113 carrying the disrupted <i>exsFB</i> gene	Kn ^r Sp ^r Er ^r	This work
pXP09	pSAL322 carrying the <i>exsFA</i> gene	Kn ^r Er ^r Sp ^r	This work
pXP10	pSAL322 carrying the <i>exsFB</i> gene	Kn ^r Er ^r Sp ^r	This work
pSP15	pFX113 carrying the disrupted <i>bclA</i> gene	Kn ^r Er ^r Sp ^r	38
Oligonucleotides			
ex1F3P	5' GATCCAATCTGTACATTCCTAGCTGTACC 3'		
ex1R1P	5' GCAGCAGCAATCATTGAAACGATTACAGG 3'		
ex2F1P	5' GATCGAAGAGCATTCCCTTAGGTACTC 3'		
ex2R1P	5' GCACACATATAAGTGTGCTTTTATGAACGC 3'		
ex1F5B	5' GGATCCTTCTCTTCTGATTGCGAATTTAC 3'		
exR5H	5' AAGCTTCTAGCTAATTTGTGCAACTG 3'		

^a Ap^r, Er^r, Kn^r and Sp^r, resistance to ampicillin, erythromycin, kanamycin, and spectinomycin, respectively.

strains, and this variation is responsible for the length variation of the filament nap covering the outer layer of the exosporium (38). More recently, two O-linked carbohydrate components attached to BclA, a 715-Da tetrasaccharide and a 324-Da disaccharide, have been identified (6). Multiple copies of the tetrasaccharide are linked to the collagen-like region of BclA, whereas the disaccharide may be attached outside of this region; each oligosaccharide may be attached to BclA via a GalNAc linker. The 715-Da tetrasaccharide is composed of three rhamnose residues and an unusual component, named anthrose [i.e., 2-O-methyl-4-(3-hydroxy-3-methylbutamido)-4,6-dideoxy- β -D-glucose]. The complete structure of this tetrasaccharide has been determined: it is a 2-O-methyl-4-(3-hydroxy-3-methylbutamido)-4,6-dideoxy- β -D-glucopyranosyl-(1 $\rightarrow$ 3)- α -L-rhamnopyranosyl-(1 $\rightarrow$ 3)- α -L-rhamnopyranosyl-(1 $\rightarrow$ 2)-L-rhamnopyranose. The 324-Da disaccharide is composed of one rhamnose residue and a component identified as 3-O-methyl-rhamnose.

Various studies have attempted to identify other individual

proteins from the exosporia of *B. cereus* and *B. anthracis* (4, 22, 33, 36, 39). Several proteins have been described to be present in or tightly associated with the exosporium, but their roles in the structural and functional organization of the exosporium remain to be elucidated. Here, we describe two exosporium proteins, ExsFA and ExsFB, which are required for the localization of BclA on the spore surface and which contribute to the stability of the exosporium crystalline layers.

MATERIALS AND METHODS

Bacterial strains, plasmids, culture media, and preparation of spores. *Escherichia coli* TG1 (23), HB101(pRK212.1) (40), and M15(pREP4) (QIAGEN) were used for cloning procedures, mating experiments, and heterologous protein production, respectively. The *B. anthracis* strains and cloning vectors used in this study are listed in Table 1.

E. coli was grown in L broth or on L agar (28). *B. anthracis* was grown in brain heart infusion medium (Difco Laboratories) and on NBY medium for spore preparation (17). Antibiotic concentrations in the media were as follows: for both *E. coli* and *B. anthracis*, ampicillin, 100 μ g/ml; kanamycin, 40 μ g/ml; and spec-

tinomycin, 100 µg/ml. For *E. coli*, erythromycin was added at a concentration of 180 µg/ml; for *B. anthracis*, the concentration was 5 µg/ml.

B. anthracis spores were prepared as previously described (37).

Preparation of exosporium and cell extracts. The exosporium from strain PF08, used for antibody preparation, was prepared as previously described (37).

