

REGULATION OF ENDOSPORE FORMATION IN *BACILLUS SUBTILIS*

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Spore formation in bacteria poses a number of biological problems of fundamental significance. Asymmetric cell division at the onset of sporulation is a powerful model for studying basic cell-cycle problems, including chromosome segregation and septum formation. Sporulation is one of the best understood examples of cellular development and differentiation. Fascinating problems posed by sporulation include the temporal and spatial control of gene expression, intercellular communication and various aspects of cell morphogenesis.

PRESPORE

Equivalent to the forespore but sometimes used specifically for the small compartment before completion of engulfment.

FORESPORE

A small compartment that is formed after asymmetric division. It is sometimes used specifically for the small compartment specifically after completion of engulfment.

MOTHER CELL

The large compartment in which the spore develops.

SPORANGIUM

The literal meaning is equivalent to the mother cell, but it is frequently used to refer to the two-compartment sporulating organism (that is, the prespore/forespore plus the mother cell).

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Specialized differentiated cell types are used by a wide range of bacteria as a method of dealing with starvation and the survival of harsh conditions. Some of the specialized cells are called spores, such as those made by actinomycetes or myxobacteria. Endospores are the toughest of these cell types and are almost certainly the longest surviving. They are formed by ancient (deeply-rooted) lineages of bacteria, such as anaerobes of the genus *Clostridium* and aerobes of several genera — the best known of which is *Bacillus*. The endospore is formed by an unusual mechanism involving asymmetric cell division, followed by engulfment of the smaller cell (PRESPORE OR FORESPORE) by its larger sibling (MOTHER CELL OR SPORANGIUM) (FIG. 1). The secret of the success of endospore formation lies in the altruistic behaviour of the mother cell, which uses all of its resources to endow the prespore with resources, particularly protective layers, thereby maximizing the chances of survival for the mature spore. Spores can survive treatments that rapidly and efficiently kill other bacterial forms, including high temperatures (even 100°C), ionizing radiation, chemical solvents, detergents and hydrolytic enzymes¹. They can remain dormant for immense periods of time, perhaps even millions of years^{2,3}. Many endospore-forming bacteria are important in industry and medicine; members of the genus *Bacillus* are responsible for the production of a large proportion of the world market of industrial enzymes and are also important biocontrol agents in agriculture⁴. *Bacillus anthracis* and various *Clostridium* spp. (for example, *Clostridium tetani*, *Clostridium botulinum* and

Clostridium perfringens) are important pathogens and their ability to form tough, resistant endospores is an important factor in this pathogenesis⁵.

The best-studied spore-forming bacterium is *Bacillus subtilis*. Used as a model system for cell fate and development for several decades, its main advantages experimentally are its extremely powerful genetics and, more recently, its tractability to the application of cell-biological methods^{6,7}. Consequently, this organism is probably better understood in terms of its general biochemistry, physiology and genetics than any other organism, with the exception of *Escherichia coli*. Sporulation in *B. subtilis* has also proved to be a very powerful tool that has improved our understanding of various basic processes in bacteria, including transcriptional regulation and the cell cycle. There have been many comprehensive reviews of sporulation (see REF. 8 for the most recent review). This review describes only the recent progress in understanding the molecular basis of spore formation, and focuses mainly on the temporal and spatial regulation of the process and the earlier morphological events.

Control of the initiation of sporulation

A detailed description of initiation of sporulation is beyond the scope of this review (see REFS 9–11 for recent reviews), but the key features of this intricate decision-making apparatus are as follows. The main stimulus for sporulation is starvation. It is also important that the population density is high. In addition, no single nutritional effect acts as the trigger. Rather, the cell has an

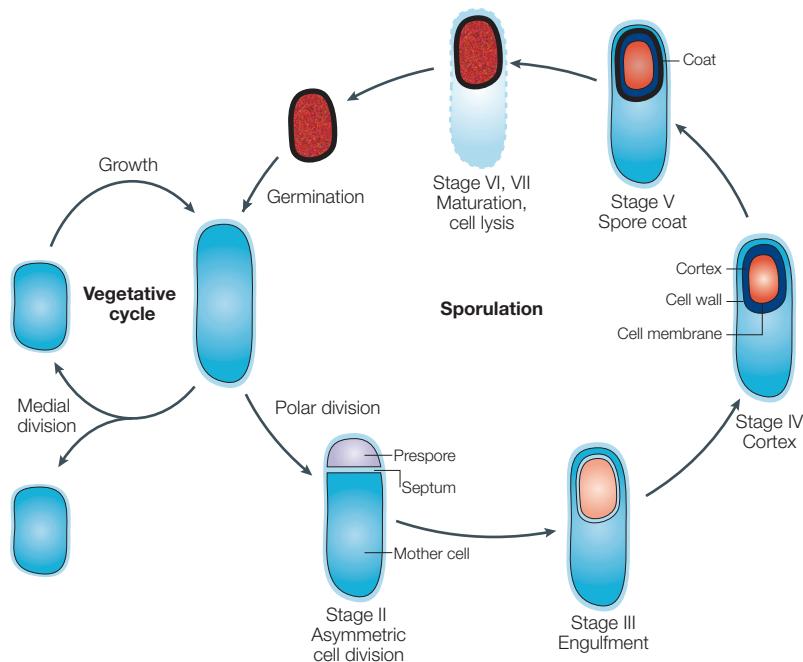


Figure 1 | The sporulation cycle of *Bacillus subtilis*. This simplified schematic shows only the key stages of the cycle.

