Characterization of the *Bacillus subtilis* Spore Morphogenetic Coat Protein CotO

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Bacillus spores are protected by a structurally and biochemically complex protein shell composed of over 50 polypeptide species, called the coat. Coat assembly in *Bacillus subtilis* serves as a relatively tractable model for the study of the formation of more complex macromolecular structures and organelles. It is also a critical model for the discovery of strategies to decontaminate *B. anthracis* spores. In *B. subtilis*, a subset of coat proteins is known to have important roles in assembly. Here we show that the recently identified *B. subtilis* coat protein CotO (YjbX) has an especially important morphogenetic role. We used electron and atomic force microscopy to show that CotO controls assembly of the coat layers and coat surface topography as well as biochemical and cell-biological analyses to identify coat proteins whose assembly is CotO dependent. *cotO* spores are defective in germination and partially sensitive to lysozyme. As a whole, these phenotypes resemble those resulting from a mutation in the coat protein gene *cotH*. Nonetheless, the roles of CotH and CotO and the proteins whose assembly they direct are not identical. Based on fluorescence and electron microscopy, we suggest that CotO resides in the outer coat (although not on the coat surface). We propose that CotO and CotH participate in a late phase of coat assembly. We further speculate that an important role of these proteins is ensuring that polymerization of the outer coat layers occurs in such a manner that contiguous shells, and not unproductive aggregates, are formed.

Complex, organized macromolecular structures are ubiquitous in biological systems. Examples include bacteriophage, flagella, and the mitotic apparatus (22, 36, 39). In each of these cases, a subset of structural proteins has especially important roles in guiding morphogenesis, while the remainder has only limited impact on the overall architecture. In this work we focus on the assembly of a multiprotein protective structure called the coat that plays an essential role in bacterial spore survival.

Spores are formed by bacilli and clostridia in order to endure extended periods of nutrient deprivation and extremes of environmental stress (42, 43). A series of protective structures that encase the spore play critical roles in survival. The coat is a proteinaceous structure present in spores of all species and has multiple complex functions, including protection from toxic molecules and facilitating germination (14, 17, 25, 27). The coat is also flexible, being able to fold and unfold as needed, to accommodate changes in the interior volume of the spore that are part of the process of spore formation and germination (7, 13, 15, 47, 55, 65). In *Bacillus subtilis*, where it is the outermost spore structure, the coat is comprised of multiple layers that are organized into two major sets: a darkly staining outer coat and a lightly staining, finely striated inner coat (Fig. 1A) (1, 63). These layers are made up of at least 50 protein species (19, 20, 33, 34, and H. Kim, P. Grabowski, M. Hahn, D. C. McPherson, R. Wang, C. Ferguson, P. Eichenberger, and A. Driks, submitted for publication). Understanding the

mechanisms that guide deposition of these proteins into the correct layer not only gives insight into the formation of complex macromolecular assemblies but may also provide strategies for the decontamination of pathogenic spore-forming species, including certain clostridia and *B. anthracis* (see, for example, reference 32). Studies in *B. subtilis* are greatly facilitated by the wealth of basic knowledge of this organism and the ease with which it can be manipulated (56).

Studies from several laboratories reveal that the coat proteins are organized into a complex interaction network (Fig. 2A, left panel) (14, 17, 25, 27). Studies of the assembly of this network have identified a relatively small subset of the coat proteins, called morphogenetic proteins, which have especially important roles in coat protein assembly. One of the earliest acting of these is SpoIVA, which attaches the coat to the underlying spore membrane (16, 46, 49, 50, 52, 58). SpoVID is also involved in attachment of the coat to the forespore; in its absence, the shell of the coat that assembles under the direction of SpoIVA does not remain connected to the forespore, with the ultimate result of a swirl of coat in the mother cell (5, 16). SpoVID binds to the morphogenetic coat protein SafA, guiding it to the forespore (44, 45). SafA is required for normal coat composition and ultrastructure and for normal germination (44, 60). The morphogenetic coat protein CotE resides at the inner coat-outer coat interface and is responsible for the assembly of the outer coat (4, 38, 68). One of the coat proteins assembled under the control of CotE is CotH, which directs deposition of an important coat protein subset (38, 41, 70). In addition to its role in coat protein assembly, CotH also stabilizes coat proteins, one of which is CotG (2, 30, 71). CotG, in turn, directs CotB deposition (41, 53, 71).

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FIG. 1. Thin-section electron and atomic force microscopic analysis of wild-type and mutant spores. Electron micrographs of (A) wild-type (PY79), (B) *cotO* (PE250), (C) *cotH* (SL639), (D and E) *cotH cotO* (MH163) sporangia as well as (F) *cotH cotO* (MH163) spores and (G) a wild-type (PY79) sporangium. The images in panels B and C are representative of the range of variation seen in both *cotH* and *cotO* mutant spores. Atomic force micrographs of (H) wild-type, (I) *cotO*, and (J) *cotH* spores, analyzed in the tapping mode, are also shown. Outer coat (OC) and inner coat (IC) are indicated in panels A and B, and cortex (CX) is indicated in panel A. Outer coat inclusions are indicated with arrows in panels D and E but not F (where the inclusion is on the left face of the spore). Bars, 0.5 μ m.

Coat protein synthesis begins after the cell has formed the asymmetrically located septum that is the early hallmark of sporulation (28). This event divides the cell into a larger mother cell and a smaller forespore. Important morphogenetic coat proteins, including most of those mentioned above, begin to be synthesized at this time, and synthesis continues, at least for some of these proteins, for most or all of the remainder of sporulation (19–21, 57, 69). Next, an endocytic-like event transforms the forespore into a protoplast that resides entirely within the mother cell. Based on extensive analysis, there is no reason to suspect that coat proteins have any role in gene expression (12, 19, 20, 41). Therefore, the control of coat formation by the morphogenetic coat proteins is most likely exerted solely at posttranslational levels, especially assembly. After a process that lasts about 8 to 10 h, the mother cell lyses and releases the spore into the environment. Interestingly, coat

assembly continues even after ejection into the extracellular environment, as some coat protein cross-linking events continue for several more hours (51).

We have been identifying coat proteins by a combination of proteomic, genetic, and cell biological methods (19, 20, 34, 38). In this work, we characterize one such protein, CotO, previously referred to as YjbX (20). We show CotO to be an important morphogenetic coat protein that shares some (although not all) of the functions of CotH. These results reveal a novel layer of complexity in coat assembly and suggest that morphogenetic coat proteins with at least partially overlapping functions are maintained during evolution.

