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The ExsY Protein Is Required for Complete Formation of the Exosporium of *Bacillus anthracis*▽

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The outermost layer of the *Bacillus anthracis* spore is the exosporium, which is composed of a paracrystalline basal layer and an external hair-like nap. The filaments of the nap are formed by a collagen-like glycoprotein called BclA, while the basal layer contains several different proteins. One of the putative basal layer proteins is ExsY. In this study, we constructed a Δ*exsY* mutant of *B. anthracis*, which is devoid of ExsY, and examined the assembly of the exosporium on spores produced by this strain. Our results show that exosporium assembly on Δ*exsY* spores is aberrant, with assembly arrested after the formation of a cap-like fragment that covers one end of the forespore—always the end near the middle of the mother cell. The cap contains a normal hair-like nap but an irregular basal layer. The cap is retained on spores prepared on solid medium, even after spore purification, but it is lost from spores prepared in liquid medium. Microscopic inspection of Δ*exsY* spores prepared on solid medium revealed a fragile sac-like sublayer of the exosporium basal layer, to which caps were attached. Examination of purified Δ*exsY* spores devoid of exosporium showed that they lacked detectable levels of BclA and the basal layer proteins BxpB, BxpC, CotY, and inosine-uridine-preferring nucleoside hydrolase; however, these spores retained half the amount of alanine racemase presumed to be associated with the exosporium of wild-type spores. The Δ*exsY* mutation did not affect spore production and germination efficiencies or spore resistance but did influence the course of spore outgrowth.

Bacillus anthracis, the causative agent of anthrax, is a gram-positive, rod-shaped, aerobic bacterium that forms endospores (or spores) when vegetative cells are deprived of certain nutrients (22). Spore formation begins with an asymmetric septation in the starved cell that produces large and small genome-containing compartments, called the mother cell and forespore, respectively (31). The mother cell then engulfs the forespore and surrounds it with three concurrently synthesized layers, called the cortex, coat, and exosporium (5). The cortex, which is the innermost and thickest of the three layers, is composed of peptidoglycan (4). The coat, which tightly covers the cortex, is composed of an undetermined but probably large number of different proteins (14). The exosporium, which is a loose-fitting, balloon-like structure enclosing the spore, is apparently composed of at least a dozen different proteins and glycoproteins (29). After spore formation is complete, the mother cell lyses to release the spore. Mature spores are dormant and resistant to harsh chemicals and physical damage, which allows them to survive in their normal soil environment for many years (18). When spores encounter an aqueous environment containing appropriate nutrients, they germinate and grow as vegetative cells (27). Germination is activated by small-molecule germinants, such as L-alanine and inosine, which are recognized by receptors located within the spore membrane that underlies the cortex (36). When *B. anthracis* spores enter a human or other mammalian host, germination and cell growth produce toxins that can rapidly cause death (17).

Recent concerns about the use of *B. anthracis* spores as a biological weapon have resulted in efforts to better understand the interactions between *B. anthracis* spores and the cells of the mammalian immune system (2, 9, 12, 23) and also to develop better detectors for these spores (25, 30, 32, 37). Both efforts require a detailed molecular description of the outermost layer of the *B. anthracis* spore, the exosporium. The exosporium serves as a semipermeable barrier that excludes large, potentially harmful molecules, such as antibodies and hydrolytic enzymes (5, 6), and it also serves as the source of surface antigens (5, 28, 32). The exosporium is composed of a paracrystalline basal layer and an external hair-like nap. Most, if not all, of the filaments of the hair-like nap are formed by a single collagen-like glycoprotein called BclA (1, 33). In contrast, the basal layer appears to be composed of at least a dozen different proteins in tight and loose associations (24, 29). The proteins include BxpB (also called ExsF), which was recently shown to be required for the attachment of BclA and the hair-like nap to the basal layer and also to play a role in suppressing spore germination (29, 34). In the exosporium, BclA and BxpB are present in stable high-molecular-mass (i.e., >250 kDa) complexes, which also contain the protein ExsY and, possibly, its homologue CotY (24, 29). It has been reported that ExsY is required for exosporium assembly in *Bacillus cereus*, which forms spores very similar to those of *B. anthracis* (35).

In this study, we constructed a Δ*exsY* mutant strain of *B. anthracis* and used this strain to examine the role of ExsY in exosporium assembly. Our results show that in sporulating cells devoid of ExsY, exosporium assembly is arrested after the formation of a cap-like fragment that covers one end of the forespore. Inspection of the resulting spores revealed new structural features within the exosporium basal layer. The

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Δ*exsY* spores could readily be stripped of apparently all exosporium. These exosporiumless spores were compared to wild-type spores to identify exosporium proteins and functions. Other characteristics of Δ*exsY* spores were examined, including variations in the outgrowth of germinated spores. A possible mechanism for the assembly of the exosporium is discussed.

MATERIALS AND METHODS

***B. anthracis* strains.** The Sterne 34F2 veterinary vaccine strain of *B. anthracis* was obtained from the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Md., and used as the wild-type strain in this study. The Sterne strain is not a human pathogen because it lacks plasmid pXO2, which is necessary to produce the capsule of the vegetative cell (8). A variant of the Sterne strain carrying a Δ*exsY* mutation that precisely deletes the entire *exsY* open reading frame was constructed by allelic replacement, essentially as previously described (3, 29). This procedure replaced the *exsY* gene with a spectinomycin resistance cassette, which was confirmed by PCR amplification and DNA sequencing of the relevant region of the chromosome. The Δ*exsY* mutant strain was called CLT325.

Preparation of spores and sporulating cells. Spores were prepared by growing strains at 37°C in liquid (with shaking) or on solid (1.5% agar) Difco sporulation medium (DSM) (19) until sporulation was complete, typically 48 to 72 h. Spores were collected by centrifugation, washed extensively with cold (4°C) sterile deionized water, sedimented through a two-step gradient of 20% and 50% Renografin (Bracco Diagnostics), and extensively washed again with cold water (10). Spores were stored and quantitated as previously described (28). Sporulating cells, also called sporangia, were obtained from cultures grown in liquid DSM at 37°C with shaking. Culture density was measured spectrophotometrically at 600 nm, and spore development was monitored by phase-contrast microscopy. Sporangia were harvested by centrifugation at 4,000 × *g* for 10 min at 4°C.

