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Assembly and genetics of spore protective structures

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Abstract. The sporulation program in *Bacillus subtilis* ends in the formation of a highly resistant endospore that can withstand extremes of heat, mechanical disruption, ultraviolet irradiation, lytic enzymes and chemical attack. These properties are attributed mainly to the unique structure of spore coat and cortex, as well as to the physical state of the spore cytoplasm. The outermost layer of the spore, called the coat, has two morphologically distinct sublayers: an electron-dense outer coat and an electron-translucent inner coat. The coat is composed of more than 2 dozen proteins of varying size. Many coat genes and coat proteins have been isolated and characterized in detail, and studies of these have identified proteins with important roles in coat assembly, resistance and spore germination. We describe here characteristics of the coat proteins and propose a model for coat assembly based on recent work.

Key words. *Bacillus subtilis*; spore; coat protein; assembly; protective structure.

Introduction

The final step in the assembly of the *Bacillus subtilis* spore is the formation of the coat. This structure is essential for resistance and dormancy, at the very least by acting as a shield. One of the best-described protective functions of the coat involves acting as a barrier against lytic enzymes [1–7]. In addition to enabling the spore to survive periods of starvation, the coat also plays a role in the rapid response to the return of nutrient to the environment, known as germination [see review by Moir, this issue].

A typical ultrathin section electron micrograph of a dormant spore is shown in figure 1. The core, at the center of the cell, and the cortex, surrounding the core, are described in the reviews by Moir and Popham [this issue]. Surrounding these structures is the spore coat, consisting of a thick, electron-dense outer layer and a thinner, lamellar-like inner layer [2, 3], with between 25 and 35 proteins ranging in size from 8 to 65 kDa [4]. Spore resistance to harsh environmental conditions is due to both the complex spore structure and the unique physiological state within the core [1, 6]. Strikingly, the cortex and coat play two apparently opposing functions. First, they are protective structures essential to resistance and dormancy [1, 3, 5]. Second, they are critical to the prompt response of spores to molecules that trigger germination [8, 9].

In this chapter, we first review the morphology and composition of the spore-protective structures. Next, we briefly discuss each of the coat proteins and the regulatory factors, including sigma factors, which control coat protein gene (*cot*) expression. Finally, we propose a possible model for coat assembly based on recent results, and discuss a strategy for future research.

Coat morphology and composition

The spore coat has two morphologically distinct layers: a thick, highly electron dense outer coat and a less electron dense inner coat that appears to be further composed of a number of layers (fig. 1). The outer coat retains its electron-dense appearance even after sequential treatments with alkaline reagents and a mixture of proteases [10, 11]. The majority of the coat is composed of proteins [1–3, 12–14]. As the resistance properties of the spore suggest, solubilization of the coat proteins is quite difficult, even with harsh treatments such as 0.1 M NaOH, heat, sodium dodecyl sulfate, dithiothreitol, urea or combinations of these conditions [15]. Following these treatments only about 70% of coat proteins are solubilized,

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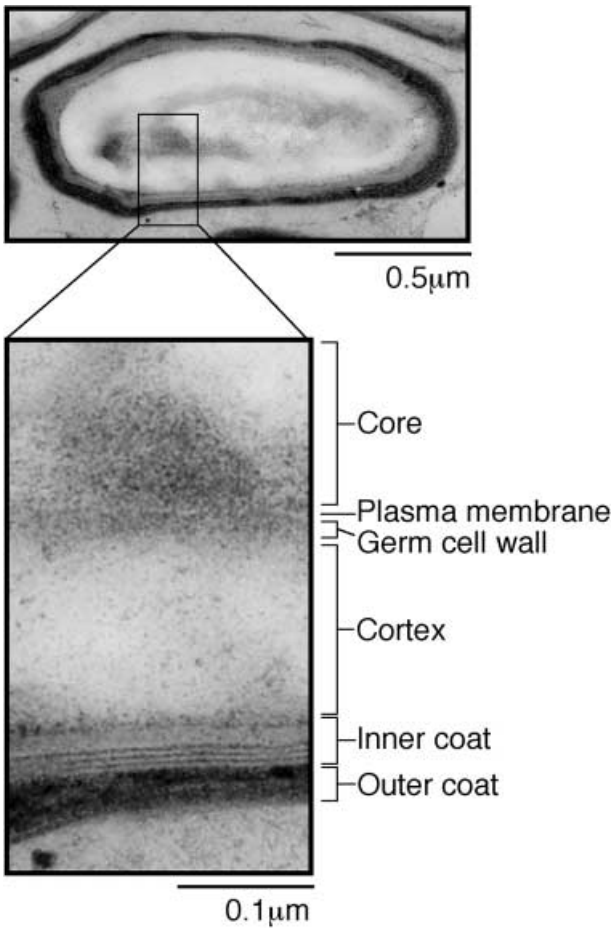

Figure 1. Fine structure of the *B. subtilis* spore. The upper panel shows a thin section electron micrograph of a dormant spore. The lower panel shows a magnified view in which the identities of the spore layers are indicated.

