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Shaping an Endospore: Architectural Transformations During *Bacillus subtilis* Sporulation

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Abstract

Endospore formation in *Bacillus subtilis* provides an ideal model system for studying development in bacteria. Sporulation studies have contributed a wealth of information about the mechanisms of cell-specific gene expression, chromosome dynamics, protein localization, and membrane remodeling, while helping to dispel the early view that bacteria lack internal organization and interesting cell biological phenomena. In this review, we focus on the architectural transformations that lead to a profound reorganization of the cellular landscape during sporulation, from two cells that lie side by side to the endospore, the unique cell within a cell structure that is a hallmark of sporulation in *B. subtilis* and other spore-forming *Firmicutes*. We discuss new insights into the mechanisms that drive morphogenesis, with special emphasis on polar septation, chromosome translocation, and the phagocytosis-like process of engulfment, and also the key experimental advances that have proven valuable in revealing the inner workings of bacterial cells.

Keywords

polar septation; chromosome translocation; engulfment; SpoIIIE; SpoIIDMP; SpoIIQ-SpoIIIAH

1 Introduction

Endospore formation (hereafter also referred to as sporulation) is a developmental process that culminates in the formation of resilient, metabolically dormant spores. It is a characteristic trait of many bacterial species that belong to an ancient and exceptionally diverse bacterial phylum, the *Firmicutes* (29, 64, 77). Although the sporulation program has diversified over time, the main developmental steps seem to be conserved in all extant endospore formers (2, 3, 65, 127), and they involve profound changes in cellular architecture. Most of our current understanding of the mechanistic basis of such architectural transformations stems from studies conducted in the gram-positive, rod-shaped model

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bacterium *Bacillus subtilis*. Therefore, unless otherwise indicated, this review focuses on aspects of *B. subtilis* sporulation.

The *B. subtilis* sporulation program is triggered by nutrient starvation and is depicted in Figures 1 and 2 (see References 74 and 160 for general sporulation reviews; specialized reviews about different aspects of sporulation are indicated below; see the sidebar titled 'Transcriptional regulation of *B.subtilis* sporulation).*B. subtilis* sporulation begins with the formation of a septum close to one cell pole (polar septation) that gives rise to a sporangium consisting of two cells with different sizes: the smaller forespore and the larger mother cell. Then, the mother cell engulfs the forespore in a process that resembles eukaryotic phagocytosis. After engulfment, the forespore is enclosed within the mother cell cytoplasm, where it matures into a spore. Spore maturation involves the synthesis of a peptidoglycan cortex between the inner and the outer forespore membranes (reviewed in 125); the assembly of a proteinaceous coat around the outer membrane (reviewed in 43, 104); saturation of the forespore chromosome with the small, acid-soluble proteins (SASPs) (reviewed in 146); partial dehydration of the forespore cytoplasm; and accumulation of Ca^{2+} dipicolinic acid. Together, these protective layers and the dehydrated core confer the spore with resistance to environmental challenges, including antibiotics, desiccation, and radiation, among others (reviewed in 149). Finally, the mother cell lyses and the mature spore is released to the environment, where it remains metabolically dormant until conditions are appropriate for germination, upon which vegetative growth resumes (reviewed in 112, 147, 148, 150).

The sporulation pathway entails dramatic cellular reorganizations, illustrating the dynamic abilities of bacterial cells. Technological advancements in the fields of fluorescence and electron microscopy, in combination with the powerful genetic tools available for *B. subtilis*, have allowed researchers to study these reorganizations with an unprecedented level of detail (Figures 2 and 3). In this review, we discuss dynamic processes that shape the developing spore during the first half of the sporulation pathway: (*a*) cellular dynamics prior to engulfment, with a focus on polar septation and chromosome translocation, and (*b*) cellular dynamics during the phagocytosis-like process of engulfment.

2 Cellular Dynamics Prior to Engulfment

The most obvious cytological marker for sporulation initiation is the formation of a septum close to one cell pole. This polar division event primes the sporangia to activate forespore- and mother cell–specific transcriptional regulators that control the expression of genes necessary for subsequent events in spore development (reviewed in 51, 75, 157; see the sidebar titled 'Transcriptional regulation of *B. subtilis* sporulation'). However, polar septation also creates a dramatic topological problem, since the polar septum traps the forespore chromosome, such that only approximately one-third of it is present in the forespore and the remainder is in the mother cell (173). The trapped chromosome is actively transported to the forespore by the SpoIIIE translocation complex, which assembles at the septal midpoint (8, 173).

In this section, we discuss aspects of polar septation and chromosome translocation that are directly relevant to the phagocytosis-like process of engulfment.

2.1 Polar Septation

In most bacteria, including *B. subtilis*, cell division involves the formation of a septum by the invagination of peptidoglycan, which constricts the cellular membrane and divides the cell into two parts (recently reviewed in 53). Septation is orchestrated by the tubulin homolog FtsZ, which forms a circumferential ring-like structure (Z-ring) at the inner side of the cytoplasmic membrane, perpendicular to the long axis of the cell, that marks the impending division site. The Z-ring consists of treadmilling FtsZ filaments that move around the division plane (19) and serve as a scaffold to recruit other proteins involved in cell division, including those that synthesize septal peptidoglycan (53). During vegetative growth, the Z-ring assembles at mid-cell, resulting in the formation of a septum that divides the cell into two parts of similar sizes (Figure 4a). However, during sporulation, the Z-ring and hence the division site dynamically relocalize closer to a pole (14, 94) (Figure 4b). This process is facilitated by interaction of FtsZ with proteins synthesized shortly before polar septation under the control of Spo0A (63, 113, 179), namely, SpoIIE and RefZ (5, 88, 167), and by an increase in the expression of the *ftsAZ* operon from a σ^H-dependent promoter (14, 67, 68). However, how these factors mediate the relocalization of the Z-ring to polar position is not fully understood. For further discussion, we refer the reader to recent reviews of cell division and polar septation in *B. subtilis* (6, 53).

Polar septation can be initiated at both poles, as illustrated by the disporic phenotype observed in mutants that fail to activate the mother cell–specific transcription factor σ^E, which form a complete septum at each pole (78, 95, 121). However, in such mutants, as well as in wild-type sporangia, there is a delay of several minutes between the completion of the first septum and the initiation of constriction in the second (95, 122, 136). It is unclear what determines which septum is formed first, but it does not seem to depend on whether it is close to the new or to the old cell pole, since septation happens with equivalent frequencies at both poles (166). Nevertheless, in wild-type sporangia, the second septation process is aborted before the septum is complete to ensure only one complete septum during sporulation (122). This is likely mediated by the combined action of two factors produced in the mother cell shortly after the first septation event: SpoIIDMP, a peptidoglycan-degradation complex that mediates the dissolution of peptidoglycan in the partial septum (30, 47, 61, 122, 134, 155) (see Section 3.1.1), and MciZ, which directly interacts with FtsZ and inhibits the assembly of new Z-rings after the first septation event (18, 71) (Figure 4b).

Although there is compelling evidence that both the polar and the vegetative septa are built using the same basic cell division machinery (9, 10, 35, 54, 93), the polar septum is thinner than the vegetative septum [~25 nm versus ~80 nm (79, 87, 136, 162)]. In fact, the polar septum is just half as thick as the vegetative septum already during constriction, before septation is completed (78; Khanna et al., unpublished) (Figure 4a,b). Interestingly, the difference in thickness between the two septa correlates with the different distribution and number of FtsZ filaments at the leading edge of the closing septa (Khanna et al., unpublished). FtsZ filaments localize uniformly around the entire leading edge of vegetative

septa, but asymmetrically toward only the mother cell side of polar septa (Khanna et al., unpublished) (Figure 4a,b). Also, the number of filaments tracking the division plane during sporulation is roughly half that of vegetative growth, suggesting that septal thickness is dictated by the organization and the amount of the cell division machinery at the septum. This is further supported by the observation that in mutants lacking SpoIIE, which form thicker, vegetative-like polar septa (7, 55, 78), FtsZ coats the entire leading edge of polar septa (Khanna et al., unpublished), similar to the situation for vegetative septa. Therefore, the vegetative and polar septa differ not only in their positions but also in the fundamental organization of their cell division machinery. A thinner polar septum likely has important physiological consequences during sporulation. Firstly, many protein complexes are proposed to cross the septum, spanning both the septal membranes during sporulation (for example, SpoIIIE and SpoIIQ-SpoIIIA complexes; see Sections 2.2 and 3.1.3), which might be facilitated by the shorter distance between the septal membranes. Secondly, the thinner polar septum might be more flexible, hence allowing it to bend and stretch during engulfment to facilitate the movement of the septal junction around the forespore (see Section 3).

2.2 Chromosome Translocation

In addition to generating asymmetry in the gene expression programs of the two cells, polar septation also creates a topological problem by trapping the forespore chromosome asymmetrically in the septum. This is a direct consequence of the organization that the chromosomes adopt before polar septation when they are remodeled into an elongated structure, called axial filament, that runs from pole to pole, parallel to the long axis of the cell (23, 85, 89, 180). The axial filament consists of two chromosomes that result from a replication event before sporulation initiation, with a well-defined spatial disposition inside the cell: The origins of replication (*ori*) of the two chromosomes are tethered to the opposite poles, the termini (*ter*) are at mid-cell, and the arms of each chromosome are organized parallel to each other between the *ori* and the *ter* (16, 169, 171, 177). This results in the polar septum bisecting the forespore chromosome, leaving ~30% of the origin-proximal portion of the chromosome in the forespore and the remaining ~70% in the mother cell (173, 176). The SpoIIIE translocation complex (173), which assembles at septal midpoint (174), uses ATP hydrolysis to then actively translocate the chromosome across the septum to the forespore (8), packing a complete chromosome (> 4 Mb) in the small volume of the forespore, which is initially only approximately one-tenth the volume of the sporangium (101) (Figure 4c). Chromosome translocation into the forespore also plays a physical role in shaping the forespore, as the import of increasing amounts of DNA plus the abundant counterions required to neutralize its charge generate an elevated osmotic pressure (72, 128) that inflates the small forespore, increasing its size and reshaping it into an ovoid (101).

SpoIIIE belongs to the FtsK family of DNA translocases (37) and has a polytopic N-terminal transmembrane domain that anchors the protein to the septal membrane, connected via an unstructured linker to a cytoplasmic motor domain with ATPase activity that moves along the DNA in the presence of ATP and is directly responsible for chromosome translocation (8). The motor domains assemble into hexameric rings (26, 27), with an inner diameter large enough to accommodate a double-stranded DNA molecule (27, 103). In vitro experiments

with purified motor domains indicate that hexameric SpoIIIE rings track the $5' \rightarrow 3'$ strand in the direction of translocation, moving through the DNA in discrete steps of two nucleotides per ATP hydrolyzed (97). Chromosome translocation is therefore an energy-intensive process that requires ~1.5 million ATP molecules to move ~3 Mb of DNA into the forespore.

In vivo, SpoIIIE initially localizes at the leading edge of the constricting polar septum (56, 59, 98), prior to assembling a focus at mid-cell (174), the stable assembly of which depends on the presence of trapped DNA (15) and the motor domain (59). Cell-specific expression and degradation of SpoIIIE (130, 152, 178) demonstrated that, in intact sporangia, SpoIIIE works as a DNA exporter that pumps the chromosome out of the cell in which it assembles. This suggests that the SpoIIIE complex assembles in the mother cell to transport the chromosome to the forespore. Studies have also demonstrated that both arms of the trapped chromosome are simultaneously transported to the forespore (22), which would require the participation of at least two SpoIIIE hexamers, one per arm. However, estimations of the number of SpoIIIE monomers at septal midpoint indicate the presence of at least four hexamers (56, 178). Interestingly, although early studies seemed to indicate that SpoIIIE preferentially assembled in the mother cell (12, 56, 152), a more recent superresolution imaging study demonstrated that the protein assembles in both cells. This could be attributed to the fact that in addition to supporting translocation of the trapped chromosome into the forespore (8, 173), the SpoIIIE complex also mediates the separation of the mother cell and the forespore septal membranes (septal membrane fission) (59, 98). The participation of SpoIIIE in septal membrane fission is most readily explained if, during the final stages of cell division, SpoIIIE subunits on both sides of the septum interact via their extracytoplasmic loops, forming a protein channel that crosses both septal membranes and excludes lipids (59, 98). To reconcile this model with the DNA exporter model, we posit that SpoIIIE assembles paired channels across the septum, with only the mother cell motor domains actively engaged in exporting the chromosome to the forespore. This model is supported by photoactivated localization microscopy (PALM) images showing the presence of two SpoIIIE foci containing enough monomers to assemble two hexamers each in the forespore and in the mother cell (178), indicating that the SpoIIIE complex might comprise two side by side paired channels that span both septal membranes (Figure 4c).

Targeted degradation of SpoIIIE subcomplexes in either cell shows that both subcomplexes are required to maintain the separation of the septal membranes but only the mother cell subcomplex is essential to transporting the chromosome to the forespore (178). Interestingly, degrading the mother cell subcomplexes results in the reverse translocation of the chromosome from the forespore to the mother cell, catalyzed by the forespore subcomplexes (178), indicating that SpoIIIE can work as a bidirectional motor. The activation of the motor domains in the mother cell is likely controlled by their interaction with octameric sequences in the DNA known as SpoIIIE recognition sequences (SRSs) (126). In vitro, the interaction of SpoIIIE motor hexamers with SRSs in the permissive orientation boosts SpoIIIE ATPase activity (17, 26), potentiating the DNA tracking ability of the hexamers. In vivo, the mother cell and the forespore SpoIIIE subcomplexes are inverted (Figure 4c), and therefore they interact with SRSs in different orientations: permissive for the mother cell hexamers and nonpermissive for the forespore hexamers. This probably contributes to the activation of the

motor domains in the mother cell and deactivation in the forespore, preventing competition between subcomplexes to export the chromosome out of their respective cells. A key question that remains to be answered is, How does the last piece of the circular chromosome enter the forespore? This probably requires fusion of the two SpoIIIE channels, which generates a larger pore to allow the passage of the terminal piece.

