A Novel Spore Protein, ExsM, Regulates Formation of the Exosporium in *Bacillus cereus* and *Bacillus anthracis* and Affects Spore Size and Shape^{▽†}

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***Bacillus cereus* spores are assembled with a series of concentric layers that protect them from a wide range of environmental stresses. The outermost layer, or exosporium, is a bag-like structure that interacts with the environment and is composed of more than 20 proteins and glycoproteins. Here, we identified a new spore protein, ExsM, from a β -mercaptoethanol extract of *B. cereus* ATCC 4342 spores. Subcellular localization of an ExsM-green fluorescent protein (GFP) protein revealed a dynamic pattern of fluorescence that follows the site of formation of the exosporium around the forespore. Under scanning electron microscopy, *exsM* null mutant spores were smaller and rounder than wild-type spores, which had an extended exosporium (spore length for the wt, $2.40 \pm 0.56 \mu\text{m}$, versus that for the *exsM* mutant, $1.66 \pm 0.38 \mu\text{m}$ [$P < 0.001$]). Thin-section electron microscopy revealed that *exsM* mutant spores were encased by a double-layer exosporium, both layers of which were composed of a basal layer and a hair-like nap. Mutant *exsM* spores were more resistant to lysozyme treatment and germinated with higher efficiency than wild-type spores, and they had a delay in outgrowth. Insertional mutagenesis of *exsM* in *Bacillus anthracis* Δ Sterne resulted in a partial second exosporium and in smaller spores. In all, these findings suggest that ExsM plays a critical role in the formation of the exosporium.**

Bacillus cereus and *Bacillus anthracis* are closely related members of the *Bacillus cereus* group (47). Although *B. cereus* is mainly an apathogenic organism, certain isolates can cause two different types of food poisoning, emetic syndrome and diarrheal disease (18). The emetic syndrome is caused by ingestion of cereulide, a heat-resistant toxin produced by vegetative cells contaminating the food (30), while the diarrheal disease occurs when spores germinate in the intestinal tract. Spores are also the infective agent in anthrax, a disease caused by *B. anthracis* (64).

B. cereus and *B. anthracis* differentiate into spores when faced with nutrient deprivation. The spore is a dormant cell type that can remain viable for decades until favorable conditions induce germination and the resumption of vegetative growth. The remarkable resistance properties of the spore result from its unique architecture, consisting of a series of concentric protective layers (51). The spore core contains the genetic material and is surrounded by the cortex, a thick layer of modified peptidoglycan that promotes a highly dehydrated state. Encasing the core and the cortex, the coat is a multilayer protein shell that provides mechanical and chemical resistance. In addition, both the cortex and coat contribute to spore germination (17). Separated from the coat by an interspace, the exosporium encloses the rest of the spore, and it is composed of an inner basal layer and an outer hair-like nap (25).

Being the most external layer of the spore, the exosporium

interacts directly with the environment and as such provides a semipermeable barrier that may exclude large molecules, like antibodies and hydrolytic enzymes (3, 23, 24, 54). However, the exosporium does not appear to contribute to the typical resistance properties of the spore (6, 35, 60). Also, the exosporium is not necessary in anthrax pathogenesis when tested under laboratory conditions (7, 27, 59), although it is able to down-modulate the innate immune response to spores and mediate adhesion to host tissues (4, 8, 43, 44). The exosporium may also help the spore avoid premature germination in unsustainable environments, since it contains two enzymes, alanine racemase (Alr) and inosine hydrolase (Iunh), that can inactivate low quantities of the germinants L-alanine and inosine, respectively (6, 48, 55, 61). However, regulation of germination by the exosporium is poorly understood. Mutation of exosporial proteins has resulted in only negligible and inconsistent germination phenotypes (2, 5, 27, 28, 52, 54).

The exosporium is composed of at least 20 proteins and glycoproteins in tight or loose association (48, 53, 57, 61, 65). These proteins are synthesized in the mother cell and always start self-assembly at the forespore pole near the middle of the mother cell, concurrently with the cortex and coat formation (42). Exosporium assembly is discontinuous and starts with a synthesis of a substructure known as the cap, which likely contains only a subset of the proteins present in the exosporium (55). After cap formation, construction of the rest of the exosporium requires the expression of ExsY (6). BclA is the main component of the hair-like nap on the external side of the exosporium, and it is linked to the basal layer through interaction with ExsFA/BxpB (54, 58). In addition, CotE participates in the correct attachment of the exosporium to the spore (27).

Despite these findings, exosporium assembly continues to be a poorly understood process, and many questions remain re-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
<i>E. coli</i>		
DH5 α	Cloning host	Invitrogen
INV 110	<i>dam dcm</i> mutant	Invitrogen
<i>B. cereus</i>		
ATCC 4342	Wild type	ATCC
MF18	<i>exsM</i> ::pMFB4	This study
MF 65	<i>exsM</i> ::pMFF3	This study
ATCC 14579	Wild type	ATCC
MF 63	<i>exsM</i> ::pMFB6	This study
<i>B. anthracis</i>		
Δ Sterne	Wild type, pXO1 [−] pXO2 [−]	J. Lederberg
MF 43	<i>exsM</i> ::pMFB10	This study
Plasmids		
pASD2	Shuttle vector, <i>E. coli</i> / <i>Bacillus</i>	14
pRS69	Source of <i>gfpmut2</i>	49
pMMF1	pASD2 + <i>gfpmut2</i>	This study
pMMF3	pASD2 + <i>exsM-gfpmut2</i>	This study
pMFB4	pASD2 + internal fragment of <i>exsM</i> (<i>B. cereus</i> ATCC 4342)	This study
pMFB6	pASD2 + internal fragment of <i>exsM</i> (<i>B. anthracis</i>)	This study
pMFB10	pASD2 + internal fragment of <i>exsM</i> (<i>B. cereus</i> ATCC 14579)	This study