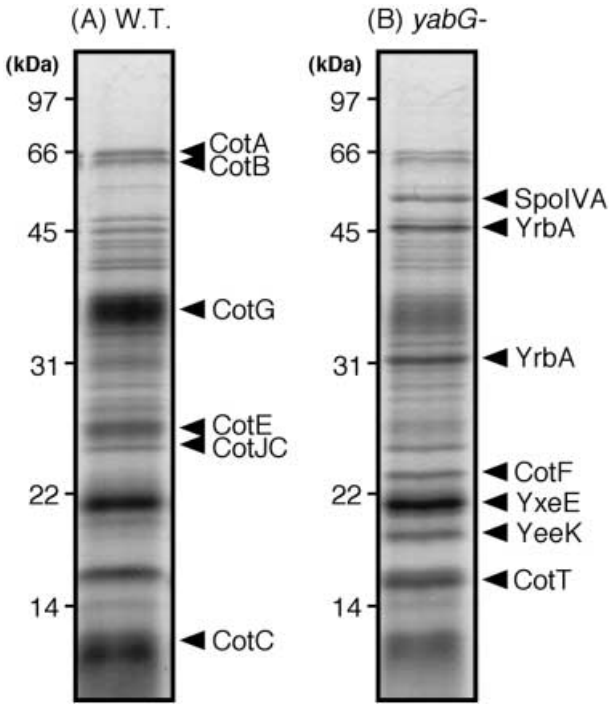

Figure 2. SDS-PAGE analysis of spore coat proteins. Spore coat proteins from wild-type (*A*) and *yabG* mutant spores (*B*) were solubilized by treatment with SDS and 2-mercaptoethanol, run on 13% acrylamide gels and stained with Coomassie Brilliant Blue. The arrowheads indicate proteins identified by N-terminal amino acid sequencing [20].

with the rest remaining in an insoluble fraction. Although the biochemical basis of the large insoluble fraction is unknown, several lines of evidence indicate the presence of diverse species of protein cross-links that may connect the coat proteins. It has been suggested that both disulfide and *o,o*-dityrosine cross-links may be present in the coat assembly [2, 16]. Intriguingly, a spore-associated transglutaminase has been identified in *B. subtilis* [17–19]. This activity directs creation of ε-(γ-glutamyl) lysine bonds between spore coat proteins. Protein cross-links could be a critical feature in spore resistance. Among the coat proteins that appear to be joined by cross-links are CotX and CotY (see below).

A typical example of the SDS-polyacrylamide gel electrophoresis (PAGE) profile of an extract of spore coat proteins from wild-type and mutant spores is shown in figure 2 [20]. Table 1 summarizes the major characteristics of the spore coat proteins of *B. subtilis*. We have chosen to designate a protein as a coat protein only when its presence in the coat has been firmly established. We discuss our reasons for employing a relatively restrictive definition for coat proteins below. Here, we simply note that some putative coat proteins are omitted from table 1.

Summary of the coat proteins

Here, we summarize what is known about the *cot* genes (and *spo* genes that encode coat proteins) and their protein products. We also describe those '*y*' genes, identified as open reading frames of unknown function during the *B. subtilis* genome project [21], which are now known to encode spore proteins with roles in coat formation.

CotA, CotB, CotC and CotD

CotA has a molecular mass of 65 kDa. The *cotA* gene is identical to a previously identified gene called *pig*, known to be responsible for sporulation-associated pigment production [22]. The expression of *cotA*, *cotB*, *cotC* and *cotD* genes (the latter three encoding proteins of 59, 12 and 11 kDa, respectively) is controlled by the sporulation-specific transcription factor σ^K and negatively regulated by the small accessory transcription factor GerE

Table 1. Only proteins known to be located in the coat are indicated. Estimates of molecular masses (MM) and isolelectric points (PI) are based on the amino acid sequences of the full-length proteins. Molecular masses (kDa) are based on migration in SDS-PAGE. Amino acids that comprise over 10% of the sequence and the presence of cell wall binding motifs (CWB) are indicated under 'sequence characteristics'. Sequence data comes from the *Bacillus subtilis* ORF database (http://bacillus.tokyo-center.genome.ad.jp).

