

Proteins Involved in Formation of the Outermost Layer of *Bacillus subtilis* Spores^{∇†}

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To investigate the outermost structure of the *Bacillus subtilis* spore, we analyzed the accessibility of antibodies to proteins on spores of *B. subtilis*. Anti-green fluorescent protein (GFP) antibodies efficiently accessed GFP fused to CgeA or CotZ, which were previously assigned to the outermost layer termed the spore crust. However, anti-GFP antibodies did not bind to spores of strains expressing GFP fused to 14 outer coat, inner coat, or cortex proteins. Anti-CgeA antibodies bound to spores of wild-type and CgeA-GFP strains but not *cgeA* mutant spores. These results suggest that the spore crust covers the spore coat and is the externally exposed, outermost layer of the *B. subtilis* spore. We found that CotZ was essential for the spore crust to surround the spore but not for spore coat formation, indicating that CotZ plays a critical role in spore crust formation. In addition, we found that CotY-GFP was exposed on the surface of the spore, suggesting that CotY is an additional component of the spore crust. Moreover, the localization of CotY-GFP around the spore depended on CotZ, and CotY and CotZ depended on each other for spore assembly. Furthermore, a disruption of *cotW* affected the assembly of CotV-GFP, and a disruption of *cotX* affected the assembly of both CotV-GFP and CgeA-GFP. These results suggest that *cgeA* and genes in the *cotVWXYZ* cluster are involved in spore crust formation.

The sporulation of *Bacillus subtilis* is initiated in response to nutrient limitations and involves a highly ordered program of gene expression and morphological changes (20, 24). The first morphological change in sporulation is the appearance of an asymmetrically positioned septum that divides the cell into a larger mother cell and a smaller forespore. Next, the mother cell membrane migrates around the forespore membrane during a phagocytic-like process called engulfment. The completion of engulfment involves the fusion of the mother cell membrane to pinch off the forespore within the mother cell. Compartment-specific gene expression brings about the maturation of the spore and its release upon the lysis of the mother cell (reviewed in reference 10). Mature spores remain viable during long periods of starvation and are resistant to heat, toxic chemicals, lytic enzymes, and other factors capable of damaging vegetative cells (17). Spores germinate and resume growth when nutrients become available (19).

The outer portions of *Bacillus* spores consist of a cortex, a spore coat layer, and, in some cases, an exosporium. The cortex, a thick layer of peptidoglycan, is deposited between the inner and outer membranes of the forespore and is responsible for maintaining the highly dehydrated state of the core, thereby contributing to the extreme dormancy and heat resistance of spores. Spore coat assembly involves the deposition of at least 50 protein species (2, 13, 14) into two major layers: an electron-dense outer layer called the outer coat and a less

electron-dense inner layer with a lamellar appearance, called the inner coat (Fig. 1) (26). These layers provide a protective barrier against bactericidal enzymes and chemicals such as lysozyme and organic solvents (17). A loose-fitting, balloon-like exosporium surrounds the spore coats of some species, including *Bacillus anthracis*, *Bacillus cereus*, and some clostridia. This structure is not observed in the *B. subtilis* spore, with the exception of a strain isolated from the human gastrointestinal tract (2, 3). Therefore, the spore coat has been thought to be the outermost structure of the *B. subtilis* spore. However, we recently found a layer located outside the outer coat composed of CgeA and CotZ (Fig. 1) (4). Using a similar method, McKenney and coworkers also identified an outermost spore layer composed of CotG, CotW, and CotZ and demonstrated that this layer, which they termed the spore crust, is absent in *cotXYZ* mutant spores (16). The identification of spore surface proteins is also becoming increasingly important for potential practical applications (9). In this study, we demonstrate that the spore crust is the externally exposed, outermost component of the *B. subtilis* spore, forming a layer that covers the spore coat. Furthermore, we identified genes involved in spore crust formation as well as additional components of the spore crust.

MATERIALS AND METHODS

General methods and bacterial construction. *B. subtilis* cells were cultured in LB medium and induced to sporulate by exhaustion in Schaeffer's medium (18) at 37°C for 24 h. Plasmid DNA for the transformation of *B. subtilis* was harvested from *Escherichia coli* strain JM109. Bacterial strains, plasmids, and primers used in this study are listed in Tables S1 and S2 in the supplemental material.

