Compartmentalization of Gene Expression during *Bacillus subtilis* Spore Formation

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INTRODUCTION

Cell differentiation is a fundamental biological process. Central to it are the coordination of gene expression with morphological change and the establishment of distinct programs of gene expression in the different cell types involved. Formation of spores by *Bacillus subtilis* is a primitive system of cell differentiation (Fig. 1), which has become a paradigm for the study of cell differentiation in prokaryotes (59, 183, 228, 231, 281). The spores formed are dormant and show greatly increased resistance to stresses such as heat and noxious chemicals compared to what is seen with vegetative cells. It was shown 25 years ago through a study of genetic mosaics that gene expression is compartmentalized during sporulation of *B. subtilis*, with different genes being expressed in the prespore and the mother cell, the two cell types involved (43, 226). In the years since, the completeness of compartmentalization has been demonstrated first by immunoelectron microscopy (48, 83, 189), then by fluorescence microscopy with immunofluorescence and green fluorescent protein (105, 170, 233, 299, 314), and most recently through the use of a two-part transcriptional probe (173).

The cell type-specific, compartmentalized programs of gene expression result from the cell type-specific activity of RNA polymerase sigma factors: σ^F and then σ^G in the prespore and σ^E and then σ^K in the mother cell (Fig. 2) (231). Compartmentalization of gene expression in each cell type is coupled to morphogenesis, with σ^F and σ^E becoming active after asymmetric division and σ^G and σ^K becoming active after engulfment of the prespore by the mother cell. Thus, the activation of σ^G and σ^K also represents compartmentalization in the sense of expression after but not before completion of engulfment. The key regulators of sporulation discussed below have been identified in all species of endospore former whose genomes have been sequenced, including the pathogens *Bacillus anthracis* and *Clostridium difficile* (277). Thus, conclusions from the study of *B. subtilis* are generally valid for members of the family *Bacillaceae* and illustrate general features of cell differentiation.

In this article we review the process of spore formation. We focus primarily on *B. subtilis* but include discussion of other species where we think it appropriate. We discuss in detail the genetic and biochemical experiments that have led to the discovery and characterization of cell-specific programs of gene expression. Since sporulation follows a distinct series of morphological and genetic stages, we detail steps in the order that they naturally occur, as though following a single cell through the entire developmental process. The cell-specific changes in gene expression that occur during sporulation are coupled to morphogenesis. The two major phases of compartmentalization are associated with two major morphological events, completion of septation and completion of engulfment. Consequently, we start with a brief description of the morphological changes during sporulation. We discuss in depth the events leading to the asymmetrically located sporulation division, which primes the organism for the compartmentalization of gene expression that follows the division. Compartmentalized gene expression is associated with the activation of the four sporulation-specific σ factors. We discuss the activation of each. We pay particular attention to regulation of σ^F because it is the first σ factor whose activity is compartmentalized during sporulation and because in vitro and in vivo analysis of its activation has progressed furthest.

Many of the loci discussed in this review were identified almost three decades ago as *spo* loci, because mutations in them blocked spore formation. Since then, our understanding of the roles of those loci has increased enormously. In general, *spo* loci encode proteins with unique roles in spore formation and, in some cases, in regulation of compartmentalization. They have provided the underpinning of much of our knowledge of compartmentalization. However, in the last decade it has become clear that there are also regulators, or regulatory mechanisms, which have overlapping rather than unique roles. The corresponding genes were generally missed in earlier studies because mutation in them had a comparatively mild effect on spore formation. Nevertheless, such overlapping regulatory mechanisms play an important part in the compartmentalization of gene expression, and they are an active area of research. For the main sections in the review, we use a historical approach in describing the development of our knowledge of
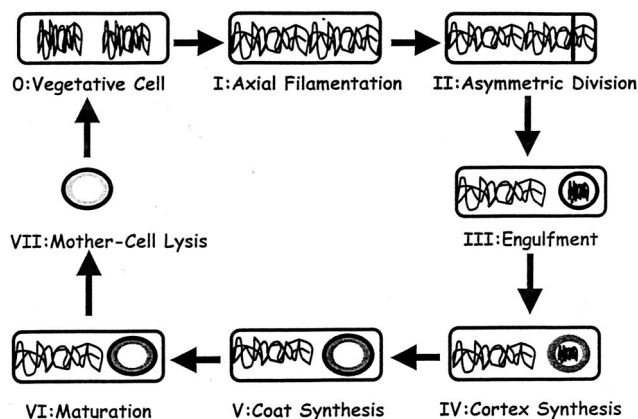
FIG. 1. Schematic representation of the stages of spore formation. A vegetatively growing cell is defined as stage 0. It is shown as having completed DNA replication and containing two complete chromosomes (represented as disordered lines within the cells), although replication is not completed at the start of spore formation. Formation of an axial filament of chromatin, where both chromosomes (or a partially replicated chromosome) form a continuous structure that stretches across the long axis of the cell, is defined as stage I. Asymmetric division occurs at stage II, dividing the cell into the larger mother cell and smaller prespore; for clarity, the septum is indicated as a single line. At the time of division, only approximately 30% of a chromosome is trapped in the prespore, but the DNA translocase SpoIIIE will rapidly pump in the remaining 70%. Stage III is defined as completion of engulfment, and the prespore now exists as a free-floating protoplast within the mother cell enveloped by two membranes, represented by a single ellipse. Synthesis of the primordial germ cell wall and cortex, a distinctive form of peptidoglycan, between the membranes surrounding the prespore is defined as stage IV and is represented as thickening and graying of the ellipse. Deposition of the spore coat, protective layers of proteins around the prespore, is defined as stage V. The coat is represented as the black layer surrounding the engulfed prespore. Coincident with coat and cortex formation, the engulfed prespore is dehydrated, giving it a phase-bright appearance, represented here as a light grey shading. Stage VI is maturation, when the spore acquires its full resistance properties, although no obvious morphological changes occur. Stage VII represents lysis of the mother cell, which releases the mature spore into the environment.

compartmentalization. We think that this historical approach is important for appreciating much of the present and past thinking about compartmentalization.

MORPHOLOGICAL STAGES OF SPORULATION

In order to discuss compartmentalization of gene expression, we first review the morphological changes in the developmental process, with which changes in gene expression are associated. Formation of heat-resistant spores from vegetative cells of *B. subtilis* takes about 7 h at 37°C. The morphological changes during sporulation were initially characterized by electron microscopy (145, 251). The basic sequence of changes is similar for all species of *Bacillus* and *Clostridium* that have been studied (79) and is illustrated in Fig. 1. Identification of successive stages by Roman numerals follows the convention introduced by Ryter (251) and now generally used. The vegetative cell is designated stage 0. Formation of an axial filament of chromatin, where two copies of the chromosome condense and elongate to form a filament that stretches across the long axis of the cell, is defined as stage I (21, 29, 308). Subsequently,

the cell divides at a subpolar site, resulting in the formation of two unequally sized daughter cells.

Completion of septation is designated stage II. At the time of asymmetric division, only approximately one-third of a chromosome is present in the smaller prespore (also called the forespore), but the remaining two-thirds is rapidly pumped in by the DNA translocase SpoIIIE (17, 303), resulting in two cells with unequal volumes but identical genomes. Next, the polar septum undergoes septal thinning, followed by bulging of the prespore into the mother cell and migration of the septal membrane around both sides of the prespore. When the migration is complete, the membranes fuse at the cell pole, pinch-
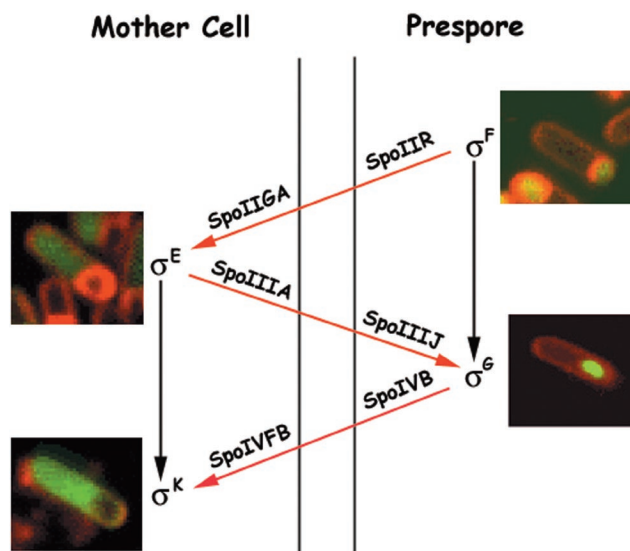

FIG. 2. Intercompartmental communication during sporulation. The parallel vertical lines represent the two membranes separating the prespore (right) from the mother cell (left). Diagonal red lines represent pathways of intercompartmental posttranslational activation, and vertical black arrows represent intracompartmental transcriptional activation. Fluorescent micrographs represent cells stained with FM4-64 (red) to visualize the cell membranes and expressing compartment-specific *gfp* fusions to *spoIIQ*, *spoIID*, *sspA*, and *gerE* for σ^F, σ^E, σ^G, and σ^K, respectively. The prespore membranes are not stained in the σ^G and σ^K images because engulfment is complete and the prespore membranes are now inaccessible to the lipophilic FM4-64 stain. σ^F, active in the prespore, is the first compartmentalized σ factor during sporulation. It triggers expression of SpoIIR, which activates the inferred receptor protease SpoIIGA, located in the asymmetric septum. Upon receipt of the signal, SpoIIGA processes the inactive precursor pro-σ^E into active σ^E in the mother cell. RNA polymerase with σ^E transcribes the *spoIIIA* operon, whose products then signal across the prespore membrane to activate σ^G, expressed in the prespore under the control of σ^F but held inactive by SpoIIAB (and probably other factors) until this signal is received. SpoIIIJ is also required for this signaling; although only required (and therefore only represented) in the prespore, it is expressed vegetatively and is presumably present in both compartments. Although not represented here, transcription of *spoIIIG* (encoding σ^G) requires an unknown signal from the mother cell as well as the SpoIIQ protein, expressed in the prespore under the control of σ^F. Once σ^G becomes active, it causes expression of SpoIVB, which is inserted into the inner prespore membrane. SpoIVB triggers processing of pro-σ^K, which is synthesized in the mother cell from the σ^E-directed *sigK* gene. The processing enzyme is thought to be SpoIVFB, which also expressed in the mother cell under the control of σ^E but does not act upon pro-σ^K until it receives the SpoIVB signal from the prespore.

ing off the prespore and releasing it within the mother cell as a free-floating protoplast that is surrounded by two membranes, one derived from each of the two cells; completion of engulfment is designated stage III (227). Engulfment is followed by the deposition of two peptidoglycan layers, the primordial germ cell wall and the cortex, in the space between the membranes surrounding the prespore (stage IV) (81). Following this deposition, a complex structure of proteins on the outside surface of the prespore, known as the coat, is constructed (stage V) (47, 109). This stage is followed by maturation of the spore (stage VI), when it gains resistance to UV radiation and high temperature (208). Lastly, the mother cell lyses (stage VII), releasing the mature spore into the environment. Germination and outgrowth, followed by a resumption of the vegetative growth cycle, occur when the spore finds itself in a nutrient-rich environment (213). Sporulation mutants are denoted by the stage in the process at which they are blocked (e.g., *spoII* mutants complete asymmetric septation but fail to complete engulfment). The names for sporulation loci include the stage of blockage caused by mutation and a distinguishing letter designation (e.g., *spoIIA*) (228, 231).

The profound morphological changes that occur during sporulation are coupled to global changes in gene expression, which are effected by activation of alternative RNA polymerase σ factors (Fig. 2) (228, 231). Activation of σ^H (and the response regulator Spo0A) in the predivisional cell leads to expression of factors important for axial filament formation, asymmetric division, and compartmentalization of gene expression. Immediately after asymmetric division, σ^F becomes active in the prespore, rapidly followed by activation of σ^E in the mother cell. The separate lines of gene expression drive engulfment of the prespore by the mother cell and result in synthesis of the late-compartment-specific σ factors. Upon completion of engulfment, σ^G becomes active in the prespore and σ^K becomes active in the mother cell. Coat and cortex synthesis, spore maturation, and mother cell lysis are driven by these late stages of cell-specific gene expression. Each step is dependent upon completion of all of the previous steps except axial filament formation (see below).

INITIATION OF SPORULATION

Gene expression becomes compartmentalized immediately after the spore septum has formed. To understand how this compartmentalization happens, it is important to explore the events leading to it. In this section we briefly discuss the activation of the master sporulation response regulator Spo0A and the alternative σ factor σ^H. For more focused reviews on the initiation of sporulation, we refer the reader to references 27 and 223.

The Phosphorelay

Spo0A is the master regulator for entry into spore formation. It is activated by the phosphorelay, a more complex version of the classic two-component system (26). In turn, Spo0A-PO$_4$ activates transcription of genes required for axial filament formation and for asymmetric division. It also activates transcription of the genes encoding the early compartmentalized σ factors, σ^F and σ^E, as well as their regulators. Sporulation is

initiated in response to a number of external and internal signals that are integrated into the phosphorelay, including signals for nutrient starvation, cell density, and cell cycle progression (27, 223, 291).

At least five kinases are involved in the phosphorelay: KinA, KinB (290), KinC (160), KinD (134), and KinE (67), of which KinA and KinB are the primary kinases for initiation of sporulation. In response to unidentified stimuli, they autophosphorylate and then donate their phosphate groups to the response regulator Spo0F. Spo0F lacks an output domain and is incapable of activating transcription; it serves only as an intermediary in the phosphorelay. The phosphotransferase Spo0B transfers the phosphate from Spo0F-PO$_4$ to Spo0A (26). The phosphorelay is also subject to negative regulation: for example, the phosphatases Spo0E, YisI, and YnzD dephosphorylate Spo0A, thereby preventing its activation (210, 221). In order to ensure that sporulation occurs only under the appropriate conditions, the phosphorelay must integrate different intracellular and extracellular signals. The mechanisms responsible for this integration are described below.

Cell density. Efficient sporulation requires high cell density (101). When cell density is low, the Rap (response regulator aspartyl phosphatase) proteins RapA, RapB (222), and RapE (133) dephosphorylate Spo0F-PO$_4$, preventing Spo0A activation. The *rapA* and *rapE* genes are cotranscribed with a downstream open reading frame encoding the signaling peptide precursors PhrA and PhrE, respectively, which are processed and exported out of the cell. As cell density increases, the processed peptides are imported by the oligopeptide permease (Opp) and inhibit the activity of RapA and RapE; similarly, the processed product of PhrC (CSF [competence- and sporulation-stimulating factor]) inhibits RapB. Inhibition of the Rap proteins prevents dephosphorylation of Spo0F-PO$_4$ and allows phosphorylation of Spo0A and the initiation of sporulation when cell density is high (133, 220).

Nutrient starvation. In addition to high cell density, nutrient starvation is also required for the initiation of sporulation. A dramatic drop in the concentration of GTP and GDP correlates with the onset of sporulation, and inhibition of GMP synthesis by decoyinine treatment induces sporulation in the absence of nutrient starvation (200). CodY has recently been identified as the key sensor of guanine nucleotide levels. Disruption of the *codY* gene allows sporulation to occur in the presence of excess nutrients, and the ability of the CodY repressor to bind DNA correlates with the GTP concentration. As a consequence, when GTP levels drop upon entry into stationary phase, CodY-regulated genes are derepressed (239). Microchip array analysis has identified *phrA*, *phrE*, and *kinB*, all positive regulators of the phosphorelay, as targets of CodY repression (204). Therefore, one way that nutrient starvation is integrated into the decision to sporulate is transcriptional regulation of phosphorelay components via CodY. In addition, sporulation is also subject to catabolite repression (117) and requires a functioning Krebs cycle (131), although the molecular basis of these dependencies is unknown.