3 Cellular Dynamics During Engulfment

Engulfment mediates a dramatic change in the topology of the sporangium, from two cells that lie side by side to a cell within a cell, the hallmark of endospore formation. After completion of engulfment, the forespore is enclosed in the mother cell cytoplasm and delimited by two membranes: an inner membrane, which is the original cytoplasmic membrane of the forespore, and an outer membrane, derived from the mother cell engulfing membrane.

Owing to the implementation of different fluorescence and electron microscopy techniques (Figures 2 and 3; see the sidebar titled ‘Early visualization studies of sporulation’), we now possess detailed descriptions of the morphological transformations that accompany engulfment at high spatial and temporal resolution (87, 101, 118). Immediately after polar septation, the septum is flat and has an average thickness (defined as the distance between the forespore and the mother cell septal membranes) of ~23 nm, tending to be thicker in the middle than at the edges. But shortly after polar septation, the septum bends toward the mother cell and becomes slightly thinner, reaching a uniform thickness of ~18 nm (87). The septum keeps stretching as the forespore grows toward the mother cell, without significant forward movement of the mother cell membrane around the forespore for ~20 min (at 30°C). Then, the leading edges of the mother cell membrane migrate toward the forespore tip by forming tiny finger-like projections, ~10–30 nm wide and ~5–20 nm long (87), slowly surrounding the forespore. Engulfment membrane migration continues for ~60 min (118), and once the leading edges meet at the tip of the forespore, they fuse, releasing the forespore inside the mother cell cytoplasm, disconnected from the mother cell membrane. During engulfment, the surface area of the mother cell increases by ~2 μm^2 (~25% of its initial value) to completely enclose the forespore (118). In addition, the forespore, initially hemispherical following polar septation, is remodeled into an ovoid and doubles in size (101).

Here, we describe engulfment as two separate processes that are controlled by different cellular machineries: (*a*) the migration of the mother cell membrane around the forespore (engulfment membrane migration) and (*b*) the separation of the mother cell and the forespore membranes through fusion of the leading edges of the engulfing membrane at the forespore tip (engulfment membrane fission).

3.1 Engulfment Membrane Migration

While engulfment may have some visual resemblance to eukaryotic phagocytosis, a key difference between the two is that during engulfment the membranes are restricted by the peptidoglycan cell wall. Specifically, the peptidoglycan septum that separates the mother cell and the forespore represents a seemingly unsurmountable steric obstacle for the

migration of the mother cell membrane around the forespore. Not surprisingly, the key cellular machineries essential for membrane migration are involved in peptidoglycan metabolism: peptidoglycan degradation (1, 28, 70, 115), carried out by a mother cell protein complex made of SpoIID, SpoIIM, and SpoIIP, and peptidoglycan synthesis (109, 118), likely carried out by forespore peptidoglycan biosynthetic machinery that tracks the leading edge of the engulfing membrane throughout migration (118). In addition, membrane migration is facilitated by a complex made by the forespore protein SpoIIQ and the mother cell protein SpoIIIAH. We describe the different factors that promote membrane migration below.

3.1.1 SpoIIDMP and a model for membrane migration based on septal peptidoglycan dissolution—

The *spoIID*, *spoIIM*, and *spoIIP* loci were identified during classical genetic studies of sporulation, as strains containing loss-of-function mutations in them are unable to form spores (61, 100, 154). *spoIID*, *spoIIM*, or *spoIIP* null mutants show a characteristic developmental blockage after polar septation, in which the mother cell membrane fails to migrate around the forespore. Rather, the septum bulges toward the mother cell as if the forespore were pushing through the middle of the septum (32, 61, 78, 154). Orthologs of the three genes are present in most sporulating *Bacillus* and *Clostridium* sequenced so far but largely absent in species that do not form endospores (2, 65). Although all three genes are essential for engulfment and sporulation in *B. subtilis*, some are dispensable in other endospore-forming bacteria. For example, in *Clostridium difficile*, SpoIIM is not required for efficient engulfment, and mutants lacking SpoIID may complete sporulation, although significantly less efficiently than the wild type (38, 129).

SpoIID, SpoIIM, and SpoIIP are transmembrane proteins encoded in separate transcriptional units under the control of σ^E (30, 61, 134, 155) (see also the sidebar titled ‘Forespore expression of SpoIIP’). The three proteins initially localize at the middle of the polar septum and subsequently relocalize to the leading edges of the engulfing mother cell membrane, where they remain until the completion of engulfment (1, 28, 70). Their localization to the septum follows a hierarchical dependence pattern in which SpoIIM recruits SpoIIP, which in turn recruits SpoIID (4, 28), strongly suggesting that the three proteins form a complex (hereafter referred to as DMP). In fact, direct interaction between SpoIIP and SpoIID has been demonstrated (4, 28). The localization of SpoIIM (and consequently SpoIIP and SpoIID) to the septum is achieved by two complementary mechanisms. The main mechanism involves a protein called SpoIIB, which is produced before polar septation, is recruited to the invaginating polar septum, and then shows a localization pattern similar to that of DMP during engulfment (4, 28, 102, 120). However, unlike mutants lacking DMP, *spoIIB* mutants are able to complete engulfment, although slowly and with some visible morphological defects such as the formation of bulges at the septum (102, 120). The second mechanism, which plays a minor role in DMP localization, depends on the proteins SpoIVFA and SpoIVFB, which are produced in the mother cell and recruited to the septum by the SpoIIQ-SpoIIIAH zipper (40, 80) (see Section 3.1.3). Mutants lacking both SpoIIB and SpoIVFAB fail to localize DMP to the septum and do not initiate engulfment (4). Thus, the engulfment proteins are localized by two redundant mechanisms, one involving SpoIIB which is recruited during cell division to serve as a septal landmark, and another involving

SpoIIQ-SpoIIIAH zipper, which assembles after the onset of cell-specific gene expression and serves as a critical landmark for the recruitment of proteins to the sporulation septum (see Section 3.1.3). It is likely that the SpoIIQ-SpoIIIAH zipper provides the primary pathway for DMP localization in bacterial species that do not contain SpoIIB (65).

The enzymatic activity of the DMP complex has been studied in some detail. SpoIID and SpoIIP both degrade peptidoglycan and are rate limiting for engulfment, indicating that their activities are critical for the process (1, 28). They also have complementary enzymatic activities. SpoIIP is both an endopeptidase and amidase that cleaves the peptide cross-links that connect contiguous glycan strands and removes the stem peptides to generate denuded glycan strands (115). SpoIID is a lytic transglycosylase that binds to and cleaves denuded glycan strands produced by SpoIIP (115, 116). SpoIID also potentiates the activity of SpoIIP (70, 115), so the two enzymes may mediate the processive degradation of peptidoglycan during engulfment. So far, no enzymatic activity has been associated with SpoIIM, and it is therefore possible that its main function is to serve as a scaffold for the assembly of DMP (4, 28).

It was traditionally assumed that the main function of DMP in engulfment was related to a previously proposed step known as septal thinning, in which it was posited that septal peptidoglycan was enzymatically thinned from the septal midpoint toward the edges. This model was supported by the enzymatic activity of DMP, and by electron microscopy studies of thin sections of fixed *B. subtilis* sporangia showing that wild-type sporangia sometimes have thinner peptidoglycan near the septal midpoint and that *DMP* mutants have thicker septa than wild type (1, 28, 78, 115). However, recent cryo–electron tomography (cryo-ET) images and staining of the septal peptidoglycan with fluorescent D-amino acids have challenged this idea by revealing that a thin peptidoglycan layer remains between the forespore and the mother cell membranes throughout engulfment (87, 101, 162). This layer is probably a remnant of the original septum, as it is still present in sporangia treated with antibiotics that inhibit the synthesis of new peptidoglycan (87). Indeed, careful measurements of the thickness of septal peptidoglycan using cryo-ET images demonstrate that septal peptidoglycan is not thinned starting at the septum midpoint but rather is uniformly and only slightly thinned as the septum transitions from flat to curved (87), which might represent the transition from relaxed to stretched septal peptidoglycan as the forespore grows and pushes against the septum. While it remains possible that the slightly thicker than normal septal peptidoglycan in the absence of DMP is due to the partial and delocalized degradation of septal peptidoglycan by DMP, this phenotype may also be due to the involvement of DMP in clearing peptidoglycan synthases from the septum, since several penicillin-binding proteins (PBPs) are retained at the septum in the absence of DMP (87). Thus, there is no longer sufficient evidence to support the previously prevalent model that septal thinning is mediated by the nearly complete dissolution of septal peptidoglycan by DMP.

These observations pose a critical question as to how the mother cell membrane is able to migrate around the forespore if DMP does not dissolve the septal peptidoglycan completely, since the junction between the septal and the lateral peptidoglycan would provide a steric block to the movement of the mother cell membrane. A hint to answer this question came

from observations that SpoIID lytic transglycosylase activity is required throughout engulfment membrane migration, and not only at the onset of engulfment (70), and that DMP is rate limiting for membrane migration (1), suggesting that it plays a role beyond dissolution of the septal peptidoglycan. The first model proposed for membrane migration was that DMP, anchored in the mother cell membrane, acts as a burnt bridge Brownian ratchet that drags the engulfing membrane around the forespore as it degrades peptidoglycan (1). This model is supported by the activity of P and D, which could allow them to form a processive enzyme complex (115). Another critical hint for the mechanism was provided by the observation that forespore peptidoglycan synthesis is also essential for engulfment membrane migration (109, 118). These observations have led to a revised model for engulfment membrane migration that is discussed in the next section.

3.1.2 Peptidoglycan synthases and a model for membrane migration based on peptidoglycan remodeling—

The observations that both peptidoglycan synthesis as well as peptidoglycan degradation are essential for forespore engulfment have evolved our understanding about the complex nature of the process, leading to a new model for membrane migration that is based on coordinated action of these two activities to move the junction between the septum and the lateral cell wall around the forespore and is depicted in Figure 5a.

It was shown that engulfment membrane migration depends on peptidoglycan synthesis by treating sporulating cultures with different peptidoglycan inhibitors, at concentrations that block other peptidoglycan-dependent processes such as septation and cell elongation (87, 118). It is critical that such processes be assessed in the same culture of sporulating bacteria, since the minimal inhibitory concentration of some antibiotics varies according to cell density and growth phase (81, 163). When peptidoglycan synthesis is inhibited, the leading edges of the engulfing membrane do not migrate to the forespore tip. However, the forespore keeps growing toward the mother cell, in a process that involves the stretching and probably also the cleavage of some peptide cross-links in the septal peptidoglycan by DMP or other peptidoglycan hydrolases, which may increase the peptidoglycan surface area without new synthesis (92). The forespore growth leads to septal curving that can resemble engulfment membrane migration, although analysis of time-lapse images clearly demonstrates that there is no net closure of the gap between the leading edges of the engulfing membrane (118). Interestingly, with some antibiotics, time-lapse microscopy demonstrates that the curved septum rotates within the sporangium, as if the septal peptidoglycan were disconnected from the lateral cell wall (87, 118).

Researchers used methods to label the location of new peptidoglycan synthesis by fluorescent antibiotics and D-amino acids (91, 161) to show that during engulfment new peptidoglycan is primarily synthesized close to the leading edge of the engulfing membrane (109, 162). Accordingly, many proteins involved in peptidoglycan polymerization track the leading edge of the engulfing membrane throughout engulfment (118, 142). Interestingly, most of these enzymes do so from the forespore side of the septum and do not localize at the leading edge of the engulfing membrane in the mother cell (118). The sole exception is PBP1, which can track the leading edge of the engulfing membrane from both the mother cell and the forespore sides, but it is not required for membrane migration (162). These

results indicate that the peptidoglycan biosynthetic machinery that is required for membrane migration localizes in the forespore, to sites that are close to the leading edge of the migrating mother cell membrane. This conclusion is further supported by the observation that MreB, a protein involved in the organization of the peptidoglycan biosynthetic machinery during cell elongation (42, 52, 66, 164), moves in the forespore at a position coincident to the leading edge of the engulfing membrane (118), suggesting that MreB-associated peptidoglycan biosynthetic machinery inserts peptidoglycan in a circumferential pattern around the forespore as the engulfing mother cell membrane migrates toward the forespore tip.

It remains unclear how the peptidoglycan biosynthetic machinery localizes in the forespore, but it seems to be independent of cell-specific gene expression (118), and we hypothesize that it may recognize a specific feature of the junction between the septal peptidoglycan and the lateral cell wall. As discussed in Section 2.1, the polar septum may be intrinsically asymmetric given the asymmetric localization of the division machinery during constriction. This could produce different structural features on each face of the septum, or it could restrict the peptidoglycan biosynthetic machinery to the forespore side of the septum. In fact, other membrane proteins such as DivIVA and SpoIIE localize to the forespore side of the polar septum after division (54). It is therefore possible that the peptidoglycan biosynthetic machinery that localizes to the polar septum during constriction (142–144) is somehow sequestered in the forespore after polar septation. Clearly, this is an interesting area for future research.