extremely complex and sophisticated decision-making apparatus, which monitors a huge range of internal and external signals. The information is channelled through several separate regulatory systems, of which the most prominent component is a crucially important transcriptional regulator called Spo0A. Microarray experiments have shown that several hundred genes (more than 10% of all *B. subtilis* genes) are directly or indirectly under the control of Spo0A¹². Spo0A synthesis is controlled transcriptionally, and the activity of the protein is regulated by phosphorylation. Transfer of phosphate to Spo0A is regulated by a complex network of interactions. There are several kinases (KinA, KinB, KinC, KinD and KinE), each of which probably responds to a different stimulus¹³. Phosphate is not transferred directly to Spo0A, but instead is transferred by two intermediates, Spo0F and Spo0B, which gives rise to the concept of a 'phosphorelay' system¹⁴. The phosphotransferase reactions, or the carriers themselves, are subject to regulation by phosphatases, which are themselves regulated by various diverse mechanisms. Phosphorylated Spo0A is an essential positive regulator of sporulation, and it works by activating the transcription of several key sporulation-specific genes, particularly the *spoIIA*, *spoIIE* and *spoIIG* genes (see below for their functions). Recent results have shown that Spo0A is probably also an important factor in sporulation during early mother-cell development¹⁵.

The other key positive regulator of sporulation is a SIGMA FACTOR, σ^H , which interacts with core RNA polymerase and directs it to initiate transcription from at least 49 promoters controlling 87 or more genes¹⁶. The σ^H and Spo0A regulatory pathways are intimately interconnected and overlap in ways that are not yet fully

resolved. Superimposed on this positive regulation are multiple negative regulators of transcription, of which the following examples are among the more interesting and important: CodY seems to be important in coupling initiation to intracellular GTP levels, and is used as the main indicator of the status of intermediary metabolism¹⁷; AbrB negatively regulates a plethora of other negative regulators¹⁸; and Soj (suppressor of *spo0J*) seems to prevent sporulation in response to a signal that is related to an aspect of chromosome segregation or replication status¹⁹.

The complexity with which the initiation of sporulation is regulated partly reflects the fact that in its natural environment (soil), *B. subtilis* is frequently faced with starvation and has a number of survival strategies available, including motility and chemotaxis, production of scavenging enzymes, scavenging of genetic material by DNA uptake and transformation, and the production of antibiotics to suppress competition and release nutrients. Sporulation seems to be the ultimate response that is used only as a last resort, perhaps because it is a long and energy-consuming process. Recently, two new systems have been described that highlight the importance of population dynamics in the control of sporulation initiation²⁰. Early sporulating cells were shown to produce both a sporulation delaying factor, which impedes the onset of sporulation in competitors, and a sporulation killing factor (to which the producer is immune), which causes lysis of the non-sporulating cells. The nutrients that are released are presumably scavenged by the factor-producing cells to delay their need to commit fully to sporulation, or to facilitate their efficient sporulation at the appropriate time.

Asymmetric cell division

Having made the decision to embark on sporulation, the first, and crucial, morphological event is asymmetric cell division. This is a modified version of the cell-division process that is used in growing cells, but which is modified by several sporulation-specific factors. Recent work has focused on two aspects of this problem. First, how the division machinery is shifted from its normal mid-cell site to a position close to the cell pole; second, how chromosome segregation is adapted to achieve the extremely asymmetrical positioning of the prespore chromosome. Cell division in bacteria is accomplished by conserved machinery that is built around a key cytosolic factor, FtsZ, which is a bacterial homologue of TUBULIN. FtsZ polymerizes to form protofilaments that congregate or assemble at the division site into a structure known as the Z-ring (FIG. 2). Positioning of the Z-ring precisely at the mid-cell is accomplished by a combination of two negative effects, called nucleoid occlusion and the Min system. Nucleoid occlusion is a mysterious effect that prevents division in the vicinity of the nucleoid²¹. The Min system comprises three proteins, MinC, MinD and either MinE or DivIVA — which act to block division near the cell poles (see REF. 22 for a recent review). MinCD acts as the inhibitor of division. In *E. coli*, MinCD exerts its topological specificity

SIGMA FACTOR
A subunit of the RNA polymerase holoenzyme that is required for promoter sequence recognition and the ability to initiate transcription.

TUBULIN
A eukaryotic cytoskeletal protein that is used to form microtubules.

