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Sporulation and Germination in Clostridial Pathogens

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SUMMARY

As obligate anaerobes, clostridial pathogens depend on their metabolically dormant, oxygen-tolerant spore form to transmit disease. However, the molecular mechanisms by which those spores germinate to initiate infection and then form new spores to transmit infection remain poorly understood. While sporulation and germination have been well characterized in *Bacillus subtilis* and *B. anthracis*, striking differences in the regulation of these processes have been observed between the Bacilli and the Clostridia, with even some conserved proteins exhibiting differences in their requirements and functions. Here, we review our current understanding of how clostridial pathogens, specifically *Clostridium perfringens*, *Clostridium botulinum*, and *Clostridioides difficile*, induce sporulation in response to environmental cues, assemble resistant spores, and germinate metabolically dormant spores in response to environmental cues. We also discuss the direct relationship between toxin production and spore formation in these pathogens.

Notably, different mechanisms exist between these organisms for forming and germinating spores. Some of these differences reflect phylogenetic differences between the Clostridiaceae (represented by *C. perfringens* and *C. botulinum*) and the Peptostreptococcaceae (represented by *C. difficile*) (1). Other differences reflect genetic diversity within each species (2–4). *C. botulinum* is the most divergent, being divided into four different metabolic groups (Groups I–IV) that effectively represent different species despite their shared production of botulinum toxin (5).

IMPORTANCE OF SPORES TO CLOSTRIDIAL PATHOGENESIS

Disease transmission by clostridial pathogens depends on their ability to form aerotolerant, metabolically dormant spores before exiting their hosts (6). Since spores are highly resistant to extreme temperature and pressure changes, radiation, enzymatic digestion, and oxidizing agents (7), they can persist for long periods of time and serve as environmental reservoirs for

these organisms (8). Spores from *C. perfringens*, *C. botulinum*, and *C. difficile* can be isolated from diverse environments, including animal gastrointestinal tracts and carcasses, wastewater, lawns, hospital rooms, and soil (8). Infections by these pathogens typically are initiated upon ingestion of spores, although *C. perfringens* can also enter the body via contaminating wounds. Upon sensing small molecule germinants, spores from these pathogens will germinate and outgrow into toxin-secreting vegetative cells.

C. perfringens

C. perfringens causes two major human diseases: food poisoning and gas gangrene (also known as clostridial myonecrosis (9)). Clostridial myonecrosis occurs when spores from the soil enter muscle tissue, typically through a wound, while food poisoning arises when spore or vegetative cells in contaminated food are ingested. Unlike most clostridial pathogens, *C. perfringens*' vegetative form can initiate infection when present at sufficiently high levels to survive passage through the stomach. *C. perfringens* causes disease by secreting a number of toxins; *C. perfringens* strains are sub-divided into toxigenic types A-E based on the combination of alpha-, beta-, epsilon, and iota-toxins they produce.

C. perfringens spores can be remarkably heat resistant, with some Type A strains surviving boiling for >1 hr (10, 11). Spores from food poisoning isolates exhibit greater resistance to heat (~60-fold), cold, and oxidizing agents (12, 13) than non-food-borne isolates, suggesting that their resistance properties facilitate their survival in undercooked or improperly held food (10).

C. botulinum

C. botulinum causes a flaccid paralysis known as botulism through the production of the potent neurotoxin, botulinum toxin (BoNT). *C. botulinum* strains are sub-divided based on their production of one or more of seven BoNT types (A-G) (14, 15). Botulism typically results from the ingestion of pre-formed BoNT in contaminated foods, but ingestion of *C. botulinum* spores in contaminated foods like unpasteurized honey can cause infant paralysis, particularly in those < 1 years old (16). Even in cases of botulism intoxication, the spore form is critical for contaminating food, where incomplete sterilization or processing, such as during home canning, can create anaerobic environments that allow *C. botulinum* spores to germinate and form toxin-producing vegetative cells that subsequently intoxicate the food (17).

C. difficile

C. difficile is a leading cause of antibiotic-associated diarrhea and pseudomembranous colitis worldwide (18, 19). *C. difficile*-associated disease occurs when spores, ingested by susceptible hosts, germinate in response to specific bile acids sensed in the mammalian gut; the resulting vegetative cells produced will secrete glucosylating toxin(s) that are the primary cause of disease symptoms (20). Notably, antibiotic exposure sensitizes individuals to antibiotic-resistant *C. difficile* by removing the colonization resistance conferred by our gut microflora (21). Spores are critical to this infection process not only because they are essential for transmitting *C. difficile* infections (6) but also because they are inert to antibiotics and resist many commonly used disinfectants in healthcare settings (22, 23).

Accordingly, spores are easily detected in healthcare-associated environments (24) in addition to numerous other sites (8).

OVERVIEW OF SPORE FORMATION

The first morphological stage of sporulation is the formation of a polar septum, which generates two morphologically distinct, but genetically identical, cells (Fig. 1, (25)). The larger mother cell engulfs the smaller forespore cell, leaving the forespore within the mother cell cytosol surrounded by two membranes. A thick layer of modified peptidoglycan known as the cortex forms between the two membranes, conferring spores with heat- and ethanol-resistance (26, 27). A series of proteinaceous layers known as the coat assembles around the outer forespore membrane, which protect the spore against enzymatic and oxidative insults. In *C. difficile*, an additional layer known as the exosporium assembles on the coat, but this layer is not present in all spore-forming organisms.

The mother cell and forespore also coordinately prepare the forespore for dormancy. The mother cell produces large amounts of dipicolinic acid (pyridine-2,6-dicarboxylic acid) complexed with calcium (Ca-DPA), which it pumps into the developing forespore in exchange for water (28). The resulting partially-dehydrated forespore cytosol prevents metabolism. The forespore produces large amounts of small acid soluble proteins (SASPs), which coat the chromosome, prevent transcription, and protect against DNA damage (26, 28). Once the forespore completes its maturation, the mother cell induces lysis and releases the metabolically dormant spore into the environment.

SPORULATION INITIATION IN CLOSTRIDIAL PATHOGENS

The decision to initiate sporulation requires that vegetative cells recognize specific environmental and nutritional signals and assimilate these cues into a robust response. All sporulating Firmicutes use the conserved transcriptional regulator, Spo0A, as a key checkpoint for integrating these signals. The response regulator Spo0A initiates spore formation by activating or repressing the expression of genes encoding early sporulation regulators (6, 29–34), and its DNA-binding activity is directly controlled by phosphorylation. In the model organism *Bacillus subtilis*, Spo0A phosphorylation is orchestrated by a complex regulatory network, known as a phosphorelay, which consists of several orphan sensor histidine kinases (KinA-E; orphan refers to histidine kinases that are not encoded beside a response regulator) and two phosphotransfer proteins (Spo0F and Spo0B) (35). The KinA-E kinases directly phosphorylate Spo0F, which subsequently transfers the phosphate to Spo0B and finally to Spo0A (Fig. 2, (36)). Anti-kinases and two different classes of phosphatases inhibit Spo0A phosphorylation levels by either blocking kinase activity or stripping Spo0A or Spo0F of its phosphate (37). The complexity of this regulatory pathway functions as a noise generator, creating heterogeneous levels of Spo0A phosphorylation within a population such that only a portion of its population commits to sporulation (38–40).

Notably, the *B. subtilis* phosphorelay is absent in clostridial pathogens, since clostridia lack orthologs of the Spo0F and Spo0B phosphotransfer proteins (41, 42). Thus, Spo0A appears

to be directly phosphorylated by histidine kinases in clostridial organisms (32, 33, 43–46), although the kinases, regulatory pathway, and environmental signals used to control clostridial Spo0A phosphorylation remain largely uncharacterized.

C. difficile

Sporulation initiation has been most extensively studied in *C. difficile*, where key Spo0A regulatory proteins have been identified (Fig. 3). An initial study identified three orphan histidine kinases with significant homology to the *B. subtilis* family of sporulation-associated phosphotransfer histidine kinases (32). Loss of one of these putative histidine kinases, CD2492, decreased spore formation ~3-fold in rich liquid media, while another kinase, CD1579, directly phosphorylated Spo0A *in vitro* (32). These results suggest that both CD2492 and CD1579 promote Spo0A activation. Loss of the third putative histidine kinase, CD1492, increases sporulation ~4-fold on solid sporulation media in a manner dependent on its conserved histidine residue (47), suggesting that CD1492 functions as a phosphatase rather than a kinase.

Interestingly, CD2492 does not necessarily always promote sporulation, since a *CD2492* mutant exhibits increased sporulation on solid sporulation media independent of its conserved histidine residue [Edwards and McBride, unpublished data]. The discrepancy in *CD2492* mutant sporulation phenotypes could be due to differences in growth conditions and/or methods for measuring spore formation. The signals that control kinase versus phosphatase activity of sporulation-associated histidine kinases are largely unknown, even in *B. subtilis*; thus, the activity observed may depend on the presence or absence of unidentified signals. Indeed, one *B. subtilis* kinase mutant exhibits varying sporulation phenotypes depending on the growth conditions used (48, 49). This contradiction highlights the importance of assessing sporulation using multiple conditions and verifying spore frequencies with at least two different methods (50).