Another aspect that remains to be elucidated is the precise identity of peptidoglycan biosynthetic enzymes that are required for engulfment. The only two known peptidoglycan-polymerizing enzymes that are essential for sporulation are the transpeptidase SpoVD (34, 165) and the putative transglycosylase SpoVE (50, 73, 106), both of which are required for cortex synthesis but not for engulfment. It has also been reported that double mutants lacking two bifunctional transpeptidase/transglycosylase PBPs, PBP2c and PBP2d, fail to maintain forespore integrity after engulfment and are therefore severely impaired in their ability to form viable spores (105). However, mutants lacking these peptidoglycan-polymerizing enzymes, individually or in combination, proceed through engulfment apparently normally (105). Thus far, there is no mutation in peptidoglycan-polymerization enzymes known to affect engulfment, likely due to the functional redundancy of peptidoglycan biosynthetic enzymes in *B. subtilis* (139, 172), which can mask the phenotypes of mutants lacking individual enzymes. Thus, the role of peptidoglycan synthesis in engulfment has been so far studied using antibiotics that block peptidoglycan synthesis, instead of specific mutations affecting the peptidoglycan biosynthetic machinery.

There is an intimate connection between peptidoglycan synthesis and the DMP proteins that degrade peptidoglycan. For example, when peptidoglycan synthesis is blocked, the localization of DMP to the leading edge of the engulfing membrane is impaired (118), suggesting that newly synthesized peptidoglycan stabilizes DMP at the leading edge of the engulfing membrane. This connection between DMP and peptidoglycan synthesis is further supported by the architecture of the leading edge of the engulfing membrane, which moves in tiny finger-like projections whose formation depends on DMP and is potentiated by

peptidoglycan synthesis (87). One model to explain the formation of these projections is that DMP tethers the engulfing membrane to the new peptidoglycan that is synthesized ahead of the leading edge of the engulfing membrane by forespore-associated peptidoglycan biosynthetic machinery, and it degrades this new peptidoglycan to make room for the engulfing membrane to advance. The limited number of DMP complexes at the leading edge of the engulfing membrane could lead to the formation of finger-like projections (70, 87) (Figure 5a).

The above results have led to a revised model for the movement of the mother cell membrane around the forespore (87, 118, 162). According to this model, the coordinated action of forespore peptidoglycan synthesis and mother cell peptidoglycan degradation at the leading edge of the engulfing membrane might move the junction between the septum and the lateral cell wall around the forespore, making room to accommodate the excess membrane synthesized during engulfment (118). In this model, new peptidoglycan is inserted ahead of the leading edge of the engulfing membrane by forespore-associated peptidoglycan biosynthetic machinery, and it is subsequently targeted by DMP for degradation (Figure 5a). As a consequence, septal peptidoglycan is extended around the forespore, providing the mold to reshape the forespore into an ovoid during membrane migration (101), and the junction between septal peptidoglycan and the lateral cell wall is moved around the forespore to complete the phagocytosis-like process of engulfment (118). This model proposes that cell wall remodeling drives engulfment via a relatively simple mechanism involving conserved enzymatic activities that are deployed in a cell-specific manner. It also accounts for the continued requirement of DMP and peptidoglycan synthesis throughout engulfment membrane migration (1, 70, 118) and explains the directional movement of the engulfing membrane, since the new peptidoglycan targeted by DMP is synthesized in the forespore, ahead of the leading edge of the engulfing membrane. Of course, many questions remain, including the mechanism by which DMP targets peptidoglycan for degradation. We posit that the enzymes might recognize either specific structural features of newly synthesized peptidoglycan or the junctional bonds that connect the septal peptidoglycan to the lateral cell wall.

3.1.3 SpoIIQ-IIIA: a zipper for the engulfing membrane—SpoIIQ (henceforth referred to as Q) is a forespore-specific protein produced under the control of σ^F (99). SpoIIIAH (henceforth referred to as AH) is encoded by the last gene of the *spoIIIA* operon, which contains eight genes, from *spoIIIAA* to *spoIIIAH*, and is transcribed in the mother cell from two σ^E-dependent promoters (69, 79). Both proteins are essential for sporulation, as they are required for the activation of the late forespore σ factor, σ^G (86, 158). However, they also play a distinct role in membrane migration.

Q and AH are integral membrane proteins that localize on opposite faces of the polar septum (in the forespore and in the mother cell membrane, respectively) and track the engulfing membrane, forming foci distributed throughout the engulfed area (20, 40, 99, 135) (Figure 5b). The two proteins interact through their extracytoplasmic domains, forming protein bridges across the septum (20, 40, 107). Accordingly, the localization of both proteins is partially interdependent, suggesting that the interaction between the extracellular domains is

important to recruit the two proteins to the septum (20, 40, 62, 132, 133). Once assembled, Q-AH complexes are stable and do not diffuse laterally in the membranes (21).

The Q-AH complex recruits other proteins to the mother cell side of the septum that are required for the subsequent activation of σ^G and σ^K (40, 41, 80). Among such proteins are the rest of the proteins encoded in the *spoIIIA* operon, SpoIIIAA through SpoIIIAG. Some of these proteins are homologous to components of bacterial secretion systems, and two of them have been shown to form homomultimeric rings in vitro (24, 41, 107, 108, 131, 182–184). It has therefore been proposed that SpoIIIA proteins in the mother cell, together with Q in the forespore, form channels connecting the two cells through which molecules required to activate σ^G are transferred from the mother cell to the forespore (25, 41, 108; see 33, 114, 185 for reviews). The Q-AH complex is also required for persistent, σ^G-independent forespore gene expression (25) and for the maintenance of the structural integrity of the forespore after engulfment (41). These observations have led to the proposal that the Q-A complex assembles channels that constitute feeding tubes through which the mother cell nurtures the forespore by providing metabolites to maintain gene expression (25), although such channels have not been observed directly yet. This model also assumes that the forespore cannot synthesize enough metabolic precursors to support its own biosynthetic activities and has to import them from the mother cell. However, this has not been established yet, and the exact nature and function of molecules, if any, that are transported through the putative Q-A channels are yet to be determined (Figure 5b).

Although the main function of Q-AH is probably related to the activation or maintenance of forespore gene expression during later stages of development, Q-AH also plays a role in engulfment membrane migration. However, the latter function depends on the culture conditions and the genetic background. Q-AH is dispensable for engulfment when sporulation is induced by resuspension in chemically defined medium (156), as the null mutants are still able to complete engulfment, albeit more slowly than the wild type (21, 158). However, the complex becomes essential for membrane migration when sporulation is induced by nutrient exhaustion (99, 141, 158) and also in sporangia with reduced DMP activity (21). Interestingly, Q-AH is essential for engulfment in sporangia wherein the cell wall is enzymatically removed using lysozyme and the mother cell and the forespore are thereby transformed into spherical protoplasts (21, 117). Under these conditions, engulfment proceeds rapidly as the cell wall is removed, completing in only a few minutes, compared to more than an hour in native conditions (21, 118). DMP is not required for protoplast engulfment, consistent with the model that DMP participates in peptidoglycan remodeling and protoplasts lack peptidoglycan. Instead, protoplast engulfment appears to rely exclusively on Q-AH and requires a minimum threshold of the Q-AH proteins to occur (21, 117). The process is independent of another protein encoded in the *spoIIIA* operon, SpoIIIAG (21), which is an integral component of the Q-A complex that connects the mother cell and the forespore (41, 131, 184), suggesting that protoplast engulfment does not rely on the assembly of a functional channel and that the zipper-like interaction between Q and AH is sufficient to wrap the mother cell membrane around the forespore when the cell wall is removed. Since Q-AH complexes are stationary and do not show lateral diffusion (21), they may serve as a ratchet to secure the mother cell membrane to the forespore, thereby preventing backward movement. Although Q-AH complexes likely play an

equivalent role in walled cells, they are not essential for membrane migration, because its function may be masked by other redundant engulfment modules. First, it is possible that DMP tethering the leading edge of the engulfing membrane to the peptidoglycan (see Section 3.1.2) also prevents backward movement (87, 118). Second, engulfment in walled cells requires a significantly longer time than that in protoplasts, and it entails an ~25% increase in the membrane surface area of the mother cell (118). This excess membrane might also contribute to membrane migration in intact, walled bacteria, by providing additional membrane surface area that can move into the space made available around the forespore by the action of coordinated peptidoglycan synthesis and peptidoglycan degradation. In keeping with this hypothesis, we have recently found that membrane synthesis is required for engulfment, although the precise role remains to be explored (101).

3.2 Engulfment Membrane Fission

Once the leading edge of the engulfing membrane reaches the forespore tip, the lipids at the leading edge rearrange and the mother cell membrane undergoes a fission event that releases the forespore into the mother cell cytoplasm, where it is now bounded by two membranes, the forespore cytoplasmic membrane and the mother cell–derived outer forespore membrane. Studies of engulfment membrane fission have been facilitated by the development of staining techniques that allow the distinction of sporangia that have undergone membrane fission from those that have not (39, 109, 151) (Figure 3). Two proteins have been implicated in this process: a mother cell–specific protein called FisB (39) and the SpoIIIE DNA translocase (151).

3.2.1 FisB—FisB is a bitopic protein with a large extracellular domain, produced in the mother cell under the control of σ^E. *fisB* mutants are not impaired in engulfment membrane migration, but the leading edges fail to fuse and release the forespore inside the mother cell cytoplasm, indicating that the protein participates specifically in membrane fission (39). This is further supported by the cellular localization of FisB, which forms dynamic oligomeric foci throughout the mother cell membrane that are stabilized at the tip of the forespore upon completion of membrane migration. In fact, FisB can also catalyze the fusion of liposomes in vitro. The extracellular domain of FisB interacts with liposomes containing cardiolipin (39), a cone-shaped phospholipid enriched in regions of bacterial membranes with negative curvature, including the inner leaflet of the engulfing membrane (13, 82, 84, 110). These results have led to the proposal that FisB might recruit cardiolipin to the positively curved membrane at the site of membrane fission, which would destabilize the membrane due to the conical shape of cardiolipin, leading to the reorganization of the lipids and the separation of the mother cell and the forespore membranes (39). Although FisB is likely to play a direct role in engulfment membrane fission, ~12% of sporangia lacking FisB are still able to complete membrane fission, albeit slowly, and produce mature spores (39), suggesting that additional factors contribute to membrane fission during sporulation.

3.2.2 SpoIIIE—As described in Section 2.2, SpoIIIE is required for septal membrane fission at the onset of sporulation (59, 98, 178). Interestingly, mutations in *spoIIIE* were identified in a genetic screen that affected the late stages of engulfment (151), implicating it in engulfment membrane fission as well. In fact, the SpoIIIE complex moves from septal

midpoint to the forespore tip after chromosome translocation, suggesting that SpoIIIE might affect engulfment membrane fission directly. However, due to the pleiotropic effects of *spoIIIE* mutations, it is also possible that SpoIIIE affects engulfment membrane fission indirectly. Firstly, *spoIIIE* deletion mutants fail to compartmentalize σ^F- and σ^E-dependent gene expression (124, 177a), probably due to failure in septal membrane fission (59, 98, 178), which results in decreased expression of some mother cell–expressed proteins such as FisB (39). However, there are specific mutations in *spoIIIE* that do not interfere with compartmentalization of gene expression (98) and yet impair engulfment membrane fission, suggesting that SpoIIIE may play additional roles in membrane fission. Secondly, it is possible that the role of SpoIIIE-mediated chromosome translocation in inflating the forespore (101) has an impact on membrane fission. Cryo-ET images of sporulating *spoIIIE* mutants show that, in the absence of chromosome translocation, an excess of membrane accumulates around the forespore in the form of folds and invaginations of the two membranes surrounding the forespore (101). This convoluted membrane topology might make the progression of engulfment and membrane fission difficult, as the leading edges of the engulfing membrane might need to travel a more convoluted pathway and thereby have trouble in meeting at the forespore tip, by that means contributing to the engulfment defects observed in *spoIIIE* mutants. However, some *spoIIIE* mutants defective in chromosome translocation show only a mild defect in engulfment membrane fission (151, 153), indicating that the roles of SpoIIIE in both processes are genetically separable. Nevertheless, further studies are required to precisely define the function of SpoIIIE in engulfment membrane fission.

In addition to FisB and SpoIIIE, additional factors may also contribute to membrane fission. For example, sporulating cells treated with antibiotics inhibiting peptidoglycan synthesis at concentrations that partially block membrane migration have a specific membrane fission defect, indicating that peptidoglycan synthesis may also play a role in membrane fission (109). It also remains possible that proteins required for membrane migration, such as DMP, may play a role in membrane fission, although such roles may be occluded by their function in membrane migration. It will be interesting to explore the roles of such proteins and of specific lipids and fatty acids that may mediate this membrane rearrangement in the final step of engulfment.

4 Outlook

Sporulation studies over the last ~150 years have shed light on fundamental questions in bacterial cell biology and have demonstrated the ability of bacterial cells to undergo dynamic architectural transformations that were, until recently, thought to be restricted to eukaryotes. In this review, we summarize some of the key processes and molecular players that contribute to the dramatic cell biological remodeling of the sporangia early during *B. subtilis* sporulation. The discoveries we highlight are critically dependent on decades of accumulated knowledge about *B. subtilis* sporulation and the development of genetic technologies to manipulate these cells. They are also a result of technical advancements in optical and electron microscopy, which have provided key mechanistic insights into the architectural transformations that occur during engulfment. Research on *B. subtilis* sporulation, aided by development of new experimental technologies, will likely continue to

contribute to our understanding of how prokaryotic cells organize and reorganize their cellular content. The development of genetic tools that work in a wide array of organisms will allow this research to expand beyond *B. subtilis*, which is only one of the many bacterial species that form endospores. We look forward to future work in other endospore-forming and engulfment-performing *Firmicutes* that will allow us to fully appreciate the diversity of sporulation programs and to understand the evolutionary origins of the cell biological transformations that underlie this process.