To construct a vector to introduce GFP (green fluorescent protein)-fused genes into the *amyE* locus, we amplified an internal fragment of *amyE* by PCR using genomic DNA of *B. subtilis* 168 as a template and primer pair AMYE980/AMYE1860R. The fragment was digested with HindIII and then cloned into

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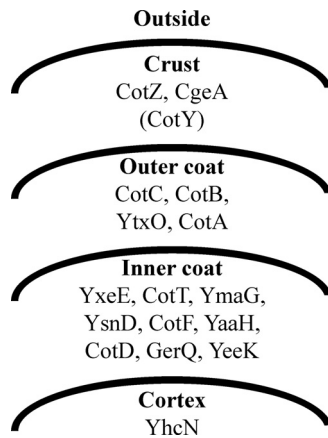


FIG. 1. Schematic representation of the outer structure of the *B. subtilis* spore and proteins assigned to each layer. With the exception of CotY (in parentheses), assignments of proteins in each layer are based on our previous data (4). The localization of CotY in the spore crust is suggested by the results of this study.

HindIII-digested pGFP7C to yield plasmid pGFP7CA, in which the *amyE* fragment was downstream of GFP and in the same orientation.

To fuse GFP to the C terminus of CotV or CotY, we first amplified each gene and its 5' promoter region (27) from *B. subtilis* 168 genomic DNA by PCR using primer pairs COTVM350/COTV383R and COTX40/COTY485R, respectively. Fragments were digested with BamHI and XhoI and then cloned into BamHI/XhoI-digested pGFP7CA to yield plasmids pCOTV8GA and pCOTY8GA. These plasmids were introduced into the *amyE* locus by a single-crossover event with selection for chloramphenicol resistance (5 μ g/ml), yielding strains COTV8GA and COTY8GA (see Table S1 in the supplemental material). To introduce the *cotZ*-GFP fusion into the *amyE* locus without the *cotY* gene, we amplified the promoter region of the *cotYZ* and *cotZ* genes from *B. subtilis* 168 genomic DNA by PCR using primer pairs COTYM200/COTYM7R and COTZ1/COTZ443R, respectively. EcoRI/BamHI-digested P_{cotYZ} and BamHI/XhoI-digested *cotZ* fragments were cloned into EcoRI/XhoI-digested pGFP7CA to yield the plasmid pCOTZ8GA. This plasmid was introduced into the *amyE* locus by a single-crossover event with selection for chloramphenicol resistance (5 μ g/ml), yielding strain COTZ8GA (Table S1). All GFP fusions used in this study contain a hexahistidine tag at their C termini, but for simplicity, these constructs are referred to as GFP fusions.

To disrupt *cotV*, *cotW*, *cotX*, *cotY*, or *cotZ*, we amplified an internal fragment of each gene from *B. subtilis* 168 genomic DNA by PCR using primer pairs COTV19/COTV220R, COTW34/COTW230R, COTX20/COTX169R, COTY21/COTY184R, and COTZ97/COTZ311R, respectively. Fragments were digested with HindIII and BamHI and then cloned into HindIII/BamHI-digested pMutin3 to yield the corresponding plasmids pCOTV5E, pCOTW5E, pCOTX5E, pCOTY5E, and pCOTZ5E. These plasmids were introduced into the *cotV*, *cotW*, *cotX*, *cotY*, or *cotZ* locus by a single-crossover event with selection for erythromycin resistance (0.5 μ g/ml), yielding strains COTV5E, COTW5E, COTX5E, COTY5E, and COTZ5E, respectively.

Immunofluorescence staining of spores. Following sporulation in Schaeffer's medium for 24 h, aliquots of cells were washed twice with phosphate-buffered saline (PBS) (pH 7.4). Pellets were resuspended in GTE-lysozyme buffer (50 mM glucose, 20 mM Tris-HCl [pH 7.5], 10 mM EDTA, 200 μ g/ml lysozyme) and incubated for 20 min at room temperature. After washing twice with PBS, pellets were resuspended in 2% bovine serum albumin in PBS (BSA-PBS) and then incubated for 30 min. Rabbit polyclonal anti-GFP antibodies (12) or rabbit polyclonal anti-CgeA antibodies (11), diluted 1:500, were added, and the mixture was incubated for 30 min at room temperature. Pellets were washed twice with PBS and incubated with a 1:1,000 dilution of Alexa Fluor 594-conjugated goat anti-rabbit IgG (Invitrogen) in BSA-PBS for 30 min at room temperature. Thereafter, pellets were washed twice with PBS and transferred onto microscope slides.