Notably, sporulation-associated histidine kinases directly control Spo0A phosphorylation through competing kinase and phosphatase activities in *C. acetobutylicum* and *C. thermocellum*, where at least one sporulation-associated histidine kinase in each species inhibits sporulation *in vivo* (44, 45), and a *C. acetobutylicum* histidine kinase has been shown to dephosphorylate Spo0A *in vitro* (45). Altogether, clostridial sporulation-associated histidine kinases appear to reversibly regulate Spo0A phosphorylation and thus the onset of sporulation.

Spo0A activity in *C. difficile* is further modulated by an RRNPP family ortholog, RstA (Regulator of Sporulation and Toxins) (51), which shares homology with the Rap phosphatases that directly dephosphorylate Spo0F in *B. subtilis*. RRNPP family members have multiple C-terminal tetratricopeptide repeat (TPR) domains that bind quorum-sensing peptides and regulate the cognate N-terminal helix-turn-helix (HTH) DNA-binding domain and/or Spo0A/Spo0F-binding domain (52, 53). RstA contains these three conserved regulatory domains, and an *rstA* mutant produces ~20-fold fewer spores than wild type, indicating that RstA promotes early sporulation events in *C. difficile* (29). Although the HTH domain appears to be dispensable for RstA to modulate sporulation (29), the putative Spo0A/Spo0F-binding domain may directly bind and control Spo0A phosphorylation and/or

dephosphorylation [Edwards and McBride, unpublished data]. Interestingly, in addition to regulating sporulation, RstA represses motility and toxin production (see below) (51). Homologs of RstA are observed in both pathogenic and non-pathogenic clostridial organisms, including *C. sordellii*, *C. perfringens*, *C. botulinum*, and *C. acetobutylicum* (51).

RRNPP systems in other spore-formers use quorum-sensing peptides to control activity of the regulator, and thus, sporulation initiation. For example, *B. subtilis* Rap phosphatase activity is inhibited by quorum-sensing peptides imported by the conserved oligopeptide permeases Opp and App, promoting sporulation (32, 34–36). Further, *C. acetobutylicum* sporulation is affected by the deletion or overexpression of genes encoding RRNPP-type regulators, which are located adjacently to putative quorum-sensing peptide genes (54). However, it is unclear whether *C. difficile* RstA activity is regulated by quorum-sensing peptides. Regardless, peptide transport coordinates the onset of sporulation in *C. difficile*, since the loss of Opp and App increases *C. difficile* sporulation ~20-fold (55). These results suggest that the peptides imported by *C. difficile* Opp and App serve as nutrients rather than quorum sensing molecules such that *C. difficile opp* and *app* mutants may initiate sporulation earlier due to decreased nutrient acquisition.

Metabolic cues regulate the initiation of *C. difficile* sporulation because the global regulators CcpA and CodY directly control the expression of genes encoding early sporulation regulators. CcpA senses carbon availability (56, 57), and CodY senses GTP and branched-chain amino acid levels (58, 59). CcpA directly represses the expression of *spo0A* and the *opp* and *sigF* operons (*sigF* encodes the first sporulation-specific sigma factor to be activated). CcpA also indirectly downregulates transcription of *sinR* (56), which encodes an early sporulation regulator that enhances sporulation (60). Not surprisingly, sporulation is increased ~10-fold in a *ccpA* mutant (56), providing further evidence that *C. difficile* initiates sporulation in response to nutrient deprivation. Similarly, CodY downregulates transcription of *sinR* and the *opp* operon, and a *codY* mutant exhibits increased sporulation (61). However, CodY's effect on sporulation is strain-specific, given that an R20291 *codY* mutant produces ~100-fold more spores than the parent strain, but the equivalent mutant in 630 *erm* produces only ~2-fold more spores (61). Interestingly, the addition of glucose reduces sporulation frequency in a CcpA-independent manner (56), revealing that additional regulatory pathways impact the timing of sporulation in response to nutritional cues.

C. difficile also uses alternative sigma factors to control sporulation initiation. SigH, which controls the transition to stationary phase, is essential for sporulation and directly drives the expression of *spo0A*, similar to *B. subtilis* (35), and *CD2492*, which modulates Spo0A phosphorylation. In contrast, SigB, the general stress response sigma factor, inhibits pre-divisional sporulation-specific gene expression and decreases spore formation (62). SigB likely decreases Spo0A phosphorylation by reducing transcription of *CD1579*, which encodes a Spo0A kinase (12), and increasing transcription of *CD1492*, which encodes a putative Spo0A phosphatase (47).

The *C. difficile* genome encodes orthologs of additional *B. subtilis* early sporulation factors, such as SinRI, Spo0J-Soj, Spo0E and KipI/KipA (see (63) for more detail). However, if the trend from recent research holds true, these uncharacterized *C. difficile* orthologs likely

function differently from those in *B. subtilis*. Indeed, recent analyses indicate that the *C. difficile* *sinR* locus encodes two *sinR* homologs, *sinR* and *sinR'*, with SinR' antagonizing SinR function, analogous to *B. subtilis* SinI's negative effect on SinR activity. In *C. difficile*, SinR functions as a DNA-binding transcriptional regulator and enhances sporulation, although the mechanism is unclear. There is some evidence that SinR regulates early sporulation events as Spo0A-dependent gene expression is significantly decreased in a *sinRR'* mutant and is not rescued by *spo0A* overexpression (60). Furthermore, additional novel *C. difficile* regulators of sporulation initiation are likely to be discovered given that *C. difficile* encounters a diverse array of environmental conditions during growth in the gut, which strongly induces sporulation gene expression relative to broth culture growth to promote survival outside of the host (64).

C. perfringens

In contrast with most spore-forming organisms, *C. perfringens* induces sporulation during exponential phase (65). Sporulation induction depends in part on sensing cell density through the Agr-like quorum-sensing system because an *agrB* mutant, which no longer makes a mature quorum-sensing peptide, has reduced Spo0A protein levels and produces ~1000-fold fewer spores than wild type (66). Like *C. difficile*, *C. perfringens* CcpA and CodY modulate sporulation (67, 68), although unlike *C. difficile*, *C. perfringens* CcpA activates rather than represses sporulation (68), with a *C. perfringens* *ccpA* mutant making ~60-fold fewer spores than wild type. Nevertheless, similar to *C. difficile*, glucose strongly reduces *C. perfringens* sporulation frequency (~2000-fold) in a CcpA-independent manner.

CodY regulates *C. perfringens* sporulation in a strain-specific manner again analogous to *C. difficile*. Loss of CodY in the type D strain CN3178 increases spore formation ~10-fold (67, 68), whereas loss of CodY in the food-poisoning strain SM101 reduces sporulation ~1000-fold relative to wild type (69). CodY likely regulates sporulation by altering expression of *abrB*, which encodes a negative regulator of sporulation that functions in a strain-specific manner (67). *C. perfringens* also employs a regulatory RNA, *virX*, to repress sporulation by decreasing the expression of genes encoding sporulation-specific factors (70).

While the kinases that phosphorylate *C. perfringens* Spo0A remain unknown, six putative orphan histidine kinases have been identified in BLAST searches (71). Given that the bile acid deoxycholate induces *C. perfringens* Spo0A phosphorylation and thus sporulation (72), these kinases may specifically respond to this bile acid. Similarly, inorganic phosphate induces *C. perfringens* sporulation (73) and could stimulate kinase activity.

C. botulinum

Unlike *C. perfringens*, *C. botulinum* sporulates during the transition from exponential to stationary phase (74). Like *C. perfringens*, the Agr-like quorum sensing system is important for sporulation initiation, with *agrB* and *agrD* *C. botulinum* mutants producing ~1000-fold less spores (75). While the impact of CcpA and CodY on *C. botulinum* sporulation remain to be studied, five putative orphan kinases have been identified as possible regulators of Spo0A (33). The CB01120 histidine kinase appears to phosphorylate Spo0A based on the observation that production of CB01120 with wild-type Spo0A, but not a non-

phosphorylatable form of Spo0A, causes lethality when heterologously produced in *B. subtilis* (33).

THE LINK BETWEEN SPORULATION AND TOXIN GENE EXPRESSION IN *C. DIFFICILE*

Although most *Clostridium* species initiate sporulation to survive unfavorable conditions, *C. perfringens* and *C. botulinum* directly couple toxin production (10, 30) to sporulation, while *C. difficile* coordinates these processes in a strain-specific manner (76).

Clostridium perfringens Enterotoxin (CPE)

C. perfringens type A causes food poisoning and non-food-borne gastrointestinal disease (3). These diseases are caused by the CPE toxin (13), which is either encoded (*cpe*) chromosomally or on a large plasmid: most food-poisoning isolates carry chromosomal *cpe*, while most non-food-borne isolates carry a plasmid-borne *cpe* (13, 77, 78). Food poisoning typically occurs when chromosomal *cpe* isolates are ingested with food, while non-food-borne disease is primarily acquired by ingesting spores. Regardless, growth of *C. perfringens* in the small intestine leads to CPE production and thus disease (3).