MATERIALS AND METHODS

General methods. *B. subtilis* and *Escherichia coli* were cultured in Luria-Bertani medium. *B. subtilis* was sporulated either by exhaustion in Difco sporu-

FIG. 2. Roles of CotH and CotO in spore coat morphogenesis. The cartoons show arcs of the coat. The outside of the spore is on the left in each panel. A) Previous (left panel) and new (right panel) understanding of the control of late events in coat assembly emphasizing the roles of CotH and CotO in morphogenesis. Arrows indicate the dependency of assembly of one protein on another. Not all morphogenetic or CotE-dependent coat proteins are indicated. CotE-independent assembly of CotH and CotO and insoluble forms of CotB, CotG, and CotS are indicated by the absence of connecting arrows. Major morphogenetic proteins are circled in the left panel. B) Speculative model of the role of CotH and CotO in preventing outer coat protein aggregation. Only the outer-most coat layers are indicated. (Left) Normally, polymerization of the outer coat proteins is biased towards formation of sheets of coat because CotH and CotO direct coat protein interactions largely, but not entirely, towards edgewise assembly. (Right) In the absence of CotH and CotO, the bias is away from interactions that direct polymerization in sheets. CotH and CotO are not indicated in the diagram.

lation medium or by resuspension (10), as indicated. Lysozyme sensitivity and tetrazolium overlay assays and transformation of *B. subtilis* were performed as described previously (10). Strain construction used standard procedures (10) and is described in Table 1. All strains are congenic with PY79. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was carried out essentially as described previously (38), using spores harvested after 24 to 28 h of sporulation by exhaustion, followed by water washing and Renografin purification (10).

Microscopy. Cells were sporulated by either resuspension at 37°C or exhaustion at 30°C and were examined either immediately (for autofluorescence microscopy) or were fixed with 3% paraformaldehyde (for immunofluorescence microscopy). For autofluorescence microscopy, sporangia were counterstained with FM4-64 (Molecular Probes) (at a concentration of 1.5 μ M) to visualize the cell membranes (48, 49). Immunofluorescence microscopy (IFM) was carried out as described previously (18) with the following modifications. Briefly, strains bearing an appropriate green fluorescent protein (GFP)-coat protein fusion were sporulated by resuspension, harvested after 24 h, washed three times in water, and applied to poly-L-lysine-coated multiwell slides. After application of 2% (wt/vol) bovine serum albumin (BSA) (Boehringer Mannheim) for 15 to 30 min, the cells were incubated at room temperature for at least 2 h with anti-GFP antibodies (Molecular Probes) at a dilution of 1:2,000 in 2% BSA, washed with phosphate-buffered saline (100 mM NaCl, 2 mM KCl, 5 mM Na₂HPO₄, 2 mM KH₂PO₄), and incubated at 4°C overnight with Alexa 568-conjugated goat anti-rabbit antibodies (Molecular Probes) at a dilution of 1:1,000 in 2% BSA. The wells were then washed and treated with Slow Fade (Molecular Probes) to reduce quenching. Images were acquired using a Leica DM IRB microscope and an Optronics Magnafire cryo-cooled camera with Magnafire 2.1 software. Images were processed using Adobe Photoshop 7.0. Electron and atomic force microscopy (EM and AFM, respectively) were performed as described previously (6, 7), using cells cultured for 18 to 24 h.

Construction of coat protein gene-*gfp* fusions. To generate translational fusions of coat protein genes to *gfp*, full-length genes (including promoters) or their 3' ends were first PCR amplified using primers listed in Table 2. These PCR products were treated with restriction enzymes that digested sites in the primers (Table 2) and ligated with either pSL36 (which integrates at *amyE*) (38) (digested with EcoRI and PmeI), pSL48 (which integrates at *thrC*) (digested with EcoRI and PmeI), pKL147 (37) (digested with EcoRI and XhoI), or pCVO119 (62) (digested with BamHI and XhoI) (the last two of which integrate by Campbell-type single reciprocal integration) (see Table 1). The resulting plasmids were then used to transform either strain PY79 or PE250 (Δ *cotO::tet*).

To generate strains DM84, DM86, and DM92 (Δ *cotO::tet amyE::cotO*, *cotO* Ω *cotO-gfp amyE::cotO*, and Δ *cotO::tet amyE::cotO Δ 179-227, respectively), we first used PCR to amplify either complete or 5'-truncated versions of the *cotO* gene, using primers OL734 and either OL735 or OL774 (to build strain DM84 or DM92, respectively). We then separately ligated each fragment with plasmid pDG364, after digestion of the PCR products and the plasmid with EcoRI and BamHI. We then linearized the resulting plasmids with BglII and used each one to transform strain PE250, resulting in strains DM84 (Δ *cotO::tet amyE::cotO*) and DM92 (Δ *cotO::tet amyE::cotO* Δ 179-227). We also used linearized plasmid pDM05 to transform strain PE371, resulting in strain DM86 (*cotO* Ω *cotO-gfp amyE::cotO*).*

RESULTS

Phenotype of *cotO* spores. We have been searching for novel morphogenetic proteins among a large collection of candidate coat proteins (19, 20, 34). Here we characterize YjbX (20), which we rename CotO. Previous studies demonstrated that CotO is synthesized under the direction of σ^E (20, 57) and localizes to the forespore under the control of CotE (20). We first characterized a *cotO* null mutant (PE250). This strain grew and entered sporulation normally and produced phase bright forespores, indicating largely normal spore core dehydration (55 and data not shown). However, transmission EM analysis revealed significant defects in coat ultrastructure in released spores. Wild-type *B. subtilis* spores possess inner and outer layers that are in close contact with each other and the underlying cortex (Fig. 1A). In contrast, *cotO* mutant spore coats had several defects. Most noticeably, the outer coat was frequently disorganized and often was missing over portions of the spore circumference (Fig. 1B and data not shown). This was true of *cotH* mutant spores as well (see below). In fact, *cotO* and *cotH* mutant spores were indistinguishable by thin-section EM. The images in Fig. 1B and C represent the range of morphological variation for both *cotO* and *cotH* mutant spores. Additionally, the inner and outer coat layers were frequently separated from each other at various points along the circumference of the spore, and the thickness of the layers varied much more than in the wild type. Interestingly, these defects were significantly less noticeable in spores yet to be released from the mother cell (data not shown). It seems that exposure of the spore to the extracellular milieu is needed for the coat defect to become fully apparent.

These morphological defects suggested that surface topography might also be significantly disturbed. To examine this possibility, we used AFM in the tapping mode to generate images of spore surfaces. Wild-type spores possess ridges, most of which span the long axis of the spore, and are well separated from each other (7, 47) (Fig. 1H). In contrast, *cotO* mutant spores frequently possessed multiple ridges in closer juxtaposition (Fig. 1I). This can be seen on the right side of the *cotO* mutant spore in the figure, in which three ridges are packed closely together.