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Literature Cited

1. Abanes-De Mello A, Su YL, Aung S, Pogliano K. A cytoskeleton-like role for the bacterial cell wall during engulfment of the *Bacillus subtilis* forespore. Genes Dev. 2002; 16:3253–64. [PubMed: 12502745]
2. Abecasis AB, Serrano M, Alves R, Quintais L, Pereira-Leal JB, Henriques AO. A genomic signature and the identification of new sporulation genes. J Bacteriol. 2013; 195(9):2101–15. [PubMed: 23396918]
3. Hutchison EA, Miller DA, Angert ER. Sporulation in bacteria: beyond the standard model. Microbiol Spectr. 2014; 2(5)doi: 10.1128/microbiolspec
4. Aung S, Shum J, Abanes-De Mello A, Broder DH, Fredlund-Gutierrez J, et al. Dual localization pathways for the engulfment proteins during *Bacillus subtilis* sporulation. Mol Microbiol. 2007; 65(6):1534–46. [PubMed: 17824930]
5. Barák I, Muchová K. The positioning of the asymmetric septum during sporulation in *Bacillus subtilis* . PLOS ONE. 2018; 13(8):1–15.
6. Barák I, Muchová K, Labajová N. Asymmetric cell division during *Bacillus subtilis* sporulation. Future Microbiol. 2019; 14(4):353–63. [PubMed: 30855188]
7. Barák I, Youngman P. SpoIIE mutants of *Bacillus subtilis* comprise two distinct phenotypic classes consistent with a dual functional role for the SpoIIE protein. J Bacteriol. 1996; 178(16):4984–89. [PubMed: 8759864]
8. Bath J, Wu LJ, Errington J, Wang JC. Role of *Bacillus subtilis* SpoIIE in DNA transport across the mother cell-prespore division septum. Science. 2000; 290(5493):995–97. [PubMed: 11062134]
9. Beall B, Lutkenhaus J. FtsZ in *Bacillus subtilis* is required for vegetative and for asymmetric septation during sporulation. Genes Dev. 1991; 5:447–55. [PubMed: 1848202]
10. Beall B, Lutkenhaus J. Impaired cell division and sporulation of a *Bacillus subtilis* strain with the ftsA gene deleted. J Bacteriol. 1992; 174(7):2398–403. [PubMed: 1551857]
11. Bechtel DB, Bulla LA. Electron microscope study of sporulation and parasporal crystal formation in Bacillus thuringiensis. J Bacteriol. 1976; 127(3):1472–81. [PubMed: 182671]
12. Becker EC, Pogliano K. Cell-specific SpoIIIE assembly and DNA translocation polarity are dictated by chromosome orientation. Mol Microbiol. 2007; 66(5):1066–79. [PubMed: 18001347]
13. Beltrán-Heredia E, Tsai FC, Salinas-Almaguer S, Cao FJ, Bassereau P, Monroy F. Membrane curvature induces cardiolipin sorting. Commun Biol. 2019; 2(1):1–7. [PubMed: 30740537]
14. Ben-Yehuda S, Losick R. Asymmetric cell division in B. subtilis involves a spiral-like intermediate of the cytokinetic protein Fts Z. Cell. 2002; 109(2):257–66. [PubMed: 12007411]

15. Ben-Yehuda S, Rudner DZ, Losick R. Assembly of the SpoIIIE DNA translocase depends on chromosome trapping in *Bacillus subtilis* . Curr Biol. 2003; 13(24):2196–200. [PubMed: 14680637]
16. Ben-Yehuda S, Rudner DZ, Losick R. Rac A, a bacterial protein that anchors chromosomes to the cell poles. Science. 2003; 299(5606):532–36. [PubMed: 12493822]
17. Besprozvannaya M, Pivorunas VL, Feldman Z, Burton BM. SpoIIIE protein achieves directional DNA translocation through allosteric regulation of ATPase activity by an accessory domain. J Biol Chem. 2013; 288(40):28962–74. [PubMed: 23974211]
18. Bisson-Filho AW, Discola KF, Castellen P, Blasios V, Martins A. FtsZ filament capping by MciZ a developmental regulator of bacterial division. PNAS. 2015; 112(17):E2130–38. [PubMed: 25848052]
19. Bisson-Filho AW, Hsu YP, Squyres GR, Kuru E, Wu F. Treadmilling by FtsZ filaments drives peptidoglycan synthesis and bacterial cell division. Science. 2017; 355(6326):739–43. [PubMed: 28209898]
20. Blaylock B, Jiang X, Rubio A, Moran CP, Pogliano K. Zipper-like interaction between proteins in adjacent daughter cells mediates protein localization. Genes Dev. 2004; 18(23):2916–28. [PubMed: 15574594]
21. Broder DH, Pogliano K. Forespore engulfment mediated by a ratchet-like mechanism. Cell. 2006; 126(5):917–28. [PubMed: 16959571]
22. Burton BM, Marquis KA, Sullivan NL, Rapoport TA, Rudner DZ. The AT.ase Spo II.E transports DNA across fused septal membranes during sporulation in *Bacillus subtilis* . Cell. 2007; 131(7):1301–12. [PubMed: 18160039]
23. Bylund JE, Haines MA, Piggot PJ, Higgins ML. Axial filament formation in *Bacillus subtilis*: induction of nucleoids of increasing length after addition of chloramphenicol to exponential-phase cultures approaching stationary phase. J Bacteriol. 1993; 175(7):1886–90. [PubMed: 7681431]
24. Camp AH, Losick R. A novel pathway of intercellular signalling in *Bacillus subtilis* involves a protein with similarity to a component of type III secretion channels. Mol Microbiol. 2008; 69(2):402–17. [PubMed: 18485064]
25. Camp AH, Losick R. A feeding tube model for activation of a cell-specific transcription factor during sporulation in *Bacillus subtilis* . Genes Dev. 2009; 23(8):1014–24. [PubMed: 19390092]
26. Cattoni DI, Chara O, Godefroy C, Margeat E, Trigueros S. SpoIIIE mechanism of directional translocation involves target search coupled to sequence-dependent motor stimulation. EMBO Rep. 2013; 14(5):473–79. [PubMed: 23559069]
27. Cattoni DI, Thakur S, Godefroy C, Le Gall A, Lai-Kee-Him J. Structure and DNA-binding properties of the *Bacillus subtilis* SpoIIIE DNA translocase revealed by single-molecule and electron microscopies. Nucleic Acids Res. 2014; 42(4):2624–36. [PubMed: 24297254]
28. Chastanet A, Losick R. Engulfment during sporulation in *Bacillus subtilis* is governed by a multi-protein complex containing tandemly acting autolysins. Mol Microbiol. 2007; 64(1):139–52. [PubMed: 17376078]
29. Ciccarelli FD, Doerks T, von Mering C, Creevey CJ, Snel B, Bork P. Toward automatic reconstruction of a highly resolved tree of life. Science. 2006; 311(March):1283–88. [PubMed: 16513982]
30. Clarke S, Lopez-Diaz I, Mandelstam J. Use of lacZ gene fusions to determine the dependence pattern of the sporulation gene spoIID in spo mutants of *Bacillus subtilis* . J Gen Microbiol. 1986; 132(11):2987–94. [PubMed: 3114421]
31. Cohn F. Untersuchungen uber Bakterien IV. Beitrage zur Biologie der Bacillen. Beitrage Biol Pflanz. 1876; 2:249–76.
32. Coote JG. Sporulation in *Bacillus subtilis*. Characterization of oligosporogenous mutants and comparison of their phenotypes with those of asporogenous mutants. J Gen Microbiol. 1972; 71(1):1–15. [PubMed: 4625072]
33. Crawshaw AD, Serrano M, Stanley WA, Henriques AO, Salgado PS. A mother cell-to-forespore channel: current understanding and future challenges. FEMS MicrobiolLett. 2014; 358(2):129–36.

34. Daniel RA, Drake S, Buchanan CE, Scholle R, Errington J. The *Bacillus subtilisspoVD* gene encodes a mother-cell-specific penicillin-binding protein required for spore morphogenesis. J Mol Biol. 1994; 235:209–20. [PubMed: 8289242]
35. Daniel RA, Harry EJ, Katis VL, Wake RG, Errington J. Characterization of the essential cell division gene ftsL(yIID) of *Bacillus subtilis* and its role in the assembly of the division apparatus. Mol Microbiol. 1998; 29(2):593–604. [PubMed: 9720875]
36. Decker S, Maier S. Fine structure of mesosomal involvement during Bacillus macerans sporulation. J Bacteriol. 1975; 121(1):363–72. [PubMed: 234941]
37. Demarre G, Galli E, Barre FX. The FtsK family of DNA pumps. Adv Exp Med Biol. 2013; 767:245–62. [PubMed: 23161015]
38. Dembek M, Kelly A, Barwinska-Sendra A, Tarrant E, Stanley WA. Peptidoglycan degradation machinery in Clostridium difficile forespore engulfment. Mol Microbiol. 2018; 110(3):390–410. [PubMed: 30066424]
39. Doan T, Coleman J, Marquis KA, Meeske AJ, Burton BM. FisB mediates membrane fission during sporulation in *Bacillus subtilis* . Genes Dev. 2013; 27(3):322–34. [PubMed: 23388828]
40. Doan T, Marquis KA, Rudner DZ. Subcellular localization of a sporulation membrane protein is achieved through a network of interactions along and across the septum. MolMicrobiol. 2005; 55(6):1767–81.
41. Doan T, Morlot C, Meisner J, Serrano M, Henriques AO. Novel secretion apparatus maintains spore integrity and developmental gene expression in *Bacillus subtilis* . PLOS Genet. 2009; 5(7):e1000566. [PubMed: 19609349]
42. Domínguez-Escobar J, Chastanet A, Crevenna AH, Fromion V, Wedlich-Söldner R, Carballido-López R. Processive movement of MreB-associated cell wall biosynthetic complexes in bacteria. Science. 2011; 333(6039):225–28. [PubMed: 21636744]
43. Driks A, Eichenberger P. The spore coat. ASMscience. 2016; 4(2):1–19.
44. Dworkin J, Losick R. Developmental commitment in a bacterium. Cell. 2005; 121(3):401–9. [PubMed: 15882622]
45. Ebersold HR, Cordier JL, Lüthy P. Bacterial mesosomes: method dependent artifacts. Arch Microbiol. 1981; 130(1):19–22. [PubMed: 6796029]
46. Eichenberger, P. Genomics and cellular biology of endospore formationBacillus: Cellular and Molecular Biology. 2nd ed. Graumann, PL, editor. Norfolk, UK: Caister Acad; 2012. 319–50.
47. Eichenberger P, Fawcett P, Losick R. A three-protein inhibitor of polar septation during sporulation in *Bacillus subtilis* . Mol Microbiol. 2001; 42(5):1147–62. [PubMed: 11886548]
48. Eichenberger P, Fujita M, Jensen ST, Conlon EM, Rudner DZ. The program of gene transcription for a single differentiating cell type during sporulation in *Bacillus subtilis* . PLOS Biol. 2004; 2(10):e328. [PubMed: 15383836]
49. Ellar DJ, Lundgren DG. Fine structure of sporulation in Bacillus cereus grown in a chemically defined medium. J Bacteriol. 1966; 92(6):1748–64. [PubMed: 4959720]
50. Emami K, Guyet A, Kawai Y, Devi J, Wu LJ. RodA as the missing glycosyltransferase in *Bacillus subtilis* and antibiotic discovery for the peptidoglycan polymerase pathway. Nat Microbiol. 2017 Jan.2
51. Errington J. Regulation of endospore formation in *Bacillus subtilis* . Nat Rev Microbiol. 2003; 1(2):117–26. [PubMed: 15035041]
52. Errington J. Bacterial morphogenesis and the enigmatic MreB helix. Nat Rev Microbiol. 2015; 13(4):241–48. [PubMed: 25578957]
53. Errington, J, Wu, LJ. Cell cycle machinery in *Bacillus subtilis*Prokaryotic Cytoskeletons: Filamentous Protein Polymers Active in the Cytoplasm of Bacterial and Archaeal Cells. Löwe, J, Amos, LA, editors. Cham, Switz: Springer Int; 2017. 67–101.
54. Eswaramoorthy P, Winter PW, Wawrzusin P, York AG, Shroff H, Ramamurthi KS. Asymmetric division and differential gene expression during a bacterial developmental program requires DivIVA. PLOS Genet. 2014; 10(8):e1004526. [PubMed: 25101664]
55. Feucht A, Magnin T, Yudkin MD, Errington J. Bifunctional protein required for asymmetric cell division and cell-specific transcription in *Bacillus subtilis* . Genes Dev. 1996; 10(7):794–803. [PubMed: 8846916]