Name	Length (a, a)	MM	SDS-PAGE (kDa)	PI	Paralogues	Sequence characteristics	Function	Gene regulation	References
CotA	513	58,499	66	6.3	–	–	unknown	SigK, GerE	22, 23, 25, 28, 65
CotB	380	42,971	34, 59	10.3	–	S20%, K12%	unknown	SigK, GerE	22, 25, 28
CotC	66	14,785	12	10.1	YnzH	K20%, Y18%, D10%	unknown	SigK, GerE	22, 25, 28
CotD	75	8,840	11	7.7	–	H23%, P11%	germination	SigK, GerE	22, 28
CotE	181	20,977	24	4.2	–	E15%, V12%	protein assembly	SigE, SigK	24, 25, 28, 33, 51, 63
CotF	160	18,725	5, 8, 19	7.9	YraD, YraF	L12%	unknown	SigK	29
CotG	195	23,939	36	11.1	–	K28%, S20%, H11%, Y11%	assembly of CotB	SigK, GerE	30, 52
CotH	362	42,812	43	6.4	YisJ	–	protein assembly	SigK	33, 52
CotJA	82	9,739	9	9.8	–	P13%	assembly of CotJC	SigE	34, 35
CotJC	189	21,695	24	5.0	YdbD, YjqC	A11%, L10%	assembly of CotJA	SigE	34, 35
CotM	130	15,222	6	4.0	–	crystallin family	protein assembly	SigK	36
CotS	351	41,052	41	7.1	YtaA	L11%, K11%	assembly of CotSA	SigK, GerE	26, 27, 37, 38
CotSA	377	42,912	43	8.1	YtcC	–	unknown	SigK, GerE	26, 27, 37, 38
CotT	82	10,130	8, 10	9.0	–	Y22%, P21%, G11%	germination	unknown	9, 66
CotX	172	18,600	24	4.3	–	V11%, A10%, L10%, D10%	protein assembly	SigK, GerE	39, 40
CotY	162	17,844	26, 52, 78	4.9	CotZ	–	protein assembly	SigK, GerE	39, 40
CotZ	148	16,533	18	5.3	CotY	–	protein assembly	SigK, GerE	39, 40
SpoIVA	429	55,156	55	4.6	–	E11%	protein assembly	SigE	24, 42, 44, 45, 63
SpoVID	575	64,958	66, 120	4.0	–	CWB motif, E21%, A10%	protein assembly	SigE	46, 49
YabG	290	33,286	33	9.6	–	–	protease	SigK	20, 21, 47
YrbA	387	43,211	30, 31, 45	6.2	–	CWB motif, P15%	protein assembly	SigE	21, 48, 49, 53

[23]. Spores from strains bearing insertional mutations in either of the four genes exhibited the wild-type pattern of coat polypeptides, except for the absence of the product of the inactivated *cot* gene. Spores bearing null alleles of *cotA*, *cotB* or *cotC* have no obvious phenotype. However, *cotD* null mutant spores germinate somewhat more slowly than do wild-type spores [22].

CotE

CotE (24 kDa) is an alkali-soluble coat protein, and its location site is probably at the junction of the inner and outer coat layer as demonstrated by immunoelectron microscopy [24]. Driks et al. demonstrated by electron microscopic observation that CotE takes up a discrete subcellular location just after the formation of the sporulation septum, and a layer of CotE appears as a ring that encircles the forespores and is separated from it by a gap of –75 nm [24]. The resulting *cotE* mutant formed spores with normal optical refractilility that were heat resistant but which were sensitive to lysozyme and somewhat impaired in germination [25]. Ultrastructural analysis indicated that the *cotE* mutant spores lacked the electron-dense outer layer of the coat but retained a normal-looking inner coat. The incorporation of CotS, located in the inner coat layer, is also dependent on *cotE* [26, 27]. Therefore, CotE is involved in the assembly of the products both into the electron-dense outer layer and lamellar inner layer of the coat. A detailed model that takes into account this complex role for CotE in coat morphogenesis is discussed below. Transcription of the *cotE* gene is governed by tandem promoters designated P1 and P2 [28]. Transcription from the P1 promoter is coregulated by *spoIIID* and is turned on during hours 3–4 of sporulation. Transcription from P2 is dependent on σ^K and is turned on during hour 5 by the appearance of the product (a small DNA-binding protein) of *spoIIID* [28].

CotF

The predicted primary product of *cotF* is a 19-kDa polypeptide which is processed to generate alkali-soluble coat proteins of 5 and 8 kDa. The *cotF* null mutation produced normal-looking spores that contained an apparently complete set of coat proteins except for the absence of the 5- and 8-kDa polypeptides. Transcription of *cotF* is under the control of σ^K [29].

CotG

The *cotG* gene encodes a polypeptide of 24 kDa that is organized into nine repeats of a 13-amino acid sequence whose consensus is H/Y-K-K-S-Y- R/C- S/T-H/Y-K-K-S-R-S [30]. CotG protein can exist in two forms, a monomeric, soluble form that can be detected by analyzing a sample of purified coat material by SDS-PAGE and a polymeric, cross-linked form that is insoluble and not amenable to electrophoretic resolution. SodA, encoding a Mn-dependent superoxide dismutase [31], promotes the oxidative cross-linking and consequent formation of the insoluble form of CotG [32]. Expression of *cotG* is under the control of σ^K and GerE. The *cotG* null mutant fails to assemble not only CotG but also CotB. As *cotB* expression is not impaired by the *cotG* mutation, CotG may be a morphogenetic protein required for the incorporation of CotB into the coat [32].