Cell cycle. In addition to factoring extracellular conditions such as cell density and nutrient availability into the decision to sporulate, the intracellular environment is monitored as well. Damage to DNA and blocking of either the initiation or progression of DNA replication prevent the initiation of sporula-

tion (126, 128, 129, 132, 165). These conditions lead to the expression of Sda (suppressor of *dnaA*) because of the presence of binding sites in the *sda* promoter region for the repressors DnaA and LexA, which no longer repress when DNA replication is blocked or DNA is damaged, respectively. Sda impairs KinA autophosphorylation, blocking the phosphorelay (28). As a result, a developmental checkpoint is established that only allows cells with undamaged, replicating chromosomes to proceed into development.

An additional mechanism is thought to link chromosome partitioning status to sporulation. Spo0J and Soj (suppressor of *spo0J*) (also known as Spo0JB and Spo0JA, respectively) are members of the plasmid-partitioning families of proteins ParB and ParA, respectively (130). Spo0J colocalizes to cell poles with the chromosomal origin of replication (175), binds to sites near the chromosomal origin (174), and is required for optimal efficiency of chromosome segregation (130). In the absence of Spo0J, Soj binds to the promoter regions of at least four Spo0A-responsive genes (*spo0A*, *spoIIA*, *spoIIE*, and *spoIIG*) and represses their transcription (33, 191, 237, 238). This effect appears to be mediated by dynamic protein localization; when Spo0J is present, Soj oscillates between sites near the poles of the cell, presumably preventing stable DNA-protein interaction and transcriptional repression. However, in the absence of Spo0J, Soj remains static and represses developmental transcription (191, 238). Although it is tempting to speculate that Spo0J and Soj sense chromosome partitioning status and regulate the initiation of sporulation accordingly, direct evidence for this model is lacking. However, consistent with a role in monitoring the cell cycle, recent studies have linked these proteins to cell division, to initiation of DNA replication, and to axial filament formation (11, 163, 209, 308).

Spo0A Regulon

The sum of all of these interactions determines if enough Spo0A-PO$_4$ has been generated to initiate sporulation. Spo0A-PO$_4$ can either activate or repress transcription by binding to a 7-bp sequence, TGNCGAA, where N is any nucleotide, in or near promoters recognized by the vegetative σ factor σ^A and the alternative σ factor σ^H (223). This binding results in global changes in gene expression, altering the expression profile of over 500 genes, which represent approximately one-eighth of the total genes in *B. subtilis* (71). Additional genomic analysis has revealed that 121 of these genes are under the direct control of Spo0A, with approximately one-third being positively regulated and the remainder being negatively regulated; 25 of the regulated genes are themselves transcription factors, indicating that many of the transcriptional changes caused by Spo0A are indirect (203).

A number of key *spo* loci are directly positively regulated by Spo0A: the *spoIIA* and *spoIIG* operons, encoding the prespore- and mother cell-specific transcription factors σ^F and σ^E, respectively; and *spoIIE*, encoding a bifunctional protein phosphatase that is required for asymmetric division and σ^F activation (231). In addition, the gene encoding the effector of axial filament formation, *racA*, is under the control of Spo0A (21, 308). Thus, Spo0A activates the synthesis of factors required for chromosome remodeling, asymmetric division, and the compartmentalized gene expression that immediately fol-

lows. The *racA* gene was identified by functional analysis of the Spo0A regulon (21), and such analyses will most likely reveal additional genes involved in sporulation.

Role of σ^H

In addition to the phosphorelay and the main vegetative σ factor σ^A, the transition-state regulator σ^H is also required for the initiation of sporulation. Regulation of σ^H synthesis and activity is not well understood but involves both posttranscriptional and posttranslational mechanisms (106, 177). σ^H regulates a number of phosphorelay genes: σ^H-dependent transcription of *spo0A* is essential for sporulation (270), and *kinA*, *kinE*, and *spo0F* also have σ^H-dependent promoters (23, 236). In addition, transcription of several *phr* genes encoding peptide precursors, some of which function to reverse phosphorelay inhibition by Rap phosphatases, is also dependent upon σ^H (197). Other σ^H-regulated genes are important for later events in sporulation: σ^H-dependent transcription of the essential cell division operon *ftsAZ* is required for efficient asymmetric septation (19, 94, 98), and the *spoIIA* operon, which encodes the prespore-specific transcription factor σ^F and its regulators, is under the control of σ^H (302). A substantial portion of the σ^H and Spo0A regulons overlap: for example, *spoIIA* and *racA* are regulated by both transcription factors. The σ^H regulon has recently been characterized by microchip array analysis (23), and functional analysis of this regulon has resulted in the independent identification of *racA* (308).

AXIAL FILAMENT FORMATION

There are several substantial differences in the events associated with the sporulation division compared with those associated with the vegetative division (114, 227). Any or all may be important for priming the compartmentalization of gene expression which follows that division. The first is the formation of an axial filament of chromatin, in which both chromosomes in the predivisional cell elongate into a filament that stretches the length of the long axis of the cell. This structure was characterized by electron microscopy (78, 188, 252) and later by fluorescence microscopy (29, 300).

Genetic Control

Standard genetic analysis failed to identify effectors of axial filament formation, and only recent genomic and cell biological studies have allowed identification of the components involved. Despite their name, none of the classic *spo0* mutations clearly prevented axial filament formation (228): *spo0H* mutants formed axial filaments (29, 94), whereas *spo0A*, *spo0B*, and *spo0F* mutants underwent an additional symmetric division during sporulation, and it was not clear if that was preceded by axial filament formation (29, 53). Some insight into the process was derived from analysis of the SMC (structural maintenance of chromosomes) protein, required for chromosome compaction and partitioning (25, 176). Mutants lacking this protein are able to activate Spo0A but are unable to form axial filaments (99), suggesting that an effector of axial filament formation is either missing or nonfunctional in this background. It was also found that asymmetric division did not
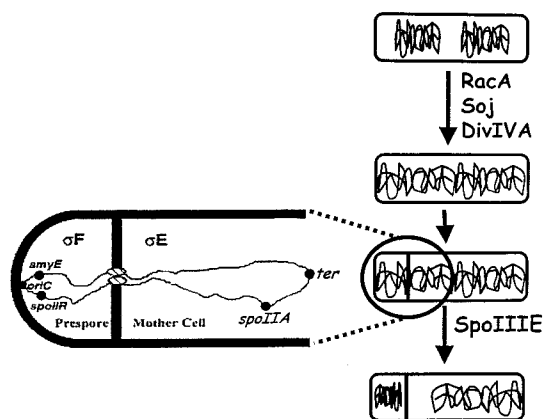
FIG. 3. Chromosome partitioning and genetic asymmetry. A single cell progressing through sporulation is represented on the right. Disordered internal lines represent the nucleoids. The earliest (topmost) cell is drawn as having two complete chromosomes, although it may contain one partially replicated chromosome. Through the action of DivIVA, RacA, and Soj, the two complete chromosomes (or the partially replicated chromosome) are remodeled into an axial filament that extends across the long axis of the cell, represented in the second cell. After asymmetric division occurs, the prespore contains only the origin-proximal one-third of a chromosome, whereas the mother cell contains one complete chromosome and two-thirds of another; this partitioning results in transient genetic asymmetry between the mother cell and the prespore. For simplicity, the septum is represented as a single line. A portion of the third cell has been expanded in order to represent the asymmetry more clearly; the hatched ovals represent the DNA translocase SpoIIIE; the locations of several genetic loci are noted; and σ^F is depicted as being active in the prespore and σ^E is depicted as being active in the mother cell. Within about 15 min of the asymmetric division, SpoIIIE pumps the remaining two-thirds of the prespore chromosome into this compartment, restoring genetic symmetry.

occur in the absence of axial filament formation, suggesting the existence of a checkpoint that functioned to couple the two events (99). Such a checkpoint would ensure that asymmetric division would trap the origin-proximal region of a chromosome in the prespore, providing a template for transcription by E-σ^F (RNA polymerase core enzyme, E, associated with σ^F) and an anchor for chromosome translocation into this compartment

DivIVA. Several lines of investigation provided insight into the genetic control of axial filament formation. The first was a study of the DivIVA protein of *B. subtilis*, considered the functional homologue of *Escherichia coli* MinE in that it restricts the division-inhibition proteins MinCD to the cell poles and so ensures mid-cell division during vegetative growth (34, 56). *divIVA* mutants have severe growth and sporulation defects, but the isolation of mutants specifically defective in sporulation suggested that DivIVA played a dedicated role in development (Fig. 3). These mutants frequently formed anucleate prespores (287), indicating that the proposed checkpoint coupling axial filament formation and asymmetric division (99) had been disrupted.

RacA. Genomic approaches made it possible to elucidate the role that DivIVA played in axial filament formation. One approach identified Spo0A-regulated genes by microchip array (71), systematically disrupted them, and screened the mutants for changes in chromosome segregation (21). In a separate study, σ^H-regulated genes were identified by microchip array,

and candidate genes for partitioning function, including those predicted to have DNA-binding domains, were disrupted and characterized (308). Both approaches resulted in identification of a gene that, when disrupted, caused an anucleate prespore phenotype. The encoded protein, RacA (remodeling and anchoring of the chromosome A), was found to bind nonspecifically to the chromosome and also to the pole of the cell, acting as a bridge connecting the two. Localization of RacA to the cell pole is dependent upon DivIVA, explaining why mutations in the corresponding genes cause similar phenotypes, although those caused by mutations in *divIVA* are more severe (21, 287, 308). From their observations, Ben-Yehuda and colleagues (21) proposed that once the origin-proximal region of the chromosome reaches the pole of the cell, RacA binds DivIVA and displaces the division inhibitor MinCD (192, 295), triggering asymmetric division (21).

Although the prospect of a checkpoint linking axial filament formation to asymmetric division is exciting, it still requires direct testing. The original observation that *smc* mutants fail to form axial filaments remains unexplained (99); it may be that RacA cannot bind to the uncondensed and disorganized chromosomes of *smc* mutants (25, 176). *racA* and *divIVA* mutants also undergo asymmetric division even though axial filament formation is impaired (21, 287, 308), whereas the *smc* mutant is deficient in both processes (99). Two plausible explanations are that RacA and DivIVA prevent asymmetric division in the *smc* mutant, enforcing the checkpoint proposed by Graumann and Losick (99) (see above) or that defects in asymmetric division and axial filament formation are independent in this background, placing the existence of this checkpoint in doubt. Epistasis tests could help distinguish between these possibilities. For example, in the former case, *racA* and *divIVA* mutations should restore asymmetric division to an *smc* mutant, whereas in the latter case they should not. More work is needed to explore the relationship between axial filament formation and asymmetric division.

Soj. The fact that *racA* mutations cause a less severe defect than *divIVA* mutations (21, 287, 308) suggested that at least one additional factor was involved in axial filament formation. Indeed, it was found that a mutant lacking RacA and the transcriptional repressor Soj (33, 191, 238) displayed a phenotype approximating the more severe phenotype caused by a *divIVA* mutation (308), a surprising result because no partitioning function had previously been attributed to Soj. However, such a phenotype could be mediated by its interaction partner, Spo0J, which is required for optimal efficiency of vegetative chromosome partitioning (130) and localizes to the origin region of the chromosome (174, 175). The RacA-Soj system is an example of redundancy in sporulation controls and is especially noteworthy because it led to the attribution of a novel function to Soj, one that was unlikely to be observed in a *racA*$^+$ genetic background.

Polar localization region. In a separate study of axial filament formation, the use of systematic chromosomal inversions identified a polar localization region, located ≈150 to 300 kbp from the origin of replication, that is required for efficient trapping of DNA in the prespore (307). Although an attractive hypothesis is that this region is the principal binding site for RacA, chromatin immunoprecipitation experiments suggest that a different region (60 to 80 kbp from the origin) is pref-

erentially bound (21). As a result, the relationship of the polar localization region to the RacA-Soj-DivIVA system remains unclear.

ASYMMETRIC DIVISION

Once a cell has formed the axial filament, the next major event in sporulation is asymmetric division. Since the cell normally divides at mid-cell with remarkable accuracy (198), this switch requires a dramatic relocalization of the cell division apparatus (61). By dividing at a polar site, the cell becomes genetically and morphologically asymmetric, and the asymmetry leads to different cell fates. The asymmetric division is critical to the establishment of compartmentalized gene expression. The division has much in common with vegetative division (61, 114), but the distinguishing features are presumptively ones that might lead to compartmentalization of gene expression. It is the distinguishing features that are considered here.

FtsZ Ring Switching

Dynamic repositioning. During vegetative growth, the essential prokaryotic tubulin homologue FtsZ forms a ring (the Z ring) at mid-cell, where division subsequently occurs (298). However, during sporulation, activation of Spo0A triggers the formation of Z rings near both poles of the cell (167). The switch has recently been characterized by deconvolution microscopy. The use of this technique revealed that, during sporulation, a Z ring initially forms at mid-cell but the FtsZ then redeploys to sites near both poles through the formation of a dynamic helical intermediate (19). This intermediate resembles the helical structures formed by two bacterial actin homologues, Mbl and MreB (137); one possibility is that FtsZ relocates by tracking along these structures.

Genetic control. Early studies showed that the *ftsAZ* operon contained three promoters, one of which was activated during sporulation by σ^H (94, 98). However, deletion of this promoter had only a moderate effect on asymmetric division (98). Similarly, disruption of the *spoIIE* locus, encoding a critical activator of σ^F in the prespore (8, 49), also had a moderate effect on polar Z ring formation (19, 149) and asymmetric division (16, 228). However, simultaneous ablation of *spoIIE* and the σ^H-dependent promoter of *ftsAZ* resulted in severe impairment of polar Z ring formation and asymmetric division. Conversely, if *ftsAZ* overexpression was combined with expression of *spoIIE*, asymmetric division could be triggered during vegetative growth (19). Therefore, *spoIIE* induction and increased *ftsAZ* expression play overlapping roles during sporulation in ensuring polar Z ring formation and asymmetric division.

SpoIIE. The discovery of a role for SpoIIE in polar Z ring formation (19, 149) reinforced previous ultrastructural studies that had implicated this protein in asymmetric division. These studies found that null mutations in *spoIIE* resulted in rare asymmetric septa that were aberrantly thick, whereas several point mutations blocked sporulation but allowed the formation of typical sporulation septa at normal frequency (16, 228). Consistent with this role, it was found that SpoIIE localizes to asymmetric division sites (10, 14) in an FtsZ-dependent manner (168). FtsZ and SpoIIE also interact in vitro and in yeast

two-hybrid assays (186). However, how this interaction assists polar Z ring formation and asymmetric division is unknown. Some possibilities include anchoring an end of the FtsZ helix to the cell membrane, antagonizing MinCD near the pole of the cell, and recognizing a polar marker (19). In addition to SpoIIE and increased *ftsAZ* expression, there is evidence that MinCD and SpoVG may play minor roles in selecting the asymmetric division site (15, 195).