Preparation of recombinant exosporium proteins. Recombinant versions of the *B. anthracis* exosporium proteins ExsY, CotY, and alanine racemase (Alr) were prepared essentially as previously described (28, 29). Briefly, the genes encoding these proteins (i.e., BAS1141, BAS1145, and BAS238, respectively [*B. anthracis* Sterne gene numbers from the Kyoto Encyclopedia of Genes and Genomes database {11}]) were amplified by PCR and inserted into the cloning site of the expression vector pET15b (Novagen). The resulting plasmids were transformed individually into *Escherichia coli* strain BL21(DE3) to express the cloned genes according to the pET system manual (Novagen). Each expressed recombinant protein contained a six-His tag and a factor Xa cleavage site immediately preceding the initiating methionine. To isolate recombinant ExsY (rExsY) and rCotY, cells expressing individual proteins were broken by sonication, and insoluble cellular material, which contained essentially all of the recombinant protein, was recovered by centrifugation at 10,000 × *g* for 20 min at 4°C. The insoluble material, the vast majority of which was the recombinant protein, was resuspended in 8 M urea and dialyzed against phosphate-buffered saline (PBS) (26) prior to use. Recombinant Alr was purified under native conditions by immobilized-metal affinity chromatography (QIAGEN), and its six-His tag was removed by factor Xa cleavage as previously described (29).

Preparation of mouse MAbs. The production and characterization of anti-BclA and anti-BxpB monoclonal antibodies (MAbs), designated EF12 and 10-23-4, respectively, were described previously (1, 28, 29). MAb 10-23-4 does not react with a paralogue of BxpB that is encoded by the BAS2303 gene and is 78% identical to BxpB (29). An anti-BxpC MAb designated FH6-1 was raised against purified Sterne exosporium by using a published procedure (28). FH6-1 was shown to bind the *B. anthracis* BxpC protein and a recombinant version synthesized in *E. coli* (C. T. Steichen and C. L. Turnbough, Jr., unpublished data). Purified rExsY and rAlr were used as antigens to raise an anti-ExsY/CotY MAb, designated G9-3, and an anti-Alr MAb, called AR-1, according to published procedures (29). In immunoblots, G9-3 reacted similarly with rExsY and rCotY, which are 85% identical; AR-1 did not react with a second *B. anthracis* alanine racemase that is encoded by the BAS1932 gene and apparently produced during vegetative growth. All MAbs were purified by affinity chromatography on protein G-Sepharose (28, 32). The MAbs represented three isotypes, i.e., immunoglobulin G1 (IgG1), κ chain (10-23-4, AR-1, and FH6-1), IgG2a, κ chain (G9-3), and IgG2b, κ chain (EF12), which were determined as previously described (13). MAbs used for flow cytometry and fluorescence microscopy were labeled by using an Alexa Fluor 488 protein labeling kit (Molecular Probes).

Gel electrophoresis and immunoblotting of spore surface proteins. Spores (3×10^8) were boiled for 8 min in 30 μl of sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 100 mM dithiothreitol, 0.012% bromophenol blue, and 10% (vol/vol) glycerol. The solubilized exosporium and other extractable proteins in the sample were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a 4 to 15% gradient polyacrylamide gel (Bio-Rad) and were visualized by staining with Coomassie brilliant blue (19). For immunoblotting, proteins were electrophoretically transferred from an SDS-polyacrylamide gel to a nitrocellulose membrane and treated as described in the manual for a Bio-Rad Immun-Blot assay kit. Briefly, each blot was blocked with gelatin, probed with a primary MAb at 5 μg/ml for 1 h, and washed. The blot was then probed with a 1:3,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (heavy plus light chains) secondary antibody for 1 h, washed, and developed with horseradish peroxidase developer solution.

Electron microscopy. Transmission electron microscopy of spores and sporangia was performed as previously described (1).

Flow cytometry. Flow cytometry was used to detect spore binding of a fluorescently (Alexa 488) labeled MAb, either the anti-BclA MAb EF12 or an equivalently labeled isotype control MAb (i.e., a MAb not exhibiting specific spore binding). Briefly, spores (10^7) were mixed with 5 μg/ml of either MAb in 20 μl of PBS containing 1% bovine serum albumin (BSA) for 1 h at room temperature. The spores were washed three times in PBS containing 1% BSA, and 2×10^4 spores were analyzed using a FACSCalibur (BD Biosciences) fluorescence-activated cell sorter with CellQuest Pro software.

Fluorescence microscopy. Slides were prepared essentially as previously described (38). Briefly, 10^6 spores were dried on slides coated with poly-L-lysine (Sigma), and the immobilized spores were treated with 1% BSA to block nonspecific binding sites and washed three times with PBS containing 0.5% Tween 20 (Sigma). The spores were then treated with either 30 μl of 5-μg/ml Alexa Fluor 488-labeled MAb EF12 or 30 μl of 5-μg/ml Alexa-labeled EF12 plus 400 nM *B. anthracis* spore binding peptide TYPLPIR conjugated to phycoerythrin (PE) (37) for 1 h at 4°C. The slides were washed as described above and examined by phase-contrast and fluorescence microscopy, using a Nikon Eclipse E600 microscope equipped with a Y-FL epifluorescence attachment. Images were captured with a Spot charge-coupled device digital camera (Diagnostic Instruments, Inc.) and displayed by using Spot (v4.0) software.