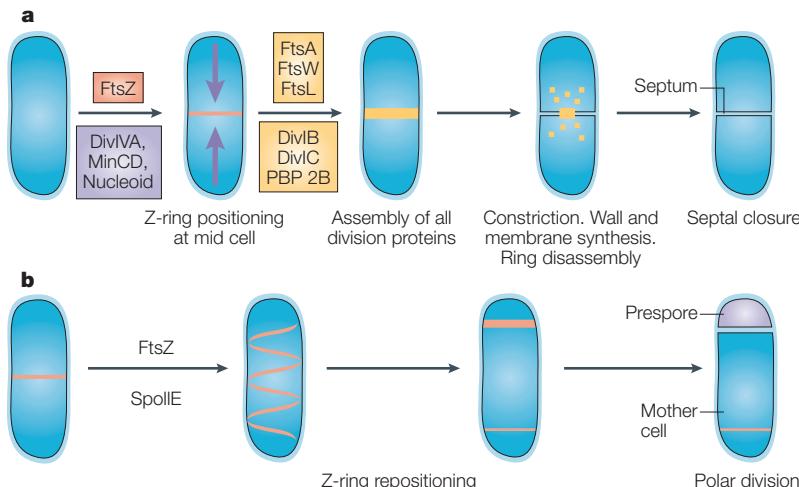


Figure 2 | Cell division during growth (a) and sporulation (b) of *B. subtilis*. The first key step in division is assembly of a ring of FtsZ protein (light red) at the future division site at the mid-cell. Positioning of the Z-ring is directed by the combined actions of the Min system and the nucleoid occlusion effect (purple arrows). Various other division proteins (yellow) are recruited to the Z-ring. At some point, the division machinery constricts the cell, coordinately organizing the synthesis of new membrane and cell-wall layers, and, in parallel, the machinery disassembles. During sporulation (b), two effects — increased FtsZ accumulation and synthesis of the sporulation-specific SpoIIIE protein — lead to the repositioning of FtsZ into two separate rings, one near each of the cell poles. The switch in position occurs by a helical redistribution of the FtsZ protein from the mid-cell to subpolar positions. The Z-rings near the two poles are usually unequal and one of them is 'chosen' for the formation of a septum, which represents the defining moment in determining the fates of the prespore and the mother cell.

over division by a remarkable pole-to-pole oscillation that is driven by the MinE protein. In *B. subtilis*, DivIVA, which is targeted to the cell poles, acts by a more straightforward mechanism, in which it recruits MinCD in a stable manner to these sites. Positioned at the mid-cell by these mechanisms, the Z-ring then recruits several other cytosolic and membrane-associated division proteins to the mid-cell. At the appropriate time, the machinery constricts, pulling in the cytoplasmic membrane and simultaneously directing synthesis of new cell-wall material in the space between the invaginating membranes²². The roles of most of the division proteins are not yet understood in detail.

During sporulation, the division machinery is redirected to positions near each of the cell poles and the ultrastructure of the septum is modified so that it contains less cell-wall material. The cell-wall material that is inserted is later removed. The mechanism by which the septum is repositioned during sporulation was recently illuminated²³. It had been known for several years that mutations in the *spoIIIE* gene substantially impair and delay the switch in division-site positioning^{24,25}. Now it seems that efficient polar division also requires an increase in the concentration of FtsZ; when a mutation that eliminates the increase in the concentration of FtsZ was combined with a *spoIIIE* mutation, the switch was virtually abolished²³. Furthermore, careful observations of FtsZ distribution during the early stages of sporulation have shown that the switch in the position of the Z-ring occurs by an unexpected mechanism in which a medial Z-ring 'disassembles' and spirals out

towards the poles²³. One of the Z-rings then constricts to bring about polar division. It is not yet understood why the two subpolar Z-rings develop at different rates so that one septum always forms ahead of the other^{26,27}.

The present challenge is to determine how SpoIIIE and increased FtsZ concentrations combine to effect this positional switch in the Z-ring, including how the normal block to division close to the cell poles that is exerted by the Min system is overcome, and how division occurs through the nucleoid, a process that is at variance with the normal nucleoid occlusion effect (see below). A full understanding of these problems might not be easy to obtain as they might require a general improvement in our understanding of the division machinery and its regulation.

Prespore chromosome segregation

The switch to polar cell division has an interesting consequence for chromosome segregation because the chromosomes are not normally positioned close enough to the cell poles to be directly accommodated in the small prespore compartment. Several years ago, it was established that prespore chromosome segregation occurs by an unusual two-step process^{28–30}. First, a segment of the chromosome that is centred roughly on the *oriC* region (where bidirectional chromosome replication is initiated) approaches the cell pole. Formation of the sporulation septum then captures about one-third of the chromosome in the small prespore compartment. The remainder of the chromosome, which is distal to *oriC*, is then transferred through the septum by a DNA transporter called SpoIIIE. SpoIIIE is a highly conserved protein that is probably used by a wide range of bacteria to protect them from the damage that would occur if part of the chromosome were trapped in the septum when it closes. In *B. subtilis* it probably has other roles associated with the late stages of sporulation³¹. Recent work on SpoIIIE has focused on the problem of the directionality of DNA transfer. The reaction is so fast (about 10 minutes) that it probably needs to be directional. There is growing evidence that this is achieved by asymmetric insertion of the complex in the septum³² (although this conclusion is controversial³³), and that the directionality might be governed by the MinD protein, which localizes in regions close to the cell poles and, at least in principle, could be enriched on one side of the nascent asymmetric septum³⁴.

In rapidly growing *B. subtilis* cells, the chromosomes are never located very close to the cell poles, so they need to be actively moved at the onset of sporulation. Recently, several components of the mechanism that moves the *oriC* region of the chromosome have been identified. One key player in this process is the DivIVA protein, which now seems to have multiple functions as a positional marker of the cell poles. In vegetative cells, DivIVA acts as the target for the MinCD system that blocks inappropriate division near the cell poles (see above). In sporulating cells, it acts as an anchor for the *oriC* region at the cell pole, in preparation for asymmetric division³⁵. Recognition of the