Spore extracts were prepared by treatment of spores with extraction buffer containing 50 mM Tris-HCl, pH 10, 8 M urea, and 2% 2-mercaptoethanol, as previously described (37). For analysis of BclA synthesis in sporulating cells, *B. anthracis* was cultivated in PA medium (20 ml) as previously described (37). Culture samples (1 ml) were taken 8 and 20 h (T_{+8} and T_{+20}) after entry into sporulation, and cells were pelleted. Cell pellets were resuspended in 1/20 of their initial volume (50 µl) in extraction buffer containing 2% sodium dodecyl sulfate (SDS) and treated as previously described (37). For supernatant analysis, the proteins were precipitated with 10% (vol/vol) trichloroacetic acid and resuspended in 1/50 of their initial volume (20 µl) in Laemmli buffer (21). For immunoblot analysis, 5 µl of cell extract and of trichloroacetic acid-precipitate material were loaded.

Immunoblotting analysis. Proteins were diluted in Laemmli buffer (21), separated by SDS-12% polyacrylamide gel electrophoresis (PAGE) or SDS-7% PAGE, and transferred onto nitrocellulose sheets (Hybond N; Amersham) with the Bio-Rad Trans-Blot system. The resulting blots were probed with the mouse anti-*ΔbclA* exosporium antiserum and with the anti-ExsFA antiserum, both of them used at a 1/2,000 dilution, or with the anti-BclA 35B8 monoclonal antibody used at 1 µg/ml (37). Blots were developed with the ECL Western blotting analysis system (Amersham), diluting the second antibody 1/20,000.

Overproduction and purification of ExsFA. The plasmid pQE30-*exsFA* was used to transform *E. coli* strain M15(pREP4) (QIAGEN) and the His₆-ExsFA protein was overexpressed as described by QIAGEN by inducing an exponential-phase culture with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h at 37°C. His-tagged ExsFA was purified as described by QIAGEN on a Ni-nitrilotriacetic acid agarose column. Purification was performed under denaturing conditions in buffer B (100 mM NaH₂PO₄, 10 mM Tris-HCl, 8 M urea, pH 8) containing 25 mM imidazole. His₆-ExsFA was eluted with buffer B containing 0.3 M imidazole. The protein was dialyzed extensively against phosphate-buffered saline and used for preparation of mouse antisera and immunoblotting analysis.

Antibody preparation. Mouse anti-*ΔbclA* exosporium and anti-ExsFA polyclonal antibodies were obtained after three injections in incomplete Freund adjuvant (purchased from Sigma), at 2-week intervals of 50 µg of exosporium preparation or 20 µg of recombinant ExsFA protein, respectively.

Plasmid constructions. The DNA fragments encoding the *exsFA* and *exsFB* genes were amplified by PCR from chromosomal DNA of *B. anthracis* 7702. ex1F3P and ex1R1P were used as primers for *exsFA*, and ex2F1P and ex2R1P were used for *exsFB* (Table 1).

The *exsFA* and *exsFB* amplicons were inserted into the HincII site of pUC19 to give pUC19-*exsFA* (pXP01) and pUC19-*exsFB* (pXP02).

pAT113-*exsFA* (pXP03), pFX113-*exsFA* (pXP04) and pFX113-*exsFB* (pXP05) containing the genes *exsFA* and *exsFB* were constructed by insertion of the *exsFA* and *exsFB* amplicons into pAT113 or into pFX113, previously digested with EcoRI and blunt-ended with Vent polymerase.

pAT113-*exsFA*-Spc (pXP06), pFX113-*exsFA*-Erm (pXP07) and pFX113-*exsFB*-Erm (pXP08) containing the disrupted genes *exsFA* and *exsFB* were constructed as follows: pAT113-*exsFA* (pXP03), pFX113-*exsFA* (pXP04), and pFX113-*exsFB* (pXP05) were digested with EcoRI, blunt ended with Vent polymerase, and then ligated to the XbaI fragment of pUC1318Spc or the SmaI fragment of pUC1318Erm containing the spectinomycin resistance and erythromycin resistance cassettes, respectively.

The *exsFA* and *exsFB* genes were also inserted into the *eag* gene as follows. The BamHI-HindIII fragments of pUC19-*exsFA* (pXP01) and pUC19-*exsFB* (pXP02) were blunt ended with Vent polymerase and ligated to pSAL322, itself digested with BamHI and blunt ended to give pSAL322-*exsFA* (pXP09) and pSAL322-*exsFB* (pXP10), respectively.