56. Fiche J-B, Cattoni DI, Diekmann N, Langerak JM, Clerte C. Recruitment, assembly, and molecular architecture of the SpoIIIE DNA pump revealed by superresolution microscopy. PLOS Biol. 2013; 11(5):e1001557. [PubMed: 23667326]
57. Fitz-James PC. Participation of the cytoplasmic membrane in the growth and spore formation of bacilli. J Biophys Biochem Cytol. 1960; 8(2):507–28. [PubMed: 13700047]
58. Fitz-James PC. Morphology of spore development in Clostridium pectinovorum. J Bacteriol. 1962; 84:104–14. [PubMed: 16561950]
59. Fleming TC, Shin JY, Lee SH, Becker E, Huang KC. Dynamic SpoIIIE assembly mediates septal membrane fission during *Bacillus subtilis* sporulation. Genes Dev. 2010; 24:1160–72. [PubMed: 20516200]
60. Francesconi SC, Macalister TJ, Setlow B, Setlow P. Immunoelectron microscopy localization of small, acid-soluble spore proteins in sporulating cells of *Bacillus subtilis* . J Bacteriol. 1988; 170(12):5963–67. [PubMed: 3142866]
61. Frandsen N, Stragier P. Identification and characterization of the *Bacillus subtilis*spoIIP locus. J Bacteriol. 1995; 177(3):716–22. [PubMed: 7836306]
62. Fredlund J, Broder D, Fleming T, Claussin C, Pogliano K. The SpoIIQ landmark protein has different requirements for septal localization and immobilization. Mol Microbiol. 2013; 89(6):1053–68. [PubMed: 23859254]
63. Fujita M, González-Pastor JE, Losick R. High- and low-threshold genes in the Spo0A regulon of *Bacillus subtilis* . J Bacteriol. 2005; 187(4):1357–68. [PubMed: 15687200]
64. Galperin MY. Genome diversity of spore-forming Firmicutes. Microbiol Spectr. 2013; 1(2)
65. Galperin MY, Mekhedov SL, Puigbo P, Smirnov S, Wolf YI, Rigden DJ. Genomic determinants of sporulation in Bacilli and Clostridia: towards the minimal set of sporulation-specific genes. Environ Microbiol. 2012; 14(11):2870–90. [PubMed: 22882546]
66. Garner EC, Bernard R, Wang W, Zhuang X, Rudner DZ, Mitchison T. Coupled, circumferential motions of the cell wall synthesis machinery and MreB filaments in *B. subtilis* . Science. 2011; 333:222–25. [PubMed: 21636745]
67. Gholamhoseinian A, Shen Z, Wu JJ, Piggot P. Regulation of transcription of the cell division gene ftsA during sporulation of *Bacillus subtilis* . J Bacteriol. 1992; 174(14):4647–56. [PubMed: 1624452]
68. Gonzy-Tréboul G, Karmazyn-Campelli C, Stragier P. Developmental regulation of transcription of the *Bacillus subtilisftsAZ* operon. J Mol Biol. 1992; 224(4):967–79. [PubMed: 1569582]
69. Guillot C, Moran CP. Essential internal promoter in the spoIIIA locus of *Bacillus subtilis* . J Bacteriol. 2007; 189(20):7181–89. [PubMed: 17693505]
70. Gutierrez J, Smith R, Pogliano K. SpoIID-mediated peptidoglycan degradation is required throughout engulfment during *Bacillus subtilis* sporulation. J Bacteriol. 2010; 192(12):3174–86. [PubMed: 20382772]
71. Handler AA, Lim JE, Losick R. Peptide inhibitor of cytokinesis during sporulation in *Bacillus subtilis* . Mol Microbiol. 2008; 68(3):588–99. [PubMed: 18284588]
72. Hansen PL, Podgornik R, Parsegian VA. Osmotic properties of DNA: critical evaluation of counterion condensation theory. Phys Rev. 2001; E64
73. Henriques AO, de Lencastre H, Piggot PJ. A *Bacillus subtilis* morphogene cluster that includes spoVE is homologous to the mra region of Escherichia coli. Biochimie. 1992; 74(7-8):735–48. [PubMed: 1391053]
74. Higgins D, Dworkin J. Recent progress in *Bacillus subtilis* sporulation. FEMS Microbiol Rev. 2012; 36(1):131–48. [PubMed: 22091839]
75. Hilbert DW, Piggot PJ. Compartmentalization of gene expression during *Bacillus subtilis*spore formation. Microbiol Mol Biol Rev. 2004; 68(2):234–62. [PubMed: 15187183]
76. Hoch JA. A life in *Bacillus subtilis* signal transduction. Annu Rev Microbiol. 2017; 71:1–19. [PubMed: 28886686]
77. Holt, JH, Krieg, NR, Sneath, PH, Staley, JT, Williams, ST. Bergey's Manual of Determinative Bacteriology. 9th ed. Philadelphia: Lippincott Williams Wilkins; 1994.

78. Illing N, Errington J. Genetic regulation of morphogenesis in *Bacillus subtilis*: roles of σ^E and σ^F in prespore engulfment. J Bacteriol. 1991; 173(10):3159–69. [PubMed: 1902463]
79. Illing N, Errington J. The spoIIIA operon of *Bacillus subtilis* defines a new temporal class of mother-cell-specific sporulation genes under the control of the σ^E form of RNA polymerase. Mol Microbiol. 1991; 5(8):1927–40. [PubMed: 1766372]
80. Jiang X, Rubio A, Chiba S, Pogliano K. Engulfment-regulated proteolysis of SpoIIQ: evidence that dual checkpoints control σ^K activity. Mol Microbiol. 2005; 58(1):102–15. [PubMed: 16164552]
81. Karslake J, Maltas J, Brumm P, Wood KB. Population density modulates drug inhibition and gives rise to potential bistability of treatment outcomes for bacterial infections. PLOS Comput Biol. 2016; 12(10):1–21.
82. Kawai F, Hara H, Takamatsu H, Watabe K, Matsumoto K. Cardiolipin enrichment in spore membranes and its involvement in germination of *Bacillus subtilis* Marburg. Genes Genet Syst. 2006; 81(2):69–76. [PubMed: 16755131]
84. Kawai F, Shoda M, Harashima R, Sadaie Y, Hara H, Matsumoto K. Cardiolipin domains in *Bacillus subtilis* Marburg membranes. J Bacteriol. 2004; 186(5):1475–83. [PubMed: 14973018]
85. Kay D, Warren SC. Sporulation in *Bacillus subtilis*: morphological changes. Biochem J. 1968; 109(5):819–24. [PubMed: 4972256]
86. Kellner EM, Decatur A, Moran CP. Two-stage regulation of an anti-sigma factor determines developmental fate during bacterial endospore formation. Mol Microbiol. 1996; 21(5):913–24. [PubMed: 8885263]
87. Khanna K, Lopez-Garrido J, Zhao Z, Watanabe R, Yuan Y. The molecular architecture of engulfment during *Bacillus subtilis* sporulation. eLife. 2019; 8:1–22.
88. Khvorova A, Zhang L, Higgins ML, Piggot PJ. The spoIIE locus is involved in the Spo0A-dependent switch in the location of FtsZ rings in *Bacillus subtilis* . J Bacteriol. 1998; 180(5):1256–60. [PubMed: 9495766]
89. Klieneberger-Nobel E. Changes in the nuclear structure of bacteria, particularly during spore formation. J Hyg. 1945; 44(2):99–108.
90. Koch R. Untersuchungen über Bacterien. V. Die Aetiologie der Milzbrand-Krankheit, begründent auf die Entwicklungsgeschichte des Bacillus anthracis. Beitrage Biol Pflanz. 1876; 2:277–310.
91. Kuru E, Hughes HV, Brown PJ, Hall E, Tekkam S. In situ probing of newly synthesized peptidoglycan in live bacteria with fluorescent d-amino acids. Angew Chem Int Ed Engl. 2012; 51(50):12519–23. [PubMed: 23055266]
92. Lee TK, Huang KC. The role of hydrolases in bacterial cell-wall growth. Curr Opin Microbiol. 2013; 16(6):760–66. [PubMed: 24035761]
93. Levin PA, Losick R. Characterization of a cell division gene from *Bacillus subtilis* that is required for vegetative and sporulation septum formation. J Bacteriol. 1994; 176(5):1451–59. [PubMed: 8113187]
94. Levin P, Losick R. Transcription factor Spo0A switches the localization of the cell division protein FtsZ from a medial to a bipolar pattern in *Bacillus subtilis* . Genes Dev. 1996; 10(4):478–88. [PubMed: 8600030]
95. Lewis PJ, Partridge SR, Errington J. Sigma factors, asymmetry, and the determination of cell fate in *Bacillus subtilis* . PNAS. 1994; 911994:3849–53.
97. Liu N, Chistol G, Bustamante C. Two-subunit DNA escort mechanism and inactive subunit bypass in an ultra-fast ring ATPase. eLife. 2015; 4:e09224. [PubMed: 26452092]
98. LiuN JL, Dutton RJ, Pogliano K. Evidence that the SpoIIIE DNA translocase participates in membrane fusion during cytokinesis and engulfment. Mol Microbiol. 2006; 59(4):1097–113. [PubMed: 16430687]
99. Londoño-Vallejo JA, Fréhel C, Stragier P. SpoIIQ, a forespore-expressed gene required for engulfment in *Bacillus subtilis* . Mol Microbiol. 1997; 24(1):29–39. [PubMed: 9140963]
100. Lopez-Diaz I, Clarke S, Mandelstam J. spoIID operon of *Bacillus subtilis*: cloning and sequence. J Gen Microbiol. 1986; 132(2):341–54. [PubMed: 3011962]
101. Lopez-Garrido J, Ojkic N, Khanna K, Wagner F, Villa E. Chromosome translocation inflates Bacillus forespores and impacts cellular morphology. Cell. 2018; 172:758–70. [PubMed: 29425492]

102. Margolis PS, Driks A, Losick R. Sporulation gene spoIIB from *Bacillus subtilis* . J Bacteriol. 1993; 175(2):528–40. [PubMed: 8419299]
103. Massey TH, Mercogliano CP, Yates J, Sherratt DJ, Löwe J. Double-stranded DNA translocation: structure and mechanism of hexameric FtsK. Mol Cell. 2006; 23(4):457–69. [PubMed: 16916635]
104. McKenney PT, Driks A, Eichenberger P. The *Bacillus subtilis* endospore: assembly and functions of the multilayered coat. Nat Rev Microbiol. 2013; 11(1):33–44. [PubMed: 23202530]
105. McPherson DC, Driks A, Popham DL. Two class A high-molecular-weight penicillin-binding proteins of *Bacillus subtilis* play redundant roles in sporulation. J Bacteriol. 2001; 183(20):6046–53. [PubMed: 11567005]
106. Meeske AJ, Riley EP, Robins WP, Uehara T, Mekalanos JJ. SEDS proteins are a widespread family of bacterial cell wall polymerases. Nature. 2016; 537(7622):634–38. [PubMed: 27525505]
107. Meisner J, Maehigashi T, Andre I, Dunham CM, Moran CP. Structure of the basal components of a bacterial transporter. PNAS. 2012; 109(14):5446–51. [PubMed: 22431613]
108. Meisner J, Wang X, Serrano M, Henriques AO, Moran CP. A channel connecting the mother cell and forespore during bacterial endospore formation. PNAS. 2008; 105(39):15100–5. [PubMed: 18812514]
109. Meyer P, Gutierrez J, Pogliano K, Dworkin J. Cell wall synthesis is necessary for membrane dynamics during sporulation of *Bacillus subtilis* . Mol Microbiol. 2010; 76(4):956–70. [PubMed: 20444098]
110. Mileykovskaya E, Dowhan W. Cardiolipin membrane domains in prokaryotes and eukaryotes. Biochim Biophys Acta Biomembr. 2009; 1788(10):2084–91.
111. Mirouze N, Dubnau D. Chance and necessity in *Bacillus subtilis* development. Microbiol Spectr. 2013; 1(1)doi: 10.1128/microbiolspectrum
112. Moir A. How do spores germinate? J Appl Microbiol. 2006; 101(3):526–30. [PubMed: 16907803]
113. Molle V, Fujita M, Jensen ST, Eichenberger P, González-Pastor JE. The Spo0A regulon of *Bacillus subtilis* . Mol Microbiol. 2003; 50(5):1683–701. [PubMed: 14651647]
114. Morlot C, Rodrigues CDA. The new kid on the block: a specialized secretion system during bacterial sporulation. Trends Microbiol. 2018; 26(8):663–76. [PubMed: 29475625]
115. Morlot C, Uehara T, Marquis KA, Bernhardt TG, Rudner DZ. A highly coordinated cell wall degradation machine governs spore morphogenesis in *Bacillus subtilis* . Genes Dev. 2010; 24(4):411–22. [PubMed: 20159959]
116. Nocadello S, Minasov G, Shuvalova LS, Dubrovska I, Sabini E, Anderson WF. Crystal structures of the SpoIID lytic transglycosylases essential for bacterial sporulation. J Biol Chem. 2016; 291(29):14915–26. [PubMed: 27226615]
117. Ojkic N, López-Garrido J, Pogliano K, Endres RG. Bistable forespore engulfment in *Bacillus subtilis* by a zipper mechanism in absence of the cell wall. PLOS Comput Biol. 2014; 10(10):e1003912. [PubMed: 25356555]
118. Ojkic N, López-Garrido J, Pogliano K, Endres RG. Cell-wall remodeling drives engulfment during *Bacillus subtilis* sporulation. eLife. 2016; 5:e18657. [PubMed: 27852437]
119. Parker GF, Daniel RA, Errington J. Timing and genetic regulation of commitment to sporulation in *Bacillus subtilis* . Microbiology. 1996; 142(12):3445–52. [PubMed: 9244562]
120. Perez AR, Abanes-De Mello A, Pogliano K. SpoIIB localizes to active sites of septal biogenesis and spatially regulates septal thinning during engulfment in *Bacillus subtilis* . J Bacteriol. 2000; 182(4):1096–108. [PubMed: 10648537]
121. Piggot PJ, Coote JG, Hill M, Estate G, Mapping G. Genetic aspects of bacterial endospore formation. Bacteriol Rev. 1976; 40(4):908–62. [PubMed: 12736]
122. Pogliano J, Osborne N, Sharp MD, Abanes-De Mello A, Perez A. A vital stain for studying membrane dynamics in bacteria: a novel mechanism controlling septation during *Bacillus subtilis* sporulation. Mol Microbiol. 1999; 31(4):1149–59. [PubMed: 10096082]
123. Pogliano K, Harry E, Losick R. Visualization of the subcellular location of sporulation proteins in *Bacillus subtilis* using immunofluorescence microscopy. Mol Microbiol. 1995; 18(3):459–70. [PubMed: 8748030]