TABLE 1. Strains and plasmids

Strain or plasmid	Genotype or description	Reference or strain construction ^a
Strain		
PY79	Wild type	66
DL067	<i>cotB::cat trpC2</i>	11
ADL318	<i>cotB::cat</i>	DL067→PY79
ADL194	<i>cotC::cat</i>	11
AD28	<i>ΔcotE::cat</i>	68
ADL04	<i>ΔcotE::cat::sp^r</i>	4
SL616	<i>ΔcotE::cat::neo^r</i>	pIK105→AD28
ER203	<i>ΔcotG::erm^r trpC2</i>	53
SL733	<i>ΔcotG::erm^r</i>	ER203→PY79
ER209	<i>cotH::cat trpC2</i>	41
SL639	<i>cotH::cat</i>	ER209→PY79
SL641	<i>cotH::cat::sp^r</i>	pJL62→SL639
PE250	<i>ΔcotO::tet^r</i>	20
CB701	<i>cotS::cat trpC2</i>	59
SL621	<i>cotS::cat</i>	CB701→PY79
SL622	<i>cotS::cat::sp^r</i>	pJL62→SL621
CB702	<i>cotSA::cat trpC2</i>	61
SL734	<i>cotSA::cat</i>	CB702→PY79
ADL867	<i>cotQΩpMM04 sp^r</i>	34
AD17	<i>gerE36</i>	9
SL637	<i>ΔcotE::cat::neo^r cotH::cat</i>	ER209→SL616
MH166	<i>ΔcotE::cat::sp^r ΔcotO::tet^r</i>	PE250→ADL04
DM88	<i>ΔcotG::erm^r cotS::cat</i>	SL621→SL733
MH198	<i>ΔcotG::erm^r cotC::cat</i>	ER203→ADL194
MH163	<i>cotH::cat ΔcotO::tet^r</i>	PE250→SL639
MH145	<i>cotC::cat ΔcotG::erm^r cotS::cat::sp^r</i>	SL622→MH198
DM103	<i>amyE::cotA-gfp cat</i>	pDM12→PY79
DM105	<i>amyE::cotB-gfp cat</i>	pDM14→PY79
CW271	<i>cotEΩpCW13(cotE-gfp) cat</i>	64
SL530	<i>amyE::cotG-gfp cat</i>	pNG01→PY79
HS172	<i>cotHΩpHS1(cotH-gfp) sp^r</i>	pHS1→PY79
PE371	<i>cotOΩpPE37(cotO-gfp) sp^r</i>	20
MMO106	<i>cotQΩpMMO12(cotQ-gfp) sp^r</i>	31a
PG80	<i>cotSΩpPG16(cotS-gfp) sp^r</i>	pPG16→PY79
MMO116	<i>amyE::cotU-gfp cat</i>	31a
PE599	<i>cotWΩpPG2(cotW-gfp) sp^r</i>	pPG2→PY79
PE388	<i>yhaXΩpPE42(yhaX-gfp) sp^r</i>	20
PE391	<i>yuzCΩpPE43(yuzC-gfp) sp^r</i>	20
PE479	<i>yutHΩpPE61(yutH-gfp) sp^r</i>	62
PE405	<i>yybIΩpPE53(yybI-gfp) sp^r</i>	20
SL546	<i>ΔcotE::cat::sp^r amyE::cotG-gfp cat</i>	SL530→ADL04
HS187	<i>ΔcotE::cat cotHΩpHS1(cotH-gfp) sp^r</i>	HS172→AD28
MH166	<i>ΔcotE::cat::sp^r cotOΩpPE37(cotO-gfp) sp^r</i>	PE371→ADL04
DM112	<i>ΔcotO::tet^r amyE::cotA-gfp cat</i>	DM103→PE250
DM114	<i>ΔcotO::tet^r amyE::cotB-gfp cat</i>	DM105→PE250
HS251	<i>ΔcotO::tet^r cotEΩpCW13(cotE-gfp) cat</i>	CW271→PE250
HS80	<i>ΔcotO::tet^r amyE::cotG-gfp cat</i>	SL530→PE250
HS189	<i>ΔcotO::tet^r cotHΩpHS1(cotH-gfp) sp^r</i>	HS172→PE250
HS122	<i>ΔcotO::tet^r cotQΩpMMO12(cotQ-gfp) sp^r</i>	MMO106→PE250
PG81	<i>ΔcotO::tet^r cotSΩpPG16(cotS-gfp) sp^r</i>	PG80→PE250
HS123	<i>ΔcotO::tet^r thrC::cotU-gfp erm^r</i>	MMO116→PE250
PG67	<i>ΔcotO::tet^r cotWΩpPG2(cotW-gfp) sp^r</i>	PE599→PE250
DM75	<i>ΔcotO::tet^r yhaXΩpPE42(yhaX-gfp) sp^r</i>	PE388→PE250
PG68	<i>ΔcotO::tet^r yutHΩpPE61(yutH-gfp) sp^r</i>	PE479→PE250
DM76	<i>ΔcotO::tet^r yuzCΩpPE43(yuzC-gfp) sp^r</i>	PE391→PE250
DM77	<i>ΔcotO::tet^r yybIΩpPE53(yybI-gfp) sp^r</i>	PE405→PE250
DM84	<i>ΔcotO::tet^r amyE::cotO cat</i>	pDM05→PE250
DM86	<i>cotOΩcotO-gfp sp^r amyE::cotO cat</i>	pDM05→PE371
DM92	<i>ΔcotO::tet^r amyE::cotOΔ179-227 cat</i>	pDM08→PE250
DM110	<i>ΔcotO::tet^r amyE::cotOΔ179-227-gfp cat</i>	pDM18→PE250
Plasmids		
pIK105	Converts <i>cat</i> to <i>neo^r</i>	29
pJL62	Converts <i>cat</i> to <i>sp^r</i>	35
pDG364	<i>amyE</i> integration vector	31
pDG795	<i>thrC</i> integration vector	23

Continued on following page

TABLE 1—Continued

Strain or plasmid	Genotype or description	Reference or strain construction ^a
pCVO119	Allows for Campbell integration of translational fusions to <i>gfp</i>	62
pKL147	Allows for Campbell integration of translational fusions to <i>gfp</i>	37
pSL36	Allows for double recombination at <i>amyE</i> of translational fusions to <i>gfp</i>	38
pSL48	<i>gfp</i> retrieved from EcoRI/BamHI digest of pSL36 and ligated into EcoRI/BamHI-digested pDG795; allows for double recombination at <i>thrC</i> of translational fusions to <i>gfp</i>	This work
pDM05	1,013 bp of <i>cotO</i> (OL734/OL735) cloned into pDG364	This work
pDM08	851 bp of <i>cotO</i> Δ179-227 (OL770/OL773) cloned into pDG364	This work
pDM12	1,758 bp of <i>cotA</i> (OL712/OL742) cloned into pSL36	This work
pDM14	1,520 bp of <i>cotB</i> (OL286/OL668) cloned into pSL36	This work
pDM18	851 bp of <i>cotO</i> Δ179-227 (OL770/OL774) cloned into pSL36	This work
pHS1	3' 274 bp of <i>cotH</i> (OL759/OL760) cloned into pKL147	This work
pNG01	<i>cotG</i> (OL196/OL197) cloned into pSL36	This work
pPG2	3' 254 bp of <i>cotW</i> (CotW-L/CotW-R) cloned into pCVO119	This work
pPG16	3' 339 bp of <i>cotS</i> (CotS-L/CotS-R) cloned into pCVO119	This work

^a Donor chromosomal or plasmid DNA was used to transform the respective strains as indicated by the arrows.

