Sporulation of *Bacillus subtilis*

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Differentiation of vegetative *Bacillus subtilis* into heat resistant spores is initiated by the activation of the key transcription regulator Spo0A through the phosphorelay. Subsequent events depend on the cell compartment-specific action of a series of RNA polymerase σ factors. Analysis of genes in the Spo0A regulon has helped delineate the mechanisms of axial chromatin formation and asymmetric division. There have been considerable advances in our understanding of critical controls that act to regulate the phosphorelay and to activate the σ factors.

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Introduction

Actively growing cells of *Bacillus subtilis* are induced to differentiate into spores by starvation for carbon, nitrogen or, in some circumstances a phosphorus source. Spore formation takes about 7 h at 37°C. Initiation signals result in activation of the master transcription regulator, Spo0A, by phosphorylation. Activated Spo0A, among other things, triggers the asymmetric sporulation division and transcription of the *spoIIA*, *spoIIE* and *spoIIG* loci, which encode key developmental regulators. The sporulation division produces two distinct cells with very different fates, the smaller prespore (also known as forespore), which develops into the spore, and the mother cell, which is necessary for spore formation but ultimately lyses (programmed cell death). Soon after the division distinct programs of gene expression are initiated in the two cell types. These are directed by sporulation-specific RNA polymerase σ factors, σ^F in the prespore and σ^E in the mother cell. About 1 h after division, the prespore is engulfed by the mother cell. On completion of engulfment, there is another substantial change in transcription, with σ^G becoming active in the prespore and σ^K in the mother cell (Figure 1). These global changes in gene regulation are coupled to morphogenesis and to each other by intercompartmental signaling, eventually leading to the development of the resistances that characterize the mature spore.

Spore formation is a very active research subject, and we discuss only some topics. For more extensive coverage, and for citations to the earlier literature we refer to fuller reviews [1–3]. Assembly of spore surface layers, spore resistance and spore germination are not considered here. Three recent reviews discuss these topics in depth [4–6].

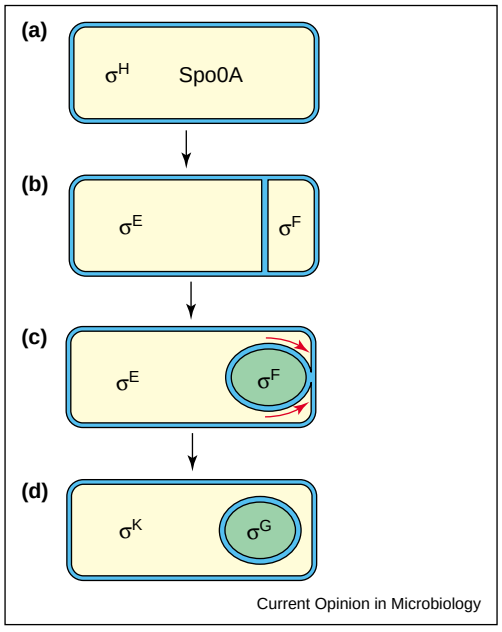
In this review, we discuss the regulation of gene expression during spore formation and the coordination of gene expression with morphological changes.

Initiation of sporulation

Genomic analysis has indicated that Spo0A directly regulates the transcription of 121 genes, with about one-third being activated and the remainder repressed. This group includes several transcription factors; a further ~400 genes are indirectly controlled by Spo0A [7,8•]. The molecular details of the interaction of Spo0A with its target DNA, the 'Spo0A box', have now been analyzed with a crystal structure [9]. Activation of Spo0A goes through several phases. Initial activation at the end of exponential growth leads to the 'transition state', which is associated with such phenomena as protease production, motility, competence for transformation [10], biofilm formation [11,12] and even cannibalism [13•]. Spore formation is thought to require increased Spo0A phosphorylation. Consistent with this requirement, analysis of the structure begins to suggest why repression of *abrB* in the transition state requires less phosphorylated Spo0A than activation of transcription of *spoIIA*, *spoIIE* and *spoIIG* early in spore formation; namely, the likely need for Spo0A~P oligomerization for the latter but not the former [9]. Following the sporulation division, Spo0A activity increases further in the mother cell and declines in the prespore; both changes are important for continued spore formation [14••]. In addition to Spo0A, entry into the transition state and sporulation requires σ^H [1].