CotH

The *cotH* gene encodes a polypeptide of 42.8 kDa [33], and the spores of *cotH* null mutant are normally heat, lysozyme and chloroform resistant but are impaired in germination. The mutant spores are also pleiotropically deficient in the assembly of several coat proteins, including the products of *cotB*, *cotC* and *cotG* genes. The ultrastructural analysis of purified spores suggests that *cotH* is needed for proper formation of both inner and outer layers of the coat [33].

CotJ proteins

The *cotJ* region reveals a tricistronic operon preceded by a strong σ^E-like promoter, and expression of *cotJ* requires σ^E. The *cotJ* operon (*cotJA, -JB, -JC*) encodes polypeptides of 9.6, 12 and 22 kDa, respectively. CotJA and CotJC are both present in spores of *cotE* or *gerE* mutant strains. The incorporation of CotJA into the coat requires CotJC and the assembly of CotJC requires the presence of CotJA. CotJB has not been found in the coat [34, 35].

CotM

The *cotM* gene is transcribed under the control of σ^K and is negatively regulated by GerE. CotM is related to the α-crystalline family of low molecular weight heat-shock proteins, members of which can be substrates for transglutaminase-mediated protein cross-linking [36]. CotM has not been detected among the extractable spore coat proteins, but is probably involved in spore outer coat assembly. Henriques et al. [36] proposed that CotM is part of a cross-linked insoluble skeleton that surrounds the spore, and that CotM serves as a matrix for the assembly of additional outer coat material and also confers structural stability to the final structure.

CotS and CotSA

The *cotS* operon consists of *cotSA* (named by *ytxN* by the *B. subtilis* genome project), *CotS* and *ytxO*, in that order [21]. *cotSA* and *cotS* encode spore coat proteins with molecular weight of 43 and 40 kDa, respectively [37, 38]. Expression of the *cotS* operon requires σ^K and GerE. CotE

is essential for the assembly of CotS in the coat. Immunoelectron microscopic observation using anti-CotS antibodies revealed that CotS is located within the coat and, in particular, is present largely in the inner coat [26]. Therefore, as will be discussed in more detail later, this result indicates that dependency of assembly on CotE cannot serve as the definition of an outer coat protein. Rather, when possible, the assignment of a coat protein to one or the other coat layer should be based on a direct localization method. CotSA is synthesized simultaneously with CotS, and its incorporation is dependent upon CotS, but not vice versa [27]. Insertional mutagenesis of the *cotS* gene resulted in no alteration of growth or sporulation, and had no effect on germination or resistance to chloroform [38], indicating that it does not affect the spore properties commonly measured in the laboratory.

CotT

CotT is synthesized as a 10.1-kDa precursor and is then processed to 7.8-kDa coat protein. It contains high percentages of proline, glycine and tyrosine, and is made up of only seven species of amino acid. The precursor form could be converted to the mature form (7.8 kDa) by a proteolytic activity similar to trypsin [9]. Insertional inactivation of *cotT* gene results in spores with morphologically altered inner coat layers, suggesting that CotT is an inner-coat protein, and causes slow germination in response to a mixture of fructose, glucose and asparagine [39].

CotV, CotW, CotX, CotY and CotZ

cotX gene is clustered with four other genes designated *cotV*, *cotW*, *cotY* and *cotZ*. *cotX* encodes an 18.6-kDa protein component of the insoluble fraction of the coat. CotV and CotX have significant sequence similarity [39, 40]. Expression of *cotX* initiates from two promoters; one directs a transcript with *cotX* alone and is absolutely dependent on both σ^K and GerE, and the other directs several multicistronic transcripts containing *cotVWX* and is transcribed under the control of both σ^K and GerE, as well as by σ^K alone. CotX appeared to be present primarily in the insoluble coat fraction.

CotY (17.9 kDa) and CotZ (16.5 kDa) are encoded by *cotY* and *cotZ*, located immediately downstream of the *cotVWX* operon. They are transcribed from a single promoter, which can be activated by either σ^K alone or both σ^K and GerE. However, CotV and CotW have not been found in mature spores. The deduced amino acid sequences of the *cotY* and *cotZ* genes are very similar. Both proteins are cysteine rich, and CotY antigen is present in spore coat extracts as disulfide cross-linked multimers. There is little CotX antigen in the soluble coat fraction, and deletion of the corresponding gene results in a 30% reduction in the insoluble fraction [39, 40]. CotZ has not yet been found in the coat.

Spores from cells bearing deletions in *cotX*, *cotYZ* or *cotXYZ* are heat and lysozyme resistant, but readily clump and respond more rapidly to germinants than do wild-type spores. Electron microscopic analysis suggests that the *cotX* null mutant spores have less densely staining outer coats, and that deletion of *cotXYZ* results in an incomplete outer coat. These proteins, as part of the coat-insoluble fraction, appear to be localized to the outer coat and influence spore hydrophobicity as well as the accessibility of the spore interior to germinants [39].