Numerous studies have demonstrated a strong correlation between spore formation and CPE production (79–82). The first genetic evidence linking *C. perfringens* sporulation with CPE synthesis came from studies showing that mutants blocked at asymmetric division during sporulation failed to produce CPE, while mutants blocked at later stages of sporulation produced CPE (81). For example, a *C. perfringens* strain SM101 *spo0A* mutant cannot produce CPE (29), while inorganic phosphate (Pi) induces both sporulation and CPE production in wild type (73).

Expression of the *cpe* gene is induced during sporulation, with the *cpe* transcript only being detected in sporulating, but not vegetative, *C. perfringens* cultures (80, 83). *cpe* is transcribed from three promoters, named P1, P2 and P3, that are either σ^E - or σ^K -dependent (83). These promoters induce high levels of CPE production during sporulation, with CPE constituting up to 20% of the total protein present (84). σ^E and σ^K are likely mother cell-specific (85), so CPE production appears to be restricted to the mother cell cytoplasm, where it reaches sufficiently high concentrations to induce paracrystalline inclusion body formation (84). However, rather than being secreted from sporulating cells, CPE is released upon mother cell lysis in the late stage of sporulation (84). Accordingly, *sigK* and *sigE* mutants of the food-poisoning strain SM101 fail to express *cpe* (65), while *sigF* but not *sigG* mutants exhibit defects in *cpe* expression and CPE production based on RT-PCR and Western blot analyses (86). Thus, only σ^F , σ^E , and σ^K are necessary for *cpe* transcription and CPE production (65), even though all four sporulation-specific sigma factors are required for *C. perfringens* sporulation (10, 65).

Since CPE production is strictly sporulation-dependent, factors that regulate early events of *C. perfringens* sporulation also regulate CPE production. Accordingly, the Agr-like quorum sensing system in the non-food-borne strain, F5603, and CodY in the food-poisoning strain SM101, activate CPE production by activating Spo0A and inducing σ^F production (66, 67).

virX, a regulatory RNA that represses sporulation, accordingly reduces *cpe* expression and CPE production (70). Notably, while sporulation is crucial for CPE production, CPE is not required for sporulation because *cpe* mutants sporulate at wild-type levels (13, 87).

***Clostridium perfringens* TpeL Toxin**

Many *C. perfringens* isolates encode a novel toxin named TpeL, which belongs to the family of large clostridial toxins (88) and is encoded both chromosomally and on plasmids (89–91). While the contribution of TpeL to *C. perfringens* pathogenesis is unknown, *tpeL* expression is specifically induced during sporulation based on transcriptional reporter studies (92). The *tpeL* promoter region contains σ^E - and σ^K -dependent sequences, and loss of σ^E strongly reduces *tpeL* expression (~100-fold) (92), indicating that *tpeL* expression also depends on σ^E similar to *cpe*. More recent analyses indicate that *tpeL* expression is induced by TpeR, a transcriptional regulator encoded in the same pathogenicity locus (PaLoc) as *tpeL*; in these analyses, TpeL production was observed under conditions that promote vegetative cell growth (93).

***Clostridium botulinum* type Neurotoxin (BoNT)**

Toxin production and sporulation coincide during the transition from exponential to stationary phase, suggesting that these processes may be co-regulated. The Agr-like quorum sensing system may coordinate these processes as in *C. perfringens*, since toxin and spore formation are reduced in *agrB* and *agrD* mutants (75). In aquatic *C. botulinum* type E strains (94, 95), sporulation and BoNT production are directly linked because loss of Spo0A prevents spore formation and reduces BoNT production >10-fold relative to wild type (30). Spo0A likely directly regulates toxin production because Spo0A directly binds the *botE* promoter *in vitro*, which contains a Spo0A box (30). Interestingly, Spo0A is the first neurotoxin regulator identified in *C. botulinum* type E, which does not encode the alternative sigma factor, BotR, that activates botulinum gene expression in other strains (96).

***Clostridioides difficile* Glucosylating Toxins**

C. difficile produces two large exotoxins, Toxin A (TcdA) and Toxin B (TcdB), which are critical for virulence (97–99). The regulatory links between *C. difficile* toxin production and sporulation are complex and appear strain-dependent. Spo0A represses toxin expression in epidemic 027 ribotypes (6, 76), does not impact toxin expression in the emerging 078 ribotype (76) and variably impacts toxin expression in the historic 012 ribotype (6, 31, 32, 76, 100). In the 630 background (012 ribotype), the stationary phase sigma factor, SigH, downregulates toxin gene expression (101), and the phosphotransfer protein, CD1492, positively affects toxin production (47) presumably through indirect means. However, as with sporulation, nutrient availability strongly influences toxin gene expression since amino acids and glucose repress toxin gene expression through the global regulators CodY and CcpA, respectively (57, 59, 102).

The most direct link between toxin gene expression and sporulation is RstA, the RRNPP family member discussed above, which inversely regulates toxin production and sporulation (51). RstA inhibits transcription of *tcdA* and *tcdB* by directly binding to the promoters and inhibiting the transcription of *tcdR* and *sigD* (Edwards and McBride, unpublished data, and

29). *tcdR* encodes the sigma factor that directly activates toxin gene expression (103), while *sigD* encodes the flagellar-specific sigma factor that also directs *tcdR* transcription (104, 105). This multi-tiered regulation suggests that RstA tightly controls toxin production. While RstA-dependent repression of toxin gene expression requires its DNA-binding domain, its regulation of sporulation does not. Thus, RstA regulates sporulation and toxin gene expression through independent mechanisms (51). However, it remains unclear if this bifunctional protein links sporulation and toxin regulation in the same cell: single-cell analyses would reveal whether toxin-producing cells also sporulate, or whether these important processes are asynchronous. Interestingly, the regulatory pathways between sporulation and toxin gene expression may be reciprocal in some *C. difficile* strains, as TcdR enhances spore formation in R20291 (027 ribotype) but not 630 (106).

Overall, several *C. difficile* regulators control both sporulation and toxin gene expression, suggesting that the coordinate regulation of both these processes is important for *C. difficile* survival.

THE SPORULATION TRANSCRIPTIONAL PROGRAM

Once Spo0A is phosphorylated, it induces asymmetric division, which eventually leads to the activation of four sporulation-specific sigma factors, σ^F , σ^E , σ^G , and σ^K . These sigma factors are essential for sporulation (25, 85, 107) because they coordinate the activation of distinct transcriptional programs within the mother cell and forespore, respectively, that culminate in the formation of a metabolically dormant spore. While the regulation of sporulation-specific sigma factors has been extensively analyzed in *B. subtilis*, the conserved sporulation sigma factors exhibit notable differences in their regulation and function in clostridial organisms relative to *B. subtilis* as well as between clostridial organisms. We first describe the activation and functions of sporulation-specific sigma factors in *B. subtilis* and compare these properties with those in *C. difficile*, *C. perfringens*, and *C. botulinum*.

B. subtilis sporulation-specific sigma factors are post-translationally activated in a compartment-specific and sequential manner. σ^F is activated in the forespore followed by σ^E in the mother cell; σ^E then activates σ^G in the forespore, which subsequently activates σ^K in the mother cell (Fig. 4, (25)). Inter-compartmental signaling regulates sporulation sigma factor activation and couples it to specific morphological changes (108). Spo0A induces the transcription of *sigF* and *sigE* in the pre-divisional cell such that σ^F and σ^E are present in both the mother cell and forespore, although both sigma factors remain inactive until asymmetric division is complete. σ^F is first activated in the forespore when the preferential activation of the SpoIIE phosphatase in the forespore leads to dephosphorylation of the anti-sigma factor, SpoIIAA (109–111), which antagonizes the anti-sigma factor, SpoIIAB. Inhibition of SpoIIAB frees σ^F to bind its target promoters, which include *sigG* and *spoIIR*. The resulting production of SpoIIR leads to σ^E activation because SpoIIR activates SpoIIGA, the protease that removes σ^E 's inhibitory pro-peptide in the mother cell (112–114).

Activated σ^E directs the transcription of genes (115) required for the mother cell to (i) engulf the forespore in a phagocytic-like process (*spoIID*, *spoIIP*, and *spoIIM*, (116–118)), (ii)

localize coat proteins to the forespore (119), (iii) activate σ^G in the forespore (*spoIIIA* operon, (120)), and (iv) produce and activate σ^K in the mother cell (*sigK*, *spoIIID*, *spoIVFA-B*, *ctpB*, and *spoIVCA* (121–125)). The SpoIIIA proteins form a complex with GerM and the forespore-specific SpoIIQ to form a channel connecting the mother cell and forespore (126–129). This channel, also known as the “feeding tube,” is required to maintain transcriptional potential in the forespore (126), and thus σ^G activity. SpoIIQ also contributes to σ^G activation by controlling the localization of SpoIIE, which likely antagonizes the σ^G -specific anti-sigma factor, CsfB (130). Thus, discrete anti-sigma factors control the activation of σ^F and σ^G , respectively, in *B. subtilis*.