Fluorescence microscopy. The fluorescence of GFP and Alexa Fluor 594-conjugated goat anti-rabbit IgG (Invitrogen) was observed by using an Olympus BX51 fluorescence microscope fitted with mirror cube units for green (U-MGFPHQ) and red (U-MWG2) (Olympus, Japan), respectively. A UPlanApo

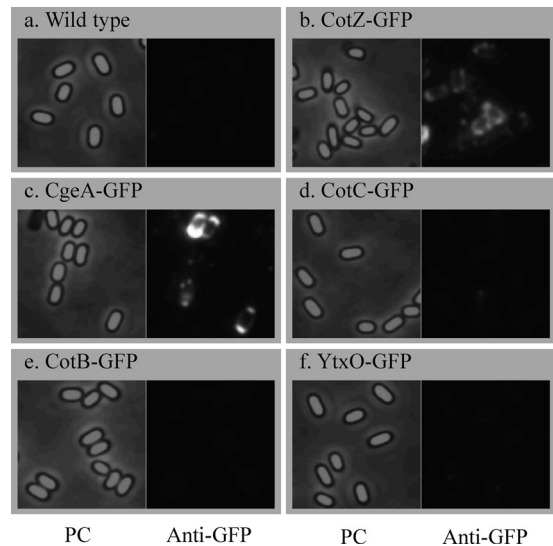


FIG. 2. Immunofluorescence microscopic detection of GFP-fused proteins in *B. subtilis* spores using anti-GFP antibodies. Strains 168 (a), COTZ8G (b), CGEA8G (c), COTC8G (d), COTB8G (e), and YTXO8G (f) were induced to sporulate by nutrient exhaustion and subjected to immunofluorescence microscopy with anti-GFP antibodies. Phase-contrast (PC) and immunofluorescence (Anti-GFP) images were captured by using exposure times of 1 s and 0.2 s, respectively.

100 \times oil Iris 3Ph objective lens and a U-TV1X-2 camera adapter were used. Images were captured by using a cooled charge-coupled-device camera (CoolSNAP ES/OL; Roper Scientific). Images were generated by RS Image Express processing software, version 4.5. Images of GFP fusion proteins were captured by fluorescence microscopy using exposure times of 0.5 to 1 s, and red fluorescence was imaged by using an exposure time of 0.2 s. The autofluorescence of spores under the experimental conditions used to capture GFP fluorescence and red immunofluorescence was obtained from exposure times of 3 s and 1 s, respectively.

RESULTS

Surface exposure of spore crust proteins. In order to obtain insights into the outermost structure of the *B. subtilis* spore, we first addressed whether the spore crust is indeed the outermost layer. The outermost layer is exposed on the surface of the spore and should thus be accessible to antibodies. We therefore carried out an immunofluorescence microscopy analysis using an anti-GFP antibody and *B. subtilis* strains expressing spore coat protein-GFP fusions described previously (Fig. 1) (4). In these strains, GFP was translationally fused to the 3' end of the gene at the native locus so that the fusion gene was the only copy that was expressed. As expected, no fluorescent anti-GFP antibody signal was detected in wild-type spores, which do not express GFP (Fig. 2a). In contrast, fluorescence surrounding spores was observed for CotZ-GFP and CgeA-GFP spores (Fig. 2b and c), indicating that anti-GFP antibodies bound specifically to GFP on the spores of these GFP fusion strains (see also Fig. S1 in the supplemental material for merged color images). However, strains expressing GFP fusions of other proteins, including four outer coat proteins, nine inner coat proteins, and one cortex protein (Fig. 1), showed no fluorescent anti-GFP antibody signal (Fig. 2 and Fig. S1). As shown in Fig. 3, a count of the number of spores with anti-GFP antibody immunofluorescence revealed that approximately