RESULTS

Spores produced by a Δ*exsY* strain of *B. anthracis* either possess a partial exosporium or are devoid of an exosporium, depending on culture conditions. To determine if ExsY is required for the formation of the *B. anthracis* exosporium, we constructed a mutant version of the Sterne strain that is unable to produce this protein. The mutant, designated CLT325, contains a chromosomal mutation (Δ*exsY*) that precisely deletes the entire *exsY* gene, the only gene in its operon according to genome annotation (11), and replaces it with a spectinomycin resistance cassette. Strain CLT325, and the Sterne strain as a wild-type control, was grown on solid and in liquid media and allowed to sporulate. Mature spores were harvested, purified, and examined for the presence of the exosporium by flow cytometry after being incubated with fluorescently labeled anti-BclA MAb EF12. The results showed that the anti-BclA MAb bound similarly and extensively to Sterne spores produced with either solid or liquid medium (Fig. 1A and data not shown) and to Δ*exsY* spores grown on solid medium (Fig. 1B), indicating the presence of the exosporium. In contrast, the anti-BclA MAb did not bind to Δ*exsY* spores grown in liquid medium (Fig. 1C), indicating the absence of the exosporium.

We further analyzed the Sterne and Δ*exsY* spores by phase-contrast and fluorescence microscopy (>100 spores inspected per sample), again following treatment with fluorescently labeled anti-BclA MAb EF12. All spores, whether grown on solid or in liquid medium, appeared similar by phase-contrast microscopy (Fig. 2A and B and data not shown). However, major differences were observed by fluorescence microscopy and from merged phase-contrast and fluorescence microscopic

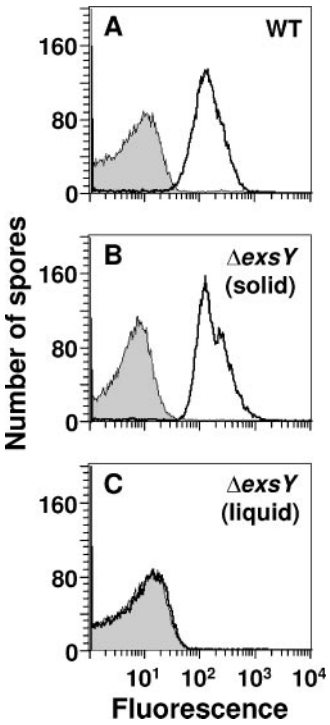

FIG. 1. Analysis by flow cytometry of binding of the anti-BclA MAb EF12 to wild-type and Δ*exsY* spores of *B. anthracis*. Spores of the wild-type Sterne strain (WT) produced in liquid medium (A) and spores of strain CLT325 (Δ*exsY*) produced on solid (B) or in liquid (C) medium were treated with fluorescently labeled EF12 (unfilled histograms with thick outlines) or an equivalently labeled isotype control MAb (gray histograms).

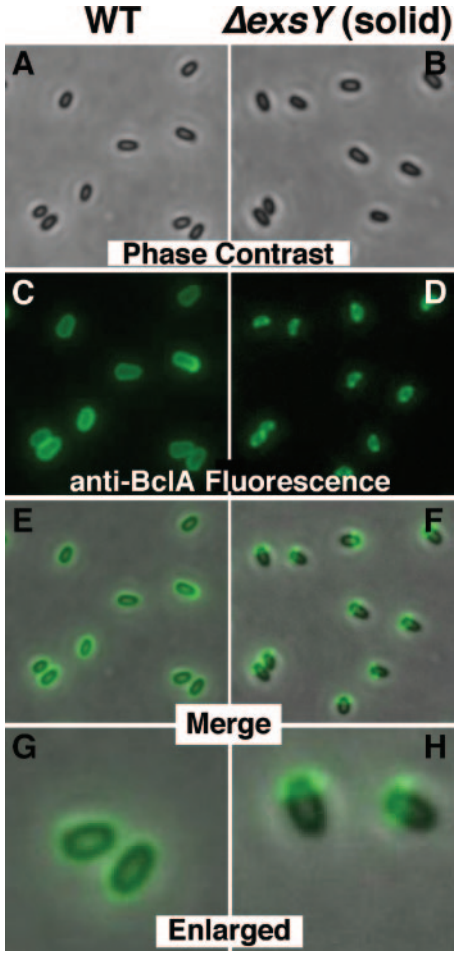

FIG. 2. Phase-contrast and fluorescence microscopic analysis of wild-type and Δ*exsY* spores of *B. anthracis* treated with fluorescently labeled anti-BclA MAb EF12. Spores of the wild-type Sterne strain (WT) were prepared in liquid medium (A, C, E, and G), and spores of strain CLT325 (Δ*exsY*) were produced on solid medium (B, D, F, and H). Phase-contrast images (A and B) and fluorescence images indicating the binding of EF12 to BclA (C and D) were used to produce merged (E and F) and enlarged merged (G and H) images.

images. Sterne spores produced on solid or in liquid medium appeared uniformly and brightly fluorescent, as expected for spores surrounded by an exosporium (Fig. 2C, E, and G and data not shown). Surprisingly, fluorescent labeling of Δ*exsY* spores grown on solid medium was restricted to a polar cap-like region, which covered about one-third of the spore (Fig. 2D, F, and H). This result suggested that only a fragment of the exosporium was present on these spores. On the other hand, no labeling by the fluorescent anti-BclA MAb was observed with Δ*exsY* spores grown in liquid medium (data not shown). This result again indicates the lack of an exosporium on these spores.

Finally, we employed transmission electron microscopy to obtain high-resolution images of the Sterne and Δ*exsY* spores (>20 longitudinal spore sections inspected per sample). The Sterne spores produced on solid or in liquid medium possessed a fully developed exosporium, with a regular basal layer and hair-like nap (Fig. 3A and data not shown). As suggested by fluorescence microscopy, the transmission electron microscopic images of the Δ*exsY* spores grown on solid medium revealed a cap-like exosporium fragment covering one end of each spore (Fig. 3B). This fragment, which we call the "cap," contained a normal hair-like nap but an irregular basal layer. As expected, the transmission electron microscopic images of the Δ*exsY* spores grown in liquid medium showed the complete loss of the exosporium from every spore examined (Fig. 3C). Other than the exosporium, the other layers of Δ*exsY* spores, whether exosporiumless or possessing a cap, appeared unaltered compared to wild-type Sterne spores.