To overproduce and purify ExsFA, a DNA fragment corresponding to the *exsFA* sequence was amplified by PCR with oligonucleotides ex1F5B and ex1R5H (Table 1). These primers added BamHI and HindIII restriction sites just 3' to the start codon and just 3' to the stop codon of the *exsFA* gene, respectively. This amplicon was inserted into the pGEM-T-Easy plasmid to give pGEM-*exsFA*. The 0.5-kb BamHI-HindIII fragment from pGEM-*exsFA* was ligated into the overexpression vector pQE30 containing a N-terminal six-His tag, itself digested with BamHI and HindIII, to give pQE30-*exsFA*.

Construction of recombinant strains. Recombinant plasmids were transferred from *E. coli* to *B. anthracis* by heterogramic conjugation (40). Allelic exchange was carried out as described by Pézard and collaborators (31).

pXP06 and pXP07 were introduced into strain 7702 to obtain the recombinant

strains PF50 (7702 *ΔexsFA*; Er^r) and PF51 (7702 *ΔexsFA*; Sp^r), respectively. pXP08 was introduced into strains 7702 and PF51 to obtain the recombinant strains PF55 (7702 *ΔexsFB*; Er^r) and PF56 (7702 *ΔexsFA ΔexsFB*; Sp^r Er^r).

The recombinant strains PF60 (7702 *ΔexsFA eag::exsFA*) and PF65 (7702 *ΔexsFB eag::exsFB*) were obtained by allelic exchange between *eag::exsFA* or *eag::exsFB* in pXP09 or pXP10 and the chromosomal *eag* locus in PF50 or PF55 strains.

pSP15 was introduced into strain SM17 to obtain the recombinant strain PF08 (7702 *Δsap Δeag ΔbclA*).

Electron microscopy. Spores were analyzed after rehydration for 15 min and negative staining as previously described (37).

RESULTS AND DISCUSSION

Isolation of an exosporium component. We previously described the BclA glycoprotein, which is the structural component of the exosporium filaments. BclA was identified by purification of the corresponding band from polyacrylamide gel and amino-terminal amino acid sequencing (37). In addition to the N-terminal sequence of BclA, another sequence (MFSSD CEFT) was obtained. This sequence was used to search for matches with proteins encoded by the genome sequence of the Ames strain of *B. anthracis* (32). It matched an open reading frame (BA1237) encoding a protein of 168 amino acids, previously named BxpB by Steichen and collaborators (36) and ExsF by Todd and collaborators (39). The corresponding gene is located in a gene cluster near the region encoding BclA. Indeed, the *bxpB/exsF* and *bclA* genes are distant (only 10 kb apart) and are separated by 13 genes. These genes include, among others, the rhamnose operon *rmLACBD*, which encodes the L-rhamnose biosynthetic enzymes, and genes that encode enzymes for polysaccharide biosynthesis (i.e., glycosyltransferase and O-methyl transferase) (6, 14, 39). All these genes, which are expressed during stage IV of sporulation when the exosporium is synthesized (22), may be involved in the BclA glycosylation.

We chose to use the name proposed by Todd and collaborators (39), and the gene was designated *exsFA*. The *B. anthracis* genome also contains a paralogue (BA2477) of this gene, from which it is distantly separated on the chromosome by about 1.1 Mb. By analogy, we named this second gene *exsFB*. The predicted ExsFB protein also contains 168 amino acids and presents 78% (131 of 168 amino acids) identity with ExsFA. The predicted molecular masses of these proteins are about 17 kDa. Most of the differences between these two proteins are in the N-terminal region. *exsF* genes are also found in the genome of the sequenced *B. cereus* and *B. thuringiensis* strains, and the corresponding proteins present between 96 and 100% identity with *B. anthracis* ExsF proteins. No similarity was found between ExsF proteins and other proteins in databases.