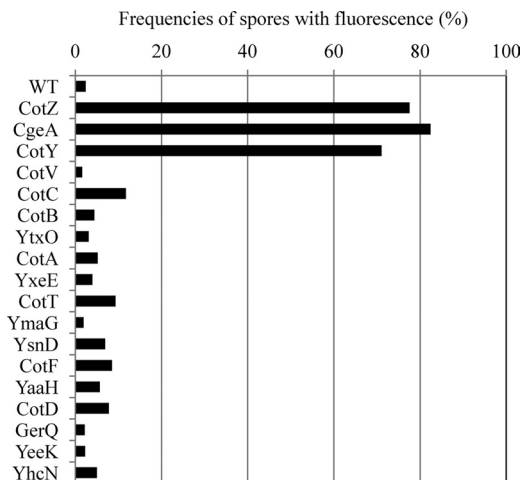


FIG. 3. Frequencies of spores stained with anti-GFP antibodies. The numbers of spores positive and negative for anti-GFP antibody staining in the experiments shown in Fig. 2 and Fig. S1 in the supplemental material were counted. The percentages of spores in the total population with immunofluorescence are shown. At least 100 spores were counted for each strain. Immunofluorescence images of all strains were captured by using an exposure time of 0.2 s. WT, wild type.

80% of spores of CotZ-GFP and CgeA-GFP strains were immunopositive (background in wild-type spores, <5%) (for CotY and CotV data, see below) (Fig. 3). In contrast, the majority of spores from strains containing GFP fusions of 14 proteins assigned to the outer coat, inner coat, and cortex exhibited no anti-GFP antibody fluorescence (Fig. 1 and 3 and Fig. S1). GFP fusion strains for all 16 proteins exhibited abundant GFP fluorescence (green) surrounding spores (4), indicating that all fusion proteins were expressed and located in spores; however, with the exception of CotZ-GFP and CgeA-GFP, none was accessible to antibodies. These results indicate that GFPs fused to CotZ and CgeA are exposed on the surface of spores, whereas GFPs fused to proteins assigned to the outer coat, inner coat, and cortex are not, consistent with the idea that the spore crust is the outermost layer of the *B. subtilis* spore.

Although CotZ-GFP and CgeA-GFP were located on the surface of spores, it was conceivable that the surface exposure of these proteins was an artifact of the fusion to GFP. To rule out this possibility, we next performed immunofluorescence microscopy of wild-type spores using anti-CgeA antibodies. Anti-CgeA antibodies bound strongly to spores of both wild-type and CgeA-GFP strains but not to a *cgeA* mutant strain (Fig. 4; see also Fig. S2 in the supplemental material for merged color images). A count of the number of spores with anti-CgeA antibody immunofluorescence revealed that 3.17%, 72.2%, and 82.6% of spores of *cgeA* mutant, wild-type, and CgeA-GFP strains were immunopositive, respectively (at least 300 spores were counted). These results indicate that anti-CgeA antibodies bound specifically to the CgeA protein on the surface of the *B. subtilis* spore. Therefore, we conclude that the spore crust composed of CotZ and CgeA is the outermost structure of the *B. subtilis* spore and forms a layer that covers the spore coat.

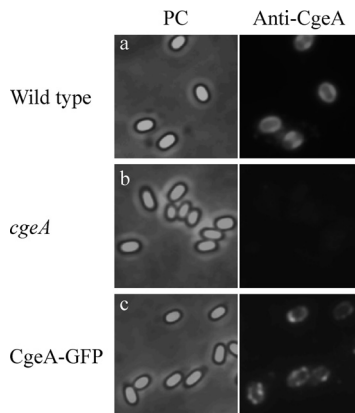


FIG. 4. Immunofluorescence microscopic analysis of *B. subtilis* spores using anti-CgeA antibodies. Strains 168 (a), MTB945 (b), and CGEA8G (c) were induced to sporulate by nutrient exhaustion and subjected to immunofluorescence microscopy using anti-CgeA antibodies. Phase-contrast (PC) and immunofluorescence (Anti-GFP) images were captured by using exposure times of 1 s and 0.2 s, respectively.