Exosporium development is arrested in sporulating Δ*exsY* cells. To analyze the defect in exosporium development caused by the lack of ExsY, we examined sporulating cells of strain CLT325 (Δ*exsY*), and of the Sterne strain as a control, by transmission electron microscopy (>100 cells inspected per sample). Cells were grown and allowed to sporulate in liquid medium. Cells were harvested and fixed for examination hourly, starting 3 h and ending 9 h after the onset of sporulation (times hereafter are designated T_n, where n is the number of hours after the start of sporulation). Between T_3 and T_4, exosporium formation was first detected, and between T_8 and T_9, mature spores were released from the mother cell. At approximately T_6, we first observed a clear difference in exosporium formation between the two strains. At this time, essentially all nascent spores within mother cells of the Sterne strain were completely enveloped by an exosporium, which

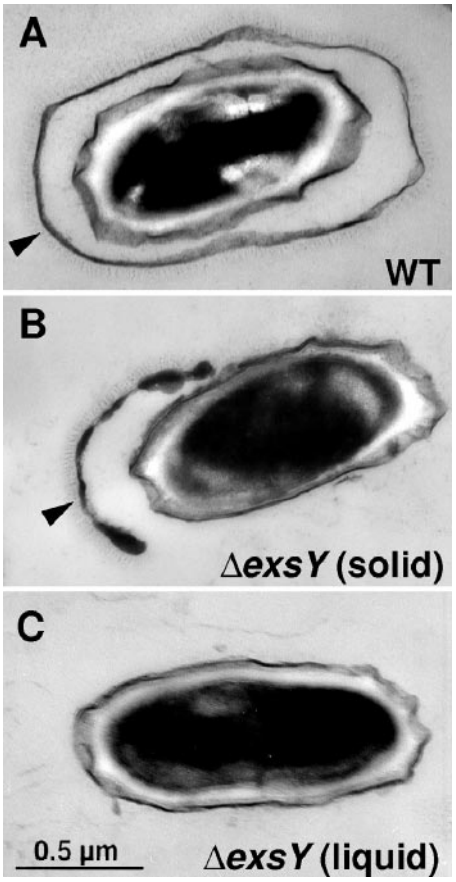

FIG. 3. Transmission electron micrographs of wild-type and Δ*exsY* spores of *B. anthracis*. Thin sections of spores of the wild-type Sterne strain (WT) produced in liquid medium (A) and spores of strain CLT325 (Δ*exsY*) produced on solid (B) or in liquid (C) medium were examined. Arrowheads point to the exosporium (A) or cap-like exosporium fragment (B). The magnifications of all image are identical.

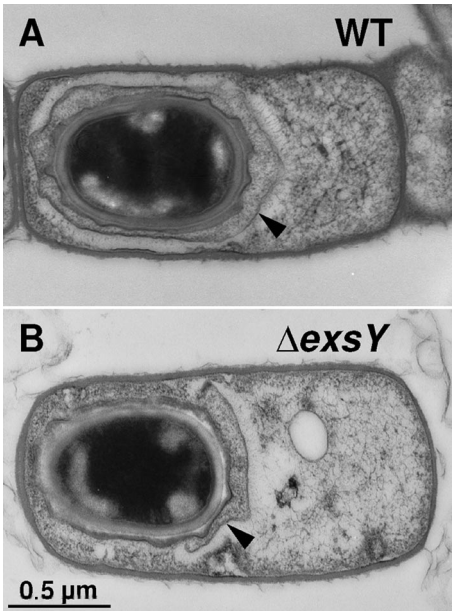

FIG. 4. Transmission electron micrographs of sporulating cells of wild-type and Δ*exsY* strains of *B. anthracis*. Thin sections of sporulating (T_6) cells of the wild-type (WT) Sterne strain (A) and strain CLT325 (Δ*exsY*) (B) were examined. Arrowheads point to the exosporium (A) or cap-like exosporium fragment (B). The magnifications of both images are identical.

contained a uniform basal layer and a normal hair-like nap (Fig. 4A). In contrast, the nascent spores within mother cells of the Δ*exsY* strain were only partially covered by a cap-like exosporium fragment (Fig. 4B). No further development of this fragment was detected at later time points, indicating a complete arrest of exosporium assembly. The T_6 exosporium fragment, which contains a normal hair-like nap, appears to be the cap found on mature spores of the Δ*exsY* strain grown on solid medium (Fig. 3B). In every T_6 cell of the Δ*exsY* strain, the exosporium fragment or cap covered the end of the spore near the middle of the mother cell. This result is consistent with previous studies showing that normal exosporium assembly begins at this location (20). The presence of a cap on all developing spores of the Δ*exsY* strain indicates that when spores of this strain are produced in liquid medium, they lose their caps. Prolonged shaking of the culture, or perhaps a presently unrecognized activity unique to liquid cultures, may dislodge the caps. Although spores of the Δ*exsY* strain do not assemble a complete exosporium, the other layers of these spores appear normal in sporangia (and also in mature spores, as indicated above).

An exosporium sublayer is revealed on Δ*exsY* spores. We previously identified a peptide, with the sequence TYPLPIR, that is capable of selectively binding to *B. anthracis* spores (37). Preliminary cross-linking experiments suggested that this peptide binds to ExsY (D. D. Williams and C. L. Turnbough, Jr., unpublished data). Therefore, we used fluorescence microscopy to examine the ability of a TYPLPIR peptide-PE conjugate (TYP-PE) to bind to purified Δ*exsY* spores. We observed no binding to spores prepared in liquid medium but did observe uniform and extensive binding to the surface of essentially every spore prepared on solid medium (data not shown). The latter result demonstrated that ExsY is not the target, or at least not the sole target, of TYP-PE binding, and it suggested that the site of TYP-PE binding on cap-containing Δ*exsY* spores is a fragile spore structure. To examine TYP-PE binding to spores with minimal damage to the presumed fragile structure, we prepared Δ*exsY* spores on solid medium without purification (i.e., spores were gently washed from plates with water). Sterne spores were prepared identically to serve as a wild-type control. A sample of each unpurified spore preparation was treated with both TYP-PE and Alexa Fluor 488-labeled anti-BclA MAb EF12 and then examined by phase-contrast and fluorescence microscopy (>100 spores inspected per sample).