garding its composition and the regulation of its synthesis. In this study, we characterized a new spore protein, ExsM, which plays a key role in assembly of the exosporium. In *B. cereus*, inactivation of *exsM* resulted in spores with an unusual double-layer exosporium, and a similar phenotype was also observed in *B. anthracis exsM* null mutant spores. Finally, double-layer exosporium spores allowed us to study the role of the exosporium in germination and outgrowth.

MATERIALS AND METHODS

General methods. *Escherichia coli* DH5 α and INV110 were used for cloning procedures. The *B. cereus* and *B. anthracis* strains and cloning vectors are listed in Table 1. *E. coli* was grown in LB medium. *B. cereus* and *B. anthracis* strains were grown in brain heart infusion medium (BHI) (BD, Franklin Lakes, NJ). Antibiotic concentrations in the media were as follows: for *E. coli*, ampicillin at 100 μ g/ml and spectinomycin at 100 μ g/ml; for *B. cereus* and *B. anthracis*, spectinomycin at 250 μ g/ml; and for all strains, kanamycin at 20 μ g/ml.

Spore preparation. *B. cereus* and *B. anthracis* spores were prepared on Leighton-Doi agar plates (37), with incubation at 30°C for 3 to 5 days. The spores were harvested by centrifugation at 15,000 $\times$ g for 15 min at 4°C, and the spore pellets were washed in ice-cold distilled water 5 to 10 times by repeated centrifugation until the samples were >95% pure. The purity of the spore samples was checked by phase-contrast microscopy. Spores were stored at 4°C in TE buffer (50 mM Tris-HCl [pH 7.2] and 10 mM EDTA [pH 8]).

Fluorescence microscopy. Sporulation of MF65 carrying the ExsM-green fluorescent protein (GFP) fusion was induced by dilution of a stationary-phase culture at 1/100 into Leighton-Doi liquid medium. The culture growth was followed by measurement of the optical density at 600 nm (OD₆₀₀), and the end of exponential growth was considered time zero of sporulation. Cell samples were taken at 60-min intervals. *B. cereus* sporangia bearing the ExsM-GFP fusion protein were stained with 1.5 μ g/ml FM4-64 (Invitrogen, Carlsbad, CA), a cell membrane stain. The cells were visualized with a Nikon Y-IM microscope equipped with a Qimaging charge-coupled device (CCD) camera. Images were obtained with the Qimage software program and processed with Adobe Photoshop 7.0 software.

Creation of *B. cereus* and *B. anthracis* mutants. To insertionally inactivate *exsM*, the first 200 bp of the *exsM* gene (bcere0010_49400; 414 bp) were amplified using PCR and appropriate primers from chromosomal DNA of *B. cereus* ATCC 4342. The amplicon was then digested with KpnI and ligated into the plasmid pASD2, an *E. coli*-*B. cereus* shuttle vector with a thermosensitive origin of replication in *B. cereus* (14). The resulting plasmid, pMFB4 (pASD2::*exsM*^{200bp}), was passed through *E. coli* INV110 (*dam dcm* mutant) to obtain unmethylated DNA and then introduced into *B. cereus* ATCC 4342 by electroporation, following a previously described protocol (62). Transformants were selected by plating on BHI agar supplemented with spectinomycin and kanamycin. To select for plasmid integration via a single crossover event (and inactivation of *exsM*), these transformants were grown at 42°C for 8 h in BHI medium without antibiotics and then plated at 42°C on BHI agar with spectinomycin and kanamycin to promote growth of the recombinants. The resulting colonies were screened by PCR with primers external and internal to the pASD2 insertion site to confirm that integration occurred. We called the insertion mutant the *exsM* mutant.

The *B. anthracis exsM* mutant was constructed by following the same protocol, with the exception that the *exsM* gene (BAS5053) fragment was amplified from the chromosomal DNA of *B. anthracis* Δ Sterne. The *B. cereus* ATCC 14579 *exsM* mutant was also constructed following the same protocol, but in this case *exsM* (BC5206) was amplified from DNA of *B. cereus* ATCC 14579.

To construct the ExsM-GFP-expressing strain, the *gfpmut2* gene was amplified from pRS69 with specific primers, digested with EcoRV and SmaI, and ligated into the plasmid pASD2. Then, the last 200 bp (lacking the stop codon) of *exsM* were amplified from *B. cereus* ATCC 4342 chromosomal DNA. The amplicon was digested with KpnI and ligated in frame to the 5' end of *gfpmut2*. The resulting plasmid (pMMF3) was then electroporated into *B. cereus* ATCC 4342. Transformants and recombinants were selected as already described for the insertion mutants.