Engulfment increases σ^G activity, which couples its transcriptional program to morphological changes (131). Activated σ^G directs the transcription of genes in the forespore (132) required to (i) modify the cortex (133), (ii) prepare the forespore for dormancy (7), and (iii) activate σ^K via regulated proteolysis by the mother cell-localized protease SpoIVFB (125). Thus, proteolytic signaling cascades induced by the forespore activate σ^E and σ^K in *B. subtilis*.

σ^K production depends on σ^E because σ^E activates transcription of *spoIIID*, which encodes the transcriptional regulator that induces *sigK* expression (134). σ^E also controls the expression of *spoIVCA*, which encodes the site-specific recombinase necessary to mediate excision of the *skin* element, a 42 kB prophage-like region that disrupts the *sigK* gene (122). Thus, multiple levels of σ^K regulation control the precise timing of its activation in *B. subtilis* to ensure proper spore assembly, since activated σ^K induces cortex and coat assembly genes (135).

Taken together, *B. subtilis* sporulation gene expression is tightly controlled both spatially and temporally, with events in the forespore being coupled to events in the mother cell. Sporulation sigma factor activation and function is further controlled by additional feedback and feedforward loops to ensure that the timing of sporulation gene expression is tightly coordinated with morphological changes (136).

Notably, while most of the gene products that control sporulation sigma factor activation in *B. subtilis* are conserved in the Clostridia, the functions of some of these gene products and the timing of their action are not always conserved. Many of the regulatory loops that fine-tune the timing of sporulation sigma factor activation and function in *B. subtilis* are not conserved in clostridial organisms (42, 71) (Fig. 4), suggesting that (i) sporulation sigma factor activity may not be as tightly regulated in clostridial pathogens relative to *B. subtilis* and/or (ii) additional regulatory pathways exist. While this review focuses on sporulation sigma factor regulation in pathogenic clostridia, this process has also been examined in detail in *C. acetobutylicum*, which revealed notable differences in the regulation of these sigma factors relative to *B. subtilis* (reviewed by Al-Hinai *et al* (85)).

C. difficile

Sporulation sigma factor activation has been best studied in *C. difficile*, where genome-wide transcriptional analyses of sigma factor mutants have defined the regulons of sporulation-specific sigma factors (137, 138). These factors were shown to function in a compartment-

specific manner similar to *B. subtilis* (Fig. 4) through the development of SNAP tag-based transcriptional reporter constructs by the Henriques group (139). Specifically, *C. difficile* σ^F and σ^G activity is restricted to the forespore, while σ^E and σ^K activity being restricted to the mother cell (139). This regulation resembles *B. subtilis* (25) with the exception that *sigG* expression does not require σ^F and is likely activated by Spo0A (137).

The mechanisms underlying both *C. difficile* σ^F and σ^E activation likely occur through a similar mechanism as *B. subtilis* based on gene conservation. Consistent with this notion, the σ^F -activating phosphatase, SpoIIE, and the σ^E -activating protein, SpoIIR, are essential for sporulation based on a transposon screen (140). Similar to *B. subtilis*, *C. difficile* SpoIIR is required for pro- σ^E processing (138). However, unlike *B. subtilis*, *C. difficile* σ^F is not essential for this proteolytic activation event because *spoIIR* is expressed from both Spo0A-dependent and σ^F -dependent promoters (138). Thus, *C. difficile* σ^E is partially active in a *sigF* mutant (137, 138).

C. difficile σ^G and σ^K activation differs markedly from *B. subtilis*. Whereas σ^G activation depends on the SpoIIQ-SpoIIIA channel complex in *B. subtilis*, *C. difficile* σ^G is active in the absence of this channel and its associated engulfment defects (141, 142). Notably, σ^G is present but inactive in the absence of σ^F (137), indicating that *C. difficile* σ^G activity is post-translationally activated in the forespore through an unknown mechanism.

Unlike most spore-forming organisms, *C. difficile* σ^K lacks the N-terminal inhibitory pro-peptide (143) that tethers *B. subtilis* pro- σ^K to the membrane (123). As a result, *C. difficile* σ^K is active upon translation, since expression of *sigK* from a tet-inducible promoter in vegetative *C. difficile* allows σ^K -dependent genes to be transcribed in contrast with *B. subtilis* (144). However, similar to *B. subtilis*, *C. difficile* *sigK* transcription depends on the excision of a large *skin* element that disrupts the *sigK* gene (143) and is activated by σ^E and SpoIIID (138, 144). In contrast with *B. subtilis*, the gene encoding the excision recombinase, *CD1231*, is constitutively expressed rather than activated by σ^E (145). Instead, CD1231 activity is post-translationally activated by the CD1234 recombination directionality factor, whose production depends on both σ^E and SpoIIID. Thus, CD1231 and CD1234 control the timing of SigK production and enhance the fidelity of spore assembly (145) similar to *B. subtilis* (146).

C. difficile sporulation sigma factors generally control similar morphological processes as *B. subtilis*, with σ^F and σ^E being required for initiating engulfment, and σ^E and σ^K being required for coat assembly (139). Unlike *B. subtilis*, *C. difficile* σ^G , but not σ^K , is required for cortex production, and *C. difficile* σ^G is required for engulfment completion at least during sporulation on solid media (137).

Taken together, *C. difficile* sporulation sigma factor mutants exhibit less inter-compartmental signaling, with the forespore line of gene expression requiring σ^F and σ^G , but not σ^E , and the mother cell line of gene expression requiring σ^E and σ^K , but not σ^G (107, 137–139) (Fig. 4). Furthermore, the timing of sporulation sigma factor activation does not to appear to be as closely coupled to morphological events as in *B. subtilis* (25, 107).

C. perfringens* and *C. botulinum

The regulation of sporulation sigma factors in *C. perfringens* and *C. botulinum* has not been characterized extensively, although gene conservation analyses indicate that the minimal machinery required for activating these sigma factors are conserved (Fig. 4) (136). *C. perfringens* σ^E and σ^K both undergo proteolytic processing during sporulation similar to *B. subtilis* and *C. difficile* (65). However, in contrast with these organisms, the *sigK* genes in both *C. perfringens* and *C. botulinum* do not contain intervening *skin* elements. Notably, σ^K appears to function at two stages during sporulation in both these organisms, with the first stage regulating sporulation initiation. Indeed, *sigK* mutants in both organisms do not appear to initiate asymmetric engulfment (65, 74), unlike in *B. subtilis* and *C. difficile*. Thus, the function of σ^K as an early and late regulator of sporulation in *C. perfringens* and *C. botulinum* exhibits similarities to *C. acetobutylicum*, which uses σ^K to initiate sporulation through its activation of *spo0A* and during late stage sporulation (147).

In *C. perfringens*, *sigK* is expressed from two promoters at different stages of sporulation: the upstream *CPR_1739* promoter activates transcription during early log phase in contrast with *B. subtilis* and *C. difficile*, while a σ^E -dependent promoter controls late-stage sporulation gene expression (65) similar to *B. subtilis* and *C. difficile*. *C. botulinum sigK* transcription is similarly biphasic, occurring during late-log phase and late-stage sporulation (74). This latter phase of gene expression occurs from σ^F - and σ^E -dependent promoters (148). Consistent with σ^K being required early during sporulation in both these organisms, *C. perfringens* σ^K activates *sigF* and *sigE* expression (65), and *C. botulinum* σ^K activates *spo0A* and *sigF* expression (74). Furthermore, *sigK* mutants in both organisms fail to complete asymmetric engulfment (65, 74), whereas *sigE* mutants are stalled at this stage (65, 148). While transcriptional analyses indicate that σ^K acts upstream of σ^F , Western blot analyses have revealed that σ^K is not detectable in the absence of σ^F (86), suggesting that the production (or stability) of these two factors is inter-dependent.

Another major difference in sporulation gene regulation relative to *B. subtilis* and *C. difficile* is that σ^E does not activate *spoIIID* expression in *C. perfringens* and *C. botulinum*. Instead, *C. botulinum spoIIID* transcription is σ^F -dependent (149). Whether σ^F regulates *spoIIID* expression in *C. perfringens* is unclear, but immunoblotting indicates that SpoIIID production does not require σ^E or σ^K (65).

These observations raise the possibility that the sporulation sigma factors exhibit differences in their compartment-specific activation in *C. perfringens* and *C. botulinum* relative to *B. subtilis* and *C. difficile*. Indeed, *sigK* is transcribed early during sporulation in *C. perfringens* (65), suggesting that σ^K may activate sporulation in predivisional *C. botulinum* cells. These observations raise the question as to whether σ^K must be proteolytically activated at this early stage. Furthermore, since *sigK* expression is regulated by both σ^F and σ^E in *C. botulinum* (148), is σ^F activity (and by extension SpoIIID activity) restricted to the forespore?