124. Pogliano K, Hofmeister AE, Losick R. Disappearance of the σ^E transcription factor from the forespore and the SpoIIE phosphatase from the mother cell contributes to establishment of cell-specific gene expression during sporulation in *Bacillus subtilis* . J Bacteriol. 1997; 179(10):3331–41. [PubMed: 9150232]
125. Popham DL, Bernhards CB. Spore peptidoglycan. Microbiol Spectr. 2015; 3(6):1–21.
126. Ptacin JL, Nollmann M, Becker EC, Cozzarelli NR, Pogliano K, Bustamante C. Sequence-directed DNA export guides chromosome translocation during sporulation in *Bacillus subtilis* . Nat Struct Mol Biol. 2008; 15(5):485–93. [PubMed: 18391964]
127. Ramos-Silva P, Serrano M, Henriques AO. From root to tips: sporulation evolution and specialization in *Bacillus subtilis* and the intestinal pathogen Clostridioides difficile. Mol Biol Evol. 2019; 36(12):2714–36. [PubMed: 31350897]
128. Raspaud E, daConceiçao M, Livolant F. Do free DNA counterions control the osmotic pressure? Phys Rev Lett. 2000; 84(11):2533–36. [PubMed: 11018928]
129. Ribis JW, Fimlaid KA, Shen A. Differential requirements for conserved peptidoglycan remodeling enzymes during Clostridioides difficile spore formation. Mol Microbiol. 2018; 110(3):370–89. [PubMed: 30066347]
130. Riley EP, Trinquier A, Reilly ML, Durchon M, Perera VR. Spatiotemporally regulated proteolysis to dissect the role of vegetative proteins during *Bacillus subtilis* sporulation: cell-specific requirement of σ^H and σ^A . 2018; Mol Microbiol. 108(1):45–62. [PubMed: 29363854]
131. Rodrigues CDA, Henry X, Neumann E, Kurauskas V, Bellard L. A ring-shaped conduit connects the mother cell and forespore during sporulation in *Bacillus subtilis* . PNAS. 2016; 113(41)
132. Rodrigues CDA, Marquis KA, Meisner J, Rudner DZ. Peptidoglycan hydrolysis is required for assembly and activity of the transenvelope secretion complex during sporulation in *Bacillus subtilis* . Mol Microbiol. 2013; 89(6):1039–52. [PubMed: 23834622]
133. Rodrigues CDA, Ramírez-Guadiana FH, Meeske AJ, Wang X, Rudner DZ. GerM is required to assemble the basal platform of the SpoIIIA-SpoIIQ transenvelope complex during sporulation in *Bacillus subtilis* . Mol Microbiol. 2016; 102(2):260–73. [PubMed: 27381174]
134. Rong S, Rosenkrantz MS, Sonenshein AL. Transcriptional control of the *Bacillus subtilis*spoIID gene. J Bacteriol. 1986; 165(3):771–79. [PubMed: 2419309]
135. Rubio A, Pogliano K. Septal localization of forespore membrane proteins during engulfment in *Bacillus subtilis* . EMBO J. 2004; 23(7):1636–46. [PubMed: 15044948]
136. Ryter A. Etude morphologique de la sporulation de *Bacillus subtilis* . Ann Inst Pasteur. 1965; 108:40–60. [PubMed: 14289982]
137. Ryter A. Structure and functions of mesosomes of Gram positive bacteria. Curr Top Microbiol Immunol. 1969; 49:151–77. [PubMed: 4980055]
138. Ryter A, Schaeffer P, Ionesco H. Classification cytologique, par leur stade de blocage, des mutants de sporulation de *Bacillus subtilis* Marburg. Ann Inst Pasteur. 1966; 110(3):305–15. [PubMed: 4955547]
139. Sassine J, Xu M, Sidiq KR, Emmins R, Errington J, Daniel RA. Functional redundancy of division specific penicillin-binding proteins in *Bacillus subtilis* . Mol Microbiol. 2017; 106(2):304–18. [PubMed: 28792086]
140. Scalettar BA, Swedlow JR, Sedat JW, Agard DA. Dispersion, aberration and deconvolution in multi-wavelength fluorescence images. J Microsc. 1996; 182(1):50–60. [PubMed: 8632447]
141. Schaeffer P, Millet J, Aubert JP. Catabolic repression of bacterial sporulation. PNAS. 1965; 54(3):704–11. [PubMed: 4956288]
142. Scheffers D-J. Dynamic localization of penicillin-binding proteins during spore development in *Bacillus subtilis* . Microbiology. 2005; 151(Part 3):999–1012. [PubMed: 15758244]
143. Scheffers D-J, Errington J. PBP1 is a component of the *Bacillus subtilis* cell division machinery. J Bacteriol. 2004; 186(15):5153–56. [PubMed: 15262952]
144. Scheffers D-J, Jones LJF, Errington J. Several distinct localization patterns for penicillin-binding proteins in *Bacillus subtilis* . Mol Microbiol. 2004; 51(3):749–64. [PubMed: 14731276]
145. Setlow B, Magill N, Febbroriello P, Nakhimovsky L, Koppel DE, Setlow P. Condensation of the forespore nucleoid early in sporulation of Bacillus species. J Bacteriol. 1991; 173(19):6270–78. [PubMed: 1917859]

146. Setlow P. I will survive: DNA protection in bacterial spores. Trends Microbiol. 2007; 15(4):172–80. [PubMed: 17336071]
147. Setlow P. Summer meeting 2013—when the sleepers wake: the germination of spores of Bacillus species. J Appl Microbiol. 2013; 115(6):1251–68. [PubMed: 24102780]
148. Setlow P. Germination of spores of Bacillus species: what we know and do not know. J Bacteriol. 2014; 196(7):1297–305. [PubMed: 24488313]
149. Setlow P. Spore resistance properties. Microb Spectr. 2014; 2(5)
150. Setlow P, Wang S, Li Y-Q. Germination of spores of the orders Bacillales and Clostridiales. Annu Rev Microbiol. 2017; 71:459–77. [PubMed: 28697670]
151. Sharp MD, Pogliano K. An in vivo membrane fusion assay implicates SpoIIIE in the final stages of engulfment during *Bacillus subtilis* sporulation. PNAS. 1999; 96(25):14553–58. [PubMed: 10588743]
152. Sharp MD, Pogliano K. Role of cell-specific SpoIIIE assembly in polarity of DNA transfer. Science. 2002; 295(5552):137–39. [PubMed: 11778051]
153. Sharp MD, Pogliano K. The membrane domain of SpoIIIE is required for membrane fusion during *Bacillus subtilis* sporulation. J Bacteriol. 2003; 185(6):2005–8. [PubMed: 12618465]
154. Smith K, Bayer ME, Youngman P. Physical and functional characterization of the *Bacillus subtilis* spoIIM gene. J Bacteriol. 1993; 175(11):3607–17. [PubMed: 8501064]
155. Smith K, Youngman P. Evidence that the spoIIM gene of *Bacillus subtilis* is transcribed by RNA polymerase associated with σ^E . J Bacteriol. 1993; 175(11):3618–27. [PubMed: 8501065]
156. Sterlini JM, Mandelstam J. Commitment to sporulation in *Bacillus subtilis* and its relationship to development of actinomycin resistance. Biochem J. 1969; 113(1):29–37. [PubMed: 4185146]
157. Stragier P, Losick R. Molecular genetics of sporulation in *Bacillus subtilis* . Annu Rev Genet. 1996; 30:297–341. [PubMed: 8982457]
158. Sun Y, Sharp MD, Pogliano K. A dispensable role for forespore-specific gene expression in engulfment of the forespore during sporulation of *Bacillus subtilis* . J Bacteriol. 2000; 182(10):2919–27. [PubMed: 10781563]
159. Sussman MD, Setlow P. Cloning, nucleotide sequence, and regulation of the *Bacillus subtilis* gpr gene, which codes for the protease that initiates degradation of small, acid-soluble proteins during spore germination. J Bacteriol. 1991; 173(1):291–300. [PubMed: 1840582]
160. Tan IS, Ramamurthi KS. Spore formation in *Bacillus subtilis* . Environ Microbiol Rep. 2014; 6(3):212–25. [PubMed: 24983526]
161. Tiyanont K, Doan T, Lazarus MB, Fang X, Rudner DZ, Walker S. Imaging peptidoglycan biosynthesis in *Bacillus subtilis* with fluorescent antibiotics. PNAS. 2006; 103(29):11033–38. [PubMed: 16832063]
162. Tocheva EI, López-Garrido J, Hughes HV, Fredlund J, Kuru E. Peptidoglycan transformations during *Bacillus subtilis* sporulation. Mol Microbiol. 2013; 88(4):673–86. [PubMed: 23531131]
163. Udekwu KI, Parrish N, Ankomah P, Baquero F, Levin BR. Functional relationship between bacterial cell density and the efficacy of antibiotics. J Antimicrob Chemother. 2009; 63(4):745–57. [PubMed: 19218572]
164. van Teeffelen S, Wang S, Furchtgott L, Huang KC, Wingreen NS. The bacterial actin MreB rotates, and rotation depends on cell-wall assembly. PNAS. 2011; 108(38):15822–27. [PubMed: 21903929]
165. Vasudevan P, Weaver A, Reichert ED, Linnstaedt SD, Popham DL. Spore cortex formation in *Bacillus subtilis* is regulated by accumulation of peptidoglycan precursors under the control of sigma K. Mol Microbiol. 2007; 65(6):1582–94. [PubMed: 17714441]
166. Veening JW, Stewart EJ, Berngruber TW, Taddei F, Kuipers OP, Hamoen LW. Bet-hedging and epigenetic inheritance in bacterial cell development. PNAS. 2008; 105(11):4393–98. [PubMed: 18326026]
167. Wagner-Herman JK, Bernard R, Dunne R, Bisson-Filho AW, Kumar K. RefZ facilitates the switch from medial to polar division during spore formation in *Bacillus subtilis* . J Bacteriol. 2012; 194(17):4608–18. [PubMed: 22730127]

168. Wang ST, Setlow B, Conlon EM, Lyon JL, Imamura D. The forespore line of gene expression in *Bacillus subtilis* . J Mol Biol. 2006; 358(1):16–37. [PubMed: 16497325]

169. Wang X, Montero Llopis P, Rudner DZ. *Bacillus subtilis* chromosome organization oscillates between two distinct patterns. PNAS. 2014; 111(35):12877–82. [PubMed: 25071173]

170. Webb CD, Decatur A, Teleman A, Losick R. Use of green fluorescent protein for visualization of cell-specific gene expression and subcellular protein localization in *Bacillus subtilis* . J Bacteriol. 1995; 177(20):5906–11. [PubMed: 7592342]

171. Webb CD, Teleman A, Gordon S, Straight A, Belmont A. Bipolar localization of the replication origin regions of chromosomes in vegetative and sporulating cells of B. subtilis Cell. 1997; 88(5):667–74. [PubMed: 9054506]

172. Wei Y, Havasy T, McPherson DC, Popham DL. Rod shape determination by the *Bacillus subtilis* class B penicillin-binding proteins encoded by pbpA and pbpH. J Bacteriol. 2003; 185(16):4717–26. [PubMed: 12896990]

173. Wu LJ, Errington J. *Bacillus subtilis* SpoIIIE protein required for DNA segregation during asymmetric cell division. Science. 1994; 264(5158):572–75. [PubMed: 8160014]

174. Wu LJ, Errington J. Septal localization of the SpoIIIE chromosome partitioning protein in *Bacillus subtilis* . EMBO J. 1997; 16(8):2161–69. [PubMed: 9155041]

176. Wu LJ, Errington J. Use of asymmetric cell division and spoIIIE mutants to probe chromosome orientation and organization in *Bacillus subtilis* . Mol Microbiol. 1998; 27(4):777–86. [PubMed: 9515703]

177. Wu LJ, Errington J. RacA and the Soj-Spo0J system combine to effect polar chromosome segregation in sporulating *Bacillus subtilis* . Mol Microbiol. 2003; 49(6):1463–75. [PubMed: 12950914]

177a. Wu LJ, Lewis PJ, Allmansberger R, Hauser PM, Errington J. A conjugation-like mechanism for prespore chromosome partitioning during sporulation in *Bacillus subtilis* . Genes Dev. 1995; 9(11):1316–26. [PubMed: 7797072]

178. Yen Shin J, Lopez-Garrido J, Lee S-HH, Diaz-Celis C, Fleming T. Visualization and functional dissection of coaxial paired SpoIIIE channels across the sporulation septum. eLife. 2015; 4:e06474. [PubMed: 25950186]

179. York K, Kenney TJ, Satola S, Moran CP, Poth H, Youngman P. Spo0A controls the σ^A-dependent activation of *Bacillus subtilis* sporulation-specific transcription unit spoIIE. J Bacteriol. 1992; 174(8):2648–58. [PubMed: 1556084]

180. Young E, Fitz-James P. Chemical and morphological studies of bacterial spore formation I. The formation of spores in *Bacillus cereus* . J Biophys Biochem Cytol. 1959; 6(3):467–82. [PubMed: 19866561]

181. Young E, Fitz-James P. Chemical and morphological studies of bacterial spore formation II. Spore and parasporal protein formation in *Bacillus cereus* var. Alesti J Biophys Biochem Cytol. 1959; 6(3):483–98. [PubMed: 13846631]

182. Zeytuni N, Flanagan KA, Worrall LJ, Massoni SC, Camp AH, Strynadka NCJ. Structural and biochemical characterization of SpoIIIAF, a component of a sporulation-essential channel in *Bacillus subtilis* . J Struct Biol. 2018; 204(1):1–8. [PubMed: 29886194]

183. Zeytuni N, Flanagan KA, Worrall LJ, Massoni SC, Camp AH, Strynadka NCJ. Structural characterization of SpoIIIAB sporulation-essential protein in *Bacillus subtilis* . J Struct Biol. 2018; 202(2):105–12. [PubMed: 29288127]

184. Zeytuni N, Hong C, Flanagan KA, Worrall LJ, Theiltges KA. Near-atomic resolution cryoelectron microscopy structure of the 30-fold homooligomeric SpoIIIAG channel essential to spore formation in *Bacillus subtilis* . PNAS. 2017; 114(34):E7073–81. [PubMed: 28784753]

185. Zeytuni N, Strynadka NCJ. A hybrid secretion system facilitates bacterial sporulation: a structural perspective. Microbiol Spectr. 2019; 7(1)doi: 10.1128/microbiolspec

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Sidebar 1

Transcriptional regulation of *B.subtilis* sporulation

The *B. subtilis* sporulation pathway is governed by the activation of distinct transcriptional regulators in different cell types at different developmental stages. These control distinct programs of gene expression that coordinate the cellular changes associated with the development of the spore (indicated in Figure 1) (reviewed in 46, 51, 75). In vegetative cells, before polar septation, the stationary phase–specific σ factor, σ^H, and the phosphorelay response regulator, Spo0A, regulate the expression of genes that control sporulation initiation (reviewed in 76, 111). After polar septation, σ^F becomes active in the forespore, followed by σ^E in the mother cell. These two σ factors control the expression of genes that are important for engulfment and for paving subsequent steps of forespore development. After engulfment, σ^G becomes active in the forespore and, finally, σ^K in the mother cell, leading to the expression of genes involved in the maturation of the spore and the lysis of the mother cell. Together, the cell-specific σ factors control the transcription of more than 500 genes (46, 48, 168), most of which are sporulation specific and not transcribed in vegetative cells.