Abortively Disporic Phenotype

In *spo*$^+$ strains, surface annular structures (cloisons) and Z rings appear at both polar sites (19, 167, 251), indicating that the cell has two potential polar division sites. However, a septum is normally formed at only one of these sites (251). In certain mutants, both potential polar division sites are utilized, resulting in a three-chambered organism consisting of two smaller prespores separated by a larger central compartment. This is the abortively disporic phenotype, which is associated with mutations in some *spoII* loci (228, 252). Mutants that demonstrate the abortively disporic phenotype can initiate sporulation but have defects in activating the mother cell-specific transcription factor σ^E (124). Three proteins expressed in the mother cell under σ^E control, SpoIID, SpoIIM, and SpoIIP, are required to prevent the second asymmetric division (57, 232). Although it was puzzling why *B. subtilis* would generate two potential division sites during sporulation, recent studies have provided some insight. Cells with anucleate prespores are frequently observed in *racA* mutant cells, which are deficient in axial filament formation. However, a substantial proportion of these cells undergo a second asymmetric division at the other end of the cell, and if they successfully capture DNA in the second prespore, they form spores (21, 308). This result suggests that the ability to divide at both asymmetric division sites is a failsafe mechanism to ensure successful sporulation even in the absence of axial filament formation.

Which End of the Cell?

An unanswered question is how the cell determines at which end of the cell it will divide. Although FtsZ rings form at both potential polar division sites, the next known protein to assemble, FtsA, localizes to only one of them (76), indicating that FtsA may play some role in selecting which of the two potential division sites is utilized. Chromosome segregation into the prespore or mother cell appears to be essentially random with respect to time of replication, and so chromosome age is presumably not a factor in determining which end becomes the prespore (43, 65). In most circumstances and in a range of species, spores are formed almost exclusively at the older pole of the cell, clearly suggesting that the pole is a determinant of asymmetry and compartmentalization (52, 112, 113). However, when the sporulation procedure involves centrifugation (with a force of perhaps 5,000 × g) and 25 mM Mg^{2+}, spore position is essentially random with respect to pole age (52). Thus, polar determinism can be lost without losing asymmetric division or compartmentalization.

Differences between Sporulation Septum and Vegetative Division Septum

The asymmetrically located sporulation division is often considered the defining early morphological event in sporulation. The machinery for asymmetric division is similar to that used for vegetative division (61). However, there are several distinctive features of the sporulation division (114, 227) in addition to those described above. Since the division is critical to the compartmentalization of gene expression that follows, it is useful to summarize those distinctive features. (i) The sporulation division septum is much thinner than the vegetative division septum. (ii) The two cells that result from the sporulation division do not separate from each other, as occurs following vegetative division. Rather, the mother cell engulfs the prespore. (iii) Autolysis of the wall material (peptidoglycan) within the sporulation septum begins in the center of the septum, and ultimately there is apparently complete loss of wall material. In contrast, autolysis of the wall material of the vegetative septum begins at the periphery of the septum and proceeds inwards. Moreover, there is little loss of wall material—the split septum provides the wall for the poles of the nascent cells (227). (iv) Prior to the sporulation division, the two chromosome origins and associated proteins move to the extreme poles of the cell rather than to a subpolar location, as in vegetative division (21, 175, 300, 308). (v) The septum is asymmetrically located, with respect to the cell poles, during sporulation but not during vegetative growth (251) (vi) Several proteins become associated with the sporulation septum that are not associated with the vegetative division septum (10, 14, 72). (vii) Complete partitioning of a chromosome into the prespore occurs after septation (303, 310), so that there is genetic asymmetry between the prespore and the mother cell when they are first formed (54, 84, 305). (viii) After the sporulation division, different programs of gene expression are initiated in the two daughter cells (231). These programs are driven by activation of cell-specific σ factors that direct RNA polymerase to transcribe different genes, which encode factors responsible for establishing the very different fates of the prespore and the mother cell.

TRANSFER OF DNA INTO THE PRESPORE

At this stage of sporulation, the two chromosomes have extended into a filament stretching along the long axis of the cell, with their origin regions near the poles (21, 300, 308). The cell divides near one of the poles, trapping the origin-proximal one third of a chromosome in the smaller prespore and leaving one chromosome and two thirds of another in the larger mother cell (Fig. 3) (305). As a consequence, the two cells are now genetically asymmetric. This asymmetry has important implications for compartmentalization of gene expression (54, 84), as discussed below. The developing organism also has a major challenge in that it must ensure that the prespore compartment receives a complete chromosome The transfer of the origin-distal two-thirds of a chromosome from the mother cell into the prespore is concomitant with the activation of different transcription factors in the prespore and mother cell. Although both of these events are thought to occur simultaneously, for the sake of clarity we will first discuss the problem of DNA

transfer and then turn to the compartmentalization of gene expression.

The critical locus for chromosome translocation is *spoIIIE*. In *spoIIIE* mutants, the origin-distal two-thirds of a chromosome remains trapped in the mother cell (305). In *spo*⁺ strains, the remaining DNA is actively pumped across the asymmetric septum from the mother cell into the prespore (303, 310). The DNA translocase SpoIIIE localizes to the center of the sporulation septum (264, 304), apparently by anchoring to the chromosome (20). SpoIIIE then uses ATP to transport the chromosome into the prespore (Fig. 3) (17).

The question remains why DNA is transported only from the mother cell into the prespore. Does the location of the chromosome origin in the prespore determine the direction of transfer? Or is it the orientation of SpoIIIE in the center of the spore septum, toward or away from the prespore? When discussing this question, it is important to note that SpoIIIE is widely conserved in nonsporeformers (267), is expressed vegetatively in *B. subtilis* (82), and is involved in chromosome partitioning in circumstances other than sporulation. For example, SpoIIIE removes trapped nucleoids from minicells, prevents chromosome bisection when DNA replication is transiently inhibited (267) and when cells lack SMC (24), and is required for efficient partitioning in mutants defective in terminating DNA replication or resolving chromosome dimers (164). These situations require either bidirectional movement or transfer out of the smaller of two cells (the minicell), suggesting that SpoIIIE lacks an inherent polarity.

Two recent studies have attempted to address the question of the polarity of SpoIIIE during spore formation by synthesizing SpoIIIE exclusively in either the prespore or mother cell (36, 265). Both laboratories agree that expression of *spoIIIE* only in the mother cell is sufficient to obtain DNA translocation into the prespore and substantially to restore spore formation. However, they disagree about the effect of expression only in the prespore. There were a number of technical differences between the two studies that could account for their different conclusions. As a consequence, more work may be necessary to fully understand how polarity of DNA transfer by SpoIIIE is regulated.

COMPARTMENTALIZATION OF GENE EXPRESSION

After axial filament formation and asymmetric division and concomitant with DNA transfer by SpoIIIE, different programs of gene expression are established in the prespore and the mother cell. These programs are directed by cell-specific σ factors whose activation is coupled to landmark morphological events. Asymmetric division triggers the activation of σ^F in the prespore, followed by activation of σ^E in the mother cell. Later in sporulation, the completion of engulfment of the prespore by the mother cell leads to the activation of σ^G in the prespore and σ^K in the mother cell. Therefore, in addition to complete spatial compartmentalization between prespore and mother cell, sporulation gene expression is also divided into temporal (pre- and postengulfment) phases. Throughout the intermediate and late stages of sporulation, the mother cell and prespore communicate with each other, sending and interpreting biochemical signals to ensure that their genetic programs are coordinated (Fig. 2).

In the sections that follow, we briefly review the historical development of evidence that gene expression is indeed compartmentalized. We then focus on the activation of the particular σ factors that have been shown to direct the compartmentalized gene expression. It is presumed that those activation mechanisms hold the key to why activation is compartmentalized. Whereas we now consider the evidence that gene expression directed by the different σ factors is compartmentalized to be compelling, our understanding of the mechanisms of compartmentalization is still incomplete. A variety of regulators of σ activation have been identified. Some of the regulators have an essential function in spore formation, so that their mutational inactivation eliminates spore formation. However, other regulators appear to have partially or completely overlapping functions, so that mutational inactivation of only one regulator may have little or no effect on spore formation. Regulators of both types may be critical for compartmentalization of the activity of the different σ factors.

Developing Evidence that Gene Expression Is Compartmentalized

The different fates of the prespore and the mother cell suggested that different genes are expressed in the two compartments. This suggestion was supported by biochemical characterization of extracts enriched for the contents of the prespore or the mother cell (5, 55, 88, 269). The first clear evidence that expression of *spo* loci was compartmentalized came from the study of genetically mosaic bacteria (43, 226). The mosaics were obtained by transforming *spo* mutants at the start of sporulation so that only one of the two copies of the chromosome became *spo*⁺. After division, mutant and wild-type alleles were distributed randomly into the prespore and the mother cell. Since the mother cell is destroyed and only the chromosome in the prespore is inherited upon spore germination, it was possible to infer the location of *spo* locus expression. For several loci, the spores obtained gave rise to *spo* mutant progeny, indicating that only the mother cell chromosome required a *spo*⁺ allele for sporulation to occur. Other loci that were tested yielded only *spo*⁺ spores, indicating that, for sporulation to occur, the allele on the prespore chromosome had to be transformed to *spo*⁺ (43, 226, 230). Subsequently, determination of β-galactosidase activity in prespore- and mother cell-enriched extracts from strains expressing *spo-lacZ* transcriptional fusions provided strong support for differential gene expression between mother cell and prespore (reviewed in reference 59).

Direct evidence that the expression of particular genes was completely compartmentalized was obtained by the use of immunoelectron microscopy with antibodies to small acid-soluble proteins (SASPs), which revealed that these proteins are found exclusively within the prespore (83). The utility of this technique was expanded by using antibodies to β-galactosidase on samples from strains expressing *spo-lacZ* transcriptional fusions (48, 189). The experiments demonstrated that the activities of σ^F and σ^G were confined to the prespore and those of σ^E and σ^K were confined to the mother cell (48, 83, 189).

Although informative, immunoelectron microscopy is time-consuming and difficult and suffers from low sensitivity. The study of compartmentalization took a leap forward through the use of immunofluorescence microscopy and then fluorescence microscopy of cells expressing transcriptional fusions to *gfp* (encoding green fluorescent protein [GFP]). These techniques provided greater sensitivity and ease of use than electron microscopy, and GFP studies have the added advantage that living cells can be analyzed. The use of these techniques demonstrated that σ^F and σ^E became active very soon after completion of septum formation and that the activities were completely compartmentalized into the prespore and mother cell, respectively, within the limits of detection. Likewise, they demonstrated that σ^G and σ^K became active very soon after completion of engulfment, also completely compartmentalized into the prespore and the mother cell, respectively (105, 169, 233, 299, 314). A two-part transcription probe provided a very different type of evidence for the completeness of compartmentalization and also provided evidence that the vegetative σ factor σ^A continues to be active in both prespore and mother cell throughout sporulation (173).

ACTIVATION OF σ^F

spoIIA Operon

Studies of the *spoIIA* locus have been critical to our understanding of compartmentalization of gene expression. The locus was found to be a tricistronic operon (80, 229) that was transcribed prior to asymmetric septation (93, 218) in a Spo0A- and σ^H-dependent manner (290, 301, 302). When the *spoIIA* operon was sequenced, none of the open reading frames bore obvious similarity to any genes in the limited database of the time. However, shortly thereafter, it became clear that the third gene in the operon, *spoIIAC*, encoded a product that was homologous to an RNA polymerase σ factor (62, 274), named σ^F (183). The first targets identified for E-σ^F action were *spoIIIG* and *gpr* (282, 285), and the site of expression of *spoIIIG* was shown to be the prespore (93, 142, 189), as it has been for all E-σ^F-directed genes analyzed subsequently (231).

Posttranslational Regulation of σ^F

Although the *spoIIA* operon is expressed prior to asymmetric division (93, 218), σ^F does not become active until after asymmetric division (93, 142). It seemed likely that σ^F was subject to some form of posttranslational regulation, and genetic analysis revealed that the other two products of the *spoIIA* operon, SpoIIAA and SpoIIAB, regulate its activity. Thus, overexpression of SpoIIAB inhibited σ^F activity, and mutation of SpoIIAB increased σ^F activity. Mutation of SpoIIAA blocked σ^F activation, but activity could be restored to these strains by mutation of SpoIIAB. Taken together, these results indicated that SpoIIAB antagonized σ^F, and that in turn, SpoIIAA might antagonize SpoIIAB (260). Consistent with an inhibitory role for SpoIIAB, a separate study showed that mutation of SpoIIAB caused hyperactivity of σ^F, blocked sporulation prior to asymmetric septation, and caused extensive lysis (38). Given that the activity of σ^F was confined to the prespore during sporulation (189), it was hypothesized that its regulation by SpoIIAA and SpoIIAB was responsible for compartmentalization.

Biochemical analysis of the interactions between members

of the regulatory pathway began to shed light on the mechanism of σ^F regulation. It was shown that the negative role of SpoIIAB was direct in that it bound σ^F, thus acting as an anti-sigma factor; SpoIIAA antagonized the action of SpoIIAB and so is an anti-anti-sigma factor (51, 199). SpoIIAB bore significant similarity to protein kinases, and it was found to phosphorylate SpoIIAA on a serine residue at position 58 (199, 207). Mutation of this serine residue to an alanine (mimicking dephosphorylation) resulted in constitutive σ^F activity, and mutation to an aspartic acid (mimicking phosphorylation) blocked σ^F activity in vivo (44), indicating that the phosphorylation state of SpoIIAA is critical for its ability to function as an anti-anti-sigma factor. Therefore, SpoIIAB inhibits σ^F both directly, as an anti-σ factor, and indirectly, by inactivating the anti-anti-σ factor SpoIIAA. SpoIIE, a membrane-bound serine phosphatase, dephosphorylates and thus activates SpoIIAA (8, 49). Therefore, it reverses the inactivation of SpoIIAA by SpoIIAB and promotes activation of σ^F. In addition, SpoIIE localizes to asymmetric division sites (10, 14), suggesting that it may mediate a link between asymmetric division and prespore-specific gene expression. The basic model of σ^F activation is illustrated in Fig. 4A.

Mechanisms of Compartmentalization

Although the known factors involved in σ^F regulation have been identified, it is still not clear why σ^F activity is confined to the prespore. Models of compartmentalization have evolved over time; we will briefly review them in roughly the order that they were proposed. Although some of them are no longer widely accepted, we think that a discussion of their supporting data and potential flaws is useful.

ATP/ADP ratio. Early in vitro experiments indicated that SpoIIAB bound σ^F in the presence of ATP, whereas it bound SpoIIAA in the presence of ADP (3, 44, 50). This result suggested that different concentrations of these nucleotides in the two compartments might be responsible for compartmentalization of σ^F activity to the prespore. However, it was not clear how the proposed differential ATP/ADP ratio was established in vivo, and direct evidence for the regulatory roles of these nucleotides was lacking. Subsequent in vitro studies of the interaction between these factors suggested that the ATP/ADP ratio was not a critical factor in determining the partner to which SpoIIAB bound (187).