SpoIVA and SpoVID

The *spoIVA* gene is transcribed by the mother-cell-active factor σ^E at about the 2nd hour of sporulation and encodes a 55-kDa protein, located on the mother cell side of the forespore membrane and which attaches the matrix to the forespore [24, 41, 42]. A *spoIVA* null allele results in an unusual defect in which the coat misassembles as swirls within the mother cell and abolishes cortex synthesis [43–45]. The *spoVID* gene was identified during a search for σ^E-dependent genes. It encodes a 64.8-kDa polypeptide required for assembly of a normal spore coat [46]. Insertion mutations in *spoVID* produce refractile spores that are resistant to heat and chloroform but are sensitive to lysozyme. Electron microscopic examination of the mutant cells reveals that development is largely normal up to about the 3rd hour of sporulation, but that later in development, the coat proteins assemble into aberrant structures [46]. Driks et al. suggested that SpoVID is also required for maintenance of the CotE ring during the later stages, when most of the proteins are assembled into the coat [46].

YabG

The *yabG* gene is transcribed 4 h after the initiation of sporulation under the control of σ^K, and encodes a polypeptide of 33 kDa [20, 47]. When a YabG-green fluorescent protein fusion is produced in sporulating cells, fluorescence is detectable around the forespores but not in the mother cell compartment using fluorescence microscopy. Inactivation of *yabG* does not affect spore resistance to heat, chloroform or lysozyme. The germination properties of the mutant spores are very similar to those of wild-type spores. Chemical extraction of the spore coat proteins from *yabG* mutant spores revealed 15-, 18-, 21-, 23-, 31-, 45- and 55-kDa polypeptides, whose abundance was low in or unextractable from wild-type spores under the same conditions. These polypeptides correspond to CotT, YeeK, YxeE, CotF, YrbA and SpoIVA, respectively, based on N-terminal amino acid sequencing (fig. 2) [20, 47]. The functions of YeeK and YxeE are unknown. Analysis of the coat proteins present in *yabG* mu-

tant spores and protease inhibition experiments in vivo and in vitro indicate that YabG is a sporulation-specific protease involved in the processing of several coat proteins, notably in the proteolysis and maturation of SpoIVA and YrbA [47].

YrbA

The *yrbA* gene is capable of encoding a polypeptide of 45 kDa (YrbA or SafA) [48]. This gene has two initiation codons in its sequence. The 5′-most of these corresponds to the original initiation site, whereas the other is located within the open reading frame such that if used, a protein of approximately two-thirds the size of the full-length product would be produced. The sizes of the potential gene products are estimated to be 45 and 31 kDa, respectively. Proteins of slightly reduced masses (43 and 30 kDa) are detectable in extracts of mature spores by Western blotting using anti-YrbA antibodies. These results strongly indicate posttranslational modification of the *yrbA* gene product during coat morphogenesis. Our examination of coat assembly in *yabG* mutant spores and protease inhibitory analysis in vivo and in vitro suggests that YrbA is synthesized as a precursor form of 45- and 31 kDa, which are processed to the matured forms during later stages of sporulation [47]. Transcription of *yrbA* requires σ^E and begins 2 h after the onset of sporulation. Insertional inactivation of *yrbA* reduces resistance to lysozyme. Electron microscopic examination reveals changes in the coat of *yrbA* mutant spores, including a reduction in the density and thickness of the outer layer and an inner coat layer-like structure around the outside of the coat. This abnormal coat structure was also observed on the outside of the developing forespores of the *yrbA* mutant. These results suggest that YrbA is involved in the assembly of coat proteins with roles in both spore lysozyme resistance and germination [47]. Ozin et al. observed an interaction between YrbA and SpoVID. They showed that YrbA and SpoVID can be coimmunoprecipitated from whole-cell extracts prepared 3 and 4 h after the onset of sporulation, and YrbA (= SafA) was found in the cortex, near the interface with the coat in mature spores using immunogold electron microscopy [49]. More recently, Ozin et al. demonstrated that SafA (known as YrbA) was localized around the forespore early during coat assembly by the observation with immunofluorescent microscopy, and that this localization was dependent on SpoVID and targeting of SafA to the forespore was also dependent on SpoVIA [50].

Assembly of the spore coat

The complex composition and structure of the coat has made understanding its assembly an important goal of sporulation research. The identification of sporulation mutants by both forward and reverse genetics has revealed several genes with crucial roles in coat assembly. Two such genes with central roles are *cotE* and *SpoIVA*, discussed above. CotE is localized in a ring that surrounds the forespore and is separated from it by a small gap [24]. The ring is the site of assembly of the outer coat, and the gap is the site of formation of the inner coat [4]. Assembly of the CotE ring depends on SpoIVA, which creates a basement layer around the forespore on which coat assembly takes place [24]. CotE protein was argued to be the site of outer coat protein deposition because a *cotE* null allele prevents formation of any detectable outer coat, but it appears not to significantly affect coat protein synthesis [25]. As described above, CotE controls the assembly of at least one inner coat protein, CotS, indicating that CotE directs both inner and outer coat protein assembly. Structure/function analysis of CotE indicates that the protein is modular and possesses a C-terminal region that directs coat protein assembly and an interior portion that guides CotE to the inner coat/outer coat interface [51]. SpoVID and YrbA also play important roles in coat formation, although they have less dramatic consequences for assembly than do SpoIVA or CotE [20, 46].