Spore protein extraction and separation. Before protein extraction, spores were sequentially washed in 1 M NaCl, 0.1% SDS-TE buffer, and TE buffer (48, 61). Later, washed spores were treated with 2% β -mercaptoethanol in 0.1 M sodium carbonate-bicarbonate buffer (pH 10) for 2 h at 37°C. The sample was centrifuged for 3 min at 15,000 $\times$ g, and the supernatant was passed through a 0.22- μ m filter to ensure that it was free of spores. Finally, the filtrate was boiled for 10 min in Laemmli sample buffer (Bio-Rad, Hercules, CA) and loaded into a 4 to 20% Tris-HCl polyacrylamide gel (Bio-Rad, Hercules, CA). Proteins were visualized by staining with colloidal Coomassie blue, GelCode Blue (Pierce, Rockford, IL), and Sypro ruby (Invitrogen, Carlsbad, CA), following the manufacturers' protocols. Protein identification of this sample by liquid chromatography-tandem mass spectrometry (LC-MS/MS) revealed the presence of several already-identified exosporium proteins with no contamination of mother cell proteins, which indicates that this extraction method is suitable for the study of the exosporium (M. M. Fazzini and V. A. Fischetti, unpublished data).

For the sonication extraction, washed spores were sonicated with a W-380 sonicator four times for 30 s (each) at 50%. The exosporium was separated from the rest of the spore by centrifugation for 3 min at 15,000 $\times$ g and further purified by passage through a 0.22- μ m membrane. To assess the efficiency of the extraction, the resulting spores were examined by scanning electron microscopy (SEM), and the exosporium fraction was studied by negative staining transmission electron microscopy (TEM). SEM revealed that most of the spores have lost the exosporium, and TEM showed the presence of exosporium fragments. In addition, LC-MS/MS analysis of the exosporium sample revealed the presence of several previously identified exosporium proteins (Fazzini and Vischetti, unpublished). The resulting sample was boiled in Laemmli sample buffer (Bio-Rad, Hercules, CA) for 10 min and loaded into a 4 to 20% Tris-HCl polyacrylamide gel (Bio-Rad, Hercules, CA). Proteins were stained with colloidal Coomassie blue, GelCode blue (Pierce, Rockford, IL), and Sypro ruby (Invitrogen, Carlsbad, CA), following the manufacturers' protocols.

To extract exosporium and coat proteins, washed spores were incubated in 50 mM sodium carbonate-bicarbonate buffer (pH 9.8), 8 M urea, 50 mM dithiothreitol (DTT), and 1% SDS for 2 h at 37°C (1, 10). The proteins were separated from the spores by centrifugation for 3 min at 15,000 $\times$ g and later were diluted 1/10 in Laemmli sample buffer and loaded into a 4 to 20% Tris-HCl polyacrylamide gel (Bio-Rad, Hercules, CA). The proteins were visualized by staining with GelCode blue (Pierce, Rockford, IL) and Sypro ruby (Invitrogen, Carlsbad, CA), following the manufacturers' protocols.

Transmission electron microscopy. Spores were fixed in 2.5% glutaraldehyde–75 mM sodium cacodylate buffer (pH 7.4) containing 0.15% Alcian blue for 4 h at room temperature. Spore pellets were then washed in sodium cacodylate buffer (pH 7.4) and postfixed for 1 h in a 1% osmium tetroxide–0.1 M sodium cacodylate buffer (pH 7.4) on ice and with 1% uranyl acetate. Dehydration involved sequential treatment with graded ethanol series. The samples

were then embedded in LR White resin. Ultrathin sections (70 nm) were cut on a Leica UltracutE instrument, and the sections were collected on uncoated 200-mesh grids and stained with 1% alcoholic uranyl acetate and Reynolds' lead citrate for 9 and 5 min, respectively. Grids were viewed with a Tecnai SpiritBT transmission electron microscope (FEI) at 80 kV, and pictures were taken with Gatan 895 Ultrascan digital camera.

Scanning electron microscopy. Spores were adhered to poly-L-lysine-coated glass coverslips. After 4 h of fixation in 2.5% glutaraldehyde in 75 mM sodium cacodylate buffer (pH 7.4) with 0.15% Alcian blue, the spores were postfixed for 1 h in 1% osmium tetroxide–100 mM sodium cacodylate buffer (pH 7.4) and were dehydrated through a graded ethanol series. The coverslips were then critical point dried and sputter coated with gold-palladium particles in a Vacuum Desk II (Denton) sputtering deposition system. Images were collected with a Leo 1550 Gemini scanning electron microscope at 2 kV.

Spore size measurement. Length and width measurements of wild-type (wt) and mutant spores were obtained from SEM photographs. The whole spore, including the exosporium, was measured. *B. cereus* wild-type and *exsM* mutant spore sizes were compared by the Mann-Whitney U test, which is appropriate to compare distributions with different variances, while *B. anthracis* wild-type and *exsM* mutant spore sizes were analyzed by unpaired Student's *t* test, since wild-type and mutant spore size distributions have the same variance.

Lysozyme resistance assay. Spores were incubated with lysozyme (250 µg/ml in phosphate-buffered saline [PBS]) for 5, 10, 20, and 40 min at 37°C, and the survivors were enumerated by plating dilutions on BHI (41). An aliquot without treatment was similarly plated on BHI and used to calculate the percentage of survival after lysozyme treatment. This experiment was performed in duplicate with three different preparations of wild-type and mutant spores, and statistical significance was analyzed by Student's *t* test.