Proteins involved in spore crust formation. The localization of CotW-GFP to the spore crust and the assembly of CotW-GFP around the spore require *cotXYZ* genes, suggesting that CotX, CotY, and/or CotZ plays important roles in spore crust formation (16). *cotVWXYZ* is a cluster of genes transcribed at the late stage of sporulation in mother cells from the promoters P_{cotVWX} , P_{cotX} , and P_{cotYZ} (27). Because CotW and CotZ are localized to the spore crust (4, 16), it is conceivable that the genes in the cluster are involved in spore crust formation. In order to identify genes important for spore crust formation, we monitored CgeA-GFP in the *cotV*, *cotW*, *cotX*, *cotY*, and *cotZ* mutant strains (Fig. 5). Because *cotW* and *cotZ* are cotranscribed with the upstream genes *cotV* and *cotY*, respectively, they are also disrupted in the *cotV* and *cotY* insertional mutant strains through a polar effect (Fig. 5). In the absence of the *cotV* and *cotW* genes, CgeA-GFP localized around the spore, indicating that CotV and CotW are dispensable for CgeA-GFP assembly on spores (Fig. 5a, b, and c). However, CgeA-GFP assembly around the spore was abolished by the *cotX* disruption; instead, CgeA-GFP was observed as spots adjacent to spores (Fig. 5d, arrows; see also Fig. S3 in the supplemental material for merged color images). This result indicates that CotX has an important role in spore crust formation. Because *cotY* and *cotZ* are paralogous genes and are cotranscribed from the promoter P_{cotYZ} , it is conceivable that the disruption of *cotY* or *cotZ* individually might not affect the formation of the spore crust. Interestingly, the disruption of either the downstream gene *cotZ* or the upstream gene *cotY* abolished the assembly of CgeA-GFP around spores, indicating that CotZ plays a critical role in the formation of the spore crust and that CotY cannot fully compensate for the loss of CotZ function (Fig. 5e and f). Misassembled materials containing CgeA-GFP appeared as dark areas adjacent to spores by phase-contrast microscopy of the *cotZ* mutant strain but not *cotY* mutants (Fig. 5f, arrows; see also Fig. S3 in the supplemental material for merged color images), suggesting that spore crust-like material formed but failed to assemble around the spore of the