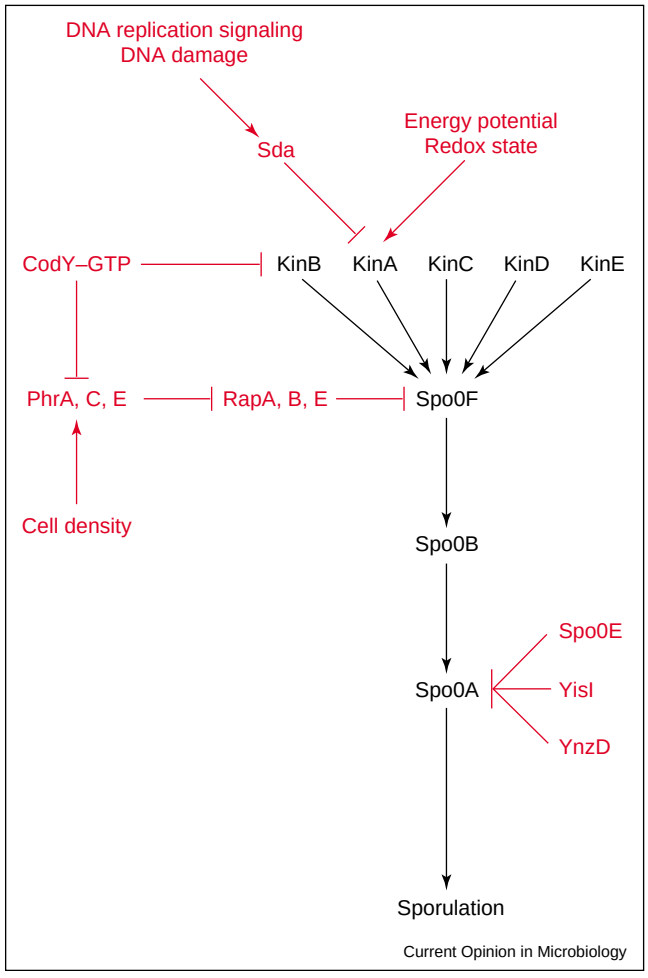
The different roles for Spo0A, presumably dependent on differing levels of phosphorylation, emphasize the importance of the phosphorelay, which is the conduit for various signals that control the phosphorylation of Spo0A. The main components of the phosphorelay and its associated phosphatases have been known for several years (Figure 2) [2,10,15]. There is increasing understanding of the many input signals.

Figure 1



Morphogenesis and gene regulation during spore formation. **(a)** Activation of Spo0A and σ^H in the predivisional cell leads to asymmetric division **(b)** and early compartmentalized gene expression with σ^F becoming active in the prespore and σ^E in the mother cell. **(c)** A series of proteins produced in the mother cell degrade the asymmetric septum and trigger migration of the membrane around the prespore, a process called engulfment, represented here by red arrows. **(d)** When the membranes fuse at the pole of the cell, the prespore is released as a protoplast in the mother cell, and a second round of compartmentalized gene expression occurs, with σ^G becoming active in the prespore and σ^K in the mother cell. These late factors activate transcription of genes that build the structural components of the spore that provide its resistance qualities.

Figure 2



Initiation of sporulation. The core phosphorelay is depicted in black, with various inputs depicted in red. Five histidine kinases, KinA–E, phosphorylate Spo0F, which transfers the phosphate to Spo0B. Spo0B in turn transfers it to Spo0A which then activates transcription of critical sporulation genes. KinA is subject to two types of regulation, inhibition by Sda and activation through one of its PAS domains. Expression of Sda is activated by impaired DNA replication or DNA damage, thereby preventing sporulation under these circumstances. By contrast, a PAS domain of KinA is thought to sense energy potential or the redox status of the cell and promote sporulation accordingly. Cell density is sensed by Phr peptides that are secreted, processed and imported back into the cell as pentapeptides where they inhibit the Rap proteins that cause dephosphorylation of Spo0F-PO_4. Transcription of *kinB* and several *phr* genes is repressed by CodY in the presence of GTP. Spo0A can be dephosphorylated by Spo0E, YisI and YnzD.