Germination assays. Spore germination was measured by assessing the loss of heat resistance or a decrease in the optical density at 580 nm (OD₅₈₀). Loss of heat resistance was measured as described by Giebel et al. (26). Briefly, spores were diluted to 4 × 10⁵ spore/ml and heat treated at 65°C for 20 min to both activate spores and kill any remnant vegetative cell contaminating the sample. Samples were then diluted 1/200 in germination buffer (10 mM Tris-HCl, pH 7.4, and 10 mM NaCl), and germination was started with the addition of 50 mM L-alanine. Aliquots were removed at 2, 5, 10, 15, 20, and 30 min and incubated at 65°C for 20 min to kill germinated spores, and later, 50 µl of each aliquot was plated on BHI agar and incubated overnight. Also, an aliquot at time zero was plated without heat treatment. The fraction of germinated spores was calculated for each time point.

To measure the loss of OD₅₈₀, spores were first heat activated at 65°C for 20 min. After heat activation, spores were resuspended in ice-cold germination buffer to an OD₅₈₀ of 0.5. After the addition of germinants, either 50 mM L-alanine or a combination of 1 mM L-alanine and 1 mM inosine (32), the OD₅₈₀ of the spore suspension was measured in a Spectramax Plus multidetection reader (Molecular Devices) using a 15-s interval for 60 min at 30°C. Before each time point, the plate was shaken for 3 s to prevent spore settling. To inhibit Alr, spores were preincubated with 1 mM D-cycloserine (DCS) for 15 min at 30°C before the addition of germinants (46). Several combination of DCS (0.1, 1, and 10 mM) and L-alanine (20, 50, and 100 mM) were assessed, with 1 mM DCS and 50 mM L-Ala being the optimal combination. In all the cases, germination in the presence of L-alanine was stimulated in a dose-dependent manner after treatment with DCS, which indicates that Alr was inhibited (29). Spores were also observed under the phase-contrast microscope at different times during germination to control that germination was not blocked.

Outgrowth assay. The tetrazolium overlay assay was performed as previously described (13, 27). The change in OD₅₈₀ was also followed to quantify outgrowth in the wild-type and *exsM* mutant samples. Spores were heat activated at 65°C for 20 min and then diluted 50% in BHI to an initial OD₅₈₀ of 0.01, following a modified protocol described previously (50). The OD₅₈₀ was followed after dilution at 2.5-min intervals for 2 h at 30°C. Before each time point, the plate was shaken for 3 min to prevent spore settling. In addition, after 60 min, spores and outgrowth cells were counted under the phase-contrast microscope. We enumerated sphere-shaped cells as spores and cylindrical cells as nascent vegetative cells (40). At least 300 cells were counted in each sample.

RESULTS

Identification of ExsM. ExsM was identified from a proteomic analysis of *B. cereus* ATCC 4342 exosporium proteins. Purified spores were first washed with high-salt and detergent solutions in order to eliminate nonspecifically adsorbed pro-

teins, including remnants of the mother cell (48). Next, the washed spores were treated with 2% β-mercaptoethanol under alkaline conditions to extract the exosporium proteins. The treated spores were later examined by scanning electron microscopy (SEM) to confirm that this gentle method of exosporium extraction did not also disrupt the coat.

The extracted exosporium proteins were separated on a one-dimensional (1-D) SDS-PAGE gel and then subjected to trypsin in-gel digestion and LC-MS/MS mass spectrometry (21). The resulting peptide sequences were matched against a database containing *B. anthracis* and *B. cereus* genomes. From the list of putative proteins obtained, an uncharacterized protein was identified within a prominent band running at 12 kDa, which also contained ExsK, CotB, and other hypothetical proteins. This protein was a good candidate to be part of the exosporium since it has no homolog in *Bacillus subtilis*, whose spores do not have a conspicuous exosporium separated by an interspace; therefore, we decided to call it ExsM, following the nomenclature established by Todd et al. (61), and pursue its study.

ExsM is a small basic protein with a theoretical molecular mass of 15.4 kDa, which differs from its apparent molecular mass of 12 kDa, possibly due to processing or partial degradation. It is encoded by a monocistronic operon and contains a CHRD domain (33). CHRD is a recently defined protein motif identified in Chordin, a key developmental protein in vertebrates that, in simple terms, dorsalizes early embryonic tissues (15). Actually, the entire ExsM sequence constitutes the CHRD domain, with a positively charged, conserved C terminus. As yet, there is no functional prediction for the CHRD domain (33).

To study the subcellular localization of ExsM, we constructed a C-terminal in-frame fusion with the GFP coding sequence (67), which was incorporated into the *B. cereus* ATCC 4342 genome by allelic replacement. The expression of the ExsM-GFP fusion was then followed at different time points before and during sporulation. To localize the forespore and estimate the stage of sporulation by phase-contrast microscopy, cell membranes were stained with the lipophilic membrane stain FM4-64. Spores bearing the ExsM-GFP fusion had no morphological differences from wild-type spores when studied by electron microscopy (data not shown).