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Sidebar 2

Nomenclature of sporulation loci

Loci important for sporulation were originally designated as *spo0*, *spoII*, *spoIII*, etc., based on the morphological stage blocked in strains containing mutations in them, determined using electron microscopy (138). For instance, mutations in *spoII* loci block sporulation in stage II (before engulfment completion), and the designations A, B, C, etc., within each category correspond to different genetic loci. In some cases, it was later discovered that a sporulation locus consisted of an operon containing several genes. A second letter was then used to distinguish the different genes within the operon (e.g., *spoIIAA*, *spoIIAB*).

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Sidebar 3

Early visualization studies of sporulation

Early observations of spore-forming bacteria via light microscopy by Cohn (31) and Koch (90) led to the conclusion that endospores assembled within the cytoplasm of another cell. This led to an ongoing debate about the mechanism of endospore assembly that was not resolved until the second half of the twentieth century, when the first evidence of engulfment was provided by electron microscopy images of fixed sporulating cells of different *Bacillus* and *Clostridium* species (57, 58, 136, 180, 181). At the same time, researchers visualized structures called mesosomes in ultrathin sections of several *Bacillus* species. These structures appeared close to the cytoplasmic membrane as invaginations and were thought to be involved in septum formation and engulfment during membrane migration (11, 36, 49, 57, 137). In fact, at that time, mesosomes were thought of as entities inherent to gram-positive bacteria with roles in DNA segregation, membrane synthesis, and endospore formation. However, later it was shown that mesosomes are artifacts formed due to chemical fixatives used for sample preparation, as they are not detected in cells processed using cryofixation and freeze substitution methods that preserve the native cellular structures (45).

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Sidebar 4

Forespore expression of SpoIIP

In addition to being expressed in the mother cell, *spoIIP* is also expressed in the forespore due to readthrough transcription from the upstream gene (44) *gpr*, which is transcribed from a promoter controlled by both σ^F and σ^G (159). However, only *spoIIP* expression in the mother cell is required for engulfment membrane migration, as indicated by the observations that forespore-specific gene expression is dispensable for engulfment (158) and that the mother cell *spoIIP* expression alone from an ectopic locus suffices for the successful completion of engulfment (1). However, expression of *spoIIP* in the forespore seems to be required for the developmental commitment of this cell (44), an event that occurs shortly after polar septation, when the forespore can no longer resume growth even if excess nutrients are provided (119). The developmental commitment of the forespore depends on the activation of σ^F and, more specifically, on the forespore-specific protein SpoIIQ, and on the forespore expression of *spoIIP* (44). The mechanisms by which SpoIIQ and SpoIIP control commitment are, however, not understood.

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Terms and Definitions

Peptidoglycan: a semirigid mesh-like structure outside the cytoplasmic membrane, consisting of glycan strands cross-linked by peptide bridges.

Deconvolution microscopy: a technique that improves the contrast and resolution in optical microscopy images by removing out-of-focus light (140).

Cryo-electron tomography: a technique to image frozen-hydrated cells in native state in three-dimension at a resolution of a few nanometers.

Cryo-focused-ion beam milling: a technique to make thin slices of biological specimens suitable for cryo-electron tomography using gallium ions.

Membrane fusion assay: an assay to distinguish between fully engulfed sporangia from those that are still undergoing membrane migration (151).

Time lapse imaging: Sequential recording of images of living specimen at fixed intervals to get temporal information about a process.

Photo-activated localization microscopy: a superresolution microscopy technique that uses photoactivable fluorophores to resolve spatial information of molecules to ~20 nm.

Single particle cryo-electron microscopy: a technique to get three-dimensional reconstruction of a purified macromolecule at near-atomic resolution.

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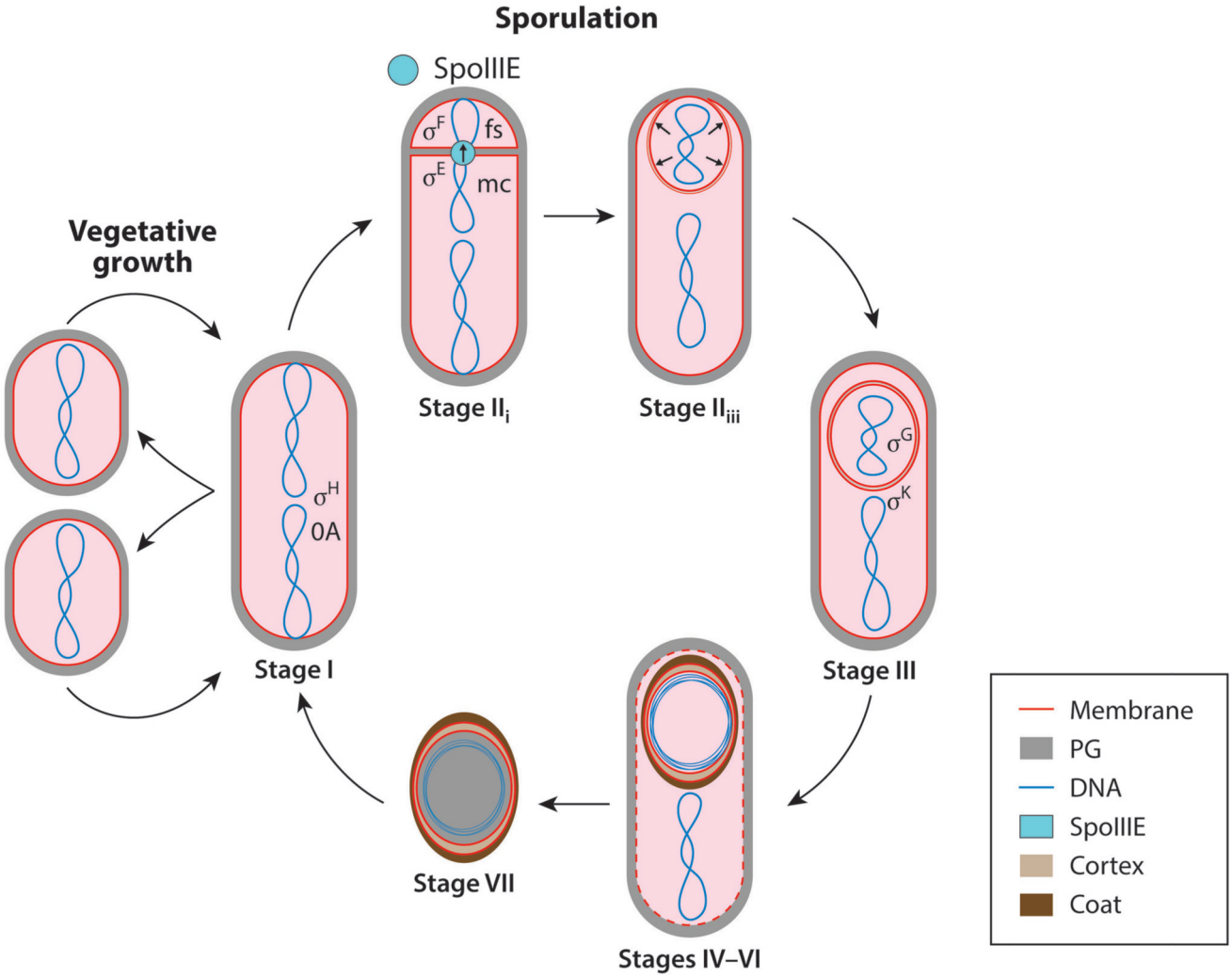

Figure 1.

Sporulation in *B. subtilis*. Schematic representation of key architectural transformations during sporulation. Membranes (*red*), peptidoglycan (*gray*), DNA (*dark blue*), SpoIIIE (*light blue*), cortex (*light brown*), and coat (*dark brown*) are indicated. The different sporulation stages are as follows (136) (see the sidebar titled 'Nomenclature of sporulation loci'): Stage I is the formation of the axial DNA filament. Stage II marks the formation of the polar septum, creating the smaller forespore and the larger mother cell. More recently, stage II has been categorized to distinguish sporulating cells with flat (stage II_i), curved (stage II_{ii}, not shown), and engulfing (stage II_{iii}) septa. During stage II, the forespore chromosome (*blue*) is translocated to the forespore by SpoIIIE (*light blue*). Stage III marks completion of engulfment of the forespore by the mother cell. Stage IV is characterized by the assembly of the cortex between the membranes of the engulfed forespore and stage V by the formation of the spore coat. In stage VI, the forespore becomes resistant to heat, and in stage VII, the mother cell lyses to release the dormant spore to the environment. The spores can germinate to produce vegetative cells when reexposed to nutrient-rich conditions. Abbreviations: fs, forespore; mc, mother cell.

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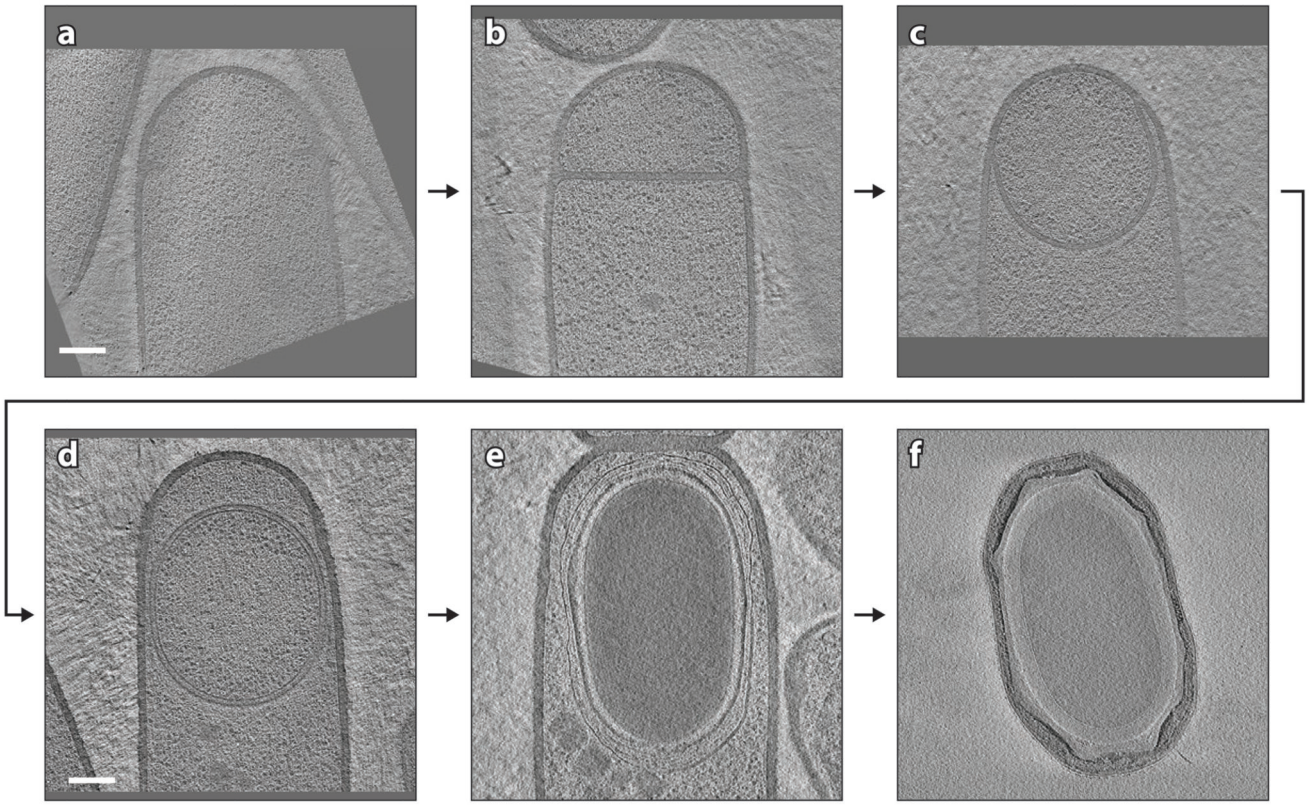

Figure 2.