Preferential inheritance. As an alternative mode of regulation, it was proposed that a factor critical to σ^F activation was preferentially segregated to the prespore during sporulation. As evidence, it was reported that in protoplasts derived from sporulating cells expressing a SpoIIE-GFP fusion protein, the fluorescent signal was found in protoplasts derived from prespores and not from mother cells; it was inferred that SpoIIE became located predominantly in the prespore (309). However, a different study in which similar experiments were performed along with careful computerized quantitation determined that the total fluorescent signal was very similar in the prespore and in the mother cell. Therefore, it was concluded that the previous result was the consequence of similar amounts of protein being present in two compartments of dramatically different sizes. In addition, time-lapse microscopy of living cells clearly demonstrated that SpoIIE-GFP is
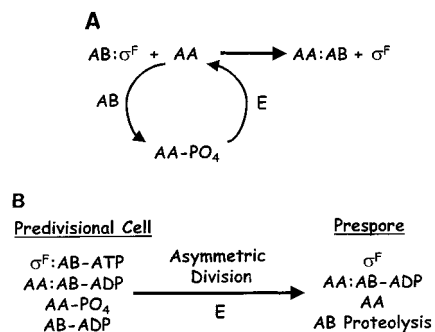

FIG. 4. Models of σ^F regulation. AA, AB, and E refer to SpoIIAA, SpoIIAB, and SpoIIE, respectively. The anti-σ factor SpoIIAB binds σ^F as a dimer but is represented here as a monomer for simplicity. (A) Basic model of σ^F regulation. The anti-σ factor SpoIIAB binds σ^F and holds it inactive. This inhibition can be reversed by the anti-anti-σ factor SpoIIAA. SpoIIAA is subject to regulation by its phosphorylation state; it is inactive when phosphorylated by SpoIIAB (a serine kinase as well as an anti-σ factor) and active when dephosphorylated by SpoIIE. Once dephosphorylated, SpoIIAA can bind SpoIIAB and liberate σ^F, activating prespore-specific transcription. In this model, the phosphorylation state of SpoIIAA is directly correlated with σ^F activity, and the fate of SpoIIAB after σ^F liberation and the nucleotide binding status of SpoIIAB are not considered. (B) Integrated model of σ^F regulation. In the predivisional cell, SpoIIAA and SpoIIAB are present in two forms: phosphorylation of SpoIIAA by SpoIIAB results in free phosphorylated (inactive) SpoIIAA and a SpoIIAA-SpoIIAB-ADP complex, while unreacted SpoIIAB-ATP forms an inhibitory complex with σ^F. As long as the level of dephosphorylated SpoIIAA remains below a certain threshold, it will be absorbed by the SpoIIAB-ADP sink. Asymmetric division triggers activation of σ^F in the prespore through three possible mechanisms: generation of excess dephosphorylated SpoIIAA so that the sink can no longer absorb all of it, sequestration of SpoIIAB in a long-lived complex with SpoIIAA, and proteolysis of SpoIIAB. Asymmetric division is thought to increase the level of dephosphorylated SpoIIAA either by activation of the phosphatase activity of SpoIIE or equivalent distribution of SpoIIE into both compartments, resulting in a much higher SpoIIE/SpoIIAA-PO_4 ratio in the prespore. The complexes listed are not intended to reflect a stoichiometric biochemical reaction; rather, they reflect the different combinations thought to be formed by these factors and how they correlate with asymmetric division and activation of σ^F. The mother cell (not shown) is presumed to resemble the predivisional cell.

present in both the mother cell and the prespore when first formed (150), indicating that preferential inheritance of SpoIIE into the prespore is not a primary determinant of compartmentalization.

Inhibitor. One assumption that had been made in models of σ^F regulation was that localization of SpoIIE to the asymmetric division site was critical for its role in σ^F activation. When the N-terminal transmembrane domains of SpoIIE were removed, the protein failed to target to the asymmetric division site, yet sporulation was only reduced by about 50% and about half of the bacteria displaying σ^F activity showed prespore-specific expression (9). This result contrasts with the effect of inactivating $spoIIE$, where sporulation is reduced at least 10^7-fold (228) and σ^F activity is abolished (9) and indicated that when the location of SpoIIE in the cell was disturbed, some other mechanism functioned to compartmentalize σ^F activity. It was suggested that a cytoplasmic inhibitor of SpoIIE was able to regulate the soluble SpoIIE protein and prevent it from becoming active in the mother cell. However, the putative inhibitor has yet to be identified. It is important to note that a separate study, using a different mutant of SpoIIE that became

solubilized during sporulation, found similar results (73) but interpreted them as supporting the preferential inheritance model (309).

Transient genetic asymmetry. Studies of chromosome partitioning during sporulation helped generate a new concept for compartmentalization studies. Analysis of the effects of *spoIIIE* mutations revealed that at the time of asymmetric division, the prespore and mother cell contained different sets of genes in that only the origin-proximal one-third of a chromosome was present in the prespore, whereas the mother cell contained one complete chromosome and the origin-distal two-thirds of another (Fig. 3) (305). It takes about 15 min before the prespore receives a complete chromosome (148, 232). It was proposed that this transient genetic asymmetry could be the key to establishing compartmentalized gene expression (84). This proposal was tested by placing the gene encoding σ^F at the *amyE* locus very near the origin of replication, so that it was present in the prespore at the time of asymmetric division, and leaving *spoIIAB* near the terminus, so that it was initially absent from this compartment (Fig. 3). This arrangement of genes supported a modest level of sporulation even in the absence of the normally essential factors SpoIIAA and SpoIIE (84). These results clearly suggested that transient genetic asymmetry could play a role in compartmentalization of σ^F activity. Fransden and colleagues favored the existence of a gene near the terminus that encoded a cytoplasmic inhibitor of SpoIIE (84), so that transient genetic asymmetry would deplete this factor from the prespore. Although this putative factor remains unidentified, the concept of transient genetic asymmetry has had a dramatic impact on studies of compartmentalization.

A separate study addressed the role of transient genetic asymmetry by moving the entire *spoIIA* operon from its normal chromosomal location near the terminus to the *amyE* locus near the origin of replication (Fig. 3). Although this relocation, in itself, had only a mild effect on sporulation (40 to 80% of the parental strain value), when it was combined with the mutant, cytoplasmic form of SpoIIE, which also had only a mild effect in itself (9), there was a synergistic effect, and sporulation was severely reduced (<1% of that of the parental strain) (54). Therefore, targeting of SpoIIE to asymmetric division sites and the natural chromosomal location of the *spoIIA* operon are partially redundant factors that contribute to activation of σ^F in the prespore. The question remained how the chromosomal position of *spoIIA* could compensate for mislocalization of SpoIIE.

SpoIIAB degradation. In a parallel line of investigation, it was discovered that a C-terminal truncation of SpoIIAB severely impaired σ^F activation and sporulation. Immunoblotting revealed that this mutant protein was much more stable than the wild type, indicating a key role for the instability of SpoIIAB in regulation of σ^F. In the absence of its binding partners SpoIIAA and σ^F, a unique C-terminal motif of SpoIIAB (215) targets the protein for degradation by the ClpCP protease complex (214). It was proposed that the natural chromosomal position of *spoIIA* near the terminus and the instability of SpoIIAB result in the anti-sigma factor's being temporarily depleted from the prespore compartment following asymmetric division. By combining this result with the transient genetic asymmetry experiments, a holistic picture be-

gan to emerge. When SpoIIE is localized to the asymmetric septum and the *spoIIA* operon is located near the origin, SpoIIAA is efficiently dephosphorylated that it can overcome the increased concentration of SpoIIAB that is present in the prespore. Conversely, when the phosphatase activity of SpoIIE is diminished by its mislocalization, the natural chromosomal position of *spoIIA* near the terminus allows SpoIIAB to be depleted from the prespore by proteolysis. In both scenarios, σ^F activation and sporulation occur with high efficiency. Only when the phosphatase activity of SpoIIE is reduced via impaired localization and SpoIIAB in the prespore is replenished by the presence of the *spoIIA* operon in this compartment is the activation of σ^F, and thus sporulation, severely impaired (54, 214).

Although genetic asymmetry is emerging as an exciting new concept, it is important to note that the genetic asymmetry is only transient. The DNA translocase SpoIIIE acts to export the remaining two-thirds of the chromosome from the mother cell to the prespore, a process that is estimated to take as little as 15 min (148, 232). Furthermore, the half-life of SpoIIAB is about 30 min (214). Thus, the decline in SpoIIAB relative to σ^F is relatively small. Nevertheless, small changes such as this are presumably sufficient to initiate prespore-specific σ^F activation. Not only that, σ^F must be activated very rapidly after septum formation and, following σ^F, σ^E must be activated in the mother cell if formation of a second septum at the other end of the organism (the abortively disporic phenotype) is to be prevented (124); the second septum may be formed as soon as 10 min after the first (77, 232). It is as if the decision to activate σ^F is balanced on a knife edge; just a minor reduction in the concentration of SpoIIAB in the prespore is presumably sufficient to trigger a very rapid cascade of events that ensure activation of σ^F in the prespore, and only in the prespore.

Cell division. An important question is how the phosphatase activity of SpoIIE is regulated (if at all) so that the level of dephosphorylated SpoIIAA remains low enough to prevent σ^F activation in the predivisional cell yet can be turned on so as to rapidly activate σ^F after asymmetric septation. An initial study utilizing a conditional mutation (*div-355*) in the late-acting essential cell division protein DivIC revealed that when asymmetric division was blocked, it prevented activation of σ^F (166). A second study found that when cell division was blocked at a very early stage in sporulating cells by depletion of FtsZ, SpoIIAA was found largely in the phosphorylated form. In contrast, when cell division was blocked at a late stage by the *div-355* mutation, there was substantial dephosphorylation of SpoIIAA. However, in neither circumstance did σ^F become active. These results suggested a two-step checkpoint: interaction with FtsZ triggered the phosphatase activity of SpoIIE, but the resulting dephosphorylated SpoIIAA did not activate σ^F until asymmetric division was complete. This checkpoint could be either enforced or bypassed by modification of SpoIIE. A mutant allele of *spoIIE*, *spoIIE48*, caused a phenotype similar to that of *div-355* in that there was substantial dephosphorylation of SpoIIAA and yet σ^F activation was blocked. Conversely, replacement of the N-terminal transmembrane domains of SpoIIE with those of the *E. coli* MalF protein resulted in delocalized protein that caused hyper-σ^F activity even when asymmetric division was prevented (150).

SpoIIE exhibits many properties expected of a "surveillance" protein that senses asymmetric division: it localizes to asymmetric division sites (10, 14) in an FtsZ-dependent manner (168), assists in the formation of polar Z rings (19, 149), and interacts with FtsZ (186). Consistent with the checkpoint model, a number of studies have found missense mutations in SpoIIE that uncouple asymmetric division from σ^F activation (32, 73, 111). Indeed, one study tested the in vitro phosphatase activity of the mutant proteins and found that it was similar to that of the wild-type protein (73), further supporting the concept that SpoIIE is subject to a phosphatase-independent regulatory step that couples asymmetric division to σ^F activation.

SpoIIAB sink. Separate studies have clearly established that when asymmetric division was prevented, dephosphorylated SpoIIAA accumulated but σ^F remained inactive (73, 150). However, how the cell prevented the dephosphorylated SpoIIAA from activating σ^F in the predivisional cell remained mysterious. A recent study has provided evidence that SpoIIAB, its ADP-bound state, can serve as a "sink" absorbing SpoIIAA in the cell until a threshold level is reached (32). Precisely why asymmetric division allows the threshold level of dephosphorylated SpoIIAA to be crossed in the prespore is unknown; some speculate that the phosphatase activity of SpoIIE is stimulated by asymmetric division (73), whereas others think that equivalent inheritance of SpoIIE in the two compartments creates a favorable SpoIIE/SpoIIAA-PO$_4$ ratio in the prespore (32). Depletion of SpoIIAB from the prespore compartment as a consequence of transient genetic asymmetry (54, 214) would logically contribute to decreasing the threshold level in this compartment. Interestingly, it was also found that in vivo, the *spoIIE48* mutation substantially impairs dephosphorylation of SpoIIAA (32), rather than not affecting phosphatase activity, as previously thought (150). Indeed, a screen for suppression of a similar SpoIIE mutation identified mutations in SpoIIAA, SpoIIAB, and SpoIIE itself, all of which restored sporulation by increasing the level of dephosphorylated SpoIIAA in the cell (32). Therefore, the second step of the checkpoint model, in which SpoIIE prevents dephosphorylated SpoIIAA from liberating σ^F (150), appears unlikely. Rather, it appears that the SpoIIAB-ADP sink fulfills this role in the cell.

Biochemical studies. Another area of ongoing research is the nature of the complexes that SpoIIAB forms with its binding partners. The basic model is that SpoIIAB inhibits σ^F activity directly by binding to it and also indirectly by acting as a serine kinase that inactivates SpoIIAA by phosphorylating it (199, 207). The phosphatase activity of SpoIIE functions to reverse this reaction (49), enabling SpoIIAA to attack the SpoIIAB-σ^F complex and allowing σ^F to become active. However, additional biochemical and genetic experiments have revealed more subtle and complex interactions between these factors.

One of the products of σ^F activation is the catalytically inactive SpoIIAB-ADP complex, and it has been reported that the replacement of ADP by ATP in this complex is an especially slow reaction (206), so that SpoIIAB-ADP functions to sequester SpoIIAB in an inactive form. In addition, similar experiments have shown that an even longer-lived intermediary is SpoIIAA-SpoIIAB-ADP (162). Although the experiments were performed in vitro, subsequent genetic analysis has revealed that different mutant SpoIIAA proteins incapable of

forming the SpoIIAA-SpoIIAB-ADP complex in vitro cannot activate σ^F in vivo; the mutant strains are Spo$^-$ (161), indicating that this mechanism of sequestering SpoIIAB is physiologically relevant. However, as described above, the same complex has been found to impair σ^F activation in the predivisional cell (and possibly mother cell) by absorbing dephosphorylated SpoIIAA (32). Understanding how the SpoIIAA-SpoIIAB-ADP complex functions both to inhibit σ^F activation by absorbing dephosporylated SpoIIAA and also to promote σ^F activation by absorbing SpoIIAB will require further study.

Structural studies of the complex formed by SpoIIAB and σ^F have allowed further elucidation of the mechanism by which SpoIIAA functions. First, it was determined that the inhibitory complex had the stoichiometry SpoIIAB$_2$:σ_1^F (30). Next, determination of the crystal structure of a complex of the *Bacillus stearothermophilus* SpoIIAB and σ^F revealed that only one of the two SpoIIAB molecules in the complex bound σ^F (31). Because of the high degree of conservation of these molecules among sporeformers (277), it is reasonable to assume that the *B. subtilis* homologues form a similar complex.

It had previously been thought that since the same residues of SpoIIAB contact both SpoIIAA and σ^F (92), SpoIIAA competed with σ^F to bind SpoIIAB. However, these new results required a reevaluation of how SpoIIAA interacted with the SpoIIAB-σ^F complex. Experiments were performed with SpoIIAB heterodimers consisting of wild-type SpoIIAB and SpoIIABR20E, a mutant deficient in binding SpoIIAA (92). The experiments revealed that SpoIIAA interacted with the molecule of SpoIIAB not bound to σ^F and induced release of σ^F from the other SpoIIAB molecule, most likely by steric displacement (115). Another interesting result from this study was that a mutant of SpoIIAB deficient in its kinase activity (SpoIIABR105A) resulted in excessive levels of σ^F activity. This result is notable in light of a previous study that found, counterintuitively, that the kinase activity of SpoIIAB was essential for σ^F activation (91). It is currently thought that the phosphorylation reaction takes place following, and therefore can be uncoupled from, σ^F liberation.

Summary. A great deal of genetic and biochemical evidence has been obtained about the pathway regulating σ^F activity. What originated as a relatively simple biochemical model (Fig. 4A) has become far more complicated (Fig. 4B). Presently, there appear to be a number of overlapping mechanisms: localization of SpoIIE to the asymmetric septum and its regulation by interaction with division proteins (9, 10, 14, 74, 75, 111, 150, 186), transient genetic asymmetry (54, 84), proteolysis of SpoIIAB (214), and a sink to absorb dephosphorylated SpoIIAA (32) that also sequesters SpoIIAB (161). The major challenge for the future is to understand how all of these mechanisms (and potentially others) are integrated so that activation of σ^F is tightly coupled to asymmetric division and completely compartmentalized to the prespore. The large number of contributing mechanism highlights how critical it is for the developing organism to efficiently regulate the earliest-acting cell-specific transcription factor.