ExsM-GFP fluorescence was detected only during sporulation, shortly after the beginning of engulfment of the forespore. Furthermore, fluorescence was restricted to the mother cell, with no visible fluorescence in the forespore. Three hours after the start of sporulation, the ExsM-GFP fusion was localized at the forespore pole facing the middle of the mother cell (Fig. 1). At 5 h, the ExsM-GFP fusion was found only at both poles of the forespore. At 6 h, as the nascent spore became visible by phase-contrast microscopy, the fluorescence intensified at the poles. At 8 h, ExsM-GFP unevenly surrounded the whole circumference of the phase-bright spore, still inside the mother cell. This ring-like pattern of localization results from the projection of a three-dimensional (3-D) image into a 2-D photograph (28) and is typical of findings for other coat and exosporium proteins when their localization was studied by use of fusion with GFP (20, 36, 38, 63). It was not possible to localize ExsM-GFP on the mature spore once it was released from the mother cell, which showed no fluorescence. Western

FIG. 1. Fluorescence microscopic localization of ExsM-GFP during sporulation. (A) Fluorescence micrographs (magnification, $\times 1,000$) of representative cells producing ExsM-GFP that were collected at 3 h (T3), 4 h (T4), 6 h (T6), and 8 h (T8) after sporulation. The cell membranes were stained with FM4-64 (red fluorescence). The figure also shows the merged images of the ExsM-GFP fusion protein and FM4-64 and the same field view under phase contrast. The brackets indicate individual sporangia. (B) Fluorescence and phase-contrast micrographs (magnification, $\times 2,000$) of ExsM-GFP carrying mature spores. The white arrows point to the spore exosporium.

blots probed with anti-GFP IgG showed that by 16 h after sporulation, the ExsM-GFP signal was lost in favor of a lower-molecular-weight band which likely corresponded to GFP alone (see Fig. S1 in the supplemental material). van Ooij et al. (63) found that sporulation carried out at lower temperatures stabilized the fluorescence signals of GFP fusions on the sur-

faces of spores. We similarly found this when sporulation was performed at 25°C, and we were able to detect fluorescence from ExsM-GFP on the exosporium of the mature spores (Fig. 1B).

The timing of expression and localization of ExsM-GFP, starting at the forespore pole closer to the middle of the mother cell and moving toward the opposite pole, is shared with other outer-spore proteins (36, 60). This agrees with the presence of a putative σ^E promoter sequence (19) upstream of *exsM* (see Fig. S2 in the supplemental material). CotE, a protein that participates during the formation of the exosporium, is also under the control of σ^E (27).

Ultrastructural analysis. To gain further insight into the role of ExsM in spore formation, we inactivated the *exsM* gene in *B. cereus* ATCC 4342 by insertional mutagenesis. Spores were prepared from the *exsM* mutant and visualized by SEM. The mutant *exsM* spores were morphologically different from wild-type spores, mainly due to changes in the mutant exosporium (Fig. 2). While wild-type spores presented an extended exosporium (Fig. 2A, shown by an arrow), *exsM* mutant spores had a closely associated exosporium which lacked this characteristic (Fig. 2B). As a result, *exsM* mutant spores were significantly shorter than wild-type spores, with no difference in width (spore length for the wt, $2.40 \pm 0.56 \mu\text{m}$, versus $1.66 \pm 0.38 \mu\text{m}$ for the *exsM* mutant [$P < 0.001$, Mann-Whitney U test]; spore width for the wt, $0.90 \pm 0.05 \mu\text{m}$, versus $0.87 \pm 0.01 \mu\text{m}$ for the *exsM* mutant; $n = 155$ [wt] or 145 [*exsM* mutant]). In addition, the wild-type spore population had a greater variability in size than the *exsM* mutant spore population, in which the length distribution was more constricted (see Fig. S3 in the supplemental material).

Thin-section transmission electron microscopy (TEM) was performed to further visualize the differences between the wild-type and mutant exosporium. While the *exsM* mutant

FIG. 2. Ultrastructural analysis of mutant spores. (A and B) Scanning electron micrographs of *B. cereus* ATCC 4342 wild-type spores showing the typical morphology (A) or *exsM* mutant spores (B). In panel A, the white arrow points to the extended exosporium. Scale bar, 1 μm . (C, D, and E) Transmission electron micrographs of wild-type (C) or *exsM* mutant (D) spores. The black arrow(s) points to a single-layer exosporium in panel C or a double-layer exosporium in panel D. Panel E shows a representative *exsM* mutant spore stained with Alcian blue to increase contrast. The brackets denote the presence of the hair-like nap on both exosporium layers. Scale bar, 250 nm.

FIG. 3. SDS-PAGE analysis of exosporium and exosporium plus coat proteins in wild-type and *exsM* mutant spores. Proteins were separated in a 4 to 20% polyacrylamide gel and stained with colloidal Coomassie blue. Panel A shows the exosporium protein profile from the *B. cereus* ATCC 4342 wild-type (lane 2) or *exsM* mutant (lane 3) spores obtained after treatment with 2% β -mercaptoethanol for 2 h at 37°C. In panel B, exosporium proteins from wild-type (lane 5) and *exsM* mutant (lane 6) spores were extracted by sonication and boiled in Laemmli sample buffer for 10 min. Panel C shows the exosporium and coat protein profile extracted with 8 M urea and 4% SDS from wild-type (lane 7) and *exsM* mutant (lane 8) spores. Lanes 1 and 4 correspond to the protein standard, and each standard molecular mass is indicated in kDa on the left. The black arrows indicate the positions of bands whose intensities changed in the *exsM* mutant.

spores showed no major disruption in any of the spore concentric layers, 77% of them were actually encased in two distinct exosporium layers (Fig. 2D). To test if each layer constituted a complete exosporium and not simply an invagination of a single exosporium, we used the contrast agent Alcian blue to distinguish the exosporium hair-like nap from its basal layer on the mutant spores. We found that the hair-like nap was present on each of the exosporium layers and facing the same direction, toward the exterior of the spore (Fig. 2E), which argues against invagination.

