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Diverse Mechanisms Regulate Sporulation Sigma Factor Activity in the Firmicutes

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Abstract

Sporulation allows bacteria to survive adverse conditions and is essential to the lifecycle of some obligate anaerobes. In *Bacillus subtilis*, the sporulation-specific sigma factors, σ^F , σ^E , σ^G , and σ^K , activate compartment-specific transcriptional programs that drive sporulation through its morphological stages. The regulation of these sigma factors was predicted to be conserved across the Firmicutes, since the regulatory proteins controlling their activation are largely conserved. However, recent studies in *(Pepto)clostridium difficile*, *Clostridium acetobutylicum*, *Clostridium perfringens*, and *Clostridium botulinum* have revealed striking differences in the order, activation, and function of sporulation sigma factors. These studies indicate that gene conservation does not necessarily predict gene function and that new mechanisms for controlling cell fate determination remain to be discovered in the anaerobic Clostridia.

Introduction

Of the many species of bacteria known to humans, only a small subset can undergo the defense strategy known as sporulation. During this developmental process, a vegetative bacterial cell differentiates into a metabolically dormant cell-type known as an endospore (referred to as a “spore” hereafter) [1–3]. By forming spores, bacteria can survive adverse environmental conditions by “reawakening” when favorable conditions return [4]. For obligate anaerobes that inhabit or transiently live in the gut, spore formation is essential for survival [5,6].

Sporulation begins with the formation of an asymmetrically placed septum, which creates two morphologically distinct but genetically identical compartments: a larger mother cell and smaller forespore ([1,3,7], Figure 1A). Migration of the mother cell membrane around the forespore during a phagocytic-like event known as engulfment leaves the forespore

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suspended in the mother cell cytosol as a double membrane-bound protoplast. A thick peptidoglycan layer known as the cortex forms between the double membranes, while a series of protective proteinaceous coat layers form around the forespore [8,9]. Upon completion of forespore maturation, the mother cell undergoes programmed lysis, releasing the mature spore into the environment [10].

Extensive studies of this developmental process in *B. subtilis* indicate that sporulation is primarily controlled at the transcriptional level by four sporulation-specific sigma factors, σ^F , σ^E , σ^G , and σ^K [11]. During sporulation, these sigma factors are post-translationally activated at specific times in specific compartments. This pattern of regulation produces distinct lines of gene expression in the mother cell and forespore that drive sporulation through its morphological stages.

While the morphological changes required to form a spore are generally conserved [2], recent studies in clostridial species have revealed that diverse mechanisms control the activation and function of the sporulation-specific sigma factors. This review summarizes these findings and highlights that there are many paths to producing a spore. While significant differences also exist in the initiation of sporulation among Firmicutes, these have been reviewed elsewhere [12,13].

Regulation of sporulation in *Bacillus subtilis*

Activation of the master transcriptional regulator Spo0A leads to the sequential activation of σ^F in the forespore, σ^E in the mother cell, σ^G in the forespore, and σ^K in the mother cell. Multiple transmembrane signaling events are required to produce this “crisscross” pattern of sigma factor activation, which links transcriptional changes to morphological changes ([11], Figure 2, black arrows).

The early sigma factors σ^F and σ^E are produced in a Spo0A-dependent manner prior to the formation of a polar septum; however, these factors remain inactive until asymmetric division is complete. In the forespore, σ^F activation begins when the phosphatase SpoIIE preferentially activates the anti-anti sigma factor SpoIIAA following polar septum formation [14,15]. Activated SpoIIAA antagonizes anti-sigma factor SpoIIAB binding to σ^F in the forespore [16], freeing σ^F to activate *sigG* and *spoIIR* expression. SpoIIR is secreted into the intermembrane space and activates the SpoIIGA protease to remove an inhibitory pro-peptide from σ^E in the mother cell [17–19].