Major changes in coat ultrastructure typically result in defects in spore function. Therefore, we measured the ability of *cotO* spores to perform two major activities of the coat: exclusion of large molecules and germination. Wild-type spores, when challenged with lysozyme, successfully protect the peptidoglycan underneath (Table 3) (1), presumably by acting as a sieve that excludes the enzyme. In contrast, some coat defects permit penetration of lysozyme (see, for example, reference 4). *cotO* spores showed a 10-fold decrease in lysozyme resistance (Table 3), indicating that the barrier formed by the coat is not fully intact. For comparison, *cotE* spores, which are missing the entire outer coat (68), showed a 100-fold decrease in lysozyme resistance, and *gerE* spores, which have greatly reduced inner and outer coat layers (40), showed a one-million-fold decrease.

To determine if *cotO* spores had a germination defect, as we would expect from the defects seen by microscopy, we used the tetrazolium overlay assay, which monitors the resumption of metabolism after germination by color changes in a dye (10). In contrast to wild-type cells, which produce a deep reddish-purple color in this assay, *cotO* spores produce a light purple that is

distinct from the pink produced by *cotE* spores and the very light pink of *gerE* spores (Table 3). Because this assay will also detect defects that appear subsequent to germination (i.e., during outgrowth), it is possible that phenotypes measured this way are due to impairments in such late events. However, since these spores possess defects in the coat which are unlikely to affect events after germination, we interpret these results as probably being due to a defect at a relatively early stage.

To investigate the molecular basis of the *cotO* phenotypes, we analyzed the coat protein composition of *cotO* spores using SDS-PAGE analysis of spore coat extracts. In an electropherogram of a *cotO* spore extract, bands at about 59 kDa, 45 kDa, 44 kDa, 40 kDa, and 36 kDa are decreased in intensity relative to the wild-type spore extract (Fig. 3A, lanes 1 and 2). We believe these bands correspond to CotB, CotQ, CotSA, CotS, and CotG, based on previously published data (11, 34, 38, 53, 59, 61, 71), and that they are absent or (in the case of CotQ) significantly reduced in intensity in strains bearing the appropriate mutations (Fig. 3A, lanes 1 to 7). Interestingly, of the two bands that correspond to CotG (36 kDa and 31 kDa) (53,

TABLE 2. Primers

Primer (gene)	Sequence (5'–3') ^a	Nucleotides
OL712 (<i>cotA</i>)	CAATTGGCGAGAAAGCGGCTGCC	–207–191
OL742 (<i>cotA</i>)	AGTACTTTTATGGGGATCAG	+1526–1539
OL286 (<i>cotB</i>)	AAAAAGAATTCCCGAGTTTCGCAAGTCCTAC	–359–341
OL668 (<i>cotB</i>)	GTTTAAACCCAAATTTACGTTTCC	+1140–1127
OL196 (<i>cotG</i>)	AAAAAGAATTCTTGATTACCTTCATCACAGC	–296–240
OL197 (<i>cotG</i>)	AAAAAAGGATCCTTAGATATCTTTGTATTTCTTTTGAC	+585–568
OL759 (<i>cotH</i>)	AAAAAGAATTCAAGGATTTAATACACTAACCGCC	+812–840
OL760 (<i>cotH</i>)	AAAAAAGCTCGAGTAAATACTTAAATGATCTTTGA	+1086–1064
OL734 (<i>cotO</i>)	GAATTCACAGCATTCTGCGCGCC	–318–300
OL735 (<i>cotO</i>)	GGATCCTTAAAAACCGAGCGGG	+684–669
OL770 (<i>cotO</i>)	AAAAAGAATTCACAGCATTCTGCGCGCC	–318–300
OL773 (<i>cotO</i>)	CGCTCTCGGCATGTTATGAGGC	+534–513
OL774 (<i>cotO</i>)	AAAAAGGATCCTTACGCTCTCGGCATGTTATGAGGC	+534–513
CotS-L (<i>cotS</i>)	CGTATCCCGGGATCCTACGATTGCGGTCAAGAGAC	+715–736
CotS-R (<i>cotS</i>)	CGTCTAGCCCTCGAGATTTCGCTCCCGATACGCTTT	+1053–1032
CotW-L (<i>cotW</i>)	CGTATCCCGGGATCCCGAAAATGCTGGTCCAAAATA	+62–83
CotW-R (<i>cotW</i>)	CGTCTAGCCCTCGAGATTGTTATCGTTTTTCCGCTCTTTG	+315–291

^a Unique restriction endonuclease sites are in boldface in each oligonucleotide sequence.

TABLE 3. Spore properties

Strain (genotype)	Lysozyme resistance ^a	Tetrazolium result
PY79 (wild type)		Reddish-purple
PE250 (<i>cotO</i>)	10	Light purple
PE371 (<i>cotO-gfp</i>)	10	Light purple
SL639 (<i>cotH</i>)	10	Light purple
MH163 (<i>cotH cotO</i>)	10 to 10 ²	Red
ADL04 (<i>cotE</i>)	10 ²	Pink
MH166 (<i>cotE cotO</i>)	10 ²	Light pink
SL637 (<i>cotE cotH</i>)	10 ²	Light pink
ADL17 (<i>gerE</i>)	10 ⁶	Very light pink
MH145 (<i>cotC cotG cotS</i>)	ND ^b	Purple
DM84 (<i>cotO amyE::cotO</i>)	ND	Purple
DM86 (<i>cotOΔcotO-gfp amyE::cotO</i>)	ND	Purple
DM92 (<i>cotO amyE::cotOΔ179-227</i>)	ND	Light purple

^a Lysozyme resistance is listed as a fold decrease in CFUs compared to the wild type.

^b ND, not determined.

71), only the 36-kDa species is reduced in intensity in the *cotO* mutant. In some cases in *cotO* mutant extracts, two bands were present immediately below the 47-kDa marker (Fig. 3A, lane 2, and C, lane 2). One or both bands are likely to correspond to CotQ, since these bands are significantly reduced in intensity in *cotQ* mutant spore extracts relative to the wild type (Fig. 3A, lanes 1 and 4). We also found that bands at about 26.5 kDa, 22 kDa, and 20 kDa were increased, and bands at about 24.5 kDa, 21 kDa, and 19 kDa were reduced in intensity relative to the wild type (Fig. 3A, lanes 1 and 2). We do not know the identities of these bands.