Of the five kinases that can phosphorylate Spo0F and so activate the phosphorelay, KinA has the major role at the onset of spore formation. It has three PAS domains that in other proteins sense changes in oxygen levels and redox potential, among other functions. The most amino-terminal of the domains in KinA is important for spore formation and has been shown to bind ATP, but is unlikely to be regulated directly by ATP levels [16]. Thus, the PAS domains provide a tantalizing clue rather than a definitive answer to KinA regulation. KinA is also subject to regulation by the Sda protein. Sda binds to KinA to form a hetero-tetramer, in which Sda is a barricade between the catalytic and phosphotransfer domains of KinA, preventing autophosphorylation [17•]. Expression of *sda* is induced by damage to DNA or by a block in DNA replication. Thus Sda prevents activation of Spo0A by the phosphorelay when DNA replication or integrity is compromised [18]. KinA is only one of many players in the phosphorelay, yet it is subject to at least two very different types of regulation.

The Rap proteins A, B and E, act to cause dephosphorylation of Spo0F∼P, and this action is inhibited by cognate pentapeptides, some or all of which may function as cell density signals. Initially, the Rap proteins, which are similar to each other, were all thought to function as phosphatases. However, study of RapC (which is associated with competence development) has indicated that it is not a phosphatase, but rather inactivates its target protein, ComA∼P by binding to the protein. The Rap proteins have in common six 34-residue (tetratricopeptide,

or TPR) repeats, which are associated with protein–protein interaction. Thus, RapA, RapB and RapE may cause dephosphorylation of Spo0F∼P by binding to it and stimulating its autophosphatase activity, rather than by functioning directly as phosphatases; it would then be the binding activities that are regulated by the pentapeptides [19•]. Consistent with this interpretation, RapA is displaced from a stable complex with Spo0F∼P by addition of its cognate pentapeptide [20]. Spo0A∼P itself is susceptible to dephosphorylation through the action of Spo0E [2,10], and two homologs, YisI and YnzD [21]; expression of each protein is increased in non-sporulation conditions [21].

The regulating pentapeptides for the Rap proteins A, B and E are derived from processing of the exported products of the *phrA*, *phrC* and *phrE* genes, respectively. Regulation of transcription of these loci is complex. Importantly, transcription of two of these genes *phrA* and *phrE* is repressed by CodY [22•]. CodY is the key sensor of guanine nucleotide levels [23]. A sharp fall in the concentration of GTP and GDP is critical to the initiation of spore formation. It occurs at the onset of sporulation and can trigger spore formation in otherwise non-sporulation conditions [10]. The fall in guanine nucleotide levels relieves the CodY-mediated repression of *phrA* and *phrE* and also of *kinB*, encoding a kinase for Spo0F [22•]. Hence CodY links a fall in guanine nucleotide levels to activation of the phosphorelay.

Variation of Spo0A activity levels within a population also impacts development. Those cells that have activated Spo0A produce an extracellular killing factor that kills cells in which Spo0A is not active. This mechanism presumably enables the producers to feed on their non-producing siblings and to delay subsequent development [13•]. An intriguing question is whether this process plays a role in competition between different *B. subtilis* populations as well as within a clonally homogenous one.