Spore protein profiles of *exsF* mutants. *exsF* knockout mutants were constructed to facilitate investigations of the role of the *exsF* gene products in spore structure: a single *exsFA* mutant, a single *exsFB* mutant, and a mutant lacking both the *exsFA* and *exsFB* genes. Protein profiles of spore extracts of the parental and mutant strains were analyzed by SDS-12% PAGE and immunodetection. No difference was observed after Coomassie blue staining (data not shown). Western blotting with polyclonal antibodies raised against the exosporium of a *ΔbclA* strain was used to characterize exosporium components other than BclA (Fig. 1A). Several protein bands were

FIG. 1. Analysis of spore extracts from strain 7702 and from *exsF* mutants. Proteins were separated by SDS-12% PAGE and analyzed by immunoblotting with polyclonal antibodies raised against $\Delta bclA$ exosporium (A) or against recombinant ExsFA (B). The same extracts were loaded in lanes 1 to 5 for panels A and B. Lanes 1, strain 7702; lanes 2, strain 7702 $\Delta exsFA$ (PF51); lanes 3, strain 7702 $\Delta exsFA$ *eag::exsFA* (PF60); lanes 4, strain 7702 $\Delta exsFB$ (PF55); and lanes 5, strain 7702 $\Delta exsFA$ $\Delta exsFB$ (PF56). For B, lane 6, recombinant ExsFA protein. The sizes of the molecular mass markers are given in kilodaltons in the left margin.

recognized in the parental spore extract (Fig. 1A, lane 1). There was also a broad strongly immunoreactive band extending from the insoluble fraction at the top of the gel (stacking gel). This band was also present in the strain in which the *exsFA* gene had been inactivated. However, several protein bands (15, 16, 17, 35, and 37 kDa) were missing from the immunoblot profile of the $\Delta exsFA$ strain (Fig. 1A, lane 2). The same extracts were also analyzed with polyclonal antibodies raised against a recombinant ExsFA protein (Fig. 1B). Different forms of ExsFA were detected in the parental spore extract: in the insoluble fraction at the top of the gel and in the three protein bands at about 17, 16, and 15 kDa (Fig. 2B, lane 1). As expected, no reactive material was detected in the extract of the $\Delta exsFA$ strain (Fig. 2B, lane 2). The band at about 17 kDa corresponded to the predicted size of the *exsFA* gene

product and ran at the same level as the ExsFA recombinant protein (Fig. 2B, lane 1 and lane 6). The two other bands, at about 15 to 16 kDa, probably corresponded to partial degradation and/or processing products of the ExsFA protein (Fig. 2B, lanes 1 and 2). In contrast, the two components around 35 to 37 kDa, which were recognized with the anti- $\Delta bclA$ exosporium antibodies, were not detected by the anti-ExsFA antibodies (Fig. 2A and B, lanes 1). Therefore, these may be spore components requiring the *exsFA* gene product for their assembly on the spore. A complementation test was performed by integration of the parental *exsFA* gene with its promoter region at the chromosomal *eag* locus encoding the S-layer protein EA1. This insertion restored the parental banding pattern to the complemented $\Delta exsFA$ strain (Fig. 1A and B, lanes 3). Therefore, the loss of ExsFA accounts for the modified protein profile of the $\Delta exsFA$ spore extract. These observations suggest that ExsFA is present in the spore as a monomer (17 kDa) and in higher-molecular-weight forms. In contrast, the strain in which the *exsFB* gene had been inactivated gave patterns indistinguishable from those of the parental strain patterns (Fig. 1A and B, lanes 4). In the double mutant in which both the *exsFA* and *exsFB* genes were disrupted, the patterns were similar to those observed for the $\Delta exsFA$ mutant (Fig. 1A and B, lanes 5). However, the intensity of the high-molecular-mass band detected with the anti- $\Delta bclA$ exosporium antibodies was significantly reduced (Fig. 1A, lane 5). Thus, ExsF proteins may be involved in relatively stable multimeric complexes, possibly including several exosporium components. These observations are consistent with those obtained by Redmond and collaborators (33), using peptide sequence analysis. They found that ExsY, CotY, and ExsF exosporium proteins are present in high-molecular-mass complexes, as well as in monomeric form.