Inspection by phase-contrast microscopy revealed refractile (i.e., phase bright) spores with a normal appearance in each spore prep (Fig. 5A and B and data not shown); however, in the preparation of Δ*exsY* spores, we also found an occasional spore-free sacculus (or sac) of approximately the size of the exosporium (Fig. 5B). Fluorescence staining by the anti-BclA MAb indicated a complete exosporium on

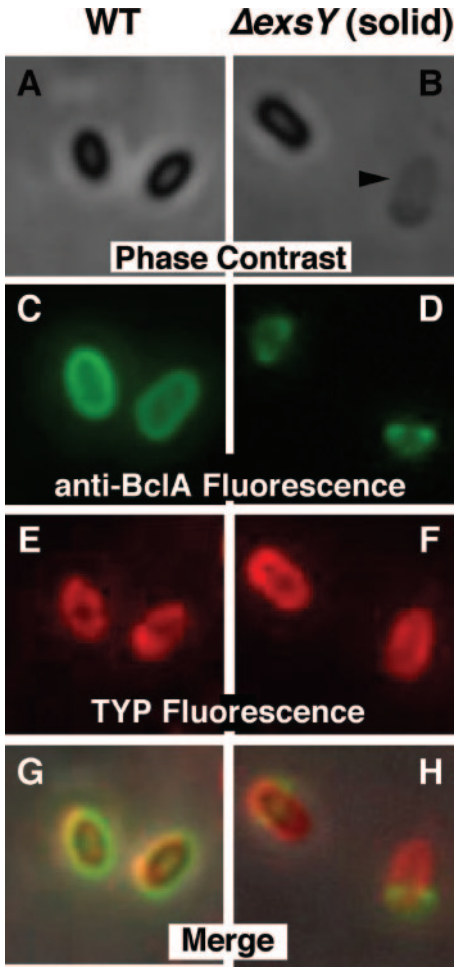

FIG. 5. Phase-contrast and fluorescence microscopic analysis of *B. anthracis* wild-type and Δ*exsY* spores treated with both Alexa Fluor 488-labeled anti-BclA MAb EF12 and TYP-PE. Spores of the wild-type Sterne (WT) and Δ*exsY* strains were produced on solid medium and harvested without purification. The phase-contrast images show two spores in the wild-type preparation (A) and one spore and one sac (indicated by an arrowhead) in the Δ*exsY* preparation (B). Fluorescence images show anti-BclA MAb staining (C and D) and TYP-PE staining (E and F) of the same material shown in the phase-contrast images. The corresponding phase-contrast, anti-BclA MAb fluorescence, and TYP-PE fluorescence images were merged to indicate overlapping staining (G and H).

Sterne spores (Fig. 5C and data not shown) and caps on all spores and nearly all sacs found in the Δ*exsY* spore preparation (Fig. 5D and data not shown). These assignments were confirmed by merging the phase-contrast images (Fig. 5A and B) with the corresponding anti-BclA fluorescence images (Fig. 5C and D) (data not shown). Fluorescence staining by TYP-PE indicated binding to the entire surfaces of Sterne spores, Δ*exsY* spores, and the sacs in the Δ*exsY* spore preparation (Fig. 5E and F and data not shown). Merged images of the fluorescence staining by TYP-PE and the anti-BclA MAb (which also include the phase-contrast images) indicated that the sacs represented a sublayer of the exosporium just under the outermost BclA-containing material in Sterne spores (Fig. 5G) and just under the caps of Δ*exsY* spores and sacs (Fig. 5H). Previous studies have suggested that the exosporium is composed of four closely packed lamellae (7), and the sublayer stained by TYP-PE may correspond to one of these.

When the Δ*exsY* spore preparation was stained with TYP-PE, many clusters of fragments of sac-like material were observed (data not shown), indicating that the sacs were easily broken and presumably corresponded to the fragile TYP-PE-binding material lost from Δ*exsY* spores grown in liquid medium. On the other hand, the same sacs within the exosporium of Sterne spores were extremely stable and apparently never sloughed from the spore. This observation is again consistent with the sac being an internal sublayer within the exosporium.

Exosporium proteins are lost from Δ*exsY* spores produced in liquid medium. Sporulation of strain CLT325 (Δ*exsY*) in liquid medium produces mature spores that have shed all observable exosporium (including the cap and sac) but maintain the other layers of the spore in an apparently normal state. Consequently, these spores are likely to be devoid of proteins incorporated solely into the exosporium and deficient in proteins incorporated in part into this layer. Presently, about a dozen proteins are known or suspected to be incorporated into the exosporium, and we have rapid and reliable assays for about half of these. To further analyze the localization of the latter group of proteins, we compared their levels in purified wild-type Sterne and exosporiumless Δ*exsY* spores. Proteins were extracted from an equal number of Sterne and Δ*exsY* spores by boiling in sample buffer and then were separated by SDS-PAGE. The gels were either stained with Coomassie brilliant blue or used for immunoblotting.

The Coomassie-stained gel was used to detect the putative exosporium protein inosine-uridine-preferring nucleoside hydrolase (IUNH). Monomeric IUNH, which has a mass of 36,276 Da, migrates with an apparent mass of approximately 50 kDa due to its low pI relative to those of protein standards. An essentially pure band of IUNH was readily detected in proteins extracted from Sterne spores (Fig. 6A). The identification and purity of the IUNH band were established by tryptic digestion and sequencing of the resulting fragments by tandem mass spectrometry (15; C. T. Steichen and C. L. Turnbough, Jr., unpublished data). In contrast, no IUNH was detectable in the proteins extracted from the Δ*exsY* spores (Fig. 6A). IUNH was not detected in this extract even when a severalfold larger sample was analyzed.