In the mother cell, σ^E activation drives the transcription of genes encoding a hydrolase complex [20,21] SpoIIM/SpoIID/SpoIIP that mediates engulfment [22,23]. By thinning the peptidoglycan layer of the polar septum, the complex permits the assembly of a channel composed of components controlled by both forespore-specific σ^F and mother cell-specific σ^E [24,25]. This channel, consisting of SpoIIQ (σ^F -controlled) and SpoIIAA-AH (σ^E -controlled), also known as the ‘feeding tube’, bridges the mother cell and forespore compartments and is necessary for σ^G activation in the forespore [26–28]. The feeding tube has homology to secretion systems and transports unknown small molecules into the forespore that are required for σ^G activation [26–28]. σ^G activity also appears to require completion of engulfment, [29] although recent studies challenge this notion [30].

Regardless, σ^G activation depends both on the status of the mother cell and forespore. Active σ^G auto-activates its own production [31] and drives a new transcriptional program encoding determinants important for late sporulation sigma factor σ^K activation in the mother cell [32].

The mother cell-specific σ^K , the last sigma factor in the sporulation pathway, is regulated by multiple mechanisms (Figure 3). *sigK* expression requires both σ^E and SpoIIID, a σ^E -regulated transcription factor [33]. σ^K production depends on σ^E - and SpoIIID-mediated activation of *spoIVCA*, which encodes the recombinase required for excising the *skin* element, a phage DNA-like insertion that disrupts *sigK* [34]. σ^K activity requires proteolytic cleavage by the intramembrane protease SpoIVFB [35], which removes an inhibitory pro-peptide from σ^K similar to σ^E . Since SpoIVFB activity depends on the σ^F -/ σ^G -dependent protease, SpoIVB [35] σ^K activation in the mother cell is coupled to σ^G activity in the forespore. Active σ^K induces the expression of genes whose products are required for cortex and coat formation [21].

Consistent with their roles in regulating engulfment, both *sigF* and *sigE* mutants arrest at asymmetric division ([36], Figure 1B). A *sigG* mutant completes engulfment but fails to make σ^K -dependent cortex or coat [27,37]. A *sigK* mutant phenocopies a *sigG* mutant [38].

Nested within this regulatory framework are a series of feedback and feedforward loops that allow the late sigma factors (σ^G and σ^K) to replace the activity of the early sigma factors (σ^F and σ^E) in their respective compartments [39]. σ^E -regulated gene products activate σ^K and down-regulate σ^E -mediated gene expression [40], while a σ^F -regulated anti- σ^F sigma factor promotes σ^G activation [41].

Thus, the *B. subtilis* sporulation cascade is organized such that the sporulation-specific sigma factors are sequentially activated through a series of intercompartmental signaling events, which in turn are coupled to morphological changes. Events in the forespore are intimately tied in space and time to events in the mother cell, and additional regulatory loops fine-tune the activity of each sporulation-specific sigma factor.

Regulation of sporulation in the Clostridia

Since the sporulation-specific sigma factors are strictly conserved among spore-forming bacteria [39,42,43], and the signaling proteins controlling their activity in *B. subtilis* are largely conserved, the sigma factors were predicted to be sequentially activated across the Firmicutes. This hypothesis, however, has been overturned by recent studies in multiple clostridial species.

(*Pepto*)*Clostridium difficile*

In the Clostridia, sporulation has been most characterized in the nosocomial pathogen *PeptoClostridium difficile* (still known as *C. difficile*) [44]. While *C. difficile* was previously classified as a *Clostridium* species in the family *Clostridiaceae*, recent phylogenetic analyses reclassified *C. difficile* as part of the *Peptostreptococcaceae* family, genus *Peptoclostridium* [45].

At the transcriptional level, *C. difficile* sporulation sigma factor gene regulation resembles that of *B. subtilis* with the notable exception that *sigG* transcription does not depend on σ^F [46,47]. *C. difficile* sigma factor activity exhibits a similar compartmentalization as *B. subtilis*, with σ^F and σ^G activity being forespore-specific, and σ^E and σ^K being mother cell-specific [48]. However, in contrast with *B. subtilis*, minimal intercompartmental signaling is observed during *C. difficile* sporulation, and sigma factor activation is not as tightly coupled to morphological changes [49].