Morphogenesis and chromosome partitioning

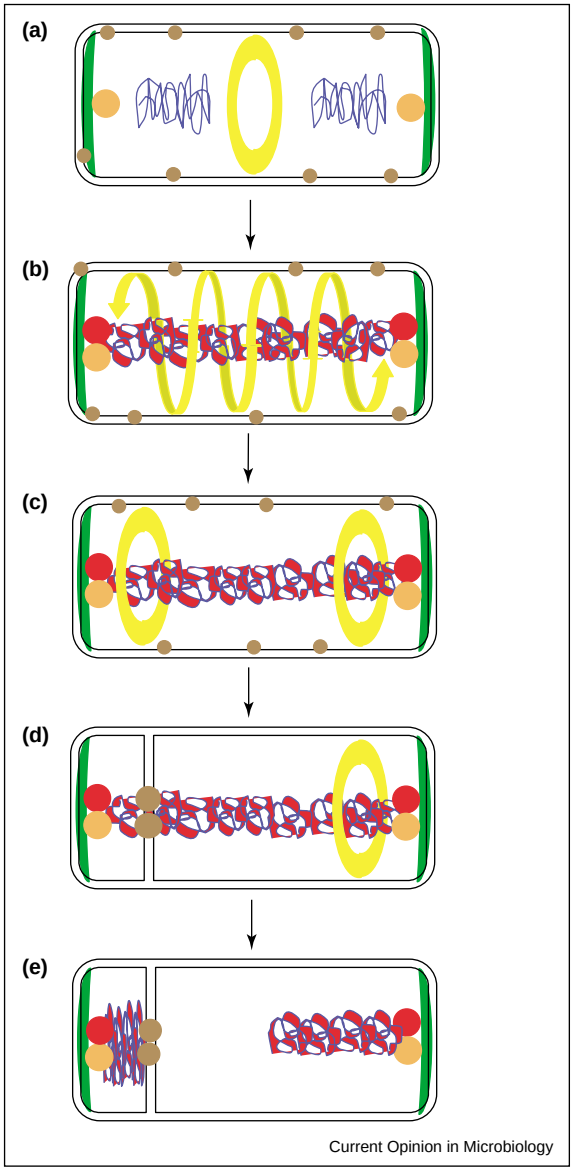
During sporulation, the bacterial cell is dramatically reorganized to generate two daughter cells of very different size and fate, the smaller prespore and larger mother cell. The nucleoids of the vegetative cell are remodeled into a continuous structure, the axial filament of chromatin, which extends the length of the cell (Figure 3). RacA has been identified as a protein produced during sporulation that binds the chromosome and the polar division protein DivIVA, acting as a bridge connecting the two; in its absence axial filaments are not formed and anucleate prespores are frequently observed [24••,25••]. The transcriptional repressor Soj also plays a role in this process, although its precise role is unclear [25••]. Concomitant with axial filament formation, the ring of the essential tubulin homolog FtsZ at midcell relocalizes, via a helical intermediate, to sites near the cell pole; the asymmetric division occurs at one of those sites [26•]. This relocalization is triggered by a σ^H-dependent burst of FtsZ expression and the Spo0A-dependent expression of SpoIIE [26•]. After division, only the origin-proximal one-third of a chromosome is present in the prespore; the remainder is then transported through the septum by the DNA translocase SpoIIIE (Figure 3) [27]. The transient genetic asymmetry between prespore and mother cell during this time, which is perhaps 15 min, is thought to play a key role in the establishment of compartmentalized gene expression [1,28•].

Early compartmentalized gene expression results in modification of the asymmetric septum, so that its peptidoglycan is removed and it begins to migrate around the prespore, finally fusing to release the prespore as a protoplast within the mother cell, a process known as engulfment (Figure 1). The cell biology of this process is beginning to be understood; it has been found that the three critical engulfment proteins, SpoIID, SpoIIM, and SpoIIP, are all produced in the mother cell and localize to the sporulation septum. It is thought these membrane-bound proteins hydrolyze peptidoglycan, thereby driving membrane migration around the prespore [29]. In the absence of the DNA translocase SpoIIIE, one or more of these proteins creates a pore in the asymmetric septum, allowing regulatory proteins to diffuse across, a testament to their degradative capacity [30]. These proteins also prevent a second asymmetric division in the mother cell; failure to do so generates a three-chambered 'abortively disporic' organism [31]. How these mother cell engulfment proteins localize to the septum is unknown; studies of a prespore engulfment protein, SpoIIQ, revealed that it is inserted and retained specifically within the migrating septum; expression of an unknown protein in the mother cell was required in order to retain SpoIIQ in the engulfing septum. Interestingly, any membrane-bound protein produced in the prespore showed a similar location, suggesting that membrane-insertion is spatially regulated in this compartment [32•].

Compartmentalized gene expression

Immediately after the asymmetric division and before a chromosome has completely partitioned into the prespore, σ^F becomes active exclusively in the prespore (Figure 1). In the pre-divisional cell σ^F is held inactivate by the anti-σ factor SpoIIAB; this inhibition is reversed by the anti-anti-σ factor SpoIIAA. SpoIIAA is regulated by its phosphorylation state; it is inactive when phosphorylated by SpoIIAB (a kinase as well as an anti-σ) and active when it is dephosphorylated by SpoIIE (Figure 4). How σ^F activation is linked to asymmetric division and confined to the prespore remains under investigation. SpoIIE localizes to asymmetric division sites and interacts with FtsZ; it has been proposed to 'sense' asymmetric division and activate σ^F in response. In support of this proposal,