We also constructed *exsM* null mutants in *B. cereus* ATCC 14579, which is the *B. cereus* type strain, and observed the same morphological changes seen with the *B. cereus* ATCC 4342 *exsM* spores. The *B. cereus* ATCC 14579 mutant *exsM* spores were shorter than wild-type spores (length for the wt, 2.46 ± 0.59 μ m, versus 1.76 ± 0.37 μ m for the *exsM* mutant [$P < 0.001$, Mann-Whitney U test]; width for the wt, 0.88 ± 0.05 μ m, versus 0.86 ± 0.06 μ m for the *exsM* mutant; $n = 154$ [wt] or 155 [*exsM* mutant]) and were composed of a double-layer exosporium (data not shown).

Spore protein profiles. Exosporium proteins from wild-type and *exsM* mutant spores were extracted with 2% β -mercaptoethanol under alkaline conditions, run on a 1-D SDS-PAGE gel, and stained with Coomassie blue (Fig. 3A) or Sypro ruby (data not shown). The resulting protein profiles showed no major differences besides an increase in the intensity of the band running at 26 kDa in the *exsM* mutant profile. When wild-type and mutant exosporiums were extracted by sonication (Fig. 3B), the *exsM* mutant protein profile presented a low-intensity band at 19 kDa and a more intense band at 45 kDa. It was not possible to precisely know which proteins were affected, since these bands contained more than one protein as determined by LC-MS/MS (Fazzini and Vischetti, unpublished). When the exosporium and coat proteins were extracted by incu-

FIG. 4. Lysozyme resistance assay. Wild-type or *exsM* mutant spores were incubated with 250 μ g/ml of lysozyme, and aliquots were taken at 0, 5, 10, 20, and 40 min. Serial dilutions of each aliquot were plated on BHI, and after overnight incubation, the survivors were enumerated. The graphic shows the mean and standard deviation (SD) of results from three independent experiments done with different preparations of wild-type and mutant spores. *, $P < 0.05$; **, $P < 0.005$ (wild-type versus *exsM* mutant; Student's *t* test).

bation with 8 M urea and 1% SDS under reducing conditions (1, 10), the protein profiles obtained were also nearly identical between wild-type and *exsM* mutant spores (Fig. 3C).

The similarity between the protein composition of wild-type and *exsM* mutant exosporiums discounts a grossly defective exosporium as the reason for duplicating this layer. However, subtle changes in the protein composition of the coat and exosporium arising from the mutation cannot be discounted. In all, our findings suggest that *ExsM* has only a small effect on the overall exosporium protein content.

Spore properties. We observed that wild-type and *ExsM*-deficient strains had very similar growth rates and sporulation efficiencies (data not shown). In addition, when the spore resistances to heat (80°C, 10 min) and organic solvents (chloroform, methanol, and phenol) were compared by following standard protocols (13), we found no differences between wild-type and mutant spores, indicating that the *exsM* mutant spore coat was not compromised, at least for these resistance properties, (data not shown). Steichen et al. (53) demonstrated that the absence of the exosporial nap moderately increased the sensitivity to lysozyme; hence, we tested spore resistance to 250- μ g/ml egg lysozyme treatment at several time points. The rapid drop seen in the wild-type spore population reflects the subset of spores that are particularly sensitive to lysozyme (35) (Fig. 4). Double-exosporium spores were more resistant to treatment with lysozyme after 5, 10, and 20 min (Fig. 4), which is consistent with the exosporium being a semipermeable barrier to harmful macromolecules (3, 23, 24).

Germination and outgrowth. The characteristic architecture of the spore contributes not only to its resistance properties but also to the resumption of vegetative growth under the right conditions. In particular, the exosporium may regulate the timing of germination, since this structure does include enzymes that modify or hydrolyze germinants. Alr converts L-alanine to its competitive inhibitor D-alanine (11, 68), and inosine hydrolase (IunH) degrades inosine and related nucleosides (6, 48, 55, 61).

FIG. 5. Spore germination in the presence of L-alanine. Heat activated wild-type or *exsM* mutant spores were incubated in the presence of 50 mM L-alanine, and germination was monitored by loss of heat resistance at selected time points. Means and SD of results from one representative experiment performed in duplicate are shown.