σ^F Regulon

Classical genetic analysis has revealed at least four *spo* loci directly regulated by σ^F: *spoIIR*, required for activation of σ^E

in the mother cell (143, 179); *spoIIIG*, encoding the late prespore-specific transcription factor σ^G (282, 283); *spoIIQ*, required for expression of *spoIIIG* and for engulfment under certain conditions (178, 284); and *spoIVB*, a regulator of the late mother cell-specific σ factor σ^K (39, 96). The gene encoding a spore catalase required for hydrogen peroxide resistance, *katX*, is part of the σ^F regulon (12), as is *gpr*, encoding a protease specific for SASPs (see below) (285). In addition, three other genes with roles during sporulation have been identified as being regulated by σ^F: *bofC*, an additional regulator of σ^K activity (97), and two regulators of σ^F activity, *lonB* (4, 263) and *rsfA* (306). Therefore, primary functions of σ^F are to couple prespore- and mother cell-specific gene expression and to direct synthesis of the late prespore transcription factor σ^G.

The σ^F regulon has been partially defined by chip array analysis. The analysis revealed 66 genes that were active during the middle part of sporulation and whose expression depended upon both Spo0A and σ^F (71). However, the approach identified genes responsive to both σ^F as well as the mother cell-specific transcription factor σ^E, so more detailed analysis may be necessary to more fully define all of the genes controlled by σ^F.

ACTIVATION OF σ^E

We now turn to the next compartmentalized transcription factor, σ^E, which is activated specifically in the mother cell following asymmetric septation. Activation of σ^E depends upon receipt of a signal from the prespore, providing the first example of communication between the two compartments during development. Activation of both σ^E and σ^F is coupled to asymmetric division. However, their activation mechanisms are very different. σ^F is synthesized in an active state and held inactive by an anti-sigma factor, in contrast, σ^E is synthesized in an inactive state and activated by proteolytic cleavage.

The first sporulation-specific σ factor be purified was σ^E, initially known as σ^{29} (103). Its isolation provided the first evidence that alternative σ factors were involved in sporulation and led to the hypothesis that a cascade of σ factors drove the developmental process (181). Immunoblotting experiments revealed that σ^E was synthesized specifically during sporulation. The anti-σ^E antiserum also detected a slightly larger protein, called P31, that was synthesized earlier than and thought to be a precursor of σ^E (292).

A combination of genetic and protein analysis revealed that σ^E and P31 (now called pro-σ^E) were the products of the *spoIIG* locus (279, 292). Pulse-chase and microsequencing experiments revealed that pro-σ^E was processed into σ^E by proteolytic removal of 27 residues from its N terminus (159, 278). Subsequently, the *spoIIG* locus was found to be a two-gene operon: the first gene (*spoIIGA*) is essential for the processing reaction (136, 147, 193), and the second gene, *spoIIGB*, encodes pro-σ^E, which is processed in a SpoIIGA-dependent manner into active σ^E. It was proposed that SpoIIGA was the processing enzyme and that its ability to act on its substrate (pro-σ^E) required the appearance of the sporulation septum, thereby constituting a developmental checkpoint (278). Mutational analysis revealed that mutants of pro-σ^E that were poorly processed could be suppressed by mutations in SpoIIGA, providing support for a direct role for the SpoIIGA protein in processing (225). However, this role has yet to be verified by in
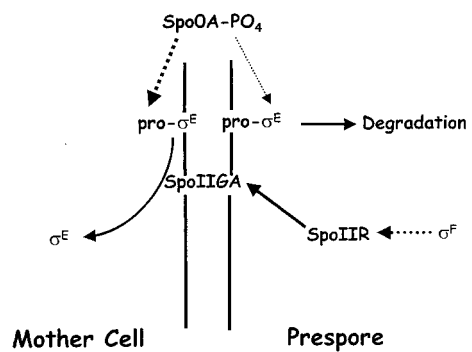

FIG. 5. Regulation of σ^E activation. Parallel vertical lines separating the prespore (right) from the mother cell (left) represent the asymmetric septum. Broken arrows represent transcriptional activation, and solid arrows represent posttranslational regulation. Spo0A-PO$_4$ is present in the predivisional cell as well as both compartments and therefore is represented above the sporulation septum. Pro-σ^E is synthesized in a Spo0A-PO$_4$-dependent manner and therefore is present in both compartments; however, recent study has indicated that Spo0A-PO$_4$-dependent transcription is largely confined to the mother cell after asymmetric division. This distinction is represented here by a thick line, indicating a high level of expression, in the mother cell and a thin line, indicating a low level of expression, in the prespore. Pro-σ^E, which is membrane bound, is processed into the active form, σ^E, by the inferred membrane-bound protease SpoIIGA. SpoIIGA becomes active in response to SpoIIR, whose expression is activated by σ^F. SpoIIGA is presumably present in both compartments, but σ^E becomes active only in the mother cell, at least in part because of the higher concentration of pro-σ^E in this compartment, as well as because of degradation of pro-σ^E in the prespore. The prespore specificity of SpoIIR expression may contribute to but is not critical for mother cell-specific activation of σ^E.

vitro experiments. Activation of σ^E was also found to depend on σ^F activity (143, 179). The pathway regulating σ^E activation is illustrated in Fig. 5.

Compartmentalization

The *spoIID* locus was found to be exclusively transcribed by E-σ^E (246). Immunoelectron microscopy of cells containing a *spoIID-lacZ* fusion showed signal exclusively in the mother cell (48). This compartmentalization of σ^E-directed gene expression has been confirmed many times in subsequent studies (reviewed in references 231 and 281). As with σ^F in the prespore, a major question to be answered was how the activity of σ^E was confined to a specific compartment. The *spoIIG* operon is expressed before septation (93, 218), suggesting that posttranslational regulation played a role in its compartmentalization. Since σ^E activation was dependent on σ^F activity (135), it was suggested that a σ^F-controlled gene encoded a signal from the prespore that triggered processing of pro-σ^E to σ^E in the mother cell (182, 268).

Intercompartmental signaling. The link between σ^F activity and σ^E activation was identified as the *spoIIR* (or *csfX*) locus (143, 179). Mutation of *spoIIR* blocked the processing of pro-σ^E to σ^E. It was found to be the only σ^F-directed locus that was required for σ^E activation, and artificial induction of *spoIIR* rendered σ^E activation independent of σ^F (179, 314). The SpoIIR protein contains a putative signal sequence, suggesting that it may be secreted from the prespore into the

intermembrane space separating it from the mother cell. As evidence for secretion, it was found that conditioned medium from bacteria engineered to express SpoIIR could trigger processing of pro-σ^E to σ^E when added to protoplasts expressing the *spoIIG* operon (119). It is thought that SpoIIR acts from the prespore to trigger SpoIIGA-directed proteolysis of pro-σ^E to σ^E, although no direct biochemical evidence yet exists to support this hypothesis.

Although the signal transduction pathway linking the compartments has now been identified, the basis for mother cell-specific activation of σ^E remains unclear. One possibility is that the prespore-specific location of *spoIIR* expression, in itself, directs that σ^E be active only in the mother cell. To test this possibility, *spoIIR* was removed from σ^F control and expressed prior to asymmetric division from the *spoIIE* promoter in strains with no σ^F. Despite this switch in time and location of SpoIIR formation, σ^E was activated only in the mother cell of the organisms that underwent asymmetric division (314). Interpretation was complicated because about half of the population did not form an asymmetrically located septum, and those organisms displayed uncompartmentalized σ^E activity. Apparently, if an asymmetric septum was formed before σ^E activation, σ^E activity was confined to the mother cell; however, if σ^E activation preceded septation, it prevented septation (314), a result consistent with the σ^E-directed block in division that normally prevents the abortively disporic phenotype (57, 124, 232). Overall, the results indicate that compartmentalization of σ^E activity in the mother cell can occur independently of σ^F and of compartmentalized *spoIIR* expression.

Protein localization. Compartmentalization studies now focused on the subcellular location of the processing reaction. It was found that fusion proteins of SpoIIGA and pro-σ^E to GFP localize to the asymmetric septum (72, 138). Fractionation experiments revealed that pro-σ^E is found primarily in the membrane, whereas processed σ^E is found primarily in the cytoplasm (118). These results suggested that the processing reaction took place at the membrane; consistent with this conclusion, it was found that a pro-σ^E mutant that failed to localize to the cell membrane was not processed (139). Immunofluorescence with anti-σ^E antibodies (which also interact with pro-σ^E) revealed a signal at the cell membrane prior to asymmetric division, a concentrated signal at the asymmetric septum upon division, and finally a signal dispersed in the cytoplasm thereafter (118). Similar immunofluorescence experiments revealed very little signal in the prespore; most of the signal was confined to the mother cell after asymmetric division (234). In addition, pro-σ^E artificially produced in the prespore was not processed (140) unless SpoIIGA was also expressed in this compartment (141). These studies suggested that exclusion of either SpoIIGA or pro-σ^E from the prespore was a mechanism of compartmentalization. In support of this model, a fusion to GFP of the N-terminal 55 residues of pro-σ^E, encompassing the prosequence, localized to the sporulation septum and, in protoplasts formed after asymmetric division, was found predominantly in the mother cell (138). These results suggested that pro-σ^E is sequestered to the mother cell face of the asymmetric septum.

However, a study assaying the expression pattern and stability of a full-length, functional pro-σ^E–GFP fusion (as opposed to the truncated version in the previous studies) found that the

fusion protein localized nonspecifically to the cell membrane. As in the previous studies, fluorographs showed a strong signal associated with the septum (86), but a similar signal was observed with the membrane stain FM4-64, indicating that there is no specific concentration of this protein at the asymmetric septum, in contrast to previous interpretations (118, 138). Rather, the strong signal probably resulted from the two septal membranes' being viewed end on, as disks (86). In addition, it was found that the fusion protein was preferentially degraded in the prespore and the *spoIIG* promoter was largely active in the mother cell after asymmetric division, providing two different mechanisms to concentrate pro-σ^E in the mother cell (86). These results provide an alternative explanation of the earlier report of pro-σ^E localizing to the mother cell face of the septum (138), namely, a large increase in mother cell-specific synthesis of pro-σ^E and prespore-specific degradation of pro-σ^E following asymmetric division. Since previous studies had shown that that *spoIIG* transcription commenced prior to asymmetric division (93, 218), a major question to be addressed was how *spoIIG* transcription became largely confined to the mother cell after asymmetric division.

Role of Spo0A. In a continuation of the study discussed above, Fujita and Losick (87) found that a substantial increase in Spo0A activity followed asymmetric division and was confined to the mother cell. This result explains the increase in transcription that they had observed (86) for the Spo0A-dependent (257, 258) *spoIIG* promoter. Disrupting the pattern of Spo0A activity by expression of a Spo0A inhibitor in the mother cell or a constitutively active form of Spo0A in the prespore severely reduced spore formation, indicating that preferential mother cell-specific Spo0A activity is important for development. A challenge now is to understand how the increase in Spo0A activity is coupled to asymmetric division and confined to the mother cell. Fujita and Losick propose that transient genetic asymmetry excludes important phosphorelay genes from the prespore at the time of asymmetric division. However, a recent study found that dephosphorylated SpoIIAA, thought to be found largely in the prespore (171), inhibits Spo0A-dependent transcription (6). This result suggests a supplemental or alternative mechanism to transient genetic asymmetry, that dephosphorylated SpoIIAA generates a feedback loop that prevents expression of Spo0A-dependent genes, such as *spoIIG*, from occurring in the prespore. Further studies will be necessary to determine the role that this feedback inhibition plays in compartmentalization of *spoIIG* transcription and of σ^E activity.

Timing of activation. Another focus of study has been the importance of timing of σ^E activation during sporulation. Although it was known that pro-σ^E processing required asymmetric septation (278) and σ^F-dependent *spoIIR* expression (143, 179), how these events coordinated proper timing of σ^E activation was unknown. It was found that expression of *spoIIR* in the predivisional cell, rather than in the prespore, had only a mild effect on spore formation (314) and on the timing of pro-σ^E processing and σ^E activity (86). Premature *spoIIG* expression from a constitutive rather than its normal Spo0A-induced promoter had little if any effect on these properties. However, constitutive expression of both *spoIIG* and *spoIIR* resulted in early processing and poor sporulation (86). Therefore, correct timing of expression of both *spoIIG* and of *spoIIR*

contributes to the proper timing of σ^E activation; they provide partly redundant mechanisms to coordinate morphogenesis and gene regulation.

Another aspect of timing to consider is that cells that fail to activate σ^E frequently undergo asymmetric division at both poles of the cell and subsequently fail to sporulate. This second division results in an abortively disporic phenotype in which there is a three-chambered structure consisting of two prespores separated by a large central compartment (124, 228). These two asymmetric divisions occur in rapid succession, with as little as 10 min separating them (77, 232). Genetic and cell biological experiments have revealed that three σ^E-directed genes that are required for engulfment of the prespore by the mother cell are also responsible for preventing the second division in the mother cell (57, 232). The rapid succession of the two divisions indicates that the σ^E-dependent mechanism to impair asymmetric division must be activated very rapidly after the first division to prevent the second. Moreover, the first division is needed in order to activate σ^F, so that *spoIIR* is transcribed and hence σ^E is activated (143, 179). Thus, some σ^E must become active in the mother cell very shortly after septation, probably before the large postseptation increase in Spo0A-directed *spoIIG* transcription can have an effect.

Regulation by gene position. Similar to the role that the chromosomal position of the *spoIIA* operon plays in σ^F activation (54), one of the ways in which the cell ensures proper timing of σ^E activation is the chromosomal location of the *spoIIR* gene. The *spoIIR* gene is located very near the origin of replication, so that it is initially present in the prespore at the time of asymmetric division and will be immediately transcribed by RNA polymerase with σ^F (143, 179) (Fig. 3). Placing *spoIIR* at a location near the terminus, so that it must be imported into the prespore by the DNA translocase SpoIIIE (17, 303, 305) to become accessible to σ^F, curtailed its expression (148, 320). This relocation also resulted in a severe reduction in σ^E activity and in sporulation, and many cells in the population exhibited the abortively disporic phenotype. Therefore, the chromosomal position of *spoIIR* is important for the proper timing of σ^E activation, prevention of the second asymmetric division in the mother cell, and efficient sporulation (148, 320).

In summary, much is now known of the signal transduction pathway governing mother cell-specific gene expression (Fig. 5). The discovery that Spo0A activity is largely confined to the mother cell after septation (87) is an important recent contribution to our understanding of compartmentalization of σ^E activity. The mechanism for the partial compartmentalization of Spo0A remains unknown. Genetic asymmetry, already known to contribute to compartmentalization of σ^F activity and timing of σ^E activation (54, 148, 320), has been suggested as a mechanism to partially compartmentalize Spo0A activity (87). Alternatively, or additionally, differential localization of phosphorelay components could play a role; a precedent for this scenario can be found in *Caulobacter crescentus* development (196). In addition, the role of a novel inhibitory feedback loop involving SpoIIAA (6) has yet to be explored. The protein(s) responsible for degradation of pro-σ^E in the prespore remains unknown; functional analysis of the σ^F regulon should facilitate its identification.

σ^E Regulon

Members of the σ^E regulon that are important for sporulation include *spoIID*, *spoIIM*, and *spoIIP*, which are required for engulfment and to prevent a second asymmetric division from occurring in the mother cell (1, 37, 57, 85, 228, 232, 246, 271, 272); *spoIVA*, *cotE*, and *spoVID*, which encode scaffold proteins for spore coat assembly (18, 245, 273, 317, 318); the *spoIIIA* operon, which is required for activation of the late-prespore specific sigma factor σ^G (125, 146); *sigK*, the composite gene for the late mother cell-specific transcription factor σ^K (155, 280); and *spoIVCA*, the gene for the recombinase that generates *sigK* via a chromosomal rearrangement (155, 235, 256). In addition, σ^E activates transcription of *spoIIID*, which encodes a regulator of some σ^E-dependent genes (102). As a result, early mother cell-specific gene expression is divided into an initial phase, when certain genes responsive to σ^E alone are expressed, including *spoIIID*, and a later phase, when SpoIIID represses some σ^E-controlled genes and activates transcription of additional σ^E-controlled genes.