Figure 3



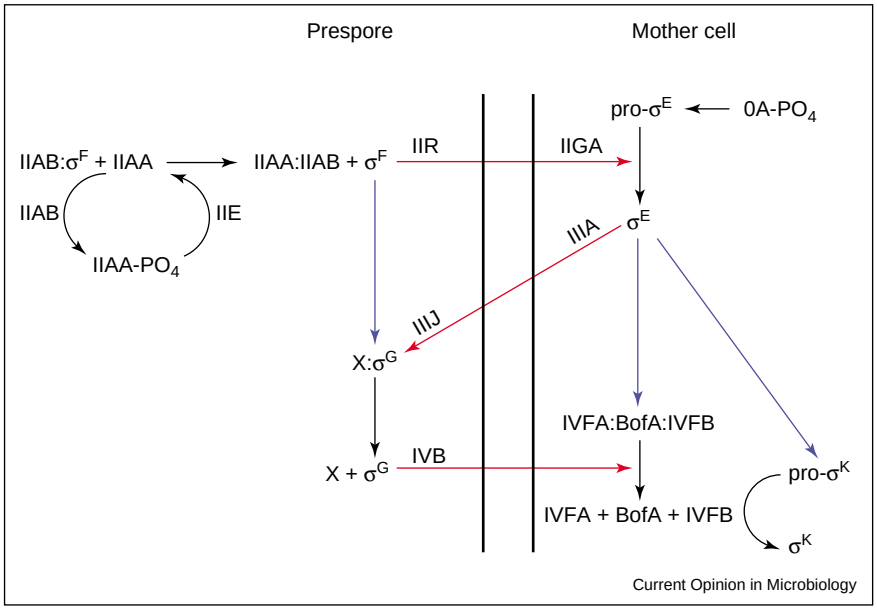
Chromosome partitioning and asymmetric division. Chromosomes are blue, DivIVA is green, FtsZ is yellow, RacA is red, Soj is orange and SpoIIIE is brown. **(a)** At initiation, the cell contains a single medial Z-ring. The cell is shown as having two distinct chromosomes at subpolar positions, although it may contain one partly replicated chromosome. Although not distinguished here, the origin of replication of both is located near the pole and their termini are located near midcell. DivIVA is located at the poles, SpoIIIE as discrete foci in the membrane, and Soj dynamically relocalizes from pole to pole, but is represented here at both for simplicity. **(b,c)** Initiation of sporulation results in expression of RacA and SpoIIE (not shown), and in an increase of FtsZ; as a consequence the nucleoids are remodeled into an axial filament coated with RacA, and the Z-ring relocalizes from midcell to subpolar locations via a helical intermediate. RacA concentrates at the origin region of the chromosome and binds to DivIVA, thereby anchoring the filament to the poles. Soj is also located at the pole and assists the process. **(d)** The division results in approximately 30% of a chromosome trapped in the prespore and the remainder, along with a second chromosome, in the mother cell. **(e)** The DNA translocase SpoIIIE relocalizes to the asymmetric septum and transports the origin-distal 70% of the chromosome into the prespore, a process that takes approximately 15 min. After asymmetric division the unused Z-ring disassembles.

several SpoIIE mutants were isolated that activate σ^F independently of asymmetric division, uncoupling the two events and impairing sporulation [33,34,35•]. The role of SpoIIAA has also been examined; dephosphorylated SpoIIAA is sequestered in an inactive, long-lived complex, or 'sink', with SpoIIAB and ADP. Different roles have been suggested for this 'sink'; there is evidence that it inhibits σ^F activation by sequestering dephosphorylated SpoIIAA in the predivisional cell [35•], and there is evidence that the complex can promote σ^F activation by sequestering SpoIIAB in the prespore [36,37]. These roles are not mutually exclusive. Structural studies have also contributed to our understanding of SpoIIAA; a key finding was that two molecules of SpoIIAB form a complex with σ^F, only one of which directly binds to it [38]. SpoIIAA interacts with the non-binding SpoIIAB molecule and induces release of σ^F from the other SpoIIAB by steric displacement [39•]. Although σ^F regulation is complex, the concentration of free dephosphorylated SpoIIAA is thought to be pivotal [35•,37]. It is presumed to be below a critical threshold in the predivisional cell, and that threshold is crossed only in the prespore. Contributory factors are likely to be an increased SpoIIE:SpoIIAA ratio [35•] and instability of SpoIIAB, combined with transient genetic asymmetry [28•,40].