Although σ^F is likely activated by a similar mechanism as *B. subtilis* based on the conservation of SpoIIE, SpoIIAA, and SpoIIAB [39,42,43], *C. difficile* σ^E activation only partially requires σ^F -regulated transmembrane proteolysis because *spoIIR* is still expressed in a *C. difficile sigF* mutant [47]. As a result, partial σ^E processing and activity is observed in the absence of σ^F . *C. difficile* σ^G activation occurs independent of both engulfment and σ^E -controlled transmembrane signaling events [46,47], since the σ^E -controlled feeding tube appears to be dispensable for σ^G activity. Instead, *C. difficile* σ^G requires σ^F for post-translational activation via an unknown mechanism [46]. σ^K activity occurs independent of the σ^G -mediated transmembrane signaling observed in *B. subtilis*, since *C. difficile* σ^K lacks an inhibitory propeptide and is active upon translation ([50], Figure 3). Production of σ^K nevertheless still requires *skin* element excision [48,51] similar to *B. subtilis*, an event that may regulate the timing of σ^K function [49].

Both *C. difficile sigF* and *sigE* mutants are blocked at asymmetric division [46,47], Figure 1B), consistent with their regulation of the peptidoglycan hydrolase genes *spoIIP* and *spoIID* [46,47]. Due to the partial activation of σ^E in a *sigF* mutant [46,47], mislocalized coat can be detected in the mother cell cytosol in *sigF*⁻ cells (Figure 1B) [46]. *C. difficile sigG* mutants have been observed to arrest either during engulfment [46] or following engulfment [47]. The variability in these phenotypes may reflect differences in the strains and growth conditions used. Nevertheless, *sigG* mutants do not make cortex, and, in contrast with *B. subtilis*, they polymerize coat around the forespore [46,48]. In further contrast with *B. subtilis*, a *C. difficile sigK* mutant produces cortex, indicating that σ^K is dispensable for cortex development [46,48] and that the forespore regulates cortex synthesis in *C. difficile*. σ^K nevertheless controls coat polymerization, similar to *B. subtilis*, since no polymerized coat is observed in a *C. difficile sigK* mutant [45,46,48].

Thus, the regulatory architecture of sporulation in *C. difficile* produces a bifurcated rather than crisscross pathway, with the forespore line of gene expression occurring largely independently of the mother cell line of gene expression (Figure 2, black arrows). Accordingly, the sequence of sigma factor activation is not as tightly controlled as in *B. subtilis*, since σ^G can be activated before σ^E and σ^K [46–48], coincident production of σ^E and σ^K during *C. difficile* sporulation does not strongly impact spore formation [50] in contrast with *B. subtilis* [52], and negative feedback loops do not function as prominently in *C. difficile* [40,50].

Lastly, the sporulation sigma factor regulons are collectively smaller in *C. difficile* than in *B. subtilis*, with global transcriptional analyses identifying ~200 genes being activated by these factors in *C. difficile* [46,47] relative to the ~450 genes in *B. subtilis* [21,32,53]. Whether

this difference is functionally significant, the sigma factors control different cellular processes during *C. difficile* sporulation relative to *B. subtilis* (Figure 1B).

***Clostridium* spp**

Studies of sporulation sigma factor function in *Clostridium* spp. have mainly been limited to *C. acetobutylicum*, *C. perfringens*, and *C. botulinum*. While most sporulation sigma factor mutants have been constructed, the mechanisms controlling sporulation sigma factor activation have not been fully characterized. Based on gene conservation, the minimal machinery for activating σ^F , σ^E , σ^G , and σ^K are conserved in these species [13,39,42,43], and both σ^E and σ^K carry a propeptide predicted to inhibit their function [39]. Despite this gene conservation, *C. acetobutylicum* SpoIIE is necessary for *sigF* expression [54] in contrast with *B. subtilis* [15] and no processing of σ^E is observed [55] in contrast with *B. subtilis* and *C. difficile*, suggesting that divergent mechanisms may control sigma factor activation in *Clostridium* spp. Indeed, the timing of σ^K activation in *Clostridium* spp. exhibits clear differences relative to *B. subtilis* and *C. difficile*, with σ^K being activated early during sporulation ([56–58], Figure 2) and independent of *skin* excision (Figure 3). In *C. acetobutylicum*, σ^K surprisingly functions at two discrete stages of sporulation ([56], Figure 2). Prior to sporulation initiation, σ^K activates *spo0A* expression, while at late stages of sporulation, σ^K activates coat protein gene expression [56]. Since σ^K carries a pro-peptide and *spoIVFB* is only expressed late during sporulation [56], it is unclear how early σ^K activity is achieved; an intriguing mechanism that has been proposed is that active σ^K is epigenetic inherited by later cell generations [56].