The main functions of σ^E are to prevent asymmetric division in the mother cell, to trigger engulfment of the prespore, to initiate spore coat assembly, and to direct synthesis of the late mother cell-specific transcription factor σ^K. It should be noted that functional conservation of σ^E has been observed in a range of species of *Bacillus* and *Clostridium* (7). In *Bacillus thuringiensis*, the *cry1A*(a) gene, encoding a protoxin crystal protein, is expressed in the mother cell under the control of σ^E as well as σ^K (2), and in *Clostridium perfringens*, the enterotoxin gene *cpe* is most likely also under the control of σ^E and σ^K (315).

The σ^E regulon has been defined by microchip array in two independent studies (58, 75). One study found that 253 genes (in 121 operons) are regulated by σ^E, and 46 of these were present in all endospore-forming bacteria whose genomes have been sequenced and absent from the genome of the related nonsporeformer *Listeria monocytogenes*. Null mutations in 12 of 98 previously undefined genes or operons caused substantial defects in sporulation; fusions of several of the encoded proteins to GFP showed localization to the outer prespore membrane (58). The other study found 171 genes under the control of σ^E, and functional analysis of this group found five novel genes required for efficient sporulation (75). The two studies agreed well and, in addition to many novel genes, identified most of the previously described σ^E-directed genes.

ENGULFMENT OF THE PRESPORE BY THE MOTHER CELL

The major morphological event that follows activation of σ^F and σ^E is engulfment of the prespore by the mother cell. After asymmetric division, the prespore and mother are adjacent and are in direct contact with the medium. Completion of engulfment results in the prespore's being entirely surrounded by the mother cell and so not in direct contact with the medium (Fig. 1). It should be noted that the word engulfment has been used in two senses in studies of spore formation, which at times can cause confusion. The first sense means the process of engulfment, and the second means the completion of engulfment (228). In terms of mutant designation, substages IIii and IIiii

are in the process of engulfment (124), whereas stage III denotes completion of engulfment (124, 228). The activation of the late cell-specific sigma factors (σ^G and σ^K) is coupled to completion of engulfment, as activation of the early factors (σ^F and σ^E) is coupled to asymmetric division (231). Therefore, a discussion of engulfment is important for an understanding of compartmentalization, because there is compartmentalization between pre- and postengulfment (in the sense of completion of engulfment) gene expression.

Initiation of Engulfment

The process of engulfment starts with changes in the sporulation septum. The first observed change is loss of wall material from the center of the septal disk, followed by loss of much of the wall material of the septum. After this autolysis has occurred, the points of attachment of the septal membrane to the peripheral cell membrane migrate to the cell pole. Engulfment is completed by membrane fusion, resulting in the detached prespore's being entirely within the mother cell (Fig. 1) (124, 178, 227). Expression of both prespore-specific and mother cell-specific loci is required for engulfment. *spoIID* (37, 228), *spoIIM* (271), and *spoIIP* (85) mutants display autolysis and membrane flexibility at the center of the spore septum but not its periphery. *spoIIQ* mutants display a block just prior to completion of engulfment (178). Loci expressed before septation are also involved in engulfment (a *spoIIB spoVG* double mutant displays aberrant septal wall autolysis) (190, 224), and the *spoIIIE* locus has been shown to be required for the membrane fusion event (264, 266).

Transcription of the *spoIID* (246), *spoIIM* (272), and *spoIIP* (85) loci is directed by σ^E, suggesting that initiation of engulfment is driven by proteins synthesized in the mother cell. Consistent with a direct role in engulfment, GFP fusions with all three of the encoded proteins localized to the asymmetric septum and engulfing prespore membrane (1, 57). In addition, these proteins degraded partial septa and prevented a second division in the mother cell (57, 232). SpoIID bears homology with a modifier of cell wall hydrolases (157, 180). All of this indirect evidence suggests that the proteins function to degrade peptidoglycan, both to enable membrane migration around the prespore and to maintain asymmetry by preventing division in the mother cell. A recent study has shown that purified SpoIID degrades bacterial cell wall in vitro (1), providing a biochemical link with the genetic and cytological data regarding this class of proteins. In addition, this study revealed that leaky *spoIID* and *spoIIP* mutants were impaired in both autolysis and membrane migration, suggesting that these two processes are mechanistically linked (1).

The *spoIIB* gene, which is expressed in the predivisional cell (190), was initially thought to be required for engulfment and sporulation (37, 228). It later became clear that it is only required in strains in which a second gene expressed in the predivisional cell, *spoVG* (319), has been disrupted (190). Single mutations in either *spoIIB* and *spoVG* cause a very mild defect, whereas the double mutant displays little autolysis and sporulates poorly (190, 224). SpoIIB localizes to the asymmetric septum and bears weak homology with an amidase, suggesting a direct role in engulfment (190, 224). The function of SpoVG in engulfment is unclear; it appears to play some role

in inhibiting asymmetric division (195, 224). The defects in double *spoIIB spoVG* mutants can be partially suppressed by mutation of a third gene, *spoVS* (241). However, the complex relationships between these genes have yet to be fully explored.

Completion of Engulfment

After autolysis and membrane migration around the prespore, the final stage of engulfment is membrane fusion. The early engulfment processes are largely driven by proteins in the mother cell. However, the SpoIIQ protein, which is expressed in the prespore from an E-σ^F-directed gene, is required for membrane fusion. SpoIIQ is predicted to be largely extracellular and has homology with endopeptidases, suggesting a direct role in engulfment. Consistent with this suggestion, an epitope-tagged SpoIIQ protein localized to the center of the asymmetric septum (178). Subsequently, SpoIIQ was found to be necessary for engulfment when sporulation was induced by nutrient exhaustion in rich medium but not when sporulation was induced by nutrient replacement in minimal medium (284). SpoIIQ is nevertheless required for sporulation in both conditions, one reason being that *spoIIIG*, encoding σ^G, is not transcribed in its absence. The link between SpoIIQ and *spoIIIG* transcription is unclear but most likely indirect (284).

A novel cytology-based screen revealed that the SpoIIIE protein, which is required for DNA transfer into the prespore (17, 303), is also required for membrane fusion (264). This result was surprising in light of earlier studies that found that *spoIIIE* mutants formed apparently fully engulfed, though unstable, prespores (121, 228, 275). While SpoIIIE initially localizes to the center of the asymmetric septum (304), it then travels as a focus along the engulfing membrane, finally coming to rest at the extreme pole of the cell, concomitant with membrane fusion at that location (264). In addition, a SpoIIIE mutant in which the putative ATP binding site is disrupted was found to be defective for DNA translocation but competent for membrane fusion, thereby uncoupling the two functions of the protein (264). Potentially, SpoIIIE establishes a checkpoint to ensure that chromosome segregation is completed prior to membrane fusion.

A final, puzzling finding is that mutations in the genes encoding the E1β and E2 subunits of the pyruvate dehydrogenase complex block sporulation just prior to and after the completion of engulfment, respectively. Although changes in medium composition can rescue other aspects of the defects in these mutants, they cannot restore sporulation, suggesting a sporulation-specific function independent of their enzymatic activity (90). More work will be necessary to determine what role these proteins play in engulfment.

Although several proteins involved in engulfment have been identified, biochemical analysis of the process has only recently been initiated. Several questions need to be addressed. What is the mechanism by which engulfment proteins, and the associated autolysis, are confined to septa so as not to damage the cytoplasmic cell wall? What ensures that autolysis starts in the middle of the septal disk and not its periphery, as during vegetative division? Does the chromosome's traversing the septum provide an initiating target for the autolysis? Lastly, it is likely that a major focus of

study will be how the cell couples the completion of engulf-ment to the late stages of cell-specific gene expression, and this is discussed below.

LATE PRESPORE-SPECIFIC TRANSCRIPTION FACTOR σ^G

Completion of engulfment of the prespore by the mother cell signals the next phase of compartment-specific gene expression. However, synthesis of the associated σ factors commences earlier. σ^F directs synthesis of the late prespore transcription factor σ^G, and σ^E directs synthesis of the late mother cell transcription factor σ^K. Since σ^G is made only in the prespore and σ^K is made only in the mother cell, compartmentalization between prespore and mother cell is not a major focus of study. Rather, the focus of study is compartmentalization in the sense of activation after completion of engulfment but not before. σ^G and σ^K are both subject to complex regulation at different levels, and their activities are tightly coordinated with each other and with cellular morphogenesis. Activation of σ^K depends on σ^G, so it is σ^G activation that is most immediately tied to completion of engulfment, providing another developmental checkpoint to coordinate morphogenesis and gene regulation.

The first insight into the role of σ^G was provided by the study of small acid-soluble proteins (SASPs). These are present in high concentrations in the mature spore, and members of a major class, the α/β SASPs, bind to and protect DNA in the spore (213). Immunoelectron microscopy revealed that, during spore formation, several SASPs were found almost exclusively in the engulfed prespore (83). This finding suggested the existence of a prespore-specific transcription factor responsible for the compartmentalized pattern of SASP expression. Prespore-enriched fractions of sporulating cells contained a protein that supported in vitro transcription of $sspE$ (encoding SASP-γ) and, when sequenced, was discovered to be the $spoIIIG$ gene product and named σ^G (283). The $spoIIIG$ gene had been previously identified as being located immediately downstream of $spoIIGB$, encoding a product with substantial homology to σ factors, and being essential for spore formation (142, 194). Consistent with the immunoelectron microscopy data, genetic analysis revealed that $spoIIIG$ is expressed in a prespore-specific pattern (93, 142). The pathway for σ^G activation is illustrated in Fig. 6.

Transcriptional Regulation of *spoIIIG*

Transcriptional regulation of the *spoIIIG* locus is complex and may play an important role in coupling σ^G activation to engulfment. The *spoIIIG* locus is transcribed from the upstream *spoIIG* promoter during the early part of sporulation, but no detectable σ^G protein is produced from this transcript (194, 282), most likely because of a predicted RNA hairpin in the intergenic region (194). Moving *spoIIIG* to an ectopic locus does not impair spore formation, indicating that readthrough from the *spoIIG* promoter is not critical to σ^G expression (282); the role of this transcript remains obscure. The *spoIIIG* locus is also transcribed from its own promoter, recognized by RNA polymerase with either σ^F or σ^G; this transcript is translated, leading to active σ^G during the later stages of sporula-
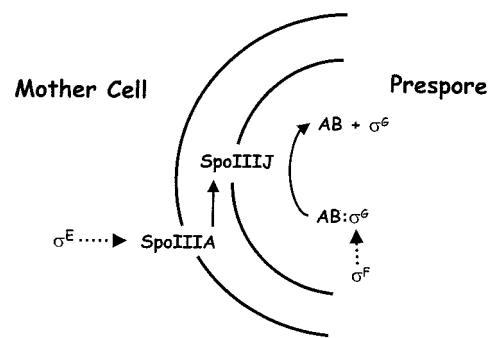

FIG. 6. Regulation of σ^G activation. Two concentric semicircles represent the inner and outer membranes of the engulfed prespore. Broken arrows represent transcriptional activation, and solid arrows represent posttranslational regulation. The structural gene for σ^G, *spoIIIG*, is transcribed exclusively in the prespore under the control of σ^F (SpoIIAB [AB] is presumably inherited from the predivisional cell). Although not represented here, transcription also requires expression of SpoIIQ in the prespore and an unknown gene in the mother cell. Activation of σ^G does not occur until engulfment is complete. Activation requires release of inhibition by SpoIIAB. This release depends on the σ^E-directed expression of the *spoIIIA* operon in the mother cell. Activation of σ^G also requires SpoIIIJ, which is expressed vegetatively and localizes to the prespore membrane but need only be expressed in the prespore. It seems likely that some mechanism distinct from SpoIIAB inhibition keeps σ^G inactive prior to engulfment. The mechanism responsible for coupling activation to completion of engulfment remains unclear.

tion (282). Since the activity of σ^F is confined to the prespore (189), the presence of a σ^F-dependent promoter ensures that σ^G is present, and therefore active, only in the prespore (83, 142).

Transcription from the *spoIIIG* promoter differs from that of other σ^F-directed promoters in a number of ways. First, its expression depends upon σ^E activation in the mother cell (218), suggestive of a signaling pathway linking the two compartments. Transcription of *spoIIIG* also depends upon expression of SpoIIQ in the prespore, suggesting a potential link between σ^G activation and engulfment (284). Lastly, consistent with dependence upon these two events, expression occurs later than that of other known σ^F-dependent promoters (144). The importance of this complex transcriptional regulation in sporulation is unclear but most likely assists in proper timing of σ^G activation relative to early compartmentalized gene expression and morphogenesis. Because σ^G can direct transcription of its own structural gene, tight regulation is presumably required to prevent inappropriate initiation of an autocatalytic loop.

Activation of σ^G

Similar to other sporulation σ factors, σ^G is subject to post-translational regulation. The first evidence was that *spoIIIJ*, a vegetatively expressed gene (60), and the *spoIIIA* operon, expressed in the mother cell (43, 125), were both required for σ^G activation but not *spoIIIG* transcription (60, 146). The anti-sigma factor for σ^F, SpoIIAB (38, 51, 260), has emerged as also acting on σ^G and indeed mediating the effects of *spoIIIJ* and *spoIIIA*. Fractionation studies revealed that SpoIIAB disappeared from the prespore at the time of σ^G activation. In addition, overexpression of σ^G resulted in toxicity that could be

suppressed by simultaneous expression of SpoIIAB (151), and disruption of *spoIIAB* led to excessive levels of σ^G activity (38). These results suggested that SpoIIAB inhibited σ^G as well as σ^F during sporulation. As direct evidence for this hypothesis, it was shown that SpoIIAB bound σ^G in vitro, and a mutant form of σ^G that SpoIIAB bound poorly in vitro, SpoIIIGE155K, bypassed the requirement for *spoIIIA* for σ^G activation in vivo but not for sporulation (146). A recent study has shown that this mutant form of σ^G bypasses the requirement for *spoIIIJ* for σ^G activation but, once again, not for sporulation (262). The ability of this mutant to restore σ^G activity but not sporulation in these backgrounds indicates that either the *spoIIIA* and *spoIIIJ* locus plays an additional role in sporulation or that the restored σ^G activity is not properly regulated.

These results suggested a model in which SpoIIAB holds σ^G inactive until a signal is received from SpoIIIJ and at least one product of the *spoIIIA* operon. The question then is how SpoIIIJ and the products of the *spoIIIA* operon function to activate σ^G. Analysis has been hampered by the fact that the eight products of the *spoIIIA* operon, all predicted to be membrane bound, are not homologous to any known proteins (281). However, it was found that although SpoIIIJ is expressed vegetatively (60), expression only in the prespore, not in the mother cell, was sufficient for spore formation. In contrast, expression of the *spoIIIA* operon in the prespore did not support sporulation (262). SpoIIIJ is a homologue of the *E. coli* YidC protein (205), which is required for insertion of proteins into the lipid bilayer (254, 261). Consistent with its expression during vegetative growth (60), *spoIIIJ* is essential if its paralogue, *yqjG*, is disrupted (205), and depletion of both encoded proteins decreases the stability of membrane-bound and secreted proteins (288). In addition, SpoIIIJ localizes to the asymmetric septum and engulfing prespore membrane (205, 262). One possible role in σ^G activation is that SpoIIIJ is required for proper insertion and/or maintenance of a receptor in the inner prespore membrane that recognizes a signal from the mother cell.