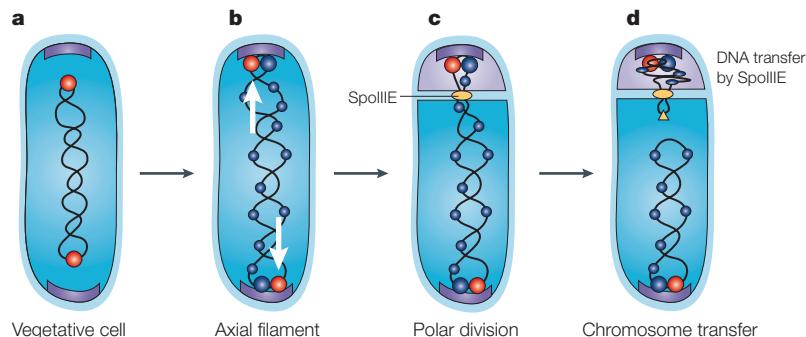


Figure 3 | Prespore chromosome segregation. **a** | In the vegetative cell, Spo0J protein binds to sites around the *oriC* regions of the chromosome to form condensed foci that are important for proper chromosome segregation. The *oriC*–Spo0J complexes (red) are typically positioned at about the one-quarter and three-quarter positions along the length of the cell. **b** | During the early stages of sporulation, the *oriC* regions move towards the cell poles and are bound to the poles by the combined action of the Soj (not shown) and RacA (blue) proteins, together with the polar anchor protein, DivIVA (purple). **c** | Asymmetric division results in trapping of the one-third of the chromosome that is attached to the *oriC* region in the prespore compartment. **d** | The SpoIIIE protein (yellow) is recruited to the leading edge of the septum, where it forms a pore through which it translocates the remaining portion of the chromosome, thereby completing the segregation of the prespore chromosome.

oriC region and its delivery to DivIVA at the cell pole involves at least three proteins with partially redundant functions. Two of these proteins, Soj and Spo0J, are encoded by adjacent genes that are located close to *oriC*. They are conserved in a wide range of bacteria, including many non-spore formers, and are closely related to proteins that are required for the stable inheritance of low-copy-number plasmids³⁶. The precise functions of these proteins are not yet clear. However, Spo0J binds to preferred sites located around the *oriC* region, and is required for efficient chromosome segregation in both vegetative and sporulating cells^{19,37–40}.

Soj is a remarkable protein that associates with the nucleoid and can jump from nucleoid to nucleoid in a highly cooperative manner. Jumping is stimulated by Spo0J, and in the absence of Spo0J, the Soj protein associates statically, and apparently nonspecifically, with chromosomal DNA and blocks the transcription of several key sporulation genes^{41–44} (see above). It seems that the Soj–Spo0J system is involved in chromosome segregation and in a possible checkpoint mechanism that prevents sporulation from being initiated under certain circumstances — presumably if some aspect of chromosome segregation has failed or has otherwise not been completed properly. Simultaneous loss of Soj and Spo0J results in an almost normal frequency of sporulation, but there is a subtle alteration in capture of the *oriC* region⁴⁵. The recently characterized RacA protein is a DNA-binding protein that associates with the chromosome, probably at dispersed sites around the *oriC* region, and which also interacts with DivIVA^{46,47}. Absence of RacA results in a failure of *oriC* capture in about 50% of sporulating cells, but this effect is greatly exacerbated if the *racA* mutation is combined with either *soj*, or *soj* and *spo0J* mutations. Loss of both Soj and RacA proteins results in a phenotype that is very similar to that of a *divIVA* mutant, which is affected specifically in prespore

chromosome segregation⁴⁷. So, these two DNA-binding proteins are probably the key effectors that are required to link the chromosome to the DivIVA protein at the cell pole. At present, our model hypothesizes that the Soj–Spo0J system is involved in organizing the *oriC* region and possibly moving it in a polar direction, allowing the RacA system to efficiently bind the *oriC* region to DivIVA at the cell pole (FIG. 3). The main problems that remain relate to ignorance of the precise biochemical functions of the Soj–Spo0J system in normal chromosome segregation. This system probably needs to be better understood before the way in which the system is modified for sporulation can be deciphered.

Establishment of cell-specific transcription

The mechanisms described above allow the sporulating cell to divide into two compartments of different sizes — the small prespore and the much larger mother cell — and to accurately segregate a chromosome into each compartment. Formation of the asymmetric septum is a key event in development. It triggers a cascade of changes in gene expression, involving different programmes of gene expression in the two cells. Two sigma factors, σ^F and σ^E , are instrumental in setting the cell-specific programmes of gene expression in motion. Both are synthesized before the septum is formed, but are held in inactive states until the septum forms. σ^F becomes active first and its activity is regulated by two distinct mechanisms, which work together to ensure that this crucially important factor is only released in a newly formed prespore compartment (FIG. 4) (see REF. 48 for a recent review). The first mechanism involves a chain of regulators; SpoIIAB is an ANTI-SIGMA FACTOR that binds directly to σ^F , and prevents it from interacting with RNA polymerase. The X-ray crystal structure of SpoIIAB in complex with part of σ^F was recently solved⁴⁹. SpoIIAB is also a protein kinase and can interact with an anti-anti-sigma factor, SpoIIAA, thereby phosphorylating SpoIIAA on a specific serine residue. Non-phosphorylated SpoIIAA interacts with the SpoIIAB– σ^F complex, directly displacing the sigma factor before the phosphorylation reaction inactivates the SpoIIAA. Release of σ^F activity depends mainly on the prespore-specific appearance of non-phosphorylated SpoIIAA. This is achieved by the SpoIIE protein, which has a phosphatase activity in its carboxy-terminal domain and can specifically dephosphorylate phosphorylated SpoIIAA. Although the temporal and spatial dynamics of this process are not completely understood, one contributing factor could be that the SpoIIE protein, which is associated with the division machinery, is delivered into the prespore compartment, thereby enriching the protein in the prespore compartment and overcoming the competing kinase activity of SpoIIAB⁵⁰. There has been some controversy over this observation, but both published studies on this subject agree that a substantially higher concentration of SpoIIE is attained in the prespore soon after septation^{50,51}. Recent work from several laboratories has shown that the phosphatase activity of SpoIIE is regulated, and that this regulation could serve to control the timing of σ^F activation, or to determine its spatial localization^{51–54}. A second mechanism that reinforces the