Because SDS-PAGE measures only extractable proteins, it will fail to detect highly cross-linked proteins. The control of both soluble and insoluble proteins by CotO is of interest. To detect insoluble proteins whose assembly is under the control of CotO, we also carried out fluorescence microscopic analysis of strains bearing both the *cotO* mutation and one of a set of coat protein gene-*gfp* fusions. Strains with inactivated *cotO* and also bearing either *cotA-gfp* (DM112) (data not shown), *cotB-gfp* (DM114) (Fig. 4A, images ii), *cotG-gfp* (HS80) (Fig. 4B, images ii), *cotQ-gfp* (HS122) (Fig. 4C, images ii), *cotS-gfp* (PG81) (Fig. 4D, images ii), *cotU-gfp* (HS123) (Fig. 4E, images ii), or one of the recently identified coat proteins *yhaX-gfp* (DM75), *yutH-gfp* (PG68), *yuzC-gfp* (DM76), or *yylI-gfp* (DM77) showed localization patterns resembling what is seen in otherwise wild-type cells (Fig. 4A, images i, to E, images i, and data not shown). As expected of fusions to CotE-controlled proteins, strains bearing CotA-, CotB-, CotG-, CotQ-, CotS-, or CotU-GFP as well as a *cotE* mutation did not form rings of fluorescence (Fig. 4B, images iii) (data not shown; 31a). We note that, using Western blot analysis and anti-GFP-antibodies, we identified 75-kDa and 90-kDa species consistent with the possibility that CotB-GFP forms a homodimer (71). These data indicate that assembly of these fusions is largely CotO independent. Therefore, while CotO controls assembly, to varying degrees, of the fractions of CotB, CotG, and CotS that are SDS-PAGE detectable, a significant presumably insoluble fraction of these proteins remains CotO independent. In contrast to those results, localization of CotW-GFP (in strain PG67) appeared to be CotO dependent by fluorescence microscopy (Fig. 4F, images ii). Instead of the rings of fluores-

cence seen in otherwise wild-type cells, in the presence of the *cotO* mutation fluorescence was present throughout the mother cell and, in some cases, was more intense at the mother cell face of the forespore than the mother cell-located fluorescence. We feel it is likely that the fluorescence at the mother cell face represents the more biologically relevant location, since when cells were cultured at 30°C, we saw only this localization and not the dispersed signal (data not shown). Taken as a whole, these results indicate that CotO partially controls assembly of a subset of CotE-controlled proteins. Because this control could be the result of partial control of CotE assembly by CotO, we examined CotE-GFP assembly in a *cotO* mutant strain. CotE-GFP localization was indistinguishable from what was seen in an otherwise wild-type strain (data not shown), consistent with the view that CotO does not control coat protein assembly through an effect on CotE deposition.

Comparison of *cotO* and *cotH* phenotypes. The *cotO* phenotypes described so far resemble those of *cotH* (41, 70). Specifically, we could not distinguish between *cotH* and *cotO* mutants by thin-section EM (see above) or by the lysozyme or tetrazolium overlay assays (Fig. 1B and C, Table 3). Mutations in other important coat protein genes, in particular *safA*, also impact on outer coat morphology (44, 60). However, to our knowledge, none of these resembles *cotO* mutant spores as strongly as does *cotH*. In contrast to the EM results, SDS-PAGE analysis revealed differences between the sets of CotH- and CotO-controlled proteins. The most obvious differences between *cotH* and *cotO* mutant extracts are that the 26.5-kDa, 22-kDa, and 19-kDa bands in *cotH* mutant extracts appear closer in intensity to the corresponding wild-type bands than do those in *cotO* mutant extracts and that the 31-kDa CotG band is absent in *cotH* mutant extracts (Fig. 3B, lanes 1 to 3). We were unable to resolve the 12-kDa CotH-controlled protein CotC (11, 41) and were, therefore, unable to determine whether its intensity differed between *cotO* and *cotH* mutant extracts. Overall, our analysis of the protein composition of *cotH* spores was consistent with previous findings (41, 70, 71). Additional differences between the CotH- and CotO-controlled proteins were revealed by fluorescence microscopic analysis of the coat proteins CotQ, CotU, and CotW (34, 67). In a separate study, we showed that CotH directs the assembly of CotQ and CotU but not CotW (31a). In contrast, in the present work we found that CotW assembly, but not that of CotQ or CotU, depends on CotO (Fig. 4C, images ii, E, images ii, and F, images ii).

The finding that the *cotO* phenotypes appear to be a subset of the *cotH* phenotypes raised the possibility that CotH controls CotO deposition. However, results from other work, in which we analyzed a *cotH cotO-gfp* strain, demonstrated that CotO assembly is independent of CotH (31a). Although the *cotH* phenotype appears more severe than that of *cotO*, it was still possible that the effect of *cotO* is a consequence of some degree of control by CotO on CotH assembly. To address this, we built a strain bearing CotH-GFP (HS172). Although this construct did not fluoresce and we could not detect it by Western blot analysis (data not shown), we were able to detect the fusion protein in released spores, but not in sporangia, by IFM (Fig. 4G, images i, and data not shown). The reason for the failure to detect fluorescence in sporangia is unclear, but we assume

FIG. 3. Coat protein composition of wild-type and mutant spores. Coat proteins were extracted from wild-type or mutant spores and fractionated by SDS-15% PAGE, and bands were visualized by staining with Coomassie blue. A) Wild-type (PY79) (lane 1), *cotO* (PE250) (lane 2), *cotB* (ADL318) (lane 3), *cotQ* (ADL867) (lane 4), *cotSA* (SL734) (lane 5), *cotS* (SL621) (lane 6), *cotG* (SL733) (lane 7), or *cotC cotG cotS* (MH145) (lane 8) spore extracts were analyzed. Arrowheads between lanes 1 and 2 indicate bands whose intensities are increased (open arrowheads) or decreased (closed arrowheads) in *cotO* mutant extracts compared to those of the wild type. Arrowheads between lanes 2 and 3, 3 and 4, 4 and 5, 5 and 6, and 6 and 7 indicate the absences of CotB (lane 3), CotQ (lane 4), CotSA (lane 5), CotS (lane 6), and the 36- and 31-kDa bands of CotG (lane 7) in extracts of the appropriate mutant spores. B) Wild-type (PY79) (lane 1), *cotO* (PE250) (lane 2), *cotH* (SL639) (lane 3), *cotH cotO* (MH163) (lane 4), *cotE* (AD28) (lane 5), *cotE cotO* (MH166) (lane 6), or *cotE cotH* (SL637) (lane 7) spore extracts were analyzed. Arrowheads between lanes 2 and 3 as well as 3 and 4 indicate bands whose intensities differ between strains. C) Wild-type (PY79) (lane 1), *cotO* (PE250) (lane 2), *cotO-gfp* (PE371) (lane 3), *cotO-gfp amyE::cotO* (DM86) (lane 4), *cotO amyE::cotO* (DM84) (lane 5), or *cotO amyE::cotOΔ179-227* (DM92) (lane 6) spore extracts were analyzed. The lanes in any given panel come from a single gel. WT, wild type.