The SpoIIAB pathway in part explains postengulfment σ^G activation, but several observations suggest that there must be some additional σ^G regulator that, minimally, prevents activation prior to engulfment (66, 262). Thus, the *spoIIIGE155K* allele that renders σ^G activation independent of SpoIIIJ and the products of the *spoIIIA* operon do not affect the timing of σ^G activation (146, 262). Furthermore, σ^F and σ^G are very similar and are both subject to regulation by SpoIIAB, with σ^F having much higher affinity for SpoIIAB in vitro; for both, binding is disrupted by SpoIIAA (66). It is difficult to see why σ^G is held inactive when σ^F is active if SpoIIAB is the only direct regulator (66). A major feature of σ^G regulation is that it only becomes active upon completion of engulfment (275, 276). The molecular basis for this linkage is unknown.

The complex transcriptional and posttranslational regulation presumably ensures that σ^G becomes active only in the prespore and only following engulfment. One possible reason for the complexity is that because the *spoIIIG* promoter is transcribed by RNA polymerase with σ^G (282), even a small amount of σ^G activity can lead to an autocatalytic loop that causes excessive levels of σ^G-dependent transcription. In support of this hypothesis, a mutation in *lonA*, encoding a putative Lon protease, resulted in high levels of σ^G activity under non-

sporulating conditions, in which σ^F-directed transcription of *spoIIIG* does not occur (259). The complexity of the control is indicated by the observation that expression was postexponential and not constitutive in the *lonA* mutant; furthermore, neither expression in sporulating conditions nor spore formation was affected by the *lonA* mutation (259). Another reason for a multitude of controls may be the presence of the transcript of *spoIIIG* that originates from the upstream *spoIIG* promoter (194, 282). The *spoIIG* promoter is strongly activated in the mother cell by Spo0A (86, 87), and the cell must inactivate any σ^G that is produced from this transcript in order to prevent a feedback loop (282) and σ^G activation in this compartment.

In summary, the mechanism of coupling σ^G activation to the completion of engulfment is largely unknown. The mechanism underlying the dependence of *spoIIIG* transcription on σ^E activation (218) is also unknown. Likewise, the specific role of the eight products of the *spoIIIA* operon, all of which are thought to be membrane bound and are not obviously homologous to any known proteins, is unclear. The role of SpoIIQ in both engulfment (178) and *spoIIIG* transcription (284) is intriguing. One reason for our ignorance may be that genetic redundancy, as seen in σ^F and σ^E regulation, is preventing the isolation of strains with a single mutation that are blocked in σ^G activation or that have uncoupled activity from engulfment. Functional analysis of the σ^F (71) and σ^E (58, 75) regulons may help to overcome this problem and identify additional factors involved in σ^G regulation.

σ^G Regulon

At least three classes of genes are present in the σ^G regulon, those involved in sporulation, in germination, and in protecting the spore from DNA damage. The genes important for sporulation include *spoIIIG*, leading to an autocatalytic loop (142, 282); *spoIVB*, a serine peptidase that signals from the prespore to the mother cell to activate σ^K (39); the *spoVA* operon, which is required for dipicolinic acid uptake into the prespore from the mother cell (63, 202, 289); *spoVT*, a regulator of σ^G-dependent gene expression (13); and *bofC*, a regulator of pro-σ^K processing (97, 296). The germination genes include the *gerA* operon, involved with germination in response to alanine; the homologous *gerB* operon, involved with germination in response to a panel of germinants (L-asparagine, glucose, fructose, and potassium ions) (213); and *pdaA*, which is required for the formation of muramic δ-lactam, a unique component of spore cortex peptidoglycan (89). The genes for protection from DNA damage include *splB*, encoding spore-photoproduct lyase, which helps protect spore DNA from UV damage (68, 219); *yqfS*, encoding a type IV apurinic/apyrimidinic endonuclease (253, 293); and *ssp* genes, which encode SASPs, the predominant proteins of the spore core (107). Some of the SASPs bind the DNA in the spore and help protect it from exposure to heat, UV radiation, desiccation, and several other adverse conditions and also provide a source of amino acids upon germination. Transcription of all the *ssp* genes (*sspA* through *sspP*), except *sspF* and *sspG*, is directed by σ^G (107). Therefore, three main functions of σ^G are to couple late prespore and mother cell gene expression, to protect the spore from hazardous conditions, and to prepare the spore for germination. For detailed discussions of spore cortex formation

and germination, we refer the reader to references 201, 213, and 286.

LATE MOTHER CELL-SPECIFIC TRANSCRIPTION FACTOR σ^K

Following the completion of engulfment and activation of σ^G in the prespore, σ^K becomes active in the mother cell. σ^K is only synthesized in the mother cell, so morphological coupling and compartmentalization into the postengulfment mother cell are the main focus of discussion. As is the case for σ^E and σ^G, activation of σ^K depends upon intercompartmental signaling (182, 231). Regulation of σ^K is similar to that of σ^E in that σ^K is synthesized as an inactive precursor, pro-σ^K, that is processed into its active form upon receipt of a signal from the prespore. However, the mechanisms of regulation are very different.

Developmental Chromosome Rearrangement

σ^K was first identified as a σ factor that could direct transcription in vitro of the late sporulation genes *spoIVCB* and *cotD* (152). However, the gene encoding σ^K was unknown. Genetic studies focused on the *spoIIIC* locus, which appeared to encode a small protein with high similarity to the C-terminal region of bacterial σ factors but lacking any corresponding N-terminal region (64). Interestingly, a different gene, *spoIVCB*, was found to encode a protein similar to the N-terminal region of a σ factor. These unusual findings were explained when it was shown that the region separating the two genes, approximately 48 kb in size, was excised during sporulation, resulting in the formation of a single, composite gene, *sigK*, encoding a full-length σ factor, σ^K (280). SpoIVCA appears to be the protein responsible for removal of the *skin* (sigma K intervening) element. It has substantial similarity to the Hin family of site-specific recombinases, binds to the processing site in vitro (235), and is the only sporulation-specific protein required for excision to occur in vivo (155). Transcription of both *spoIVCA* and the *sigK* gene is confined to the mother cell, initiated from a σ^E-dependent promoter requiring SpoIIID (102, 156, 255, 256).

Since the rearrangement generating *sigK* occurs only in the mother cell chromosome, which is not inherited, it could potentially be a novel mechanism of compartmentalizing gene expression. However, the presence of an intact *sigK* gene during growth and sporulation had no effect on development (155). Consistent with the dispensability of *skin* excision, other strains of *B. subtilis* and other species of sporeformers lack any inserted element in their *sigK* gene (2, 255, 277) with the notable exception of *Clostridium difficile* (see below) (104). However, similar to some other sporulation-regulatory elements, the role of *skin* excision was obscured by a genetic redundancy.

σ^K is synthesized as an inactive precursor, pro-σ^K (40, 152, 185). Removal of the prosequence (resulting in constitutively active σ^K) combined with artificial removal of the *skin* element resulted in some σ^K activity during vegetative growth and impaired sporulation (211). The vegetative expression is most likely because, like that of *spoIIIG* in the prespore (282), expression of the *sigK* gene is autocatalytic, being driven by a σ^K-dependent promoter (211). Interestingly, the *sigK* gene in *Clostridium difficile* also contains an insertion element, but it
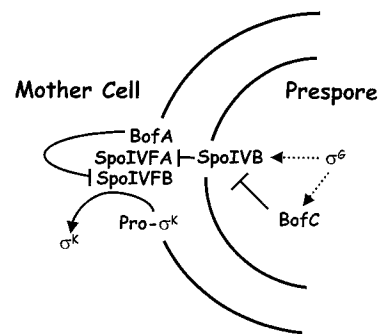

FIG. 7. Regulation of σ^K activation. Two concentric semicircles indicate the inner and outer prespore membranes surrounding the engulfed prespore. Broken arrows represent transcriptional activation, and solid arrows represent posttranslational regulation. SpoIVB is expressed in the prespore under the control of σ^G and is thought to be inserted into the inner prespore membrane, where it undergoes autoproteolysis. σ^G also directs expression of BofC, which is an inhibitor of SpoIVB. In the mother cell, BofA, SpoIVFA, SpoIVFB, and pro-σ^K are all produced under the control of σ^E. SpoIVFB is thought to be the processing enzyme that acts upon pro-σ^K to generate active σ^K. BofA inhibits SpoIVFB. This inhibition is mediated by SpoIVFA, which acts to bring these proteins in contact with one another. Signaling by SpoIVB relieves inhibition of SpoIVFB, possibly by proteolysis, and so triggers pro-σ^K processing. Pro-σ^K is tethered to the outer prespore membrane by its N terminus, and it is thought that the processing reaction with SpoIVFB occurs within the membrane.

differs from the *skin* element in size, orientation, sequence, and site of insertion. The *sigK* gene of *C. difficile* differs from its *B. subtilis* counterpart in that it does not encode a prosequence and σ^K appears to be synthesized in an active state. Consistent with the *skin* element and prosequence playing redundant roles in regulation, it was found that expression of an intact *sigK* gene in *trans* in *C. difficile* resulted in poor sporulation (104). Presumably, regulation of σ^K occurs primarily via chromosomal rearrangement in this organism, whereas in *B. subtilis* there is redundancy in regulation; either prosequence processing or *skin* excision suffices to ensure the proper time and location of σ^K activation. Consistent with this reasoning, the chromosome of *C. difficile* lacks obvious homologues to known pro-σ^K processing components (277).

Pro-σ^K Processing

Based on the nucleotide sequence of *sigK* and the N-terminal sequence of σ^K, it was inferred that that the N-terminal 20 amino acid residues are removed from the *sigK* gene product of *B. subtilis* to generate active σ^K (152, 280). Immunoblotting revealed that pro-σ^K appeared approximately an hour earlier than processed σ^K, and processing depended upon gene expression in the prespore (40, 185). Therefore, similar to regulation of σ^E (159, 278, 279), it appeared that intercompartmental signaling resulted in an inactive proprotein's being processed into an active σ factor. This signaling pathway has been identified and characterized in detail (Fig. 7).

Mother cell processing components. Genetic analysis revealed two mother cell-expressed loci important for regulation of pro-σ^K processing (41, 127). The first, *spoIVF*, is a bicistronic operon. *spoIVFA* encodes an inhibitor of pro-σ^K processing, whereas *spoIVFB* encodes a factor critical for process-

ing (40, 41, 185). The second locus, *bofA*, also encodes an inhibitor of pro-σ^K processing (243). The three encoded proteins, SpoIVFA, SpoIVFB, and BofA, identified in these early studies remain the three main mother cell-expressed proteins known to be involved in pro-σ^K processing. SpoIVFB is considered the enzyme that cleaves the prosequence from pro-σ^K, and BofA and SpoIVFA act to negatively regulate this process (248).

There are multiple lines of evidence suggesting that SpoIVFB is the processing enzyme. Most compelling among these is the finding that expression of SpoIVFB is sufficient to trigger a low level of pro-σ^K processing during vegetative growth in *B. subtilis* as well as in *E. coli* (184, 242). SpoIVFB has been shown to share two motifs with mammalian site 2 protease, a zinc metalloprotease whose catalytic center is most likely membrane embedded. Mutations in conserved residues in these motifs in SpoIVFB abolished pro-σ^K processing, suggesting that pro-σ^K processing occurs within the membrane (247, 311). Consistent with this conclusion, biochemical and cytological experiments have indicated that pro-σ^K, SpoIVFB, SpoIVFA, and BofA all localize to the cell membrane (100, 240, 249, 294, 312). Models of how BofA and SpoIVFA effect the negative regulation of SpoIVFB action have undergone a series of changes, perhaps reflecting the complexity of the process (41, 100, 153, 242, 249). The most recent study suggests that SpoIVFA acts to bring BofA and SpoIVFB together in a heteromultimeric complex localized to the outer prespore membrane and that BofA is the direct inhibitor of SpoIVFB processing activity (249) (Fig. 7).

Prespore signaling. Processing of pro-σ^K into mature σ^K was blocked in a strain lacking the late prespore transcription factor σ^G (40, 185), suggestive of signaling between the compartments. Several lines of evidence indicate that the signal is encoded by the *spoIVB* locus. Disruption of *spoIVB* blocks pro-σ^K processing, and transcription of *spoIVB* is largely dependent upon σ^G (39, 96). Transcription of *spoIVB* is the only function of σ^G that is required for pro-σ^K processing (95). The SpoIVB protein has a serine peptidase domain and a PDZ domain for protein-protein interactions; both domains are important for pro-σ^K processing and sporulation (116, 297).

It is thought that SpoIVB is inserted into the inner prespore membrane, where it undergoes autoproteolysis (297) to release signaling fragments that can diffuse across the intermembrane space and interact with the SpoIVFA-SpoIVFB-BofA complex inserted in the outer prespore membrane (249). A recent study found that SpoIVB degrades SpoIVFA in vitro (45), providing a possible mechanism of action. If this reaction occurs in vivo, it would release SpoIVFB from inhibition by BofA and trigger pro-σ^K processing (Fig. 7) (249). Interestingly, *spoIVB* mutants that show pro-σ^K processing but are Spo$^-$ have been obtained, suggesting that SpoIVB has an additional, unknown function in sporulation (212). Two other factors that play accessory roles in regulating pro-σ^K processing have been identified. The BofC protein, expressed in the prespore, inhibits SpoIVB autoproteolysis and thus pro-σ^K processing (296). The CtpB protein is similar to SpoIVB in that it contains PDZ and serine peptidase domains, although it is expressed in the mother cell under the control of σ^E. CptB is required for optimal efficiency of pro-σ^K processing (216).

In summary, the signaling pathway regulating σ^K activation is now well understood, and yet a number of issues remain. Processing of pro-σ^K has not been achieved in vitro. The proposal that SpoIVFA is proteolyzed by SpoIVB (45) needs to be tested in vivo. In addition, it is not known how the mother cell processing components localize specifically to the outer prespore membrane. It has been proposed that SpoIVFB is initially inserted into both the cell and prespore membranes and subsequently diffuses to and is retained by SpoIVFA only in the latter (250). However, the mechanism underlying SpoIVFA targeting to the outer prespore membrane remains unknown.

σ^K Regulon

The known genes in the *B. subtilis* σ^K regulon are involved in formation of the spore coat, spore maturation, and regulation of σ^K-dependent transcription. The regulon includes 14 *cot* genes, which encode proteins that make up the protective spore coat (47, 109); *spoVD* and *spoVK*, required for spore maturation (42, 69); and the structural gene for σ^K itself, leading to an autocatalytic loop (155). Expression of the transcriptional regulator *gerE* is directed by σ^K, dividing σ^K-dependent promoters into three classes: those solely regulated by σ^K are expressed throughout the late mother cell-specific stage, those that are repressed by GerE and are expressed early after σ^K activation, and those that require GerE and σ^K for activation and are expressed later (316). Detailed reviews of coat formation have appeared elsewhere (46, 47, 109). As mentioned previously, in other species expression of toxin genes is directed by σ^K: in *B. thuringiensis*, the *cryIA*(a) gene, encoding a protoxin crystal protein, is expressed in the mother cell under the control of σ^K as well as σ^E (2), and in *C. perfringens*, the enterotoxin-encoding gene *cpe* is most likely under the control of σ^K as well as σ^E (315).