ExsF products are required for BclA localization on the spore surface. As *exsF* gene products may affect high-molecular-mass spore protein complexes and because ExsFA has been copurified with the high molecular BclA protein, spore extracts of the corresponding mutants were analyzed for their BclA content. The protein profiles of spore extracts of the parental and *exsF* mutant strains were analyzed by SDS-7% PAGE and Coomassie blue staining. Under these conditions, BclA was observed as a high-molecular-mass (>250 kDa) component (Fig. 2A). There was less of this component in $\Delta exsFA$ spores (Fig. 2A, lane 2) than in wild-type spores (Fig. 2A, lane 1). Its abundance was not affected in the $\Delta exsFB$ mutant spores (Fig. 2A, lane 4), but it was totally absent from the spores of the double mutant $\Delta exsFA$ $\Delta exsFB$ (Fig. 2A, lane 5). These findings were confirmed by immunoblot analysis with anti-BclA monoclonal antibody: BclA was present in the parental and $\Delta exsFB$ strains (Fig. 2B, lanes 1 and 4), less abundant in spore extracts of the $\Delta exsFA$ (Fig. 2B, lane 2), and totally absent from those of $\Delta exsFA$ $\Delta exsFB$ mutants (Fig. 2B, lane 5). In the complemented $\Delta exsFA$ strain, BclA was restored to spore extracts (Fig. 2A and B, lanes 3). These results indicate that inactivation of the *exsF* genes affects the BclA content of spores.

To investigate whether ExsF proteins are involved in BclA biosynthesis or in the localization of BclA on the spore surface, we followed the synthesis of BclA in the parental $\Delta exsFA$ and $\Delta exsFA$ $\Delta exsFB$ strains during the late stages of sporulation.

FIG. 2. Analysis of spore extract proteins from strain 7702 and from *exsF* mutants after separation by SDS-7% PAGE. The same extracts were loaded for analysis by Coomassie brilliant blue staining (A) and by immunoblotting using anti-BclA monoclonal antibody (B). Lanes 1, strain 7702; lanes 2, strain 7702 $\Delta exsFA$ (PF51); lanes 3, strain 7702 $\Delta exsFA$ *eag::exsFA* (PF60); lanes 4, strain 7702 $\Delta exsFB$ (PF55); and lanes 5, strain 7702 $\Delta exsFA$ $\Delta exsFB$ (PF56). The sizes of the molecular mass markers are given in kilodaltons in the left margin.

FIG. 3. Analysis of BclA synthesis in strains 7702, 7702 Δ *exsFA* (PF51), and 7702 Δ *exsFA* Δ *exsFB* (PF56). Proteins were separated by SDS-7% PAGE and analyzed by immunoblotting with anti-BclA monoclonal antibody. Lane 1, 7702 mature spores; lane 2, PF51 mature spores; lane 3, PF56 mature spores; lane 4, 7702 cell extract (T_{+8}); lane 5, 7702 supernatant (T_{+8}); lane 6, PF51 cell extract (T_{+8}); lane 7, PF51 supernatant (T_{+8}); lane 8, PF56 cell extract (T_{+8}); lane 9, PF56 supernatant (T_{+8}); lane 10, 7702 cell extract (T_{+20}); lane 11, 7702 supernatant (T_{+20}); lane 12, PF51 cell extract (T_{+20}); lane 13, PF51 supernatant (T_{+20}); lane 14, PF56 cell extract (T_{+20}); and lane 15, PF56 supernatant (T_{+20}). The sizes of the molecular mass markers are given in kilodaltons in the left margin.

BclA is produced in the mother cell during sporulation (37). The growth and sporulation curves of the mutant strains were similar to those of the parental strain (data not shown), and culture samples were taken 8 and 20 h (T_{+8} and T_{+20}) after entry into sporulation (T_0). At T_{+8} , >95% of the sporulating cells contained a prespore; at T_{+20} , only mature free spores