The relative levels of six other exosporium proteins were determined by immunoblotting using previously described mouse MAbs EF12 (anti-BclA), 10-23-4 (anti-BxpB), and FH6-1 (anti-BxpC) and mouse MAbs G9-3 (anti-ExsY) and AR-1 (anti-Alr), which were produced and characterized in this study, as described in Materials and Methods. In immunoblots, all MAbs except G9-3 react specifically with their designated target proteins, as either recombinant or spore proteins (data not shown). G9-3 reacts equally well with ExsY and its homologue CotY, again as either recombinant or spore proteins; for example, G9-3 reacts with CotY extracted from cap-containing Δ*exsY* spores and with ExsY extracted from Δ*cotY* spores (data not shown). ExsY and CotY have similar masses (i.e., 16,146 and 16,842 Da, respectively) and migrate as a doublet band during SDS-PAGE (data not shown). The immunoblot of Sterne spore proteins with G9-3 as the probe

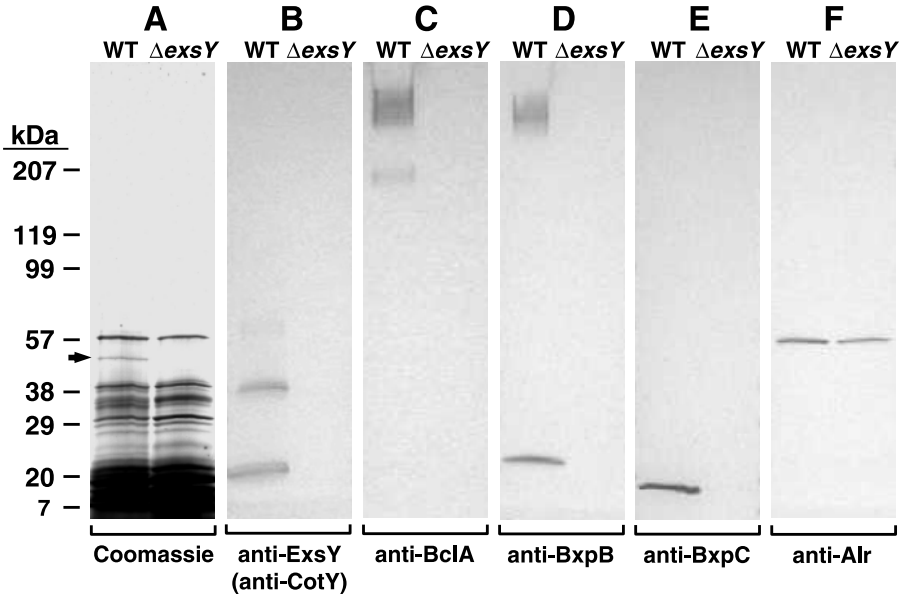

FIG. 6. Detection of proteins extracted from Sterne (WT) and exosporiumless Δ*exsY* spores prepared in liquid medium. Proteins were separated by SDS-PAGE and visualized by staining with Coomassie brilliant blue (A) or detected by immunoblotting with mouse MAbs G9-3 (anti-ExsY/CotY) (B), EF12 (anti-BclA) (C), 10-23-4 (anti-BxpB) (D), FH6-1 (anti-BxpC) (E), and AR-1 (anti-Alr) (F). The arrow in panel A indicates the band corresponding to IUNH. The gel locations and masses (in kDa) of Bio-Rad protein standards are indicated on the left.

revealed doublet bands with masses expected for monomers, dimers, and trimers of ExsY and CotY (Fig. 6B). The same pattern of oligomerization was observed with rExsY and rCotY (data not shown). The apparent absence of ExsY and CotY in >250-kDa complexes is discussed below. The immunoblot of Δ*exsY* spore proteins with G9-3 failed to detect either ExsY or CotY (Fig. 6B). The immunoblots with EF12 (anti-BclA), 10-23-4 (anti-BxpB), and FH6-1 (anti-BxpC) as probes yielded similar results (Fig. 6C, D, and E). The blots showed the presence of BclA, BxpB, and BxpC in extracts of Sterne spores but failed to detect these proteins in extracts of Δ*exsY* spores. When extracted from Sterne spores, BclA and BxpB were included in large part in >250-kDa complexes (Fig. 6C and D), as previously reported (29), and a significant fraction of BxpB (17,331 Da) and all BxpC (14,379 Da) migrated as monomeric proteins (Fig. 6D and E). ExsY, CotY, BclA, BxpB, and BxpC were not detected in Δ*exsY* spore extracts, even when several-fold larger samples were analyzed. Thus, BclA, BxpB, BxpC, and CotY, along with IUNH, appear to be lost when the exosporium is shed from the Δ*exsY* spores.

In contrast, the immunoblot with AR-1 (which reacts with the spore-associated Alr protein but not with a second, vegetative-cell alanine racemase of *B. anthracis*) detected monomeric (43,662 Da) Alr in extracts of both Sterne and Δ*exsY* spores. However, the level of Alr from Δ*exsY* spores was reproducibly about half that from Sterne spores, as determined by densitometry (Fig. 6F and data not shown). Thus, Alr appears to be present in the exosporium plus at least one other extractable spore location, or alternatively, Alr is normally found solely in the exosporium but is aberrantly localized in Δ*exsY* spores.

It should be noted that the failure to detect BclA, BxpB, BxpC, and CotY in extracts of exosporiumless Δ*exsY* spores was not due to an inability to synthesize these proteins in sporulating Δ*exsY* cells. Using fluorescence microscopy and fluorescently labeled MAbs as probes, we detected high levels of BclA, BxpB, BxpC, and CotY in the caps of Δ*exsY* spores grown on solid medium (Fig. 2 and data not shown). Additionally, fluorescence microscopy with the fluorescently labeled MAb AR-1 detected Alr on the surfaces of Sterne spores, confirming the presence of this protein in the exosporium (data not shown).