TEMPORAL CONTROL AND COMPARTMENTALIZATION

The primary emphasis of our discussion has been on the spatial compartmentalization of gene expression associated with two key morphological events, completion of septation and completion of engulfment. However, an important aspect of development is temporal regulation of gene expression. Temporal control is exercised in part by coupling activation of σ factors to morphological events: σ^F, and hence σ^E, to septation and σ^G, and hence σ^K, to engulfment, as discussed above. The two prespore σ factors, σ^F and σ^G, have overlapping promoter specificities (4, 107), suggesting three temporal promoter classes in the prespore: those recognized by σ^F only, those recognized by σ^F and σ^G, and those recognized by σ^G only. A similar argument can be made for the mother cell, where σ^E and σ^K also have overlapping promoter specificities (107).

Additional layers of temporal control are exercised by activator and repressor proteins under the control of and associated with these σ factors. Such regulators have been more fully characterized for the mother cell. The SpoIIID protein acts as an activator of some and a repressor of other genes in the σ^E regulon and is itself a member of that regulon. Thus, SpoIIID divides the regulon into an early phase, when some genes are expressed, and a late phase, when other genes are expressed,

and some genes of the first phase are turned down or off (102, 125, 152, 154). GerE effects a similar division of the σ^K regulon (122, 316). Further temporal division of the σ^K regulon was shown to result from the combined action of GerE and SpoIIID: SpoIIID repressed and so delayed the expression of some GerE-activated genes but not others (123). GerE also appears to be regulated, in part, through the action of the spore coat protein SpoVIF (158).

Different temporal classes are also found in the σ^F and σ^G regulons, but the mechanisms do not seem so clearcut (107). For example, the *spoIIIG* locus is expressed about 40 min later than other members of the σ^F regulon (144) and requires expression of *spoIIQ* (284) and some component(s) of the σ^E regulon (218). The gene for a repressor-activator, RsfA, has been identified in the σ^F regulon (306), but its role is less well defined than that of SpoIIID or GerE. Finally, in the σ^G regulon, the *sspF* locus is transcribed about 1 h after other members of the regulon (217). Two transcription regulators of σ^G-directed transcription have been identified, SplA and SpoVT (13, 68); they do not appear to have the substantial roles exercised by SpoIIID or GerE in their regulons and are not known to regulate *sspF*. In summary, there is a complex temporal progression of gene expression in both the prespore and the mother cell.

Analysis of expression of the σ^F-directed *spoIIR* locus indicated a very short period of transcription (148, 320), suggesting that transcription of some σ^F-directed genes stops before σ^G becomes active. This supposition was confirmed by using a two-part probe to test for compartmentalization of gene expression within individual cells (173). The result also provides clear evidence for *spo* gene expression being switched off during spore formation. It is not known if the temporal compartmentalization applies to other σ^F-directed promoters, or if it is tied to a morphological event such as the completion of engulfment, and the mechanism remains unknown. A similar switch was detected in the mother cell between the σ^E-directed *cotE*P1 promoter and σ^K-directed *gerE* expression. The mechanism is also unknown but is distinct from SpoIIID-directed repression (173). In general, the mechanisms of turning gene expression off during spore formation are much less well understood than those for turning expression on; their importance remains to be established.

SPORULATION OF COCCI

Sporulation has been studied almost exclusively in rod-shaped bacteria. However, one way to study the relationship between morphogenesis and gene expression is to extend studies to organisms with different shapes, notably cocci. Although coccal mutants of *B. subtilis* have been too sick to form spores (108), the coccal species *Sporosarcina ureae* is a naturally occurring sporeformer. *S. ureae* fits, by most criteria, into the genus *Bacillus* (70). The spores formed by *S. ureae* are structurally very similar to those of bacilli and clostridia (120). The sporulation septa of *S. ureae* resemble the sporulation septa of bacilli and clostridia in their ultrastructure and are very different from vegetative septa (244). However, the sporulation division in *S. ureae* is medially located with respect to cell poles, in contrast to the gross asymmetry of its location for bacilli and clostridia (244, 313). Nevertheless, compartmentalization of

gene expression is observed in *S. ureae*, and it is inferred that spatial asymmetry is not required for compartmentalization of gene expression in this species (313). Thus, one plausible factor in the establishment of compartmentalized gene expression, gross volume asymmetry, is eliminated.

Despite the symmetry of location of the sporulation division, *S. ureae* has a homologue of the *spoIIIE* gene (35, 36) that encodes a DNA translocase critical for sporulation in *B. subtilis* (17, 303). This homologue is also essential for sporulation in *S. ureae* and can complement *B. subtilis spoIIIE* mutants (35, 36). Presumably the DNA translocase function of SpoIIIE is still required in *S. ureae*. Although the cells formed at division are similar in volume, successive division planes are at right angles to each other (22, 313). This change in direction requires that the chromosome reorient at each division. This reorientation may set the stage for a distinction in chromosome movement between the prespore and the mother cell. Conjecturally, transient genetic asymmetry (54) is still a factor in the establishment of compartmentalized gene expression in *S. ureae*. This conjecture raises related questions. Does trapping of the chromosome terminus region in a particular cell at septation determine that the cell will be the mother cell? Is that location predetermined by division history, or is the choice random? One intriguing possibility is that in the absence of volume asymmetry, genetic asymmetry becomes the predominant determinant of compartmentalization, so that its disruption would have a much more dramatic effect than is observed in *B. subtilis* (54).

DISRUPTION OF COMPARTMENTALIZATION

The spatial compartmentalization between prespore and mother cell of the activities of σ^F, σ^E, σ^G, and σ^K is, when first established and for the duration of spore formation, essentially complete. However, there are several mutant backgrounds in which this compartmentalization is disrupted. In all known examples, the mutants are impaired in their ability to form spores. This result is strong circumstantial evidence for the conclusion that complete compartmentalization of the activities of the four σ factors between prespore and mother cell is essential for spore formation, although it does not establish the conclusion unequivocally. The barrier that ordinarily prevents activation of σ^K before completion of engulfment appears to be important but not essential; mutants that show activation before completion of engulfment show reduced rather than no spore formation (40, 211). Below we discuss different mutations that disrupt the prespore-mother cell compartmentalization. The analysis of *spoIIAB* mutations (38, 260) was an important landmark in the elucidation of the regulation of σ^F activity. Otherwise, mutations disrupting prespore-mother cell compartmentalization have not been studied extensively; it may be that additional types of mutation can be identified and substantially more can be learned through their study.

The components for σ^F activation are present before septum formation, but σ^F normally only becomes active following septation. However, as discussed earlier, control of σ^F activation appears to be on a knife edge. Thus, mutations that inactivate SpoIIAB and cause hyper-σ^F activity (38, 260) result in uncompartmentalized σ^F activity. Indeed, the sporulation septum is not formed (38). Similarly, certain mutations in *spoIIE* result in hyper-uncompartmentalized σ^F activity and also prevent

septation and spore formation (73, 111). Clearly, in these mutants σ^F becomes active before septation, and indeed a σ^F-directed gene appears to prevent formation of the sporulation septum (111).

Mutations that disrupt the *spoIIIE* locus result in loss of compartmentalization (171, 173, 234, 303–305). In contrast, many missense mutations in *spoIIIE* do not disrupt compartmentalization but rather result in hyper-prespore-specific expression of σ^F-directed genes located in the origin-proximal third of the chromosome and no expression of such genes located elsewhere in the chromosome (303–305). Both classes display a similar block in DNA translocation. However the mutant SpoIIIE protein of the latter class (class I) is located in the middle of the spore septum, as is wild-type SpoIIIE; no localization, and often no SpoIIIE protein, is detected with the former class (class II) of mutant (304). These observations led to the suggestion that SpoIIIE might form an effective seal around the DNA as it traversed the septum. In the absence of this seal, in class II mutants, small molecules, such as σ^F or SpoIIAA, could traverse the septum, resulting in a loss of compartmentalization (304). However, a separate study found that β-galactosidase could not diffuse across the septa of class II mutants and proposed that persistence of SpoIIE in the mother cell rather than a hole in the septum was responsible for the loss of compartmentalization (234). Our recent studies support the existence of a small hole permeable to σ factors or their regulators and to GFP (110).

A further puzzling observation was that whereas σ^F-directed gene expression was uncompartmentalized in class II *spoIIIE* mutants, it was again compartmentalized when a *spoIIG* mutation was introduced into such mutants (171, 234, 309), blocking σ^E activity. Given that σ^E-directed genes are required to cause septal wall autolysis during engulfment (1, 37, 57, 85, 228, 232, 246, 271, 272), it seemed plausible that similar activity might enlarge a breach in the septum resulting from loss of SpoIIIE. Indeed, σ^E-directed transcription of *spoIID* and *spoIIP* is required to disrupt compartmentalization in *spoIIIE* class II mutants (110). Presumably, autolytic activity associated with SpoIID and SpoIIP enlarges the putative hole sufficiently to allow passage of σ^F or its regulators and of GFP and possibly of σ^E.

Mutations in the *spoIIIA* and *spoIIIJ* loci have been known for some time to prevent activation of σ^G and hence σ^K (60, 146, 281). These mutations have recently been found to also cause a loss of compartmentalization of σ^F and σ^E activities (172). The activities of these σ factors are initially compartmentalized in the mutants, and compartmentalization is only lost after completion of engulfment of the prespore by the mother cell. Thus, it appears to be a secondary consequence of the sporulation defect rather than the cause of the stage III blockage. It is likely that the loss of compartmentalization is a consequence of instability of the engulfed mutant prespores (172).

CONCLUSION AND FUTURE DIRECTIONS

Spore formation in *B. subtilis* is a simple developmental process (Fig. 1) that lends itself to genetic and cell biological analysis. *B. subtilis* monitors its intracellular and extracellular environment, integrating this information into a phosphorelay that regulates the initiation of sporulation. When conditions

are appropriate, the action of the phosphorelay results in the phosphorylation and so activation of the master response regulator Spo0A. Active Spo0A and the transition regulator σ^H trigger global changes in gene regulation, setting the stage for formation of the axial filament and the asymmetrically located sporulation division. Formation of the axial filament requires the DNA-binding proteins RacA and Soj as well as the division protein DivIVA (Fig. 3). The asymmetric division requires greatly increased expression of *ftsZ* and induction of *spoIIE*, and as a consequence the cytokinetic protein FtsZ spirals from mid-cell to sites near the poles; division occurs at one of these sites, yielding the smaller prespore and the larger mother cell.

Compartmentalized activity of two sporulation-specific sigma factors commences very soon after the sporulation division, and it is now well established that compartmentalization is, within the limits of detection, complete: σ^F in the prespore and σ^E in the mother cell (Fig. 2). The first to become active is σ^F. A complex regulatory pathway has been elucidated for σ^F, centered on the anti-sigma factor SpoIIAB, the anti-anti-sigma factor SpoIIAA, and the protein phosphatase SpoIIE, which activates SpoIIAA by dephosphorylating it (Fig. 4A). Activation is associated with completion of the sporulation division septum and is confined to the prespore. When the sporulation septum is first formed, only the origin-proximal one-third of a chromosome is present in the prespore. This genetic asymmetry is resolved by SpoIIIE, which transfers the remaining portion of the trapped chromosome into the prespore (Fig. 3). It takes perhaps 15 min for the origin-distal two-thirds, including the genes for SpoIIAA, SpoIIAB, and σ^F, to be transferred. The transient genetic asymmetry during this time, the instability of SpoIIAB, the long-lived SpoIIAA-SpoIIAB-ADP complex sequestering dephosphorylated SpoIIAA, and the localization of the SpoIIE phosphatase to the septum have been shown to be important contributors to the prespore specificity of σ^F activation (Fig. 4B), although there is considerable redundancy in their contributions. What is not yet clear is why activation occurs so rapidly after septation and why σ^F activity is absolutely confined to the prespore. It is as if the system is very delicately balanced with respect to the prespore, and a slight push ensures prespore-specific activation, yet the system is robust in that normally there is no activation before septation and none in the mother cell.

Activation of the next factor, σ^E in the mother cell, requires prior activation of σ^F. Activation is by cleavage of a prosequence from pro-σ^E, probably through the action of the putative protease SpoIIGA. The activation of σ^E must occur rapidly (<10 min?) after formation of the sporulation septum in order to prevent the formation of a second asymmetrically located septum. Minimally, in that time σ^F becomes active and directs transcription of *spoIIR*; SpoIIR triggers the processing of pro-σ^E; σ^E directs transcription of *spoIID*, *spoIIM*, and *spoIIP*, whose products act to prevent the formation of the second septum. It is not clear how σ^E activation is achieved so rapidly and exclusively in the mother cell, although prespore-specific proteolysis of pro-σ^E is probably a contributory factor. Postseptation enhancement in the mother cell of Spo0A-directed transcription of *spoIIG* (encoding pro-σ^E) is an important contributing factor to the production of σ^E in the mother cell (Fig. 5), but it is not clear why Spo0A activity (and perhaps phosphorelay activity) is greatly enhanced in the mother cell

and curtailed in the prespore, nor is it clear if this increased transcription could account for the rapidly induced, σ^E-directed suppression of septation.

Activation of pro-σ^E requires expression of the σ^F-directed *spoIIR* locus. This exemplifies a recurring theme, that compartment-specific gene expression is controlled by intercompartmental regulatory signals, a theme often referred to as crisscross regulation. Mother cell-specific activation of σ^E does not require that *spoIIR* be expressed in the prespore. Thus, the mother cell specificity of σ^E activity can be independent of the prespore specificity of σ^F action. These sigma factors direct compartmentalized gene expression but not the process of compartmentalization.

Completion of engulfment of the prespore by the mother cell starts the next phase of compartmentalized gene expression. It is associated with the action of a second pair of sporulation-specific sigma factors, σ^G and σ^K (Fig. 2) The first of these to become active, σ^G, does so exclusively in the prespore. Its prespore specificity can be explained, at least in part, by σ^F-directed transcription of *spoIIIG*, the structural gene for σ^G. However, it is not clear why activation occurs only after completion of engulfment. A chain of regulators involves the products of *spoIIIA* (made in the mother cell) and *spoIIIJ* (needed only in the prespore) antagonizing the anti-σ^G activity of SpoIIAB (Fig. 6), and yet the action of SpoIIAB is inadequate to explain why σ^G is not activated before completion of engulfment and why its activation is tied to that event. It is thought that a critical regulator remains to be identified. The morphological coupling of the activation of σ^F and σ^G to completion of septation and engulfment, respectively, is clear; the mechanisms are not.

The final σ factor to be activated during sporulation, σ^K, is also subject to complex regulation. First, in *B. subtilis* strain 168, though not in other strains or species (with the exception of *C. difficile*), there is mother cell-specific excision of an insertion element in *sigK*, the structural gene for pro-σ^K. There is then σ^E-directed transcription of *sigK*, also ensuring the mother cell specificity of σ^K. Finally, as with its predecessor in the mother cell, σ^E, σ^K is activated from an inactive prosequence form. It requires a complex of proteins synthesized in the mother cell together with a σ^G-directed signal from the prespore (Fig. 7). Mechanistically, the activation of pro-σ^K is very different from that of pro-σ^E. The final processing enzyme is thought to be a membrane-embedded metalloprotease, SpoIVFB.

The compartment-specific σ factors provide the bare framework of compartment-specific gene expression. However, within each σ regulon are several temporal classes of genes, some requiring additional activators and some subject to repressors. Some genes are clearly turned off before others become active. For key regulators, timing is critical. For example, accelerating the expression of σ^K or delaying the expression of *spoIIR* greatly impairs spore formation. The concept of transient genetic asymmetry emphasizes the importance of timing in the establishment of compartment-specific gene expression.

Compartmentalization of gene expression during bacterial spore formation is but part of the process of forming a heat-resistant, dormant spore. In our discussion of each of the sigma factors, we have only briefly mentioned the roles that their regulons play in building the mature spore. There is more to

be done to understand compartmentalized gene expression during spore formation. There is very much more to be done to fully understand how this "simple" cell differentiation is achieved.

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