Different stages of sporulation as visualized by cryo-FIB (focused ion beam) microscopy–coupled cryo–electron tomography. Slices through high-resolution tomograms of cryo-FIB-milled *B. subtilis* sporangia during different stages of spore formation as outlined in Figure 1. (*a*) A sporangium with a nascent polar septum being formed. (*b*,*c*) Sporangia with flat and engulfing septa. (*d*) A sporangium with a fully engulfed forespore. (*e*) A sporangium with developing coat and cortex. (*f*) A mature spore. Scale bars represent 200 nm. See Reference 87 for materials and methods. Panels *b* and *c* are reproduced from Reference 87.

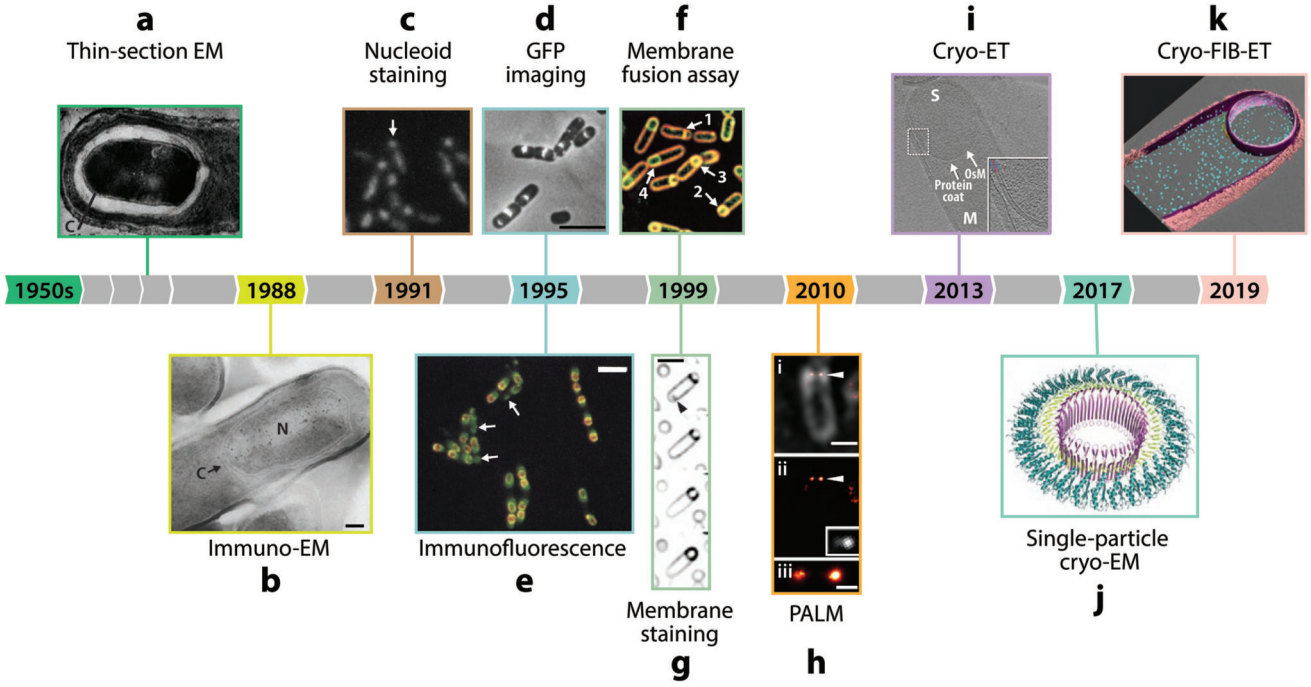

Figure 3.

Timeline of key imaging techniques to study *B. subtilis* sporulation. (*a*) Thin-section EM micrograph of fixed *B. subtilis* sporangium. C indicates the cell membrane surrounding the endospore. Reproduced from Reference 136 (*b*) Immuno-EM micrograph of fixed *B. subtilis* sporangium treated with anti-SASP-α/β antibody. C and N indicate spore cortex and forespore nucleoid respectively. Reproduced with permission from Reference 60. (*c*) Fluorescence micrograph of *B. subtilis* sporangia wherein the DNA is stained with 4′,6-diaminodino-2-phenylindole (DAPI), with a forespore chromosome indicated by the white arrow. Reproduced with permission from Reference 145. (*d*) Fluorescence micrograph of *B. subtilis* sporangia indicating the localization of the coat protein CotE tagged with GFP. Reproduced with permission from Reference 170. (*e*) Immunofluorescence micrograph of *B. subtilis* sporangia wherein the coat protein SpoIVA is localized with Cy3-labeled secondary antibodies (*red*) and CotE with fluorescein-labeled secondary antibodies (*green*). The arrows indicate misassembled examples of CotE and SpoIVA. Reproduced with permission from Reference 123. (*f*) Fluorescence micrographs of *B. subtilis* at different stages of sporulation, showing a medial focal plane after optical sectioning, deconvolution microscopy (140). Membranes are stained with FM4–64 (*red*) and MitoTracker Green (MTG) (*green*) to distinguish cells that have completed membrane fusion (Arrow 4) from those that have not (Arrows 1–3). Adapted from Reference 151. (*g*) Time-lapse images of a *B. subtilis* sporangium after optical sectioning; deconvolution microscopy showing different stages of engulfment. Membranes are stained with FM4–64. The arrowhead points to a partial septum being formed in the mother cell. Reproduced with permission from Reference 122. (*h*) PALM image of *B. subtilis* sporangium showing SpoIIIE assembling during cell division (59). PALM images of SpoIIIE-tDEOS in (ii) were overlaid with FM 5-95 stained membranes (gray) in (i) to indicate localization of SpoIIIE at dividing sporulation septum.

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(iii) indicates the enlarged PALM image of the inset in (ii); subsequent imaging making use of a mutant with thicker septal peptidoglycan was able to demonstrate that the SpoIIIE subcomplex is present in both the forespore and the mother cell (178). (*i*) Cryo-ET image of a skinny *ponA* mutant sporangium of *B. subtilis*. S indicates spore, and M indicates mother cell. The inset represents a zoomed-in view of the black square with the forespore and the mother cell membranes shown in blue and red dots indicating the peptidoglycan sandwiched between them. Adapted with permission from Reference 162. (*j*) Single-particle cryo-EM reconstruction of *B. subtilis* SpoIIIAG, a component of the putative Q-A channel, at 3.5 Å resolution. Reproduced with permission from Reference 184. (*k*) Annotated image of *B. subtilis* sporangium obtained via cryo-ET coupled with cryo-FIB milling. The forespore membrane (*pink*), the mother cell membrane (*magenta*), peptidoglycan (*peach*), and ribosomes (*blue*) are highlighted. Adapted from Reference 87. Abbreviations: EM, electron microscopy; ET, electron tomography; FIB, focused-ion beam; GFP, green fluorescent protein; PALM, photoactivated localization microscopy.

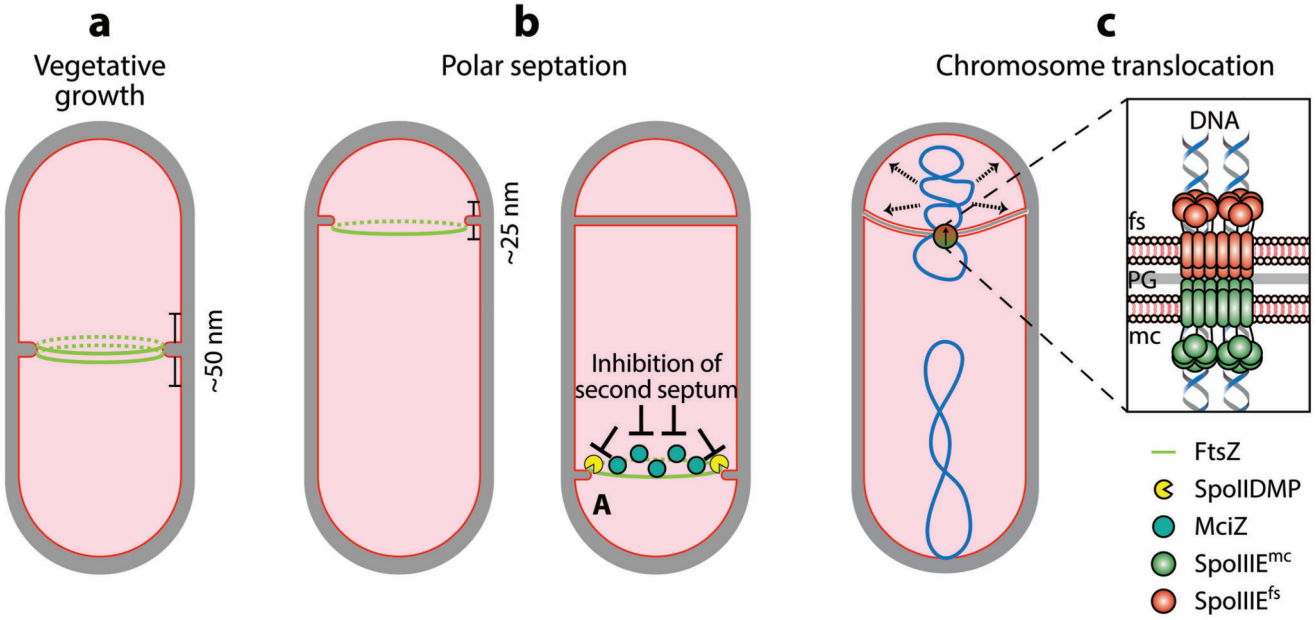

Figure 4.

Key processes mediating cellular dynamics prior to engulfment. Membranes (*red*) and peptidoglycan (*gray*) are indicated. (*a*) During vegetative growth, FtsZ (or Z-rings, *green*) localize uniformly around the leading edge of the invaginating medial septum, and (*b*, *left*) during sporulation, they localize only on the mother cell side of the invaginating septum (K. Khanna, unpublished). The thicknesses of both the medial (a) and the polar septa (b) are indicated. The number of Z-rings during vegetative growth is almost twice the number during sporulation (K. Khanna, unpublished). (*b*, *right*) After the completion of the first septum, the formation of additional septa is inhibited by combined action of SpoIIDMP (*yellow*) and MciZ (*cyan*) (47, 71, 122). (*c*) SpoIIIE (*red-and-green circle*) at the middle of the septum mediates the translocation of the forespore chromosome (*blue*) from the mother cell to the forespore (173). (*Inset*) The paired SpoIIIE channel showing the assembly of four hexamers (178), two each in the forespore (*red*) and in the mother cell (*green*), with only the mother cell complexes being active to export the chromosome to the forespore.
Abbreviations: fs, forespore; mc, mother cell; PG, peptidoglycan.

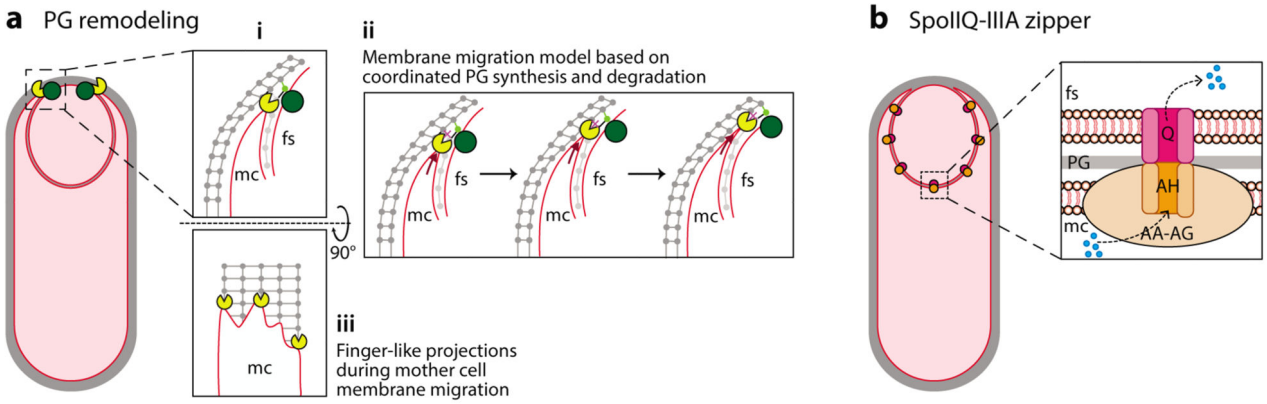

Figure 5.

Key processes mediating cellular dynamics during engulfment. Membranes (*red*) and peptidoglycan (*gray*) are indicated. (*a*) Model of membrane migration based on the coordinated action of peptidoglycan degradation by DMP (complex of SpoIID, SpoIIM, and SpoIIP proteins, *yellow*) on the mother cell side and peptidoglycan synthesis by enzymes (*dark green*) on the forespore side. (*i*, *ii*) Zoomed-in view of the leading edge wherein new peptidoglycan (*light green*) is synthesized by peptidoglycan synthases (*dark green*) that track the leading edge of the engulfing membrane from the forespore, and bonds between the old septal peptidoglycan (*light gray*) and lateral peptidoglycan (*dark gray*) are hydrolyzed by DMP (*yellow*) on the mother cell side, providing room for membrane migration (118), indicated by a maroon arrow in subpanel *ii*. (*iii*) The mother cell membrane migrates in finger-like projections (87) that might emerge by tethering of the mother cell membrane by a limited number of DMP complexes to peptidoglycan synthesized above. (*b*) Arrangement of Q-AH zipper as discrete loci distributed throughout the engulfing membrane. Inset shows Q (*pink*, on the forespore side) and AH (*orange*, on the mother cell side) interacting in a zipper-like fashion. AA-AG proteins (*light orange*) are recruited to the complex via AH on the mother cell side. The complex may comprise a channel that mediates the transport of certain metabolites (*blue*) from the mother cell to the forespore. Abbreviations: fs, forespore; mc, mother cell; PG, peptidoglycan.

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