Activation of σ^F in the prespore is rapidly followed by activation of σ^E in the mother cell (Figure 2). On receipt of a signal (SpoIIR) from the prespore, the inactive membrane-bound precursor, pro-σ^E, is processed to an active state, most likely by the putative protease SpoIIGA (Figure 4). Expression of the gene encoding pro-σ^E, *spoIIGB*, is greatly enhanced by the master response regulator Spo0A; it has been recently found that a burst of Spo0A activity takes place in the mother cell following asymmetric division. As a consequence, the level of pro-σ^E becomes much higher in this compartment than in the prespore, contributing to compartmentalized σ^E activity [14••]. Microchip arrays have been used to define the σ^E regulon. Some 253 genes (in 157 operons) appear to be directly controlled by σ^E, giving a clear indication of the extent of genetic reprogramming in the mother cell [41•,42•]; further reprogramming occurs following engulfment and the activation of σ^K. Although reprogramming is extensive, it is important to note that the main vegetative factor, σ^A, continues to be active in both the mother cell and the prespore both before and after engulfment [43].

σ^G is synthesized in the pre-engulfment prespore and held inactive before the completion of engulfment (Figure 4); it was previously thought that SpoIIAB was responsible for

Figure 4



Compartmentalized gene expression and intercompartmental signaling. All Spo proteins are represented by only their roman numeral and letter designation. Red arrows are intercompartmental signals, blue arrows indicate intracompartmental transcriptional activation, black arrows are intracompartmental posttranslational activation, and two dark lines represent the membranes separating the two compartments. σ^F is held inactive by SpoIIAB; the inhibition is reversed by SpoIIAA which is active when dephosphorylated by SpoIIE and inactive when phosphorylated by SpoIIAB. σ^F activates expression of σ^G and SpoIIR. SpoIIR triggers processing of pro-σ^E in the mother cell by SpoIIGA. Spo0A activity in the mother cell contributes to greatly enhanced σ^E activity in the mother cell. σ^E activates expression of SpoIIIA, SpoIVFA, SpoIVFB, BofA and pro-σ^K. SpoIIIA releases σ^G from inhibition by an unknown factor, 'X'; this pathway is mediated by SpoIIIJ, expressed vegetatively but only required, and thus represented, in the prespore. σ^G activates expression of SpoIVB, which signals across the prespore membrane and disrupts the SpoIVFA–BofA–SpoIVFB complex, releasing SpoIVFB to process pro-σ^K into active σ^K.

the inhibition. However, a mutant form of σ^G that is not bound by SpoIIAB does not become active in the prespore until engulfment is complete [44•]. Further, although SpoIIAB binds to and inhibits σ^G *in vitro*, the inhibition is reversed by dephosphorylated SpoIIAA, which is present in the prespore before engulfment [45•]. Both studies suggest SpoIIAB-independent regulator(s) that couple σ^G activation in the prespore to the completion of engulfment; it may be that SpoIIAB fulfils a partly redundant role in preventing activation in the mother cell [44•]. The products of the *spoIIIA* and *spoIIIJ* loci are required for σ^G activation; whereas SpoIIIJ is normally produced vegetatively, it can support sporulation if only produced in the prespore. By contrast, *spoIIIA* is expressed only in the mother cell and switching it's site of expression to the prespore impaired spore formation [46]. These results suggest that a signal is transmitted from one or more products of the *spoIIIA* operon to SpoIIIJ in the prespore to release σ^G from inhibition, although additional factors are likely to be involved (Figure 4).

The late mother cell σ factor σ^K is synthesized as an inactive precursor. Genetic evidence indicated that pro-σ^K is processed by the putative protease SpoIVFB, and that this reaction is inhibited by SpoIVFA and BofA (Figure 4). The roles of these proteins are beginning to be clarified; processing of pro-σ^K by SpoIVFB has been demonstrated in *E. coli* and found to be inhibited by BofA [47•]. SpoIVFA mediates this inhibition by bringing BofA and SpoIVFB together in a multimeric complex localized to the outer prespore membrane [48]. Protein localization is also an area of interest. In contrast to the directed insertion that occurs in the inner prespore membrane [32•], SpoIVFB is initially inserted into both outer prespore and peripheral membranes non-specifically, and then is retained only in the outer prespore membrane by SpoIVFA, a process termed 'diffusion and capture' [49]. Processing of pro-σ^K requires a signal, SpoIVB, from the prespore. SpoIVB has been found to proteolyze SpoIVFA *in vitro*; if this reaction occurs *in vivo* it would separate SpoIVFB from its inhibitor BofA and thereby trigger processing [50•].