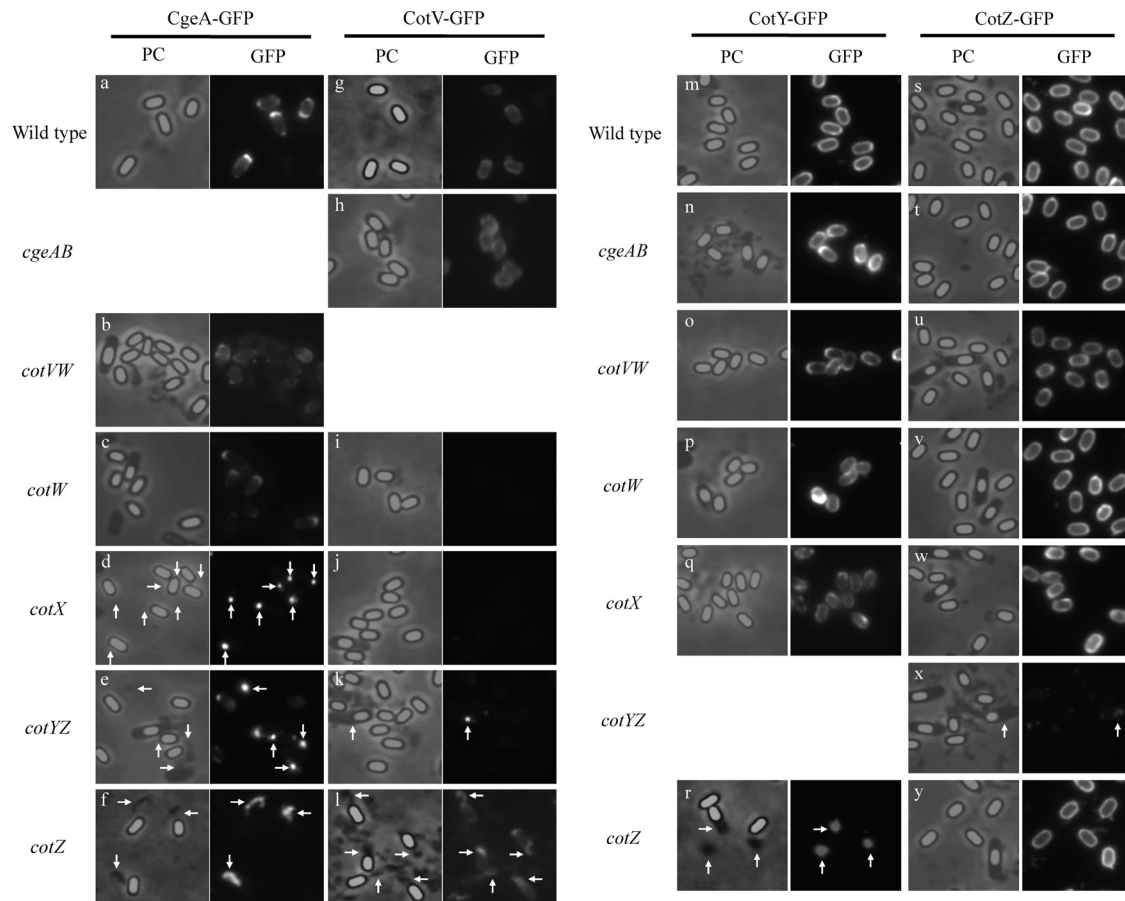


FIG. 5. Localization of proteins in wild-type and mutant spores. Strains expressing *cgeA*-GFP (a to f), *cotV*-GFP (g to l), *cotY*-GFP (m to r), or *cotZ*-GFP (s to y) and those with insertional mutations in *cgeA* (h, n, and t), *cotV* (b, o, and u), *cotW* (c, i, p, and v), *cotX* (d, j, q, and w), *cotY* (e, k, and x), or *cotZ* (f, l, r, and y) were induced to sporulate by nutrient exhaustion. (Left) Phase-contrast images; (right) GFP fluorescence images. Arrows indicate misassembled materials.

cotZ mutant. The disruption of *cgeA*, *cotV*, *cotW*, *cotX*, *cotY*, or *cotZ* did not affect the localization of the other 14 proteins assigned to the outer coat, inner coat, or cortex (Fig. 1 and data not shown). These results indicate that among the tested genes, at least *cotX* and *cotZ* have important roles in spore crust formation but not in spore coat formation.

Because CotW and CotZ are located in the spore crust (4, 16), we constructed strains expressing C-terminal GFP fusion proteins of CotV, CotX, or CotY. We introduced *cotV*-GFP, *cotX*-GFP, or *cotY*-GFP into the *amyE* locus (27). Therefore, the intact gene remains at the native locus in these strains. Although the fluorescence intensity of the CotX-GFP strain was below the level of detection (data not shown), CotV-GFP and CotY-GFP fluorescence located around the spores was clearly evident (Fig. 5g and m). CotV-GFP fluorescence was partially detached in *cotZ* mutant spores (Fig. 5l, arrows), indicating that normal CotV assembly requires CotZ. The localization of CotY-GFP around the spore was abolished in the *cotZ* mutant (Fig. 5r, arrows). Misassembled material that appeared dark by phase-contrast microscopy in *cotZ* mutant spores also contained CotV-GFP and CotY-GFP (Fig. 5l and r, arrows; see also Fig. S4 and S5 in the supplemental material for merged color images). We also carried out immunofluo-

rescence microscopy of CotV-GFP and CotY-GFP strains using anti-GFP antibodies (Fig. 3 and Fig. S1). Although CotV-GFP was not exposed on the surface of spores, antibodies bound efficiently to spores of the CotY-GFP strain. Taken together, these data indicate that CotV-GFP partially depended on CotZ for its localization on the spore, whereas CotY-GFP, which is exposed on the surface of the spore, requires CotZ to surround the spore. These results further suggest that CotY and, possibly, CotV are additional components of the spore crust.