FIG. 4. Subcellular localization of coat protein-GFP fusions. Sporangia bearing *cotB-gfp* (A), *cotG-gfp* (B), *cotQ-gfp* (C), *cotS-gfp* (D), *cotU-gfp* (E), or *cotW-gfp* (F) in either wild-type (i), *cotO* (ii), or *cotE* (iii) backgrounds were examined by autofluorescence microscopy at approximately the sixth hour of sporulation at 37°C (*cotB-gfp*, *cotG-gfp*, *cotS-gfp*, and *cotW-gfp*) or 24 h following inoculation of cultures grown at 30°C (*cotU-gfp* and *cotQ-gfp*). Spores bearing CotH-GFP (G) in wild-type (HS172) (i), *cotO* (HS189) (ii), or *cotE* (HS187) (iii) backgrounds were reacted with anti-GFP antibodies and examined by immunofluorescence microscopy.

that, somehow, the fusion partner or the interactions made by CotH obscure the antigen. We then built a *cotO cotH-gfp* strain (HS189) and examined the resulting spores by IFM to examine control of CotH assembly by CotO. We were able to detect a signal by IFM in this strain (Fig. 4G, images ii). Therefore, it appears that CotH assembly is largely CotO independent, and CotO assembly is largely CotH independent.

To gain more insight into the control of CotH deposition, we examined released spores of a *cotE cotH-gfp* strain (HS187) by IFM. Although no outer coat is visible in thin-section electron micrographs of *cotE* spores (68), it is possible that some outer coat proteins are present in *cotE* spores but at too low a level to generate an electron-dense structure. As in the wild-type and *cotO* backgrounds, we detected CotH-GFP associated with *cotE* spores (Fig. 4G, images iii). This is consistent with previous findings suggesting a CotE-independent function for CotH (41, 70). This is also consistent with the view that this CotH is in an insoluble form, as CotH is not detected by Western blot analysis of SDS-PAGE-resolved coat proteins from a *cotE* mutant (70).

We found that the CotH-GFP-bearing strain was indistinguishable from a *cotH* null mutant by EM (i.e., a partial outer coat was present) and SDS-PAGE analysis of spore proteins (data not shown). We note that this does not mean the fusion protein is entirely inactive, as the fusion still localizes to the forespore. Previous results indicate that CotH is likely to be present in both coat layers or at the inner coat-outer coat interface (70). Our data are consistent with either possibility. The finding that both *cotH* and *cotO* mutant spores have a defective coat structure prompted us to reexamine *cotH* spores by AFM. As in our previous AFM analysis, we found that these spores possess ridges (7). However, in contrast to our previous observation, our current results indicate that in a significant number of spores the ridges appeared less distinct and sometimes branched (Fig. 1J and data not shown).

Characterization of *cotH cotO* spores. The results cited above do not exclude the possibility that CotO functions, at least in part, in a CotH-independent manner. To address this, we tested whether the *cotH cotO* mutant phenotype (in strain MH163) differs from that of *cotH*. Using the tetrazolium overlay assay, we found that a *cotH cotO* strain produced a red color, in contrast to the light purple color generated by *cotH* or *cotO* strains, and distinct from the pink color produced by the *cotE* strain (Table 3). SDS-PAGE analysis of *cotH cotO* spore extracts showed that a band of about 20 kDa was reduced in intensity, and a band of about 23 kDa was increased in intensity, in the *cotH cotO* strain but not in either of the single mutant strains (Fig. 3B, lane 4). Therefore, it is very likely that CotO function is not entirely dependent on CotH.

Thin-section EM showed that released *cotH cotO* spores had a significant coat defect that was comparable to what was seen for either of the single-mutant strains (Fig. 1F). Also like the case of the single mutants, the morphology of the coats of the double mutant spores still encased in the mother cell appeared much closer to the wild-type morphology (Fig. 1D, E, and G). However, a striking difference between the single- and double-mutant strains was that, in about 80% of *cotH cotO* mutant sporangia (and in about 10% of released spores), we detected inclusions appearing to contain outer coat material based on

their staining and the fact that they were often continuous with outer coat material still associated with the forespore (Fig. 1D to F). Possibly, these structures represent an alternate stacking arrangement of outer coat layers that is prevented by CotH and CotO. From this perspective, CotH and CotO appear to help tip the balance slightly away from stacking and towards polymerization along the plane of the forespore surface, so that the shell of the outer coat closes around the forespore (Fig. 2B).

Additionally, the *cotH cotO* double mutant was reduced in resistance to lysozyme by a factor of 10 to 100, depending on the experiment, in contrast to the 10-fold reductions seen with either of the single mutants (Table 3). Light microscopic examination revealed that liquid suspensions of *cotH cotO* spores occasionally clump, unlike either single mutant. It is plausible that clumping affects lysozyme sensitivity and, therefore, could account for the variability in this experiment. Taken together, these data suggest that, in addition to controlling some common functions, CotH and CotO also act independently to some degree.

Previously, it was shown that CotO assembly is CotE controlled (20). However, it was still formally possible that CotO can act in a CotE-independent manner, either because CotO localizes to the inner coat (in a manner undetected by current experimental approaches) or because it can exert an effect on the coat without localizing. Previously, to address the role of CotH in inner coat function, Naclerio and colleagues examined *cotE cotH* double mutants (41). They reasoned that a more severe phenotype in the double mutant would suggest a role in inner coat assembly, since the entire outer coat is absent in *cotE* spores (68). Because they did, in fact, find that the double mutant had a more severe phenotype than *cotE* alone, based on lysozyme resistance and germination assays (41), those authors were able to demonstrate a CotE-independent function of CotH. We used the same approach to search for a CotE-independent function for CotO. To do this, we compared the tetrazolium overlay phenotypes of *cotE* (in strain ADL04) and *cotE cotO* (in strain MH166) mutant spores. We found that the *cotE cotO* mutant strain produced a lighter pink than that of *cotE* alone, as did the *cotE cotH* spores, indicating a more intense phenotype than that of *cotE* and, therefore, a CotE-independent role for CotO in addition to those roles of CotO that depend on localization by CotE (Table 3). We did not, however, detect a difference in lysozyme sensitivity between either *cotE* and *cotE cotO* or *cotE* and *cotE cotH*, contrary to previous results (41). This is likely due to differences in strain backgrounds, which we have found to significantly influence the results of this assay in the presence of *cotE* and *cotH* mutations (data not shown).