ANTI-SIGMA FACTOR

A negative transcriptional regulator that acts by binding to a sigma factor and preventing its activity. An anti-anti-sigma factor, in turn, counteracts the action of an anti-sigma factor.

compartment specificity of σ^F activation occurs as a result of the relative instability of SpoIIAB^{55,56} and the position of its gene (*spoIIAB*), which is almost opposite the *oriC* region on the circular chromosome. At this position, *spoIIAB* is one of the last genes to be transferred into the prespore compartment by the SpoIIIIE DNA transporter (see above). Presumably, during the period of about 10 minutes when the prespore has no *spoIIAB* gene, the concentration of the SpoIIAB protein falls substantially, relative to the more stable σ^F and SpoIIAA proteins. The combination of these two mechanisms — SpoIIIIE activity regulation and *spoIIAB* chromosome positioning — provides a high-fidelity system that ensures correct temporal and spatial control of σ^F activity⁵⁷.

Mother-cell commitment

Immediately after activation of σ^F in the prespore compartment, σ^E becomes active in the mother cell. This factor is synthesized as an inactive pre-protein, which is activated by proteolytic processing — probably by SpoIIGA, which has a putative serine protease activity⁵⁸. SpoIIGA requires the action of a prespore-specific protein, SpoIIR, which is controlled by σ^F (REFS 59,60). SpoIIR is a secreted protein that can exit the prespore and, in principle, can contact the membrane-bound SpoIIGA protease on the outer face of the opposing septal membrane of the mother cell. However, what makes this signal vectorial — why SpoIIGA in the prespore septal membrane is not activated — is not yet clear. Recent results indicate that there is more than one mechanism helping to confine σ^E activity to the mother cell⁶¹. First, it seems that pro- σ^E is selectively degraded in the prespore compartment, a process that is perhaps facilitated by a hypothetical σ^F -dependent protease. Second, the synthesis of both SpoIIGA and σ^E is probably maintained in the mother cell but not in the prespore by the compartmentalized activity of Spo0A¹⁵ (see above). So, although Spo0A is initially active in the predivisional cell, its activity seems to be maintained after septation, specifically in the mother-cell compartment. It is additionally possible that pro- σ^E is selectively delivered to the mother-cell face of the septum at division⁶², although it is possible that this apparently active redistribution process might in fact be due to one or both of the factors described above⁶¹. These complex regulatory pathways ultimately result in the establishment of active forms of σ^F and σ^E in the prespore and mother-cell compartments, and these powerful regulators set in motion profound changes in gene expression that result in the differentiation of the two cell types.

One final, crucial step in cell-fate determination remains. Mutations that prevent the activation of either σ^F or σ^E result in a phenotype in which the mother-cell compartment undergoes another ‘asymmetric’ division, which forms a second prespore-like compartment at the opposite pole. Catastrophically, the prespore chromosome segregation machinery then transfers the ‘mother-cell’ chromosome into the second prespore, leaving no chromosome in the central compartment. This ‘disporic’ phenotype indicates that the sporulating cell is initially prepared for division near either of its cell poles and that

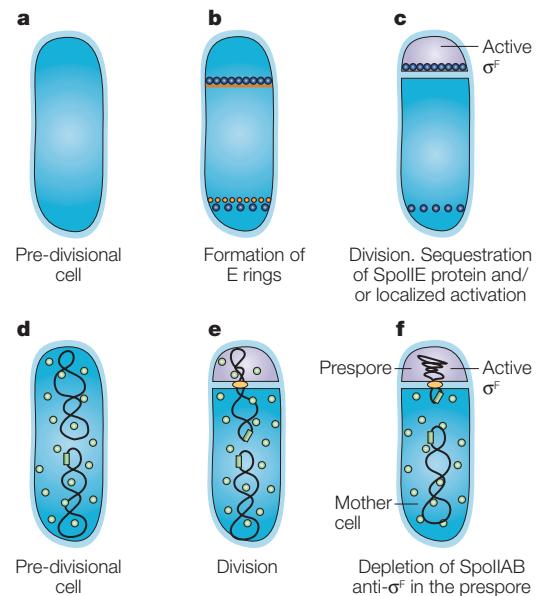


Figure 4 | Cell-specific activation of σ^F . Two distinct mechanisms help to ensure the correct compartmentalization of σ^F activity in the prespore. **a–c** | The SpoIIIE protein (blue spots) has a phosphatase activity that can overcome the negative regulation of σ^F by the SpoIIAB protein. SpoIIIE is also required for the correct formation of the prespore septum (FIG. 2). SpoIIIE is recruited to the FtsZ rings by a direct interaction with FtsZ. The development of the two rings is asymmetrical and the ring that contains the most SpoIIIE protein (upper in this case) usually achieves division first. During or following division, it seems that the SpoIIIE protein becomes enriched in the prespore compartment, greatly enhancing the likelihood of σ^F activation in that compartment. SpoIIIE phosphatase activity also seems to be regulated and it is possible that this regulation responds in some way to formation of the septum. **d–f** | A chromosome position effect operates in a quite different way to compartmentalize the release of σ^F activity. The SpoIIAB anti- σ^F factor (green circles) is an unstable protein that is encoded by a gene that is located at an *oriC*-distal part of the chromosome (green boxes). During the period immediately after formation of the polar septum (**e**), and before the *spoIIAB* gene is translocated into the prespore (**f**) (FIG. 3), SpoIIAB protein concentrations decrease, allowing the more stable σ^F protein to become active in the small compartment.

the σ^F/σ^E activation cascade culminates with one or more genes that fix the fate of the mother cell by blocking the second potential polar division²⁶. Recently, it was established that blocking the second polar-division step requires the concerted action of three different σ^E -dependent genes — *spoIID*, *spoIM* and *spoIP*⁶³. These genes presumably encode cell-wall lytic enzymes that degrade the material in the developing septum. The same enzymatic activities are probably important in the functioning of the proteins in engulfment.