Several coat proteins with minimal affects on the overall structure of the coat are, nonetheless, required for the assembly of one or a few coat protein species. For example, CotH is required for incorporation of CotG and CotB into the coat [52]. CotG, CotJC and CotS are required for assembly of CotB, CotJA or CotSA, respectively [27, 30, 35]. For the two pairs of CotJ and CotS proteins, the genes encoding each pair are in an operon or are very close in the chromosome [27, 35]. Thus, it is tempting to speculate that, at least in these examples, coat formation is guided by events at both the transcriptional, translational and posttranslational stages. The molecular events that direct specific interactions between coat proteins remain unknown. No consensus amino acid sequences have been detected among coat proteins. As mentioned above, a variety of protein cross-links may be present in the coat and, most likely, participate in generating the rigid coat.

Although many, and probably most, of the coat proteins have now been described, and a reasonably detailed model for coat assembly can be proposed (see below), our tools for analysis of the coat remain relatively primitive and there is still the possibility for significant confusion using current methodologies. In particular, the varying operational definitions of a coat protein have sometimes led to confusion in the analysis of the coat – for several reasons. First, not all proteins that are readily extracted from dormant spores are components of the coat. For example, YrbB is present mainly in the cortex layer, but is extracted along with the spore coat proteins [53]. This and other examples clearly indicate that some compo-

nents of the outer forespore membrane or cortex are often coextracted with spore coat proteins by the harsh solubilization conditions typically employed. The second source of confusion comes from one common approach to deciding whether a protein is a component of the inner or outer coat layer. Often, this assignment is based on whether assembly of a given coat protein is CotE dependent. Since the outer coat is apparently missing in *cotE* mutant spores, CotE-dependent assembly has been taken as an indication of an outer coat location. However, CotS is a CotE-dependent inner coat protein [26]. This example serves as a caution and emphasizes the need for direct localization studies. We suggest that although such experiments can be difficult (and, in fact, have been carried out for only a few coat proteins), the assignment of some coat proteins to specific layers should be reconsidered.

A protein interaction network controls coat protein assembly

Based on more recent studies, we tentatively propose that a network of interactions connects the coat proteins, as shown in figure 3. SpoIVA, which becomes active 2 h after the onset of sporulation (T_2), is responsible for some of the earliest interactions between the coat proteins. SpoIVA controls assembly of SpoVID directly or indirectly [24, 45]. SpoVID, in turn, participates in CotE assembly, interacts with YrbA and, most likely, other coat proteins [24, 49]. The relative position of each protein in the network hierarchy is inferred from relative differences in the corresponding mutant phenotypes, as well as biochemical and structural experiments. An intriguing possibility, highlighted by this model, is an interaction between SpoIVA and SpoVID, which remains to be tested. Once these early connections are in place, CotE can direct the assembly of the large set of proteins whose deposition is under its control, including CotS, CotA, CotC and CotG.

A more recently identified player is YabG, whose assembly into the coat depends on SpoIVA. YabG cleaves or degrades SpoIVA, YrbA, CotT and CotF, as well as several proteins that are still designated by the names assigned by the genome sequencing project [20, 47]. Previous reports provided evidence of coat protein processing of CotT [9] and 5- and 8-kDa polypeptides [29]; however, our characterization of YabG is the first case in which both a processing factor and its coat protein substrate(s) are known [20, 47].

Previously, Driks proposed a model for spore coat formation consisting of four major steps in which SpoIVA, SpoVID, CotE and YrbA play crucial roles [4]. Here, we propose a more comprehensive model that takes into account the most recent findings (fig. 4). During steps 1 and 2, SpoIVA first binds to the mother cell side of the forespore membrane at the asymmetric division site, 2 h after the onset of sporulation (T_2), and then covers the surface of the forespore membrane that appears as engulfment continues [42]. SpoVID, YrbA and CotE, which are also expressed along with SpoIVA under the control of σ^E, localize at the layer of SpoIVA on the forespore surface. These proteins comprise the basement layer on which coat proteins will assemble in subsequent stages. At the completion of engulfment at step 3, σ^K is activated by a signal from σ^G, and CotA and other coat proteins are syn-