Summary of Sporulation Regulation—Clearly, major differences exist in the functions and regulation of conserved sporulation sigma factors in *C. difficile*, *C. perfringens*, and *C. botulinum* relative to the pathways defined in *B. subtilis*. *C. difficile*'s regulatory

architecture exhibits greater similarity to *B. subtilis* than to *C. perfringens* and *C. botulinum*, which appear relatively similar to each other. Further study of *C. perfringens* and *C. botulinum* is needed to define the order of sporulation sigma factor activation, their specific regulons, and location of their activity. Such studies could provide insight into the evolution of diverse sporulation networks.

SPORE ASSEMBLY

The mechanisms by which spores are physically assembled in clostridial organisms remain poorly defined, although some progress has been made in *C. difficile*. This section focuses on factors required for engulfment, coat, and exosporium assembly in *C. difficile* and the functions of specific coat and exosporium proteins. We also outline the role of the spore-specific small molecule, calcium dipicolonic acid (Ca-DPA), during *C. perfringens* and *C. difficile* spore formation.

Engulfment

The second major morphological event after asymmetric division is engulfment, whereby the smaller forespore is encircled by the mother cell, leaving the forespore free-floating in the mother cell cytosol surrounded by two membranes (Fig. 1). Engulfment is mediated by peptidoglycan synthesis machinery in the inner forespore membrane working in concert with peptidoglycan degradation machinery localized in the mother cell-derived outer forespore membrane in *B. subtilis* (150, 151). The degradation complex consists of the SpoIIP and SpoIID peptidoglycan hydrolases (152, 153) in complex with the transmembrane scaffolding protein, SpoIIM (154). Loss of any of these *B. subtilis* components prevents engulfment completion and thus heat-resistant spore formation (116–118). Interestingly, *C. difficile* SpoIIM is largely dispensable for engulfment and heat-resistant spore formation in contrast with *B. subtilis*, while *C. difficile* SpoIIP and SpoIID are critical for these processes similar to *B. subtilis* (155, 156). While it is unclear why SpoIIM is dispensable for *C. difficile* engulfment, *spoIID* and *spoIIP* are expressed in different cellular compartments in *C. difficile* relative to *B. subtilis* (156), which may obviate the need for SpoIIM to bring SpoIID and SpoIIP together. *C. difficile* SpoIIP and SpoIID nevertheless have conserved enzymatic activities relative to *B. subtilis* (152, 155, 157), although SpoIIP undergoes site-specific cleavage in *C. difficile* (156) in contrast with *B. subtilis* (158).

The conserved SpoIIQ-SpoIIIA channel is also required for *C. difficile* engulfment unlike *B. subtilis*. In *B. subtilis*, the channel (also known as the “feeding tube”) connects the mother cell and forespore (120, 126, 128) and serves as a back-up mechanism for engulfment (159). However, in both organisms the channel is essential for maintaining forespore health because the forespore collapses on itself in channel mutants (127, 141).

Coat Assembly in *C. difficile*

The coat consists of ~80 proteins in *B. subtilis* (119) that localize to the forespore and form a series of concentric proteinaceous shells around the forespore. However, given that only 25% of these proteins have homologs in *C. difficile* (160), different pathways likely control

coat assembly in these two organisms. Furthermore, *C. difficile* encodes only two of the nine coat morphogenetic proteins that function to recruit coat proteins to the *B. subtilis* forespore.

The two coat morphogenetic proteins shared between *B. subtilis* and *C. difficile* are SpoVM and SpoIVA. SpoVM functions as a landmark protein in *B. subtilis* by recognizing the positive curvature of the forespore membrane and embedding itself within this membrane (161, 162). SpoVM recruits SpoIVA to the forespore and is required for SpoIVA to use its ATPase activity (163) to polymerize around the forespore (161). SpoIVA preferentially localizes SpoVM to the outer forespore membrane (164) and recruits the coat morphogenetic protein, SpoVID, to the forespore by binding SpoVID's C-terminal Region A (164). These three proteins form the basement layer of the *B. subtilis* coat (165). SpoVID in turn recruits the inner coat morphogenetic protein, SafA (166), and outer coat morphogenetic protein, CotE (167). *B. subtilis* *spoIVA* and *spoVM* mutants mislocalize coat and fail to make cortex, so they cannot produce heat- or chloroform-resistant spores (168–170). Furthermore, *spoIVA* and *spoVM* mutants are actively lysed by a bacilli-specific (171) quality control mechanism mediated by CmpA (172).

C. difficile *spoIVA* mutants resemble a *B. subtilis* IVA mutant in mislocalizing coat and failing to produce heat-resistant spores (173, 174). However, in contrast with *B. subtilis*, *C. difficile* *spoIVA* mutants produce cortex (170, 174), consistent with the absence of CmpA in the clostridia (175). Nevertheless, it is unclear why *C. difficile* *spoIVA* mutants are heat-sensitive given that they produce visible cortex.

Unlike SpoIVA, *C. difficile* SpoVM is largely dispensable for spore formation. *C. difficile* *spoVM* mutants exhibit a ~3-fold defect in heat- and chloroform-resistant spore formation, make cortex, and properly localize SpoIVA around the forespore in most cells in contrast with the 6-log defect in heat- and chloroform resistance observed in a *B. subtilis* *spoVM* mutant (169, 173). While it is unclear whether SpoVM encases the *C. difficile* forespore as it does in *B. subtilis* (161), *C. difficile* SpoVM directly binds SpoIVA in recombinant co-affinity purification analyses (173), so *C. difficile* may use a redundant factor to substitute for loss of SpoVM.

While SpoVM is largely dispensable for *C. difficile* spore formation, the clostridial-specific CD3567 was shown to function as a coat morphogenetic protein because it is essential for proper coat localization and heat-resistant spore formation although not cortex formation (174). CD3567 was identified based on spore proteomic analyses (176) and targeted mutagenesis (174) and directly binds to SpoIVA through CD3567's C-terminal LysM domain. Thus, it was renamed SipL (SpoIVA Interacting Protein L) (174). Fluorescent protein fusion analyses indicate that SipL is necessary for SpoIVA to encase the forespore, while SpoIVA is necessary for SipL to localize to the forespore (Touchette & Shen, unpublished data). Thus, even though Clostridia-specific SipL (174) lacks sequence homology with bacilli-specific SpoVID (41), aside from their C-terminal LysM domains, the two proteins are functional homologs because they directly bind SpoIVA, encase the forespore, and recruit coat proteins to the forespore (164, 174).

C. difficile SpoIVA and SipL likely comprise the coat basement layer (Fig. 5), but the specific coat proteins they recruit to the forespore are unknown. Analyses of SpoIVA and SipL localization in engulfment mutants indicate that SpoIVA and SipL can localize to the forespore membrane in the absence of engulfment (156), while the outer coat protein CotE (CD1433) localizes to the cytosolic polymerized coat visible by phase-contrast microscopy (141)). These results indicate that SpoIVA and SipL can adhere to the forespore independent of engulfment but that outer layer coat proteins require engulfment completion to stay associated with the forespore.

Additional coat proteins have been identified in spore proteomic analyses, some of which have been shown to be surface-localized (176–179). While coat proteins are enriched in these analyses, cytosolic contaminants inevitably become encased as the outer layers are assembled in the mother cell cytosol (176–179). The functions of many of these coat proteins have not been determined, although enzymatic activities have been determined for several coat proteins (180, 181), and CotA has been implicated in spore assembly and heat- and ethanol-resistance (180). For example, alanine racemase interconverts L- and D-alanine (as well as L- and D-serine) and alters the sensitivity of *C. difficile* spores to the D-alanine co-germinant (181).

C. difficile CotE, which is unrelated to *B. subtilis* outer coat morphogenetic protein, CotE (119), degrades mucin and promotes spore binding to intestinal epithelial cells (182). Loss of CotE or its C-terminal mucinase domain reduces virulence in a hamster model of infection, indicating that the spore surface actively regulates *C. difficile* colonization and disease (182). Given that *C. difficile* *cotE* mutant spores do not have obvious ultrastructural or resistance property defects *in vitro* (180), these analyses highlight the importance of analyzing spore function in the context of infection.

Exosporium Assembly in *C. difficile*

The *C. difficile* exosporium layer directly contacts the coat unlike the exosporium of *Bacillus cereus* group spores, which have an interspace gap between the exosporium and the coat (160, 183, 184). Since the *C. difficile* exosporium is closely associated with the spore coat (Fig. 5), it has been challenging to identify specific components. However, by sonicating spores, the Paredes-Sabja lab enriched for exosporium proteins (178) such as the i) cysteine-rich proteins, CdeC (CD1067) (185), CdeM (CD1581) (64), and CdeA (CD2375) and ii) collagen-like proteins, BclA1, BclA2 and BclA3 (186, 187). Traces of coat proteins such as CotA, CotB, CotD and CotE were also observed, which may indicate that they are part of the coat/exosporium interface (178).