To investigate the response of *exsM* mutant spores to L-alanine (50 mM), we assessed both the loss of the spore heat resistance properties and the fall in optical density at 580 nm (OD_{580}) after the addition of the germinant. Both methods reflect the loss of the spore core content and rehydration, and the loss of OD_{580} also represents cortex hydrolysis (45). Compared to wild-type spores, ExsM-deficient spores showed a low rate of germination, but the efficiency of germination after 15 min was ultimately equal (Fig. 5 and 6A). To study if the lower rate in germination was due to the action of Alr or due to a

slower passage of L-alanine through the double-layer exosporium, we preincubated the spores with DCS (1 mM) to inhibit Alr before adding L-alanine (50 mM). We monitored germination by following the change in OD_{580} . The pretreatment with the Alr inhibitor increased the germination rate of *exsM* mutant spores to wild-type levels and also the germination efficiency to levels higher than those of the wild type (Fig. 6B). While these data suggest that *exsM* mutant spores have more Alr than wild-type spores, more experiments are needed to study the levels of Alr. In all, these results (Fig. 5 and 6) showed that double-exosporium spores have altered germination kinetics compared to wild-type spores, which suggest that the exosporium plays a key role in germination. In this regard, when germination was induced with L-alanine (1 mM) and inosine (1 mM), we observed an overall enhanced germination response from wild-type and mutant spores (12, 22, 34, 66). Also, *exsM* mutant spores exhibited a faster and more complete germination than wild-type spores (Fig. 6C). There was no effect on the germination rate when either spore population was pretreated with DCS (Fig. 6D), which agrees with a previous report that Alr has no effect when germination is activated by L-alanine (1 mM) and inosine (1 mM) (11).

The resumption of metabolism after germination of wild-type and *exsM* mutant spores was tested using a variant of the semiquantitative tetrazolium (TTZ) overlay assay (13). TTZ turns red once it is reduced by active dehydrogenases present in the germinated spore and is an indicator of outgrowth. After

FIG. 6. Effect of DCS on spore germination. Loss of OD_{580} was measured as an indicator of spore germination. Wild-type and *exsM* mutant spores were germinated in the presence of 50 mM L-alanine (A) or were pretreated with 1 mM DCS and then germinated with 50 mM L-alanine (B). Also, spores were germinated with 1 mM L-alanine and 1 mM inosine (C) or were pretreated with 1 mM DCS and then germinated with 1 mM L-alanine and 1 mM inosine (D). Means and SD of triplicate measurements of representative experiments are shown.

FIG. 7. Spore outgrowth in complete medium. (A) A photograph of a representative tetrazolium overlay assay of *exsM* mutant and wild-type strains is shown. (B) Changes in the OD₅₈₀ were used to assess wild-type and *exsM* mutant spore germination and outgrowth in BHI medium. Means and SD of results from one representative experiment performed in triplicate are shown.

2 h of incubation at 37°C, *exsM* mutants were less red than the wild type (Fig. 7A), although when the incubation was held for longer times (16 h), the color intensity was the same as that of the wild type (data not shown), which suggested that spore outgrowth might be slow but not blocked in the mutants. We compared germination and outgrowth of spores in BHI by monitoring changes in the OD₅₈₀. The drop in OD₅₈₀ observed

was not as high as in the germination assays because in this case spores were resuspended at a lower initial OD₅₈₀ (Fig. 7B). However, when a higher initial OD₅₈₀ was used to study germination in BHI, we obtained a drop of OD similar to that seen before (data not shown). It has been demonstrated that the extent of germination is positively correlated with spore density (9), and in the case of *B. cereus*, it results from the release of L-alanine by germinating spores, which in turn stimulates germination of the whole spore population (16). *ExsM*-deficient spores showed a more efficient germination on BHI but a lower rate of vegetative growth (Fig. 7B), confirming the results obtained with the semiquantitative assay. Since outgrowth can be visualized as a change from a spherical (spore) to a cylindrical (outgrowing cell) shape (40), we used phase-contrast microscopy to assess the percentage of outgrowing cells. After 60 min of incubation in BHI, the wild-type sample contained 76% $\pm$ 1.7% of outgrowing cells versus 44.5% $\pm$ 2.5% in the *exsM* mutant sample. Thus, the escape mechanism of the nascent cell may not be forceful enough to rapidly burst from the mutant double-layer exosporium, which may be reflected as a diminishing outgrowth rate.

B. anthracis ExsM-deficient mutants. We also inactivated *exsM* in *B. anthracis* Δ Sterne by insertional mutagenesis and studied the resulting mutant spores by SEM and TEM. The morphological differences seen between *B. anthracis* wild-type and *exsM* mutant spores were not as large as the ones seen between *B. cereus* wild-type and *exsM* mutant spores, because *B. anthracis* spores do not have an extended exosporium (Fig. 8A and B). Still, as with *B. cereus*, *B. anthracis exsM* mutant spores were shorter and rounder than wild-type spores (spore length for the wt, = 1.63 $\pm$ 0.21 μ m, versus 1.30 $\pm$ 0.21 μ m for the *exsM* mutant [P < 0.001, Student's t test]; spore width, 0.97 $\pm$ 0.08 μ m, versus 1.04 $\pm$ 0.11 μ m for the *exsM* mutant; n = 99

FIG. 8. Ultrastructural analysis of *B. anthracis exsM* mutant spores. (A to C) Scanning electron micrographs of *B. anthracis* Δ Sterne wild-type (A) or *exsM* mutant (B and C) spores. In panel C, the white arrows indicate spores that have partially lost their exosporium. Scale bar, 500 nm. (D and E) Transmission electron micrographs of *B. anthracis* Δ Sterne wild-type (D) or *exsM* mutant (E) spores. The black arrows points to a single-layer exosporium in panel D and a double-layer exosporium in panel E. Panel F shows a representative *exsM* mutant spore stained with Alcian blue to increase the contrast. The brackets denote the presence of the hair-like nap on top of the exosporium in both layers. Scale bar, 250 nm.