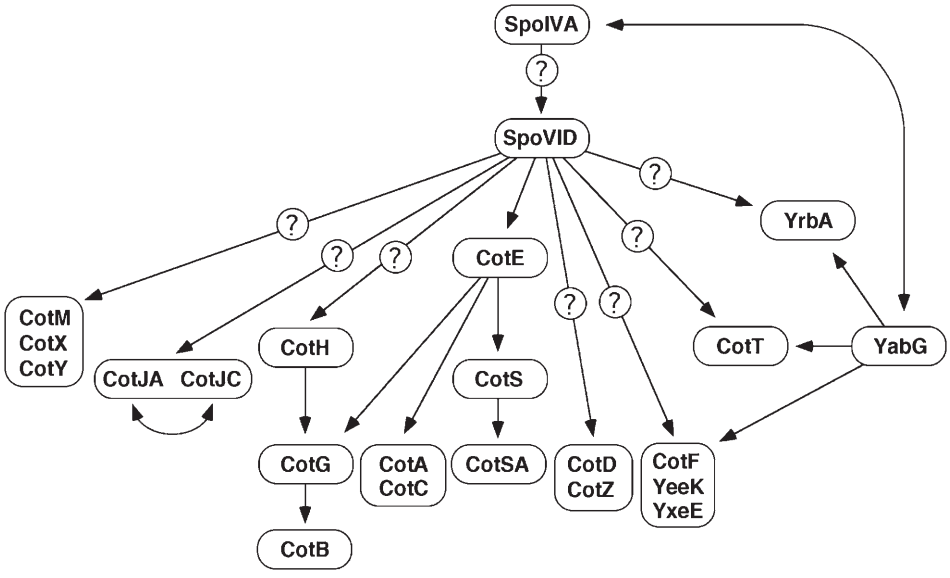

Figure 3. Network of interactions between spore coat proteins. Assembly or processing of each coat protein is regulated by the protein immediately above it in the hierarchy. The question marks indicate the likely involvement of unidentified factor(s) in each step. See the text for detailed discussion.

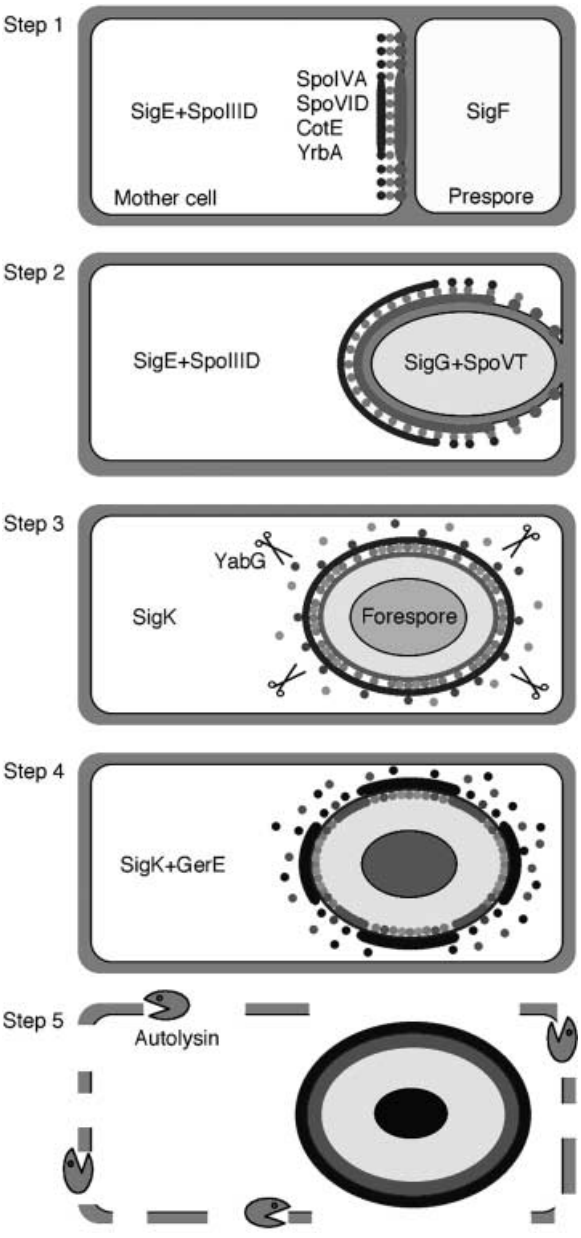

Figure 4. A model for spore coat assembly. Assembly of the coat is illustrated in five steps. See the text for detailed discussion.

thesized during T_3 to T_4. YabG is also synthesized at this step and integrated into the coat layer. It gradually cleaves SpoIVA, YrbA and other coat proteins. At step 4, σ^K and GerE-dependent coat proteins are synthesized and incorporated into coat layers during T_5–T_6. At the final step, mature spores are released from the surrounding mother cell by the action of autolysins, which degrade the mother cell envelope.

A major gap in our understanding of coat assembly, and indeed of sporulation in general, is how the cell ensures that certain mother-cell-synthesized proteins are correctly localized. For example, how is SpoIVA directed solely to the septum and not to the remainder of the mother cell inner membrane? After engulfment, when the cytoplasmic membrane encasing the mother cell membrane is separated from the membrane surrounding the forespore, it is likely that other spore-associated proteins localize specifically to the forespore. Therefore, the key point to resolve is the nature of the difference between the cytoplasmic membranes surrounding mother cell and forespore.