In *C. acetobutylicum*, differences in sporulation sigma factor gene expression are also observed. While σ^F induces *sigG* expression similar to *B. subtilis*, *C. acetobutylicum* σ^F activates *sigE* expression [59] in contrast with *B. subtilis* and *C. difficile*. Late during sporulation, σ^E induces *sigK* expression [56] similar to *C. difficile* and *B. subtilis*, whether SpoIIID is required for this induction is unclear.

In contrast with *B. subtilis* and *C. difficile*, *C. acetobutylicum* *sigK*, *sigF*, and *sigE* mutants are blocked prior to asymmetric division ([55,56,59], Figure 1B). Since σ^F activation depends on polar septum formation in *B. subtilis* [16], and *C. acetobutylicum* σ^E is required for asymmetric division [59], *C. acetobutylicum* σ^F may require σ^E for activity. While a *C. acetobutylicum* *sigG* mutant completes engulfment similar to *B. subtilis*, coat fragments are visible around the forespore [55] in contrast with *B. subtilis* but similar to *C. difficile*. In contrast with both organisms, a *C. acetobutylicum* *sigG* mutant makes cortex (although to a lesser extent than wild type, Figure 1B, [55]). Notably, artificial expression of *spo0A* in a *sigK* mutant allows sporulation to proceed past engulfment but prior to coat and cortex formation [56].

The mechanisms controlling *C. perfringens* have not been characterized, although both σ^E and σ^K undergo proteolytic processing [57]. Consistent with the early function of σ^K during sporulation, *C. perfringens* σ^K is required for *sigE* expression, even though σ^E induces late stage σ^K production [57]. Whether *C. perfringens* σ^K activates *spo0A* expression, and whether SpoIIID activates *sigK* expression, remains to be tested. However, in contrast with

B. subtilis and *C. difficile*, *C. perfringens* *spoIIID* expression occurs independent of σ^E [57]. While Western blot analyses indicate that σ^F controls σ^E production [60] similar to *C. acetobutylicum*, semi-quantitative RT-PCR analyses suggest that σ^K up-regulates *sigF* [57], highlighting the need for further studies in *C. perfringens*.

The morphological stage(s) at which *C. perfringens* *sigF* and *sigG* mutants are arrested are unknown, although a *sigK* mutant is blocked before asymmetric division [57] similar to *C. acetobutylicum*. In contrast with *C. acetobutylicum*, a *sigE* mutant arrests after asymmetric division ([57], Figure 1B).

In *C. botulinum*, studies of sporulation sigma factor regulation have been restricted to transcriptional analyses. *sigK* exhibits a similar two-stage expression profile [61] as *C. acetobutylicum* and *C. perfringens*, with *sigK* being expressed early during sporulation but rising dramatically in a σ^E - and σ^F -dependent manner late during sporulation [61]. Similar to *C. acetobutylicum*, σ^K activates *spo0A* and *sigF* [58]. Whether σ^K also regulates late stages of sporulation needs to be determined, as do the regulatory mechanisms controlling σ^F and σ^G function. Intriguingly, *spo0A* transcripts are under-expressed in *sigF*, *sigE*, and *sigG* mutants via an unknown mechanism [61].

C. botulinum *sigF* and *sigE* mutants arrest at asymmetric division [61] in contrast with *C. acetobutylicum*, while a *C. botulinum* *sigG* mutant completes engulfment and forms coat fragments but no cortex [61]. Thus, σ^G regulates cortex but not coat formation similar to *C. difficile* and *C. acetobutylicum*. Whether a *sigK* mutant is arrested at an earlier stage of sporulation than *sigF* and *sigE* mutants is unclear.