[wt] or 102 [*exsM* mutant] [see Fig. S4 in the supplemental material]). Additionally, we observed that the exosporium of the mutant spores was partially or fully detached from the spores (Fig. 8C). Thin-section TEM revealed that 30% of the *exsM* mutant spores had a partial double exosporium, versus only 4% for the wild-type spores, with both layers of exosporium presenting a basal layer and a hair-like nap in the correct orientation (Fig. 8E and F).

This partial phenotype could be due to BxpC, a paralogue of ExsM present in *B. anthracis*, which has been previously identified as a component of the *B. anthracis* exosporium (53). While inactivation of this gene alone did not affect the structure of the exosporium (data not shown), it could be compensating for the function of ExsM during exosporium formation in the mutants. BxpC has 40% identity and 62% similarity to ExsM. The main difference between ExsM and BxpC is in the C terminus, which is positively charged in ExsM but not in BxpC. Interestingly, ExsA and CotE, two proteins that participate in the attachment of the exosporium to the spores, have a negatively charged C terminus (2, 27). Alternatively, the *B. anthracis exsM* partial phenotype may be due to insufficient production of enough exosporium protein to construct two complete layers. *B. cereus* spores have larger exosporium; thus, there is more protein available for a double-layer exosporium. Additional studies with ExsM and BxpC double mutants are necessary to distinguish between these two options.

DISCUSSION

Several proteins have already been identified as components of the *B. cereus* and *B. anthracis* exosporium, and their absence during sporulation usually results in an exosporium that is incomplete, damaged, or unattached, depending on the specific protein that is missing (31). In this study, we describe a new exosporium protein, ExsM, and a new phenotype, the double-layer exosporium that resulted from its absence. These two separate layers that surrounded the spore possessed a hair-like nap facing the exterior of the spore, implying that the double-layer exosporium is not the product of simple invagination, yet we cannot discard that the double layer configuration could have been the result of one convoluted layer, which may appear like two layers under the electron microscope. While exosporium formation occurs without a visible connection to the rest of the spore, some communication through the interspace between these two structures is necessary to obtain a closed layer (27). It is possible that ExsM forms part of this communication system and its absence promotes disorder in the interspace that leads to the construction of a second exosporium. Alternatively, ExsM might inhibit the spontaneous formation of an extra exosporium layer. Since exosporium formation depends on a series of morphogenetic proteins that direct the assembly of structural proteins (31), a tight regulation of this process is needed to avoid deposition of premade exosporium proteins at the wrong time or place during sporulation. In this regard, a low percentage of wild-type *B. anthracis* spores presented lamellas in their interspace (data not shown), indicating the importance of this kind of regulation. Another possibility is that ExsM may be necessary for formation of the cap. In this case, absence of a defined cap during sporulation of *exsM* mutant spores can lead to a lack of polarity of the exosporium and result in duplication of this structure.

Although we need additional experiments to distinguish between these different options, studies with ExsM-GFP showed that ExsM is present at the correct time and initial site of exosporium formation, suggesting that it actively participates in this process.

The lower rate of germination displayed by *exsM* mutant spores in the presence of L-alanine was the result of the action of Alr present on the double exosporium. It was not due to a slower diffusion of L-alanine through two layers of exosporium, since the germination rate was improved with pretreatment with DCS. However, it is not clear if the Alr inhibitory effect seen with *exsM* mutant spores was due to an increased amount of this enzyme or its particular localization in two consecutive layers. Two percent β -mercaptoethanol extraction of the mutant exosporium showed an increased amount of protein running at 45 kDa, which could reflect an increase in Alr, whose theoretical molecular mass is 43.7 kDa, but this increment was very subtle, since it was not observed in the protein profiles from exosporium extracted by sonication.

Besides the inhibitory effect of Alr, we observed that the presence of an extra layer of exosporium increased the extent of germination with L-alanine and inosine and in complete medium. These results suggest that the exosporium not only affects the timing of germination by degrading small amounts of germinants but also promotes germination in the presence of the right amount of nutrients, which agrees with observations that the absence of a functional exosporium affects germination (27, 28).

Steichen et al. (55) showed that outgrowing cells always escape from the spore shell through the exosporium cap, which may be a weakened spot to facilitate such escape. Using this escape mechanism, the new vegetative cell is able to suddenly burst and separate from its exosporium in less than 1 s (55). We have shown that the presence of the double-layer exosporium slowed down outgrowth, probably because it presented a greater physical barrier to the escape process. It would be interesting to see how this result fits the "bottle cap model" (55) by investigating if the mutant double exosporium possesses one cap on each layer or if any of the layers are homogeneous.

In summary, we demonstrated that the exosporium provides protection against lysozyme, enhances germination, and participates in the outgrowth of the nascent cell, which are processes that need to occur under the right conditions to maximize the survival of the nascent vegetative cell and are central to the pathogenesis of both *B. cereus* and *B. anthracis* (39, 56).

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