The answer to this question is not known, but an interesting possible clue comes from the finding that at least five spore proteins contain cell wall binding motifs (CWB motif), which are common in proteins in bacteria and phages [48, 54, 55]. Included in this group are two old friends, YrbA and SpoVID, which form a complex early in coat assembly and are found in the cortex [48, 49, 54, 55]. A third member of the group, YaaH, has two repeats of the CWB motif and is a coat protein required for the L-alanine-stimulated germination pathway [54]. The region including the CWB motif in YaaH appears to be sufficient for protein assembly to the forespore, because a fusion protein of β-lactamase and CWB motif of YaaH was extractable from mature spores [54]. These results suggest a general role for CWB motifs in coat protein localization. A further mechanism that could participate in directing proteins to the forespore membrane would be a novel form of targeted secretion.

Not all early assembled spore proteins possess CWB motifs or potential membrane-spanning regions. The lack of a CWB motif in SpoIVA means that this potential mechanism cannot explain the targeting of SpoIVA to the forespore. SpoIVA has no likely membrane-spanning regions or a signal peptide, apparently excluding the likelihood of a mechanism based on secretion. Within SpoIVA, the C terminus is essential for localization [42]. These findings suggest that targeting of SpoIVA is probably dependent on receptors in the forespore membrane. In this regard it is interesting that SpoIVA localization depends, in large part (although not entirely), on the small mother-cell-synthesized protein SpoVM [42, 56]. Another example of a protein that localizes specifically to the mother cell side of the septum is pro-σ^E, the inactive form of σ^E [57, 58]. It will be interesting to learn whether these two localization events depend on septum-specific proteins, some other characteristic of the septal membrane, or a combination of the two.

Strategies for future studies

Genomic sequencing

We are now standing at the entrance way to a new era in research in *B. subtilis*, as well as in other living cells. Until now, identification of coat proteins has been driven by traditional technologies, including screening of mutants or reverse genetic analysis of extracted spore proteins.

However, the environment for spore research has been drastically changed by the completion of the sequencing of *B. subtilis* chromosome in 1997. Of the ~4100 estimated genes, about half are of unknown function [21]. Some of these are expressed during sporulation and involved in spore morphogenesis and germination and are indeed coat proteins [20, 48] (see table 1). The precise functions of these proteins remain to be determined.

DNA array techniques

The availability of DNA array technology permits us to identify the total set of gene expressed at any given stage of sporulation. Fawcett et al. analyzed the transcriptional profile of the early-to-middle stages of sporulation in *B. subtilis* by DNA arrays and demonstrated global changes in gene transcription [59]. The information that can be acquired by micro- and macroarray analysis may be more useful in understanding the global control of gene expression than that obtained by Northern blotting or fusions of *lacZ* to individual genes. However, these array techniques are still inferior to conventional transcription analysis methods in terms of quantitative accuracy of the data, and careful verification will be necessary.

Proteome analysis

Recently proteome analysis was carried out in *B. subtilis*. Hirose et al. resolved the proteome of the extracellular proteins of *B. subtilis* 168 by two-dimensional (2D) gel electrophoresis and identified more than 100 protein spots due to secreted proteins [60]. Hecker et al. analyzed the phosphate starvation response in *B. subtilis* using 2D gel electrophoresis and carried out proteome and transcriptional analysis [61]. By proteome analysis, we could identify the entire set of coat proteins and gain considerable insight into the network of interactions that guide the coat assembly process. Furthermore, 2D gel electrophoresis can readily detect protein modification; for example, a modification that alters the isoelectric point but not the mass will produce a significant change in migration. This kind of profiling of specific gene products may give insight into the function of protein whose absence fails to generate a phenotype, as is often true of the coat proteins. As the information coming from such studies increases in volume, bioinformatic analysis of transcriptional and proteomic data will become an essential tool. We may even look forward to the day when we can simulate sporulation and formation of the resistant spore on a computer.

Visualization of spore proteins

Immunoelectron microscopy, immunofluorescence microscopy and autofluorescence microscopy using green fluorescent protein (GFP)-fusion-bearing cells are being widely applied to determine directly the locations of spore proteins [42, 57, 62–64]. GFP-fusion technology is particularly exciting as it permits visualization of dynamics of protein localization in living cells. This method will undoubtedly continue to have a major impact on future work.

Conclusions

The dormant spore has several morphologically unique structures. Of these, the spore coat plays an especially important role as a protective structure that resists harsh conditions such as heat, chemicals and mechanical disruption. We have summarized the characteristics of the spore coat proteins and the factors regulating expression of these coat-related genes. We also proposed an updated model for coat assembly, where SpoIVA, CotE and the novel protease YabG play important roles. Finally, we have outlined how novel approaches provide a new strategy for research in spore morphogenesis in the coming years.

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