While it is unclear how the *C. difficile* exosporium is assembled, genetic analyses have identified exosporium morphogenetic proteins. The cysteine-rich proteins, CdeC and CdeM have been implicated in exosporium morphogenesis (Paredes-Sabja, unpublished data). Spores deficient in CdeC have a defective coat permeable to lysozyme, higher core water content and are more susceptible to ethanol and heat than wild-type spores (185). Interestingly, cysteine-rich proteins are essential for the morphogenesis of the outer crust of *B. subtilis* and exosporium of *B. cereus* group (188–190). These *Bacillus* spp. cysteine-rich proteins self-assemble into 2D crystalline layers (189, 191) that correlate with the 2D-

crystalline basal layer underneath the hairy nap (extensions) on *B. anthracis* spores. Both CdeC and CdeM form dimers, trimers, and higher molecular weight complexes (178, 185, 192), suggesting that a similar self-assembly mechanism might govern the assembly of the outer layers of *C. difficile* spores.

The *C. difficile* BclA proteins have also been implicated in exosporium assembly: spores lacking either BclA1, BclA2 and BclA3 produce defective exosporiums (186) and exhibit heat-resistance defects (186, 193). The BclA proteins likely comprise the hair-like extensions of *C. difficile* spores (187), since these proteins produce hair like-projections on *B. anthracis* spores (160). Furthermore, like *B. anthracis* BclA (194), *C. difficile* BclA3 is glycosylated (193). *C. difficile* BclA orthologues also appear to have a similar topology to *B. anthracis* BclA because both require N-terminal domains to localize to the spore surface (187).

Exosporium Function in *C. difficile*

As the outermost layer of *C. difficile* spores, the exosporium may contact host-component(s) that contribute to the persistence of *C. difficile* spores in the host (195, 196). In TEM analyses of clinically relevant *C. difficile* strains, the exosporium layer appears as electron-dense “bumps” on the spore surface with hair-like extension (185, 196). Notably, two exosporium morphotypes are observed in clonal populations: thin and thick electron-dense layers, although both have hair-like extensions (197, 198). This observation raises the possibility that the different morphotypes may have different roles during *C. difficile* infection such as spore persistence and immune evasion. Consistent with this hypothesis, loss of the exosporium protein, CdeM (CD1581), decreases *C. difficile* fitness in gnotobiotic mice (64), and loss of individual BclA proteins, particularly BclA1, results in decreased colonization in mice (186).

CLOSTRIDIAL SPORE GERMINATION

Overview of Spore Germination and Outgrowth

During spore germination, metabolically dormant spores lose their resistance properties and transform into metabolically active cells. The low water content of the spore cytosol, known as the core, (~25–40%) is critical to this resistance because it prevents metabolism (7). Ca-DPA transport is essential for dehydrating the core, while the modified peptidoglycan cortex layer is essential for maintaining this partially dehydrated state. Thus, spore germination requires the removal of this cortex layer to allow core hydration and metabolism to resume (28).

Spore germination begins when spores sense small molecules termed germinants, which trigger a signaling cascade that leads to cortex degradation, release of Ca-DPA, core hydration, and degradation of SASPs bound to the chromosome. Although many germination-related proteins are conserved in the clostridial pathogens, notable differences in their function and mechanisms of action have been identified in *C. difficile*, *C. perfringens*, and *C. botulinum*. As discussed below, the order in which cortex hydrolysis and core hydration occurs differs between these species and is even strain-specific in the case of

C. botulinum. Furthermore, *C. difficile* spore germination has several unique features to its signaling pathway that are specific to *C. difficile* and/or Peptostreptococcaceae family members.

Germinant Sensing and Signaling

Environmental signals—In most spore-forming bacteria, germinants are nutrient signals such as amino acids, monosaccharides, nucleosides, salts, and organic acids (28). In contrast, *C. difficile* responds to cholate-derived bile acids, which are produced exclusively in the mammalian gut (199, 200). Taurocholate is the most potent of the cholate-derived germinants, while chenodeoxycholate is an efficient competitive inhibitor of taurocholate-mediated germination (201). Bile salt-induced spore germination may not be unique to *C. difficile*, since taurocholate can enrich for clostridial species from fecal samples (202), and spores from *Paenibacillus sordellii*, a Peptostreptococcaceae family member, germinates in response to some bile acids (203). Notably, amino acid and calcium ion co-germinants enhance taurocholate-induced *C. difficile* spore germination, with glycine and calcium ions being the most potent of these small molecules (199, 204, 205).

In *C. perfringens* and *C. botulinum*, germinant specificity is species- and strain-specific. Universal germinants for *C. perfringens* food-poisoning and non-food-borne isolate spores include L-cysteine, L-serine, L-threonine and a mixture of L-asparagine and KCl, while unique germinants for food-poisoning isolate spores are L-asparagine, L-glutamine, KCl, and the co-germinants Na⁺ and Pi (206–208). Bicarbonate is a unique co-germinant for non-food-borne spores (209). L-alanine, L-cysteine, L-methionine, L-serine, L-phenylalanine, and glycine can induce spore germination of Group I proteolytic *C. botulinum*, although L-lactate and bicarbonate ions can act as co-germinants (210, 211). Spores of Group II non-proteolytic *C. botulinum* germinate in response to L-alanine, L-cysteine, L-serine, L-threonine, and glycine, with L-lactate serving as a co-germinant (211, 212).

Germinant selectivity is likely influenced by adaptation to specific environmental niches. For example, the responsiveness of *C. perfringens* food-poisoning isolate, but not non-food-borne isolates, spores to KCl or NaPi implies that food-poisoning isolates have adapted to food niches (i.e., processed meat products) where KCl and NaPi are highly abundant (10). Also, the finding that bicarbonate is a unique co-germinant for non-food-borne spores, which germinate better than food-poisoning isolates spores in the presence of cultured intestinal epithelial cell (196, 210), suggests that non-food-borne isolate spores are better adapted to germinate in the host's intestinal epithelium environment where bicarbonate is more prevalent (213). Similarly, the responsiveness of *C. difficile* spores to taurocholate may allow *C. difficile* to sense favorable conditions in the gut, since taurocholate levels are increased in the dysbiotic gut during antibiotic treatment (21).

While classical germinants are directly sensed through germinant receptors, non-nutrient germinants can artificially trigger germination of many different bacterial species independent of germinant receptors. These include the cationic surfactant dodecylamine (28, 214), lysozyme (196), and Ca-DPA (215). While Ca-DPA activates *C. sporogenes* (a proxy for *C. botulinum* Group I, (5)) spore germination, it does not activate *C. difficile* spore germination (205, 216, 217) and is a relatively weak activator of *C. perfringens* spore

germination (218). Interestingly, although high hydrostatic pressure can induce *Bacillus* spp. spore germination independent of germination receptors, high pressure alone is not sufficient to induce spore germination of *C. sporogenes* (219), *C. perfringens* (220), and *C. difficile* (220). For *C. perfringens* and *C. difficile*, the ineffectiveness of high pressure to induce germination is likely due to differences in their germination mechanism relative to *Bacillus* spp. as discussed below.

Transmembrane germinant receptors in *C. perfringens* and *C. botulinum*

Almost all bacterial spores sense environmental signals through germinant receptors localized in the spore's inner membrane; the exception to this rule is *C. difficile* as described below. Germinant receptors usually consist of three protein subunits (A, B and C) encoded in a tricistronic operon (*gerABC*). GerA and GerB are transmembrane proteins, and GerC is a lipoprotein. Loss of any one of these components generally eliminates germinant receptor function (28, 196).

Many spore-forming bacteria encode multiple tricistronic operons, which are thought to determine the germinant specificity of a given strains. While *B. subtilis* encodes three major germinant receptors, there are diverse arrangements for *ger* genes and operons in the *Bacillales* and *Clostridiales* (see (28, 196)). For example, *C. perfringens* has a monocistronic *gerAA* distant along with a *gerK* locus, which encodes a bicistronic *gerKA-KC* operon upstream of the oppositely-oriented monocistronic *gerKB* gene (196, 206). While many *Clostridium* species encode monocistronic germinant receptors genes, studies in *C. perfringens* provided the first evidence that functional germinant receptors can consist of a single subunit. Indeed, the lipoprotein GerKC is the sole and essential germinant receptor for all known nutrient and non-nutrient induced germination of SM101 and F4969 spores (221, 222), in contrast with *B. subtilis*, where all three subunits are essential for functional germinant receptor formation. Nevertheless, although GerAA or GerKB can play auxiliary roles in food-poisoning strain SM101 spore germination (206, 222, 223), GerAA is required for non-food-borne strain F4969 spore germination.

Canonical GerA family germinant receptors localize to the spore's inner membrane in several species including *B. subtilis* (224) and *C. botulinum* (225). The GerKC subunit is present in the inner membrane of *C. perfringens* spores at ~250 molecules/spore (221); the abundance and relative stoichiometry of the GerKA, GerKB and GerAA subunits is unclear.

gerA family operons can also contain an additional *gerAD* gene, which encodes a novel protein of 50–80 residues with two highly conserved transmembrane sequences in some *Bacillales* and *Clostridiales* species (196). Although GerAD is required for functional germinant receptor formation in *Bacillus* spp. (226, 227), their role in *Clostridiales* spore germination remains unclear.