Previous results demonstrated that forespore-localized autofluorescence from CotO-GFP is entirely CotE-dependent (20). To test whether CotO-GFP localized to the forespore in the absence of CotE below the level of detection of autofluorescence, we applied anti-GFP antibodies to strain MH166 (bearing *cotE cotO-gfp*) and examined the cells by IFM. Although we saw no autofluorescence signal, we did detect CotO-GFP by immunofluorescence (Fig. 5C, image iii), indicating that some CotO-GFP localizes to the forespore in the absence of CotE, albeit at a level that is probably much lower than that in the presence of CotE.

FIG. 5. Subcellular localization of CotO-GFP. Spores bearing *cotOΔcotO-gfp* (PE371) (A and B), *cotE cotOΔcotO-gfp* (MH166) (C), or *cotOΔcotO-gfp amyE::cotO* (DM86) (D and E) were examined by bright-field microscopy (i) or autofluorescence microscopy (ii) or were reacted with anti-GFP antibodies and examined by immunofluorescence microscopy (iii). Sporangia bearing *cotOΔcotO-gfp* (PE371) (F) and *ΔcotO::tet amyE::cotOΔ179-227-gfp* (DM110) (G) were examined by autofluorescence microscopy at approximately the eighth hour of sporulation.

Since both CotH and CotO act in CotE-independent ways, we compared extracts of *cotE cotO* and *cotE cotH* spores by SDS-PAGE to further compare the roles of these proteins in coat assembly. We found that *cotE cotO* spore extracts were indistinguishable from *cotE* spore extracts (Fig. 3B, lanes 5 and 6). Therefore, the CotE-independent action of CotO is not revealed by SDS-PAGE. In striking contrast, we found that

cotE cotH spore extracts had many bands not found in extracts of *cotE* or wild-type spores (Fig. 3B, lane 7). The additional bands might be spore core or coat proteins that become readily extracted in the double mutant, or they could be proteolytic products of those proteins. Regardless, these data point towards a significant difference between the roles of *cotO* and *cotH* (in addition to their common functions), at least in the context of a *cotE* mutation.

The ultrastructural analyses described above allowed us to examine the contributions of specific CotH- and CotO-controlled proteins to coat morphology. The similarities of *cotH* and *cotO* spores by EM exclude major morphological roles for CotQ and CotU (whose assembly is CotH, but not CotO, dependent [see above]). This left CotB, CotC, CotG, CotS, and CotSA, proteins known to be CotH controlled (38, 41) and, except for CotC, partially CotO controlled (this work), as candidates for coat proteins with morphological roles. Since mutations in any of these genes individually do not resemble *cotH* or *cotO* mutations (11, 53, 59), we generated a strain in which *cotC*, *cotG*, and *cotS* are inactivated (MH145) (the loss of CotG and CotS also affect CotB and CotSA assembly, respectively [53, 61]). The coat protein composition of this strain resembled that of the wild type (except for the absence of bands corresponding to the products of the deleted genes as well as CotB and CotSA) (Fig. 3A, lane 8). MH145 spores produced a slightly lighter color than the wild type in the tetrazolium overlay assay but not as light as either the *cotH* or *cotO* mutant strain (Table 3). Therefore, the phenotypes of *cotH* and *cotO* mutant spores must be due either to interactions between as-yet untested combinations of coat proteins or, perhaps more likely, as-yet unknown coat protein(s) under the control of CotH and CotO.

Discrete functional regions of CotO. Spores of the *cotO-gfp* fusion-bearing strain (PE371) were indistinguishable from *cotO* null mutant spores as measured by the pattern of SDS-PAGE-resolvable coat proteins, resistance to lysozyme, or the resumption of vegetative growth after germination, as measured by the tetrazolium overlay assay (Fig. 3C, lanes 2 and 3, Table 3). Since autofluorescence microscopy shows that CotO-GFP localizes to the forespore (Fig. 5F) (20), the presence of GFP at the CotO C terminus largely, if not entirely, prevents CotO-dependent coat protein assembly without preventing CotO deposition. It was possible that the addition of GFP to CotO rendered it temperature sensitive, as has been seen for GFP fusions to SpoIVA (50) and CotQ (31a). However, this appears not to be the case, as the SDS-PAGE banding patterns seen after sporulation at 30°C or 25°C were indistinguishable from those seen when sporulation was carried out at the usual temperature of 37°C (data not shown). We also note that the *cotO-gfp* mutant phenotype is largely a result of the allele itself and not the nature of the chromosomal disruption, as it (and the *cotO* null strain [PE250]) is complemented by placement of a wild-type copy of *cotO* at the *amyE* locus (in strains DM84 [$\Delta cotO$ *amyE::cotO*] and DM86 [*cotO-gfp* *amyE::cotO*]). We detected restoration of both the wild-type pattern of coat protein deposition (Fig. 3C, lanes 4 and 5) and of germination, as detected by the tetrazolium overlay assay (Table 3). We interpret this as evidence that the effect of GFP on CotO is the consequence of some role for the C terminus in CotO-dependent coat protein localization.

To more directly test for a role in coat assembly for the CotO C terminus, we generated a strain (DM92; $\Delta cotO::tet amyE::cotO\Delta 179-227$) bearing a version of CotO missing the C-terminal 49 residues of this 227-amino-acid protein. This strain resembled the *cotO* null mutant in its pattern of extractable coat proteins (Fig. 3C, lane 6) and in germination (Table 3). To exclude the possibility that loss of the C-terminal 49 amino acids prevented CotO deposition, we also built a strain producing a version of CotO in which the C-terminal 49 residues were replaced with GFP (DM110) and confirmed that this protein localized to the forespore during sporulation (Fig. 5G).

Location of CotO. The finding that the *cotO-gfp* strain resembled the *cotO* null mutant but still assembled CotO-GFP raised the possibility that CotO-GFP was present on the surfaces of PE371 spores. To test this, we applied anti-GFP antibodies to spores of this strain and examined them by fluorescence microscopy. This experiment demonstrated that CotO-GFP is accessible to antibodies and, therefore, is surface located (Fig. 5A, image iii, and B, image iii). Taken together with our thin-section electron microscopic analysis, which indicates that at least some outer coat is present in a *cotO* strain, we suggest that CotO is present to a significant degree in the outer coat and, furthermore, defines a layer within the outer coat that is exposed in a *cotO-gfp* strain. The use of IFM to detect CotO presented an interesting but not necessarily surprising additional result: the fluorescence pattern surrounding each spore had areas of bright fluorescence interrupted by faint fluorescence or even no fluorescence at all (Fig. 5A, image iii, and B, image iii). This is in contrast to the autofluorescence produced by the CotO-GFP fusion that is most intense at the spore poles (Fig. 5A, image ii, and B, image ii). We interpret this as a consequence of the variability in outer coat assembly in the *cotO-gfp* bearing strain. In this view, enough outer coat has formed in some regions to occlude antibody decoration of CotO-GFP, but in other places the fusion is exposed, resulting in patchy fluorescence.