Engulfment — a phagocytosis-like process

Asymmetric cell division is followed by a second, unusual morphological event — prespore engulfment. The cell-wall material in the septum is degraded, normally beginning at the centre where septal closure occurs. Then, the edges of the pair of septal membranes migrate around the prespore cytosol. The migrating

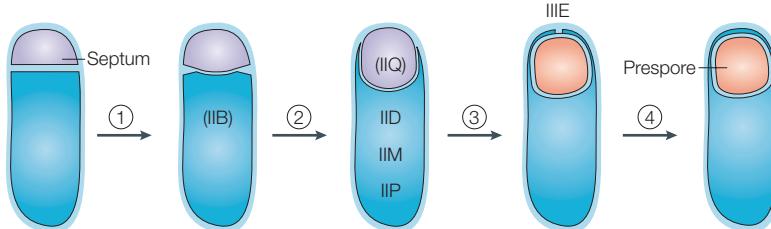


Figure 5 | Four steps in prespore engulfment, and the proteins involved in each step. The protein names have been abbreviated by omitting the Spo prefix. SpoIIIB and SpoIIQ are placed in parentheses as their functions are not completely essential for engulfment. SpoIID, SpoIIM and SpoIIP (also probably SpoIQ) are needed throughout stages 2 and 3. SpoIID, SpoIIM and SpoIIP are needed to drive regression of the second polar septum, as well as for engulfment. SpoIIIIE is involved in the final step of membrane fusion. Modified with permission from REF. 64 © (2002) Cold Spring Harbor Laboratory Press.

membranes meet at the apex of the cell, where they fuse, releasing the prespore as a free protoplast that is completely enclosed in the mother-cell cytoplasm and separated from it by two membranes of opposite topology⁶⁴ (FIG. 5). The first step in engulfment — degradation of the wall material at the centre of the septum — is probably catalysed by the SpoIIIB protein (FIG. 5b). *spoIIIB* mutants attain almost normal levels of sporulation, but recent analysis of the mutant phenotype has shown that they are severely delayed at the first step of engulfment⁶⁵. Mutations of *spoIIIB* were first identified in mutants that were blocked in engulfment, but it has since been shown that these mutants have a second mutation, in the *spoVG* gene⁶⁶. Single mutants in *spoVG* have a variety of subtle effects on sporulation, including precocious septation and minor defects in cortex synthesis⁶⁷. It is not yet understood why combination with *spoVG* enhances the engulfment defect of *spoIIIB* mutants.

Three proteins that are required for membrane migration during engulfment are the σ^F -dependent proteins that were mentioned above, SpoIID, SpoIIM and SpoIIP (FIG. 5c). All three proteins have a transmembrane domain with a main extracytoplasmic domain^{68–70}. They all localize initially at the centre of the septum and then track around the prespore at the leading edge of the migrating membranes⁶⁴. SpoIID at least has cell-wall hydrolytic activity, so these proteins probably act to hydrolyse linkages in the cell wall, and/or between the cell wall and membrane. Hydrolysis of cell-wall material in the septum is clearly needed to allow any movement of the septal membranes. The same, or closely related hydrolytic activities might be involved in allowing membrane migration around the apex of the mother cell to progress to engulfment. Abanes de Mello *et al.*⁶⁴ have proposed an interesting model in which one or more of these proteins uses the energy of hydrolysis of the cell wall to pull the membrane domain towards the pole.

The role of the prespore compartment in engulfment is not yet clear. Genetic data are consistent with there being at least two σ^F -dependent genes involved in engulfment. One of these genes is *spoIIQ*⁷¹, but recent results have questioned the importance of this gene, owing to the curious discovery that the penetrance of

the engulfment phenotype of *spoIIQ* mutants is dependent on the media conditions that are used to induce sporulation⁷². Nevertheless, *spoIIQ* encodes a membrane protein with an extracellular domain that has a metalloendopeptidase domain, which is consistent with it being a hydrolytic enzyme that facilitates engulfment.

The final step of engulfment involves membrane fusion at the apex of the cell. Surprisingly, this event has been shown to require the amino-terminal multiple transmembrane domain of the SpoIIIIE protein^{73,74}. SpoIIIIE is therefore yet another protein that has two distinct roles in sporulation. As described above, it can effect DNA translocation into the prespore immediately after septation. The protein then migrates to the pole, where it probably has a direct role in membrane fusion. The molecular basis of membrane fusion is not yet understood, but it is an interesting function that is more often associated with eukaryotic cells than with prokaryotic cells.