While further analyses are clearly needed to elucidate the mechanisms controlling the activity and compartmentalization of *Clostridium* spp. sporulation sigma factors, the control of the master transcriptional regulator Spo0A by σ^K defined in *C. acetobutylicum* may be broadly conserved in *Clostridium* spp., since a *C. perfringens* *sigK* mutant arrests at an earlier stage of sporulation than a *sigE* mutant. Nevertheless, since variation in sporulation sigma factor mutant phenotypes are observed between *Clostridium* spp. (Figure 1B), divergent mechanisms may control sigma factor function in different *Clostridium* spp. Transcriptional profiling of sigma factor mutants in multiple *Clostridium* spp. would provide critical insight into these questions. Lastly, it should be noted that σ^K regulates additional cellular processes beyond sporulation in these species: in *C. botulinum* it controls stress responses [62], and in *C. acetobutylicum* it controls solventogenesis [56].

Conclusions

In *B. subtilis* an intricate dialogue between the forespore and mother cell controls the cellular differentiation program of sporulation. However, this intercompartmental dialogue is not broadly conserved in (*Pepto*)*clostridium difficile* and *Clostridium* spp. despite the conservation of many signaling proteins. Instead, sporulation-specific sigma factors are activated with minimal intercompartmental signaling in *C. difficile* and in a different sequence in *Clostridium* spp. The sigma factors also control different morphological processes during sporulation in these clostridia. Accordingly, determining the mechanisms

controlling sporulation sigma factor activation in different Firmicutes will undoubtedly lead to new insights into how differential gene expression is coordinated to produce different cellular fates. Even in *B. subtilis*, open questions remain, such as what is the feeding tube transporting, and why is σ^G activation coupled to engulfment completion? In *C. difficile*, what is the mechanism for post-translationally activating σ^G ? To what extent do sporulation studies in *C. difficile* extend to other *Peptoclostridium* spp.? In *Clostridium* spp., how is early σ^K function achieved? How are the remaining sporulation sigma factors regulated? More broadly, what evolutionary pressures might explain this diversification in regulatory architecture?

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Highlights

- Conserved sporulation sigma factors are differentially regulated in the Firmicutes.
- The order in which sporulation sigma factors function is not conserved.
- The mechanisms controlling σ^K activation are particularly divergent.
- *C. difficile* sigma factor activation is not strictly tied to morphological changes.

Figure 1. Morphological Phenotypes of Sigma Factor Mutants in *Bacillus subtilis*, *(Pepto)Clostridium difficile*, *Clostridium acetobutylicum*, *Clostridium perfringens*, and *Clostridium botulinum*

utations in sporulation specific sigma (σ) factor genes, *sigF*, *sigE*, *sigG*, and *sigK*, result in a variety of phenotypes. **A.** Morphological stages of sporulation (I–VII) defined by transmission electron microscopy (TEM) studies in *B. subtilis* [7]. Stage I, decision to sporulate (no morphological phenotype); Stage II, completion of asymmetric division; Stage III, completion of engulfment; Stage IV cortex formation (pink) between the inner and outer forespore membrane; Stage V, coat assembly around the outer forespore membrane (blue); Stage VI, spore core maturation, e.g. chromosome condensation; Stage VII, release of fully mature spore following mother cell lysis. Key structures that comprise a mature spore are labeled. **B.** *B. subtilis* *sigF*⁻ and *sigE*⁻ complete asymmetric division (Stage II), while *sigG*⁻ and *sigK*⁻ complete engulfment but fail to make coat or cortex (Stage III). *C. difficile* *sigF*⁻ completes asymmetric division; it also makes coat that mislocalizes to the mother cell cytosol (blue); *sigE*⁻ arrests at asymmetric division (Stage II); *sigG*⁻ does not complete engulfment in strain JIR8094 (shown, [46]), although it does in strain 630 *erm* [48]. Regardless, both *sigG*⁻ strains fail to make cortex, despite producing coat (blue) that localizes around the forespore; *sigK*⁻ produces cortex (pink) but no detectable coat. *C. acetobutylicum* *sigF*⁻, *sigE*⁻, and *sigK*⁻ do not complete asymmetric division (Stage I), although a small population (< 2%) of *sigE*⁻ mutants complete asymmetric division [55]; *sigG*⁻ completes engulfment and makes some cortex along with coat-like fragments that localize next to the forespore. *C. perfringens* *sigE*⁻ is blocked at asymmetric division (Stage

II), while *sigK*⁻ does not initiate asymmetric division (Stage I); *sigF*⁻ and *sigG*⁻ phenotypes have yet to be analyzed by TEM. *C. botulinum sigF*⁻ and *sigE*⁻ complete asymmetric division (Stage II); *sigG*⁻ completes engulfment and appears to make coat fragments that localize around the forespore; *sigK*⁻ TEM has not been performed.