To investigate whether CotO-GFP would be on the surface of an otherwise wild-type spore, we carried out IFM on spores produced by the *cotO Ω cotO-gfp amyE::cotO* strain (DM86) in which the null phenotype of the *cotO-gfp* allele is corrected (Fig. 3C, lane 4). While most of these spores autofluoresced (indicating that the fusion was successfully incorporated into the spores) (Fig. 5D, image ii, and E, image ii), we found that the number of spores detected by IFM was reduced to about 10% of that of the *cotO-gfp*-bearing strain (PE371). In those spores that were bound by antibody, localization was largely restricted to the spore poles (Fig. 5D, image iii, and E, image iii). We interpret the reduction in the number of fluorescing spores as due to blockage of CotO-GFP by assembly of CotO-dependent coat proteins (driven by the wild-type *cotO* allele). Therefore, we take this experiment as evidence that CotO is, for the most part, not a surface protein. Nonetheless, some of the fusion protein is accessible at the spore poles. This may be evidence of a subset of surface-located CotO or, alternatively, a consequence of the GFP moiety disrupting the coat to the point where some usually buried proteins become surface exposed. Either way, it is intriguing that fluorescence is polar in this case.

DISCUSSION

The architectural plan of the bacterial spore coat, like that of other bacterial cellular structures such as the flagellum and the pilus, is guided, to a significant degree, by a relatively small subset of structural proteins (39, 54). Elucidating the roles of these morphogenetic proteins reveals major steps in the molecular control of coat protein assembly (12). In this work, we characterize the novel morphogenetic protein CotO. We show that CotO is a largely (but not entirely) CotE-controlled coat protein that acts at an intermediate point in coat assembly.

CotO is involved in the assembly of at least five coat proteins, including CotB, CotG, CotS, CotSA, and CotW, and is needed for appearance of a morphologically normal outer coat, as judged by thin-section EM and AFM. Our data suggest that a significant amount of CotO resides within the outer coat. We assume that at least its CotE-dependent functions are exerted from this location. It is plausible that a significant amount of CotH is at the same location, as CotH-GFP-bearing spores are phenotypically null, and the fusion is detectable on the spore surface. We propose that, to a large degree, CotO and CotH act at a late stage of coat assembly from within the outer coat to direct maturation of this structure. Nonetheless, either or both proteins could have inner coat locations as well from where they might exert their CotE-independent functions. In this regard, an important precedent is the control of the outer coat protein CotG by the CotE-independent, morphogenetic coat protein SafA, which is likely to be located at the cortex near the interface with the inner coat (44, 60). It is noteworthy that CotH has been shown to stabilize cytoplasmic CotC and CotG (2, 30, 71). This finding raises the possibility that, at least in part, the roles of CotH and CotO that we have described are due to protection against a protease. Stabilization of CotC and CotG takes place in the cytoplasm (2, 30, 71). This raises the further possibility that CotH and CotO carry out their functions from a cytoplasmic location. These interesting possibilities remain unexplored.

We propose that CotO and CotH work in conjunction to direct assembly of CotG (which, in turn, directs CotB assembly [53]) and CotS (which, in turn, directs CotSA assembly [61]). Nonetheless, our data indicate that these proteins have separate functions as well. The sets of proteins under their control overlap, but there are some differences as well; CotQ and CotU assembly depends on CotH and not CotO, and CotW assembly depends on CotO but not CotH (31a). We suggest that additional differences remain undiscovered, as it is very unlikely that our data explain the full set of phenotypic differences among *cotH*, *cotO*, and *cotH cotO* strains. An interesting clue to the as-yet unknown functions of CotH and CotO is the appearance of aggregates of outer coat in the mother cell cytoplasm of *cotH cotO* sporangia. This observation raises the possibility that directing protein-protein interactions along productive routes and/or preventing formation of nonproductive aggregates is tightly controlled (Fig. 1D to F). It is, perhaps, unexpected that mutants of precisely this type have not been identified previously among coat protein gene mutants. Interestingly, however, similar aggregates have been identified in sporulating cells bearing a mutation in *spoVT*, encoding a homologue of the transcription factor AbrB (3). Bagyan et al. showed that SpoVT both positively and negatively regulates a

large number of σ^G -controlled genes in the forespore (3). Forespore gene expression under the control of σ^G is coupled to activation of σ^K in the mother cell and, therefore, to expression of a large number of coat protein genes (8). As a result, it is likely that formation of aggregates in a *spoVT* mutant strain is an indirect effect of the mutation on coat protein synthesis.

Taken as a whole, our findings suggest a modified picture of coat assembly in which the events that occur after CotE deposition are more complex than previously envisioned (Fig. 2A, compare right and left panels). We propose that assembly of CotH and CotO to the spore, under the partial control of CotE, results in the localization of CotH and CotO within the outer coat and below the coat surface (in addition, potentially, to locations in the inner coat, or inner coat-outer coat interface, as suggested for CotH by others [41, 70]). Together, CotH and CotO direct at least two distinct events. First, they direct deposition of specific coat proteins, including some under control of both CotH and CotO. Second, due to some factor that is under the redundant control of CotH and CotO, the tendency of outer coat protein layers to stack is partially inhibited such that polymerization of the layers into closed shells takes place (Fig. 2B, compare right and left panels). The variations in thickness of the outer coat along the circumferences of wild-type spores suggests that the balance between stacking and edgewise polymerization is a delicate one even in normal cells. This model is not meant to suggest that other coat proteins do not also have important roles in outer coat formation. SafA, CotG, CotM, and at least some of the proteins encoded by the *cotXYZ* cluster participate in outer coat assembly (24, 26, 60, 67). To fully understand this phase of coat formation, the roles of these proteins will need to be integrated into the model.

We do not know how many morphogenetic coat proteins are still to be identified. We expect the number to be small and to remain a small percentage of the total number of coat proteins, based on ongoing work in which we are characterizing each mother cell protein by genetic and cell biological methods (19, 20, 31a). Nonetheless, it is likely that at least a few more will be found.

The complexity of events taking place under the direction of CotH and CotO is striking. It will be interesting to discover if similarly complex late events occur in other species. The distributions of morphogenetic coat protein homologues in the currently sequenced *Bacillus* genomes may give some insight into this question. While homologues of SpoIVA and CotE are encoded in the genomes of all the sequenced bacilli, homologues of CotH and CotO are present in only a subset of species. Therefore, while certain major steps in assembly may be similar among all species, assembly is also likely to be fine-tuned in a species-specific manner. Characterizing coat assembly in a variety of species should give us a better understanding of the range of available solutions to the challenge of coat formation and insight into how morphological variation between species is achieved.

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