Regulation of spore development — σ^G and σ^K

The completion of engulfment is a key event governing the later stages of spore development. In the prespore, a third sporulation-specific sigma factor, σ^G , becomes active at this time⁷⁵, and this sigma factor controls the final stages of development inside the spore. Regulation of σ^G takes place on several levels (FIG. 6a). Transcription of the *spoIIIG* gene, which encodes σ^G is spore-specific because it requires the σ^F -form of RNA polymerase, but also depends on a mother-cell factor or event because it does not occur in *spoIIIG* (σ^E) mutants⁷⁶. Curiously, it also seems to require the SpoIIQ engulfment protein, although how this dependence could be mediated is by no means clear⁷². Finally, the σ^G protein is regulated post-transcriptionally and remains inactive if the genes that affect engulfment are deleted (for example, by a *spoIID* mutation⁷⁶). Although it is possible that the products of these genes have a direct effect on σ^G activity, it seems more likely that this dependence reflects a mechanism that couples activation of σ^G to completion of engulfment — a morphological event. σ^G activity is also blocked by mutations in the eight-gene *spoIIIA* operon, or in the *spoIIIJ* gene, both of which block sporulation after engulfment^{76,77}. The basis for these dependencies has not been resolved. The *spoIIIA* operon is expressed in the mother cell and encodes proteins that might comprise a transport system to move molecules into, or out of, the space between the prespore membranes⁷⁸. *spoIIIJ* encodes a protein that is essential for membrane protein biogenesis in a wide range of organisms. It is not essential in *B. subtilis* because there is a homologous protein, YqjG, that can support growth in its absence⁷⁹. However, YqjG cannot substitute for the sporulation function of SpoIIIJ. Recent work has shown that the SpoIIIJ membrane protein biogenesis function is required in the prespore compartment, indicating that insertion of one or more proteins into the prespore membrane is needed for σ^G activation⁸⁰. An alternative view of these effects could be that the SpoIIIJ or SpoIIIA proteins are needed to maintain the metabolism of the engulfed

prespore, and that if this does not occur, gene expression is affected indirectly.

An interesting candidate for the immediate effector of the regulation of σ^G activity is the anti- σ^F factor, SpoIIAB. σ^G is closely related to σ^F , and both *in vitro* and *in vivo* experiments have shown that SpoIIAB is capable of regulating σ^G (REFS 77,81,82). However, it remains unclear whether this is physiologically relevant in sporulating cells, not least because SpoIIAB is thought to be inactivated by both degradation and the action of the SpoIIAA anti-anti-sigma factor before σ^G is produced (see above). Clearly, there is still quite a lot of work to be done to understand the regulation of σ^G .

The final mother-cell-specific sigma factor, σ^K , is regulated at multiple levels. First, *sigK*, the gene encoding σ^K , is interrupted by a 40-kbp DNA element comprising an integrated prophage⁸³. Excision of the prophage is required to join the two coding pieces of the *sigK* gene together. The site-specific recombinase (SpoIVCA) is transcribed specifically in the mother cell under the control of σ^E RNA polymerase. Second, the *sigK* gene is under the control of σ^E (REF 84). Third, the σ^K protein is subject to post-transcriptional regulation: like its mother-cell predecessor, σ^E , it is translated as an inactive precursor that requires proteolytic processing to remove an amino-terminal pro-sequence⁸⁵.

SpoIVFB is probably the membrane-bound protease that is responsible for proteolytic activation of σ^K in the mother cell^{86,87}. Genetic analysis has identified two regulators of SpoIVFB, known as SpoIVFA (encoded by a gene that is co-transcribed with *spoIVFB*) and BofA⁸⁸. Both regulators are also membrane proteins. Recent work has shown that these three proteins form a complex that is targeted to the outer spore membrane by a 'diffusion/capture'-type mechanism^{89,90}. The membrane proteins are inserted into the cytoplasmic membrane at undefined positions. They then diffuse and are captured at their required site in the outer spore membrane. SpoIVFA seems to be responsible for the targeting, or capture, although this highlights the question of how SpoIVFA is targeted. BofA is the immediate regulator of SpoIVFB⁹⁰. The final crucial step in this complex regulatory pathway is interesting, because it again involves an intercellular signal, in this case from the spore. The SpoIVB protein is made specifically in the spore because its expression is dependent on the σ^G form of RNA polymerase. SpoIVB is secreted into the space between the inner and outer membranes surrounding the spore. Recent work has shown that SpoIVB is a protease of the trypsin family, which self-cleaves into several active fragments^{91,92}. At present, the simplest model for SpoIVB action is that it proteolytically cleaves BofA and/or SpoIVFA. This then triggers the intercompartmental signal by releasing SpoIVFA activity so that σ^K processing can proceed. Interestingly, like many of the proteins described above, SpoIVB probably has at least one secondary activity because when the requirement of SpoIVB for σ^K processing is bypassed, *spoIVB*-null mutants are still defective in sporulation.

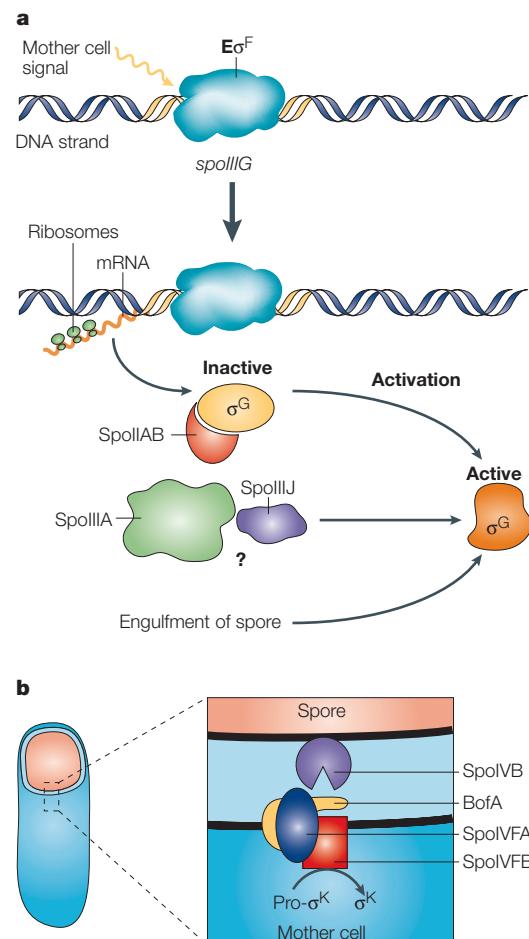


Figure 6 | Regulation of the late stages of sporulation.