Figure 2. Conserved sporulation-specific sigma factors regulate sporulation in diverse ways in the Firmicutes

In *B. subtilis*, sigma factor activity is regulated in a “crisscross” pattern: σ^F , σ^E , σ^G , and σ^K are sequentially and alternately activated in the forespore and mother cell compartment, respectively. After Spo0A-mediated activation of *sigF* and *sigE*, σ^F becomes active in the forespore. Expression of the σ^F regulon leads to the activation of σ^E in the mother cell. Products of the σ^F and σ^E regulon activate σ^G in the forespore compartment. σ^G auto-activates its production and induces σ^K activation in the mother cell. σ^F and σ^E are required for *sigG* and *sigK* expression in the forespore and mother cell, respectively. In *C. difficile*, after Spo0A-mediated transcriptional activation of *sigF* and *sigE*, σ^F activates σ^G via an unknown post-translational mechanism. σ^F also activates σ^E , although the requirement is only partial (dotted black line) due to σ^F -independent expression of *spoIIR*. σ^E activates *sigK* transcription; σ^K is active following translation. *sigG* expression is activated by Spo0A and may be subject to auto-activation. In *C. acetobutylicum*, σ^K activates *spo0A* expression. Spo0A in turn activates *sigF* and *sigE* transcription. The mechanisms controlling the activity of σ^E , σ^G , and/or σ^K are unknown (question marks), although initial studies suggest that σ^G may be dispensable for late σ^K function. It is hypothesized that σ^K may be epigenetically inherited so that Spo0A can be activated during vegetative growth ([56], red arrow). Green arrows designate transcriptional activation; black arrows designate post-translational activation; dotted arrow indicates partial requirement for activation; question marks indicate that more work needs to be done to determine the mechanisms controlling sigma factor gene transcription and/or sigma factor activation.

Figure 3. Regulation of σ^K activity differs in the Firmicutes

σ^K is regulated by transcriptional and post-translational mechanisms during sporulation. In *B. subtilis* and *C. difficile*, a phage DNA-like *skin* element must excise from genomic DNA prior to *sigK* transcription. In *B. subtilis*, after *skin* element excision, σ^E and the σ^E -regulated transcription factor SpoIIID activate *sigK* expression. σ^K is produced with an inhibitory pro-peptide that undergoes proteolytic activation via SpoIVFB. In *C. difficile*, σ^K regulation is similar to *B. subtilis* with the exception that σ^K lacks a pro-peptide and is active upon translation. In *C. acetobutylicum*, σ^K acts at two stages during sporulation. Early σ^K function has been hypothesized to occur via epigenetic inheritance or by early activation of *sigK* transcription by σ^A [56]. However, these hypotheses have not been tested. Late *sigK* transcription is mediated by σ^E , although the involvement of SpoIIID for transcriptional activation remains to be determined. It is unclear whether σ^K undergoes proteolytic activation, although SpoIVFB would be predicted to cleave σ^K . In *C. perfringens*, early *sigK* transcription appears to occur via read-through transcription of *CPR_1739* [57], while late transcription is mediated by σ^E . Whether SpoIIID activates *sigK* transcription has not been determined, although σ^E does not control *spoIIID* expression. While SpoIVFB is conserved, it is unclear if it removes the pro-peptide from σ^K ; whether cleaved σ^K is the active form has also not been determined. In *C. botulinum*, factors controlling *sigK* transcription are unknown. Whether σ^K undergoes proteolytic activation is also unclear. Based on gene conservation, it is hypothesized that σ^E and SpoIIID activate *sigK* transcription, and SpoIVFB activates σ^K pro-peptide cleavage.