were present, as determined by microscopic examination of the culture samples. Cell fractions and culture supernatants were tested for the presence of BclA (Fig. 3). At T_{+8} , BclA was detected in the sporulating cells of all strains (Fig. 3, lanes 4, 6, and 8) and was absent from the corresponding supernatants (Fig. 3, lanes 5, 7, and 9). At T_{+20} , BclA in the parental strain was only found associated with the free spores, as expected (Fig. 3, lanes 10 and 11). In the Δ *exsFA* culture sample, BclA was found in the supernatant and also associated with the free spores (Fig. 3, lanes 12 and 13). In contrast, BclA was absent from the spore fraction of the mutant Δ *exsFA* Δ *exsFB* strain and was found exclusively in the culture supernatant (Fig. 3, lanes 14 and 15). Therefore, BclA is produced by the mutants. However, it was partially released into the culture supernatant after mother cell lysis of the Δ *exsFA* strain and totally released by the Δ *exsFA* Δ *exsFB* strain. Thus, ExsFs products seem to be required for the correct localization of BclA to the spore surface.

***exsF* mutants form spores with altered exosporium ultra-structure.** BclA is the structural component of the exosporium filaments, and Δ *bclA* mutants are devoid of filaments (37, 38). The surface of the *exsF* mutant spores was examined by electron microscopy and compared to that of the parental strain (Fig. 4A). The abundance and the length of filaments on the Δ *exsFB* mutant were similar to those on the parental strain

FIG. 4. Electron micrographs of the spore surface of parental and mutant *exsF* strains after negative staining. (A) Strain 7702, (B) strain 7702 Δ *exsFA* (PF51), (C) strain 7702 Δ *exsFB* (PF55), and (D) strain 7702 Δ *exsFA* Δ *exsFB* (PF56) are shown. Scale bar, 100 nm.

FIG. 5. Electron micrograph of exosporium crystalline arrays sloughed off in strain 7702 Δ *exsFA* (PF51) after negative staining. The white and black arrows indicate the two distinct arrays, respectively. Scale bar, 100 nm.

(Fig. 4C). In contrast, the spore surface of the Δ *exsFA* mutant had considerably fewer exosporium filaments, and their length was similar to that of the parental strain (Fig. 4B); the spore surface of the Δ *exsFA* Δ *exsFB* double mutant was totally devoid of filaments (Fig. 4D). Complementation of the Δ *exsFA* mutation restored filaments to the spore surface (data not shown). These observations were consistent with the SDS-PAGE and immunodetection analyses. Thus, the abundance of filaments on the spore surface was directly related to the amount of BclA protein detected in spore extracts. In addition, electron microscopy analysis of the Δ *exsFA*, Δ *exsFB*, and Δ *exsFA* Δ *exsFB* spore preparations showed that inactivation of *exsF* genes generated instability in the organization of the exosporium basal layer, which contains several crystalline sublayers (2, 12, 16, 43). For all three mutants, this instability

resulted in the crystalline sublayers being sloughed off from the spore surface, interestingly revealing the presence of two distinct arrays (Fig. 5). The instability phenomenon, observed with fresh spore preparations, increased with storage time. No such phenomenon was detected with the parental or the complemented Δ *exsFA* and Δ *exsFB* strains, even after prolonged storage time of the spore stocks. This direct visualization of the sublayers in the Δ *exsF* mutants spore preparations may facilitate further investigation of the exosporium sublayer structure.

In conclusion, we show that both ExsF proteins are exosporium components required for the proper localization of BclA filaments on the spore surface and for the stability of the crystalline layers of the exosporium. These proteins are probably organized in multimeric complexes with other exosporium components, including BclA. The protein ExsFA may interact

with BclA and ensures that it is localized on the spore surface. The contribution of ExsF is less clear, but one hypothesis is that ExsFB and ExsFA interact with the components of both arrays of the crystalline basal layer and ensure its stability. Alternatively, the absence of two spore components (35 to 37 kDa) in mutant strains suggests that ExsFs proteins may be required for the assembly of exosporium structural components or their maintenance on the spore. Such a role would then resemble that described for some morphogenetic coat proteins, such as SpoIVA and SpoVID (11), which are involved in *B. subtilis* coat assembly. The role and the contribution of the ExsF proteins to the structure and ultrastructure of *B. anthracis* exosporium are issues that will be elucidated in further investigations.

ACKNOWLEDGMENTS

We thank Agnès Fouet for interest and helpful discussions during this work and for critical reading of the manuscript.

This work was supported in part by grant no. 62500 from the CHORI and by grant no. 04-34-025 from DGA.

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