In general, components of the developing spore are synthesized in the compartment where they are needed. There is one striking exception, where a spore component appears to be made in the wrong compartment. Dipicolinic acid (DPA) is made in the mother cell by the products of σ^K-controlled *spoVF* locus [3]. However, DPA (probably as a 1:1 complex with Ca^{2+}) is required in

the spore, where it constitutes ~10% of the spore dry weight and is important for heat resistance [51]. The products of the σ^G-directed *spoVA* locus are required for transport of DPA into the prespore [52]. It is not clear why the system is organized in this surprising way. A possible explanation is that DPA is secreted early in germination, and it may be that the same SpoVA complex is somehow required for this secretion, as well as for the prior DPA import into the prespore [52]. This (partial) explanation remains to be tested.

Conclusions

The key players in the pattern of gene expression are Spo0A, σ^H, σ^F, σ^E, σ^G and σ^K. A good understanding of their complex regulation is developing (Figures 2 and 4). Microarray analysis has identified RacA, which is critical for the previously neglected Stage I of spore formation, axial filament formation. The mechanism of polar FtsZ ring formation has become clearer, although the choice of location remains to be explained (Figure 3). We are beginning to understand how guanine nucleotides, DNA perturbation, and perhaps redox potential act on the phosphorelay to control Spo0A activation; we have a better idea about how dephosphorylation is controlled (Figure 2). Nevertheless, much remains unknown about the numerous controls that regulate the phosphorelay. We now know that Spo0A activity is largely confined to the mother cell after asymmetric division but not how this is achieved; is it possible that other phosphorelay components or regulators have compartmentalized patterns of localization or activity?

σ^F is the first transcription factor to show compartment specificity, and is the most studied. Its direct regulator is SpoIIAB. A long-lived SpoIIAA–SpoIIAB–ADP complex has emerged as critical in sequestering key regulators; somehow the balance is tipped in favor of σ^F activation in the newly formed prespore. Increased formation of SpoIIAA from its inactive phosphorylated derivative, instability of SpoIIAB, and transient genetic asymmetry are all factors that favor prespore-specific activation. But how does it all happen so quickly and so specifically? The activation of σ^E in the mother cell compounds the problem. Both σ factors are activated after septation and are needed to prevent a second septum being formed. Yet the second septum is thought to form within 10 minutes of the first if not prevented through the action of three σ^E-directed engulfment proteins. How does the entire path from σ^F to *spoIIR* to σ^E to *spoIID*, *M* and *P* become active so quickly yet remain so tightly regulated? Potentially, additional regulators await identification.

σ^G is very similar to σ^F in structure and is also inhibited by SpoIIAB. However, it has become clear that SpoIIAB does not regulate σ^G activation in the prespore. Activation requires completion of engulfment, and also expression of the *spoIIIJ* and *spoIIIA* loci. The critical link to engulfment remains unidentified. Completion of engulfment cuts the prespore off from contact with the medium. Might this result in a sudden oxygen, or energy, or other, deficiency in the prespore that somehow triggers σ^G activation? σ^K is similar to σ^E, and both are activated from a pro-σ, although the controls are very different (Figure 4). Processing has not been achieved *in vitro* for either, but pro-σ^K processing has been achieved in *E. coli*; this system offers some of the potential of *in vitro* analysis.

Specific protein localization plays an important role in development, yet the subject is only beginning to be studied in detail. Membrane proteins in the prespore, in general, localize to the septum whereas septal localization in the mother cell appears to require specific mechanisms. Preferential protein localization in bacteria is poorly understood; spore formation provides an excellent model to study the problem. Spore formation continues to provide insight into how the bacterial cell dynamically coordinates metabolism, gene expression, chromosome partitioning and morphogenesis during development.

Acknowledgements

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