The Δ*exsY* mutation does not affect cell growth, efficiency of spore production, or spore resistance. To characterize the physiological effects of the Δ*exsY* mutation, we compared the growth rates, cell yields, and sporulation efficiencies of cultures of the Sterne and CLT325 (Δ*exsY*) strains grown in liquid DSM at 37°C with shaking. We observed no significant differences between the two cultures, with both growing with maximum doubling times of ~25 min and sporulating with >95% efficiency (data not shown). *Bacillus* spores are resistant to heat, lysozyme, and organic solvents, such as chloroform, methanol, and phenol. Using standard protocols (19), we compared the levels of resistance to these treatments of purified preparations of Sterne and CLT325 (Δ*exsY*) spores produced in liquid DSM. We found no significant difference in the survival rates of Sterne and Δ*exsY* spores (data not shown).

The Δ*exsY* mutation does not affect the efficiency of spore germination but can alter the process of outgrowth. We also measured the effects of the Δ*exsY* mutation on spore germination and outgrowth. Spores of either the Sterne or CLT325 (Δ*exsY*) strain were dried on a coverslip, which was placed in a microscope chamber maintained at 37°C. After allowing the coverslip to warm to 37°C, the chamber was filled with prewarmed (37°C) growth medium containing RPMI 1640 with L-glutamine (Cellgro) and 0.2% brain heart infusion medium (Difco) (32). Spore germination and outgrowth were monitored by phase-contrast microscopy and time-lapse photogra-

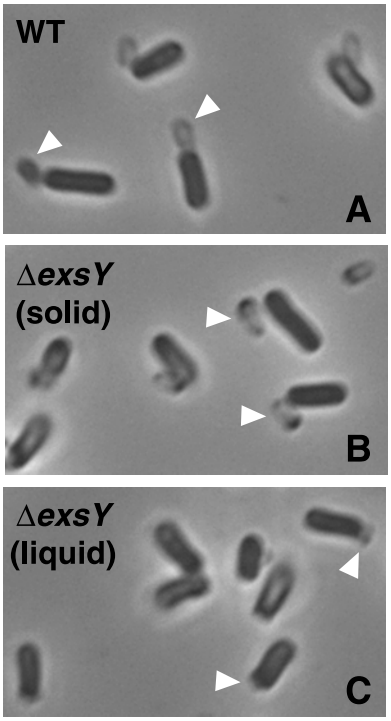

FIG. 7. Analysis of outgrowth of Sterne and Δ*exsY* spores. Wild-type Sterne (WT) spores produced in liquid medium (A) and Δ*exsY* spores produced on solid (B) or in liquid (C) medium were allowed to germinate and outgrow in growth medium for 55 min at 37°C and then examined by phase-contrast microscopy. White arrowheads indicate some of the shells that encapsulated the outgrowing cells.

phy as previously described (29). The marker for germination was the change in spore appearance from refractile to phase dark, which occurs upon rehydration of the core and lysis of the cortex (21). The marker for outgrowth was the popping of the vegetative cell from its exosporium-containing "shell" (29). Previous studies indicated that this shell also includes the outer layer of the spore coat (16). Purified spores produced with both solid and liquid media were examined.

For all preparations, ≥95% of the spores germinated between 3 and 20 min of incubation in the growth medium. Most spores that did not germinate within this time period remained phase bright for the duration of the experiment (i.e., 90 min). The median time for germination of Δ*exsY* spores produced on solid or in liquid medium was approximately 6 min. The median time for germination of Sterne spores produced on solid or in liquid medium varied from 6 to 9 min. This variation was not related to the method of preparation but to differences observed with spores prepared under the same conditions. The source of this variation is unknown. For all spore preparations, essentially all outgrowth occurred between 35 and 60 min of incubation. Although it was not possible to reliably compare the median times of outgrowth for the four spore preparations due to a lack of synchrony, these preparations exhibited interesting differences during outgrowth. Both Sterne preparations outgrew identically, with popping of the vegetative cell from its shell within 10 s (i.e., the time between photographic images). Typically, this popping resulted in the complete escape of the outgrowing cell from its shell (Fig. 7A and data not shown). Although the shell often remained close to the outgrowing cell immediately after popping, a clear separation between them generally occurred within a few minutes. The Δ*exsY* spores produced on solid medium (i.e., cap-containing spores) exhibited popping essentially identical to that observed with Sterne spores (Fig. 7B). With these Δ*exsY* spores, the cap could be seen as a dark region on one end of the shell left behind by the outgrowing cell (Fig. 7B). This shell presumably contains the sac, perhaps other elements of the exosporium basal layer, and the outer spore coat. In contrast, the Δ*exsY* spores produced in liquid medium (i.e., devoid of all detectable exosporium) did not pop during outgrowth. Instead, the outgrowing cell gradually emerged from the shell, which presumably contained only the outer spore coat. Typically, this shell was only partially dislodged from the outgrowing cell (Fig. 7C). Apparently, complete escape of the outgrowing cell from its shell requires a more forceful exit.

DISCUSSION

The results of this study demonstrate that ExsY plays a critical role in the formation of the exosporium of *B. anthracis*. The absence of ExsY during sporulation causes an arrest in exosporium formation after the assembly of an exosporium fragment called the cap. The cap covers one end and approximately one-third of the forespore. The cap is assembled on the end of the forespore that is near the middle of the mother cell, which is also the site at which normal exosporium assembly is initiated (20). Thus, the cap presumably represents an early stage in exosporium development. The cap produced in the absence of ExsY contains a normal hair-like nap but an irregular basal layer, as judged from electron micrographs of purified spores. Therefore, ExsY is required not only to complete exosporium assembly after cap formation but also to form a normal cap. These requirements may reflect a checkpoint during exosporium development for proper cap formation or the absence of an essential structural element for continued exosporium assembly.

Although ExsY may be incorporated into a normal cap, its structural role in cap formation appears to be less important than its role in the assembly of the last two-thirds of the exosporium. Perhaps there is another protein that can substitute for ExsY early in exosporium assembly but not later in this process. A potential surrogate for ExsY during cap formation is CotY, which is 85% identical to ExsY and is present in cap-containing Δ*exsY* spores. The *cotY* gene (actually the *cotY-bxpB* operon) is apparently transcribed from a promoter recognized by the early mother cell sigma factor σ^E (29). The *exsY* gene (or *exsY* single-gene operon) appears to be transcribed from a promoter recognized by the late mother cell sigma factor σ^K (i.e., a σ^K-like promoter sequence is located 40 bp upstream of the *exsY* gene). Accordingly, CotY could be synthesized early in sporulation and incorporated into a nascent cap, while ExsY could be synthesized later and participate as an essential structural element for the last two-thirds of the exosporium. Confirmation of such a model will require the demonstration that normal exosporium assembly is discontinuous and/or that the exosporium contains segments with different protein compositions.