a | Regulation of σ^G synthesis and activity. Transcription of the *spoIIIG* gene is directed by the σ^F form of RNA polymerase ($E\sigma^F$). It also requires an as-yet-undefined signal from the mother cell, which is dependent on σ^F . σ^G protein is initially inactive (yellow), possibly due to the action of the SpoIIAB anti- σ factor. Activation depends on the completion of prespore engulfment, as well as on the SpoIIIA and SpoIIJ proteins.

b | Regulation of σ^K activity. σ^K is synthesized as a precursor protein with an amino-terminal pro-sequence. It is processed by a membrane-associated protease, SpoIVFB, which is recruited to the outer prespore membrane by SpoIVFA protein. BofA is also recruited to this complex by SpoIVFA. BofA is probably an inhibitor of SpoIVFB. Processing is released by an intercellular signal that comes from the σ^G -dependent SpoIVB protein. SpoIVB is secreted into the space between the two membranes and is a protease that probably acts by cleaving BofA and/or SpoIVFA to release the SpoIVFB pro- σ^K processing enzyme.

Other transcriptional regulators

Superimposed on the global regulation that is exerted by the sporulation sigma factors are a number of minor transcriptional regulation mechanisms that fine-tune the timing and expression levels of a large number of sporulation genes. At least one such regulator operates in parallel with each of the sigma factors. RsfA was discovered serendipitously as a regulator of σ^F -dependent gene expression⁹³. Mutations in the *rsfA* gene have positive or negative effects on the transcription of various

σ^F -dependent genes. The protein is associated with the nucleoid, but direct evidence for regulation at the level of transcription is presently lacking. Later in prespore development, another transcriptional regulator SpoVT (which is related to AbrB; see above) modulates genes in the σ^G regulon⁹⁴. SpoIID is probably the best understood auxiliary transcriptional regulator. It positively or negatively regulates a wide range of genes in the σ^E regulon during the early phase of mother-cell development⁹⁵. Finally, GerE has an analogous role to that of SpoIID in the process of later mother-cell development that is controlled by σ^K . The crystal structure of GerE has been solved, and regions of this small (74 amino acid) transcriptional regulator that is required for DNA binding and transcriptional regulation are now well defined^{96,97}.

Spore morphogenesis – core, cortex and coat

The interior of the spore undergoes marked changes in physicochemical properties as it develops. Low-molecular-weight proteins are synthesized in large amounts to coat the DNA, providing protection against several kinds of DNA damage. The same proteins are broken down during germination to provide a source of amino acids. Large amounts of dipicolinic acid are synthesized in the mother cell and taken up by the prespore, together with divalent cations (usually Ca^{2+}), which leads to the dehydration and mineralization of the spore. Meanwhile, the spore cortex, a modified cell wall, is synthesized outside the spore protoplast membrane. Finally, a multilayered proteinaceous coat is assembled outside the cortex. In some spore formers (for example, *B. anthracis*), an exosporium is present as an extreme outer layer^{98,99}. As the spore is being constructed, its ability to respond to specific germinants, to shed its protective layers and to rehydrate and resume vegetative growth are also built into the structure. The regulation of most of these activities is underpinned by the later prespore and mother-cell sigma factors, which work together with the auxiliary regulators described above. Details of the nature of the proteins and assembly functions have been described in detail in recent reviews^{100,101}.

Conclusions and future challenges

Sporulation in *B. subtilis* is probably the best-understood example of cellular differentiation and development in developmental biology. The pathways of gene expression have been detailed, and it is likely that most of the important regulators have been identified. A number of interesting models have emerged that might be relevant to development in other bacteria and higher organisms. From a microbial point of view, the detailed facets of sporulation are relevant to the persistence of these organisms in the environment and, for pathogens, in the host. From this perspective, the main structures of the *B. subtilis* spore have been identified and catalogued in detail. The central mechanisms of dormancy and germination are beginning to be understood in molecular detail. This knowledge will provide powerful tools of applied microbiology for elucidating the mode of action of disinfectants and sporocides. Although much of our knowledge relates to a single species, *Bacillus subtilis*, the completion of several genome sequences has provided the opportunity for comparative studies of other organisms of applied and basic importance. It is becoming apparent that many of the central regulators controlling *B. subtilis* sporulation are found throughout the endospore formers. However, there are exceptions. The control of initiation seems to be substantially divergent in different organisms, reflecting adaptation to different environments and niche-specific challenges¹⁰². Some of the largest bacteria known — for example, the 1 mm long *Epulopiscium* — are endospore forming bacteria. These differ from the better-known spore formers because they produce multiple spores — apparently as a means of propagation¹⁰³. There are even organisms that are spherical (for example, *Sporosarcina*), and which therefore lack the asymmetry that underpins many key features of endospore development in *B. subtilis*, but which can still form endospores using the same general regulators and machinery¹⁰⁴. Clearly, then, there are several good reasons for studying a diversity of spore formers. Nevertheless, *B. subtilis* continues to offer a powerful system for delving into the basic mechanisms of spore development, and it will probably provide interesting problems for many more years.

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