Genomic analyses of *C. botulinum* strains have identified four different *gerABC* sub-types (*gerX* type1–4) (211). *gerX* indicates that the germinant recognized by the receptor encoded in the locus is unknown. Notably, although group II *C. botulinum* (gIICb) encodes only one predicted canonical germinant receptor despite being able to respond to many amino acids, deletion of *gerBAC* from gIICb strain NCTC 11219 does not affect germination in response

to nutrient or non-nutrient germinants (228). Thus, unidentified germinant receptors would appear to mediate spore germination in gIICb in the absence of *gerABC*.

Germinant receptor-mediated spore germination is heat-activatable through a mechanism that is thought to involve conformational changes in the inner membrane germinant receptors that potentiates their activation (28). Accordingly, both *C. perfringens* and *C. botulinum* spore germination can be enhanced by a transient heat shock (229, 230). In contrast, *C. difficile* spore germination is not heat-activatable consistent with the absence of germinant receptor genes in its genome (217, 231).

A Pseudoprotease, CspC, is the bile salt germinant receptor in *C. difficile*

Since *C. difficile* lacks germinant receptors, it was unclear how it senses bile salt germinants, which are structurally distinct from the nutritional germinants sensed by germinant receptor-encoding spore-formers. Joseph Sorg's group identified the elusive *C. difficile* germinant receptor using a genetic selection for germination-defective mutants (232). This screen identified 7 point mutations in *cspC*, which encodes a pseudoprotease from the subtilisin-like serine protease family, and two nonsense mutations in *cspBA*, which encodes a fusion protein consisting of CspB and CspA subtilisin-like serine proteases (Fig. 6). While the CspB protease is catalytically active, both *C. difficile* CspA and CspC are pseudoproteases because they harbor two point mutations in their catalytic triads (233). Subsequent work revealed that CspBA is required for CspC incorporation into spores such that the *cspBA* nonsense mutations identified in the screen effectively lead to loss of CspC (234, 235).

CspC was further implicated as the direct receptor for bile salts using a second genetic screen for altered spore germinant specificity. By selecting for spores that germinate in response to the germination inhibitor, chenodeoxycholate (201), the Sorg group determined that a single point mutation in CspC (G457R) could change the ability of *C. difficile* spores to sense cholate versus deoxycholate derivatives (232). Although direct binding of bile acids to CspC has yet to be demonstrated biochemically, germinant binding to germinant receptors has not been established in any organism.

ACTIVATION OF CORTEX HYDROLYSIS IN CLOSTRIDIAL PATHOGENS

Just as clostridial pathogens use two different mechanisms for sensing germinant, these organisms use two different mechanisms to degrade their cortex: (i) proteolytic activation of the cortex hydrolase, SleC, and (ii) DPA-mediated activation of the cortex hydrolases CwlJ and/or SleB (Fig. 7, (28)). *C. perfringens* and *C. difficile* use the SleC pathway, while *C. botulinum* uses either mechanism in a strain-specific manner, with Groups I and III using CwlJ/SleB (211, 230, 236), and Groups II and IV presumably using the SleC pathway. While cortex hydrolases are thought to specifically recognize the muramic- δ -lactam modification that characterizes cortex peptidoglycan (237), the CspB-SleC cortex hydrolysis pathway is only found in the Clostridia, whereas the CwlJ/SleB system is present in the Bacilli and some members of the Clostridia (196).

Proteolytic Activation of the SleC Cortex Hydrolase in *C. perfringens* and *C. difficile*

SleC was first identified as a cortex hydrolase in biochemical fractionations of germinating *C. perfringens* exudates (238, 239) and was subsequently shown to have lytic transglycosylase and amidase activities (240). SleC's long N-terminal predomain acts as an intramolecular chaperone to ensure proper folding of its hydrolase domain (241). The predomain is removed by proteolysis in a YabG-dependent manner (at least in *C. difficile* (235)) to generate the pro-SleC zymogen. This immature form remains in mature spores until germinant signaling induces the proteolytic removal of SleC's inhibitory pro-peptide to generate active SleC (242).

The proteases responsible for activating SleC were identified as CspA, CspB, and CspC in biochemical fractionations of *C. perfringens* strain S40 germinating exudates (242, 243). These three proteases are members of the subtilisin-like serine protease family (243) and are encoded by a tricistronic operon immediately upstream of the *sleC* gene in *C. perfringens* strain S40 (244). While non-food-borne *C. perfringens* strains encode this tricistronic operon, *C. perfringens* food-poisoning strain SM101 encodes *cspB* alone upstream of *sleC* (4, 245, 246). Genetic analyses in SM101 revealed that *C. perfringens* CspB is sufficient to proteolytically activate SleC, since *cspB* mutant spores exhibit 10^4 -fold germination and cortex hydrolysis defects relative to wild type (245) and fail to process pro-SleC into active SleC in response to germinant (245).

SleC is the major cortex hydrolase in *C. perfringens*, since *sleC* mutant spores have a 10^3 -germination defect relative to wild type (218). The SleM cortex hydrolase (247) functions as an accessory cortex hydrolase, since a *sleM* mutant exhibits wild-type germination, but a *sleC sleM* double mutant has a 100-fold more severe germination defect than the *sleC* single mutant (218).

The CspB protease and SleC cortex hydrolase have similar functions in *C. difficile*, since loss of CspB leads to a $\sim 10^3$ - 10^5 germination defect (233, 234), and complementation with wild-type, but not a catalytic mutant, *cspB* restores germination and pro-SleC cleavage (233). Loss of *sleC* also results in a similar $\sim 10^3$ -fold germination defect relative to wild type (234) and an inability to hydrolyze cortex (216, 248). However, in contrast with the tricistronic *cspA-cspB-cspC* operon in some *C. perfringens* strains, *C. difficile* strains (and other Peptostreptococcaceae family members) universally encode *cspB* fused to *cspA*, with *cspC* encoded downstream of the *cspBA* fusion gene (*cspBA-cspC*) (235).

SleC and Csp proteins are produced in the mother cell of both *C. perfringens* and *C. difficile* (137–139, 249). Western blot analyses of spore fractions in *C. perfringens* and *C. difficile* indicate that CspB and SleC localize to a coat-extractable fraction (248, 250). However, *C. perfringens* SleC can be visualized in the cortex region of mature spores using immunoelectron microscopy (251), indicating that fractions previously assumed to comprise coat proteins alone extract proteins from the cortex region. Interestingly, neither *C. perfringens* nor *C. difficile* SleC harbor signal sequences, raising the question as to how they are targeted to the intermembrane space (assuming Csp proteins are targeted to this region).

The crystal structure of *C. perfringens* CspB confirmed that Csp proteins consist of three main domains: a long N-terminal prodomain, a subtilisin fold, and an internal jelly roll domain (233, 243). Like other subtilisin-like proteases, *C. perfringens* and *C. difficile* CspB have a catalytic triad consisting of Asp, His, Ser, and the prodomain undergoes autoprocessing (233, 243). However, unlike almost all previously-studied subtilisin-like serine proteases, both *C. perfringens* and *C. difficile* CspB remain bound to their prodomain following autoprocessing *in vitro* (233, 243). The prodomain sterically inhibits recombinant CspB activity (233), but it is unclear whether it stays bound to CspB in mature spores. Regardless, the prodomain presumably must be removed during spore germination to allow CspB to bind its presumed substrate, SleC. The central jelly roll domain within the subtilase domain is another unique feature to CspB; this domain markedly increases the conformational rigidity of CspB *in vitro* and is necessary for stable CspB production in *C. difficile* (233).

CspB activity has been proposed to require calcium based on the observation that calcium is a critical co-germinant during taurocholate-induced *C. difficile* spore germination (205). Depletion of calcium *in vitro* and in intestinal extracts reduces wild-type *C. difficile* spore germination and abrogates spore germination in a strain lacking CD3298, an ATP/GTP binding protein required for releasing calcium during germination (205). While this data indicates that calcium is a key adjuvant during *C. difficile* spore germination, the precise stage regulated by calcium is unclear, since it could affect CspB activity, CspC activation, germinant permeability or as-yet-unidentified events. Whether calcium is required for *C. perfringens* spore germination remains to be tested.

Collectively, germinant signaling triggers CspB-dependent processing of inactive pro-SleC into active SleC, which subsequently degrades the cortex in both *C. perfringens* and *C. difficile*. It is unclear how germinant signaling leads to CspB activation, especially since these two organisms use different germinant receptors. The topology of these receptors also differs between these organisms, with Ger receptors being embedded in the *C. perfringens* forespore-derived membrane, and soluble *C. difficile* CspC being made in the mother cell (Fig. 6) (28, 200). *C. difficile* CspC has been hypothesized to be transported across the mother cell-derived membrane into the intermembrane space so that it can activate CspB through protein-protein interactions (200, 234, 252, 253). CspB in *C. perfringens* and *C. difficile* is presumably also transported into the intermembrane space so that it can activate its substrate SleC, which has been localized to this region in *C. perfringens* by immunoEM (251). Clearly, many open questions regarding the molecular details of this process remain to be addressed.