We next attempted to identify genes important for the assembly of CotV-GFP and CotY-GFP on spores. Because *cgeA* and *cgeB* are cotranscribed as an operon, an insertional mutation in *cgeA* affects *cgeB* expression through a polar effect. The disruption of *cgeAB* did not affect the localization of CotV-GFP or CotY-GFP (Fig. 5h and n). CotV and CotW were dispensable for CotY-GFP assembly around the spore, but CotV-GFP failed to localize on spores of the *cotW* mutant, as indicated by the absence of obvious fluorescence (Fig. 5i, o, and p). Likewise, CotX was required for the localization of CotV-GFP, but not CotY-GFP, on spores (Fig. 5j and q). The mutation in *cotY*, which affects the expression of both *cotY* and *cotZ*, abolished CotV-GFP localization; fluorescence was ob-

served as a spot in the mother cell (Fig. 5k, arrow). Misassembled material containing CgeA-GFP, CotV-GFP, and CotY-GFP, observed adjacent to free spores of the *cotZ* mutant (Fig. 5f, l, and r, arrows), was not found for the *cotY* mutant (Fig. 5e and k). Thus, CotY is presumably needed to form the misassembled spore crust-like material observed for *cotZ* mutant spores.

To investigate the importance of CotY, we introduced *cotZ*-GFP and the promoter region of *cotZ* upstream of *cotY* (without the *cotY* gene) into the *amyE* locus. CotZ-GFP expressed from the *amyE* locus surrounded spores in the absence of *cgeAB*, *cotVW*, *cotW*, and *cotX*, indicating that CotZ is able to localize on the spore independent of CgeA, CgeB, CotV, CotW, and CotX (Fig. 5s, t, u, v, and w; see also Fig. S6 in the supplemental material for merged color images). CotZ-GFP also localized appropriately on spores in the absence of the *cotZ* gene at the native allele (Fig. 5y). CgeA-RFP (red fluorescent protein) was detached from spores in the *cotZ* mutant strain, but this detachment was suppressed by the introduction of *cotZ*-GFP into the *amyE* locus (data not shown). These data indicate that CotZ-GFP is functional at least in terms of spore crust formation. Therefore, we could assess the importance of CotY using the strain possessing a *cotYZ* disruption and *cotZ*-GFP at the *amyE* locus (Fig. 5x). CotZ-GFP failed to localize around the spore, and only faint fluorescence in the mother cell was observed (Fig. 5x, arrow). These results indicate that CotY and CotZ depend on each other to localize on spores, and both proteins have critical roles in spore crust formation. Taken together, our results suggest that (i) CgeA, CotY, and CotZ are located in the spore crust; (ii) CotY and CotZ play critical roles in spore crust formation; (iii) CotW has an important role in the assembly of CotV-GFP; and (iv) CotX has an important role in the assembly of CgeA-GFP and CotV-GFP.

DISCUSSION

A loose-fitting, balloon-like exosporium surrounds the spore coats of some bacterial species, including *B. anthracis*, *B. cereus*, and some clostridia, whereas this feature is not observed for the *B. subtilis* spore, with the exception of a strain isolated from the human gastrointestinal tract (2, 3). The gene product of *B. anthracis* *exsY*, the *B. subtilis* *cotY* and *cotZ* ortholog, was identified in purified exosporium (21). Taken together with our finding that CotY and CotZ are components of the spore crust, this suggests the possibility that the spore crust of *B. subtilis* is a cognate of the *B. anthracis* exosporium layer. Some exosporium proteins of *B. anthracis* are glycosylated or associated with glycosylated material (23). Spore crust was stained by ruthenium red under an electron microscope, suggesting that the spore crust is composed of polysaccharide in addition to proteins (16). These observations suggest the possibility that the composition of the spore crust is similar to that of the exosporium. *cgeA* is part of the *cgeABCDE* cluster, whose transcription is controlled by σ^K and GerE, and *spsA*, the paralog of *cgeD*, encodes glycosyltransferase (22, 24). Since *cgeABCDE* and *spsA*, as well as *cotVWXYZ*, are expressed late during sporulation in the mother cell, these gene products might also be involved in spore crust formation.