Previous studies demonstrated that ExsY is present in a

high-molecular-mass complex that also contains BclA, BxpB, and possibly other proteins. This complex is highly stable and migrates with an apparent mass of >250 kDa during SDS-PAGE (24, 29). These observations suggested that ExsY plays an important role in exosporium assembly as part of the >250-kDa complex. However, the results of this study indicate that nearly all ExsY extracted from spores is in the form of monomers, dimers, and trimers. Apparently, only a small fraction of ExsY is stably incorporated into the >250-kDa complexes, which we confirmed by immunoblotting with the anti-ExsY/CotY MAb G9-3 and large amounts of extracted spore proteins (data not shown). Thus, the primary role of ExsY in exosporium assembly may not include the formation of highly stable complexes with BclA and BxpB.

Spores produced on solid medium by strain CLT325 (Δ*exsY*), i.e., in the absence of ExsY, retain the cap even after purification. Staining with a fluorescent conjugate of the spore binding peptide TYPLPIR (i.e., TYP-PE) and inspection by fluorescence microscopy revealed that these Δ*exsY* spores possess a layer called the sac. The sac appears to be a sublayer of the exosporium that underlies the cap while also covering the remainder of the spore. In wild-type spores, the sac appears to underlie the outermost BclA-containing sublayer of the exosporium. Thus, the sac may correspond to an inner layer of the four closely packed lamellae that were previously reported to make up the basal layer of the exosporium (7). The sac appears to be stable in wild-type and cap-containing Δ*exsY* spores. However, when the sac is shed from Δ*exsY* spores, it is extremely fragile. Spores produced in liquid medium by strain CLT325 lose the cap and also the sac. Whether these losses reflect physical damage caused by shaking of the sporulating culture or an activity uniquely associated with the liquid culture remains to be determined. Although the sac layer was readily observed on cap-containing Δ*exsY* spores by fluorescence microscopy, electron micrographs of these spores or of sporangia in which these spores were being produced did not reveal an exosporium-like layer (i.e., the sac) surrounding the entire spore. Possibly, the sac is refractile to the staining employed for electron microscopy or the sac was destroyed during sample preparation. The protein composition of the sac is presently under investigation.

The observation that purified CLT325 (Δ*exsY*) spores produced on solid medium retain their caps suggests a connection between the exosporium and the rest of the spore. Such connections could direct the assembly of the exosporium around the developing forespore. In sporulating Δ*exsY* cells, both ends of the cap bend towards, and are close to, the rest of the forespore (Fig. 4B). Perhaps these clamp-like ends represent the proposed connections. These or related connections could persist in the mature spore and provide structural stability. At present, no connections between the exosporium and other layers of the mature spore have been detected.

The Δ*exsY* spores produced in liquid medium lacked an exosporium and presumably had lost the proteins used to assemble this layer. Therefore, we examined these spores for the loss of several confirmed and putative exosporium proteins for which we have good assays. Our results showed that BclA, BxpB, BxpC, CotY, and IUNH were undetectable, indicating that these proteins are present solely in the exosporium. The level of another putative exosporium protein, the spore-associated protein Alr, was reduced to approximately 50% of that found on wild-type spores. This result suggests that Alr is present in the exosporium and at least one other spore location or that Alr is aberrantly localized in Δ*exsY* spores. We are currently comparing the protein contents of wild-type and Δ*exsY* spores by using proteomic and other analytical techniques to further characterize exosporium proteins.

Deletion of the *exsY* gene did not significantly alter cell growth, the efficiency of sporulation and germination, or the resistance of spores to heat, lysozyme, and organic solvents. These observations indicate a lack of involvement in these activities of exosporium features lost from Δ*exsY* spores. The median times for germination and outgrowth of Δ*exsY* spores, whether produced on solid or in liquid medium, were either the same or a few minutes shorter than those observed for similarly prepared wild-type spores. This result was unexpected because Δ*exsY* spores, at least those grown in liquid medium and devoid of an exosporium, lack BxpB. This protein was recently shown to delay spore germination and outgrowth (29). This apparent discrepancy will require further investigation. Our observations of outgrowth of Δ*exsY* spores provided a surprise. We expected that germinating Δ*exsY* spores would not exhibit the popping phenomenon observed when germinating wild-type spores suddenly escape their exosporium encasement. However, Δ*exsY* spores containing a cap exhibited popping essentially identical to that of wild-type spores. Apparently, the cap-containing Δ*exsY* spores are surrounded by a structure, presumably one that includes the sac, that also abruptly ruptures following sufficient enlargement of the germinating spore. In contrast, exosporiumless Δ*exsY* spores did not exhibit popping during outgrowth but exhibited a gradual emergence of the outgrowing cell from a shell that presumably contained only spore coat material. It appears that this material does not constitute a persistent physical barrier to spore germination and outgrowth.

The exosporium is conserved among all pathogenic *Bacillus* species, including *B. anthracis*, the opportunistic human pathogen *B. cereus*, and the insect pathogen *B. thuringiensis*. The high energetic cost of maintaining an elaborate exosporium is presumably offset by survival advantages that contribute to spore viability and virulence. However, these advantages remain to be established. Recent studies have shown that the mechanical removal of the exosporium from wild-type *B. anthracis* spores results in increased killing by murine macrophages (12) and by nitric oxide (23). It will be interesting to determine whether exosporiumless Δ*exsY* spores exhibit similar susceptibilities. In addition, these exosporiumless spores should be useful tools for new studies to evaluate the roles of the exosporium, perhaps in spore survival in harsh environments or during interactions with the cells of the mammalian immune system.

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