The exosporium is dispensable for most laboratory tests of

spore resistance; thus, its function is not well understood. It was suggested previously that the exosporium has a role in protecting spores of *B. anthracis* from macrophage-mediated killing (7). Also, in *Bacillus thuringiensis*, the exosporium may protect insecticidal crystal toxins (often enclosed within the structure) from the environment, thus enhancing pathogenicity (1). The function of the spore crust is also not yet clear, since *cotZ* mutant spores, which lack a spore crust, are resistant to heat and lysozyme treatment (data not shown). Zhang et al. reported previously that *cotXYZ* mutant spores have an unusual outer coat that is readily disrupted during fixation, embedding, or sectioning for electron microscopy (28), suggesting that the spore crust might contribute to the tight packing of the outer coat. *cotXYZ* mutant spores readily clump upon purification (28), and *cotZ* mutant spores also tended to clump during our experiments (data not shown). In addition, our attempts to identify proteins exposed on the surface of *cotZ* mutant spores by immunofluorescence microscopy failed, since antibodies bound nonspecifically to these spores (data not shown). These observations suggest that spores lacking a spore crust are abnormally adhesive; thus, in a natural environment, the spore crust might help to prevent clumping and allow spores to distribute.

Since there is no obvious phenotype for a crust-defective spore and the disruption of any one gene encoding a spore coat protein typically has little or no effect on spore resistance, morphology, or germination, it is difficult to know whether the GFP fusions used in this study are functional. Anti-CgeA antibody covered all around wild-type spores (Fig. 4a), whereas anti-GFP (Fig. 2c) and anti-CgeA (Fig. 4c) antibodies did not uniformly stain spores of the CgeA-GFP strain. These observations suggest the possibility that the GFP fusion affects CgeA localization or CgeA exposure on the surface of spores. Therefore, we cannot rule out the possibility that the proteins used in this study were affected by the fluorescent protein fusions. However, at least the localization and surface exposure of CgeA on spores were confirmed for the wild-type strain (Fig. 4a), and CotZ-GFP was functional in terms of forming the spore crust. Immunofluorescence microscopy of wild-type spores using anti-CotV, anti-CotY, and anti-CotZ antibodies or Western blot analysis of proteins extracted from purified spores of GFP fusion strains using specific antibodies for spore coat and spore crust proteins is desirable to check whether the GFP fusion of proteins used in this study is affecting the assembly of the coat or crust component.

Although *cotY* and *cotZ* are paralogous genes, their gene products could not compensate for each other in spore crust formation and instead depended on each other to assemble on spores (Fig. 5r and x). There are several possible reasons why these proteins required each other to assemble on spores. One possibility is that the proteins might have distinct, essential roles in spore crust formation and need the spore crust to localize on the spore. Alternatively, these proteins may form hetero-oligomers; therefore, both proteins are necessary to form a protein polymer in the spore crust. The latter possibility could be tested by using yeast two-hybrid experiments or pull-down assays, which would help reveal the interaction network of proteins in the spore crust.

The identification of spore surface proteins is becoming increasingly important for potential practical applications (9). It

was proposed previously that CotA, CotB, CotC, and CotG are externally exposed on the surface of spores (6, 8, 15, 25), whereas CotA-GFP, CotB-GFP, and CotC-GFP were not efficiently exposed on spores in this study (Fig. 3). There are several possible reasons for the discrepancy. Purification procedures used in previous studies may have partially removed the crust and exposed the outer coat. GFP was fused to the C-terminal end of the coat and crust proteins in this study; however, GFP fused to the N-terminal end of these proteins might be exposed on spores more efficiently, as was the case in a previous study (5). The frequencies of spores with fluorescence signals in immunofluorescence microscopy using anti-GFP antibodies of CotA-GFP, CotB-GFP, and CotC-GFP strains were slightly higher than that of the wild type (Fig. 3). These results suggest that some outer coat proteins are present in the spore crust in small amounts that are at least detectable by methods used in previous studies (e.g., each antibody exhibits different sensitivities) (6, 8, 15, 25). However, our data demonstrated that the spore crust proteins CgeA, CotY, and CotZ were clearly more efficiently exposed externally on the spore surface than outer coat proteins (Fig. 3). Accordingly, CgeA, CotY, and CotZ would be preferred candidates for anchoring heterologous proteins to display on the surface of *B. subtilis* spores.

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