DPA-mediated Activation of Cortex Hydrolases in Group I *C. botulinum*

Gene conservation predicts that *C. botulinum* Groups II and IV activate cortex hydrolysis via CspB-mediated proteolytic activation of pro-SleC (211), but Group I (and likely Group III based on homology) use the partially redundant cortex hydrolases, CwlJ and SleB, to remove their cortex layer (211, 230, 236). Both hydrolases are produced in their mature form, but their activation appears to be tied to Ca-DPA release as observed in *B. subtilis* (28, 254). *C. sporogenes* (a proxy for Group I *C. botulinum*) *cwlJ* mutant spores do not

germinate in response to Ca-DPA unlike *sleB* mutant spores (230, 255) similar to analogous mutants in *B. subtilis* (256). In *B. subtilis*, SleB activity is directly inhibited by YpeB (254), but the mechanism by which SleB is activated during germination is unknown. *C. botulinum* Group I *sleB* and *ypeB* mutants exhibit 2–3-log defects in spore germination based on colony forming units, whereas *cwlJ* mutant spores produce wild-type levels of colonies on rich media (236). In contrast, *cwlJ* and *sleB* mutant spores in *C. sporogenes* have similar germination defects in single-spore germination analyses (230). Regardless, CwlJ and SleB likely have partially overlapping functions during Group I *C. botulinum* spore germination, so that a double mutant lacking both enzymes would presumably exhibit a severe germination defect similar to *B. subtilis* (257). Interestingly, both Group I and Group III *C. botulinum* spp. can encode multiple SleB homologs (211), suggesting that there may be additional redundancy in regulating cortex hydrolysis.

Dipicolinic acid (Ca-DPA) and Spore Formation vs. Germination

Ca-DPA is a spore-specific molecule that makes up 5–15% of spore dry weight (256). It is synthesized in the mother cell from lysine biosynthesis intermediates (258) and transported across the outer and inner forespore membranes by SpoVV (259) and SpoVAC (260, 261), respectively, in exchange for water. While the partial dehydration of the core by Ca-DPA transport into the forespore confers heat resistance to mature spores (256, 261, 262), Ca-DPA is differentially required for spore formation. In *B. subtilis*, mutants defective in synthesizing or transporting Ca-DPA cannot stably form spores due to premature germination and cortex hydrolase activation (256, 259, 260). Similar to *B. subtilis*, *C. sporogenes* *spoVA* mutants also produce unstable spores, presumably due to premature SleB activation (211). In contrast, *C. difficile* mutants defective in synthesizing or transporting Ca-DPA can stably form spores, although they are less dense than wild-type spores (261). Interestingly, Ca-DPA synthesis is necessary for *C. perfringens* spore formation (263), but Ca-DPA transport is not (262). Since Ca-DPA does not strongly induce *C. perfringens* germination (262) presumably because it uses the CspB-SleC pathway to activate cortex hydrolysis, it is unclear why DPA is required for spore formation in *C. perfringens* but not *C. difficile*. This difference may reflect the different mechanisms used by these organisms to synthesize Ca-DPA. While *C. difficile* uses dihydro-dipicolinate synthase (DpaAB or SpoVFAB) to produce Ca-DPA, *C. perfringens* uses an electron transfer flavoprotein, EftA, to make this molecule. Since *C. botulinum* lacks SpoVFAB homologs and likely uses EftA, DPA-less *C. botulinum* spores likely will not be stably produced.

Cortex Hydrolysis Precedes Ca-DPA Release in *C. difficile*

While Ca-DPA release activates cortex hydrolysis in Group I *C. botulinum* and other CwlJ/SleB-controlled systems, cortex hydrolysis is necessary for Ca-DPA release in *C. difficile* (261). Cortex hydrolysis activates the mechanosensitive SpoVAC channel (264) to release Ca-DPA from the core, since *C. difficile* spores germinated in high osmolyte solutions degrade their cortex normally but release Ca-DPA more slowly (265). Furthermore, *C. difficile* *sleC* mutant spores do not release Ca-DPA (216, 217). A similar mechanosensing phenomenon may occur in *C. perfringens*, since a *sleC-sleM* double mutant and a *cspB* mutant exhibit major defects in Ca-DPA release (218, 245). Thus, the CspB-SleC cortex

degradation pathway likely induces Ca-DPA release, while the order of these events is reversed in spores that use CwlJ and SleB to degrade their cortex (28).

Events After Cortex Hydrolysis and Ca-DPA Release

While recent studies have provided critical insight into the early stages of germination in clostridial pathogens, namely germinant sensing, cortex hydrolysis, and Ca-DPA release, little is known about subsequent events. Ca-DPA release in *C. difficile* leads to core hydration based on the observation that the optical density of germinating *dpaAB*⁻ and *spoVAC*⁻ spores decreases by ~50% relative to wild type, whereas the optical density of *sleC*⁻ spores remains unchanged (261). Core hydration allows metabolism to resume, and transcript levels increase within 15 min of germination (231).

In *B. subtilis*, Ca-DPA release activates the Gpr germination protease (266), which degrades SASPs coating the nucleoid. The amino acids liberated by SASP degradation provides substrates for both catabolism and metabolism (267). It is currently unclear whether Ca-DPA activates Gpr homologs in clostridial pathogens.

Loss of Resistance Properties During Germination

The hydration of the core during spore germination allows metabolism to resume while concomitantly leading to loss of resistance. This is because the resistance properties of spores can largely be attributed to the (i) low water content of metabolically dormant spores and (ii) coating of the genetic material by small acid-soluble spore proteins (SASPs), which collectively prevent metabolic activity in the spore core. Indeed, *B. subtilis* mutant spores with higher water content or lacking SASP proteins are more sensitive to heat (256, 268), and in the latter case, are more sensitive to UV irradiation (269, 270). Although *B. subtilis* produces three major types of SASP proteins (α , β , and γ), which are encoded by multiple genes (269), SspA and SspB (α and β , respectively) are the major SASPs coating the chromosome and the primary contributors to *B. subtilis* spores' resistance to UV and solar radiation (270).

Similar to *B. subtilis*, *C. difficile* and *C. perfringens* mutant spores with higher spore water content are more susceptible to heat (261, 262, 271) and UV irradiation possibly because increased hydration reduces SASP binding to DNA (262, 271). Consistent with this idea, *C. perfringens* food poisoning isolates with extreme resistance properties (e.g. type A chromosomal *cpe* and type C Darmbrand strains) produce an Ssp4 variant (also known as SASP4) that binds DNA with higher affinity than Ssp4 variants from more heat-sensitive *C. perfringens* isolates (272, 273). Mutational analyses indicate that this Ssp4 variant is a major determinant controlling the extreme heat resistance of certain food poisoning strains. Interestingly, Ssp4 exhibits only ~20% sequence identity relative to the three other SASP proteins (Ssp1–3) produced by *C. perfringens* strains, suggesting that this Ssp4 variant may be a specific adaptation of food poisoning isolates (e.g. SM101). Indeed, it should be noted that Ssp1–3 have been shown to confer UV resistance to *C. perfringens* food poisoning strain SM101 spores using anti-sense RNA silencing methods (274).

C. botulinum encodes 4 SASPs, CBO1789, CBO1790, CBO3048, and CBO3145. This latter protein exhibits the lowest sequence conservation and is dispensable for resistance to nitrous

acid (275), and thus may function similar to *B. subtilis* SASP γ in acting as a food source for the outgrowing spore (276). Mutational analyses indicate that CB01789 and CPB01790 are primarily responsible for conferring resistance to nitrous acid, an oxidizing agent (275).

The role of SASPs in conferring resistance to *C. difficile* spores has not been examined, although RNA-Seq analyses suggest that SspA and SspB are the predominant SASPs produced (137). *C. difficile* spores are less resistant to heat than *B. subtilis* and *C. perfringens* spores, since *C. difficile* spores are readily killed at temperatures above 80°C (261).

Conclusions and Unique Features of *C. difficile* Spore Germination

Diverse pathways are clearly used by Group I *C. botulinum*, *C. perfringens*, and *C. difficile* to mediate spore germination. The pathway used by Group I *C. botulinum* spore germination most resembles *Bacillus spp.* germination, with germinant receptors sensing amino acids and stimulating Ca-DPA release followed by cortex hydrolysis by CwlJ and SleB (28). While *C. perfringens* spore germination also uses germinant receptors to sense nutrient germinants like amino acids, germinant sensing activates the CspB-SleC cortex hydrolysis pathway, after which most Ca-DPA is released. *C. difficile* spore germination is the most divergent, with its unique germinant receptor, the CspC pseudoprotease, sensing bile acids. Like *C. perfringens*, germinant sensing activates the CspB-SleC cortex hydrolysis pathway, and the change in pressure caused by the resulting cortex hydrolysis induces SpoVAC to release Ca-DPA.

Although CspA, CspB, and CspC are conserved in many clostridial organisms, they are produced as single protease domains in the Clostridiaceae and Lachnospiraceae families and as a CspB-CspA fusion protein in the Peptostreptococcaceae family (235). Furthermore, CspB is the only active serine protease, with CspC and the CspA domain being pseudoproteases. Since CspA is critical for incorporating the germinant receptor and pseudoproteases, CspC, into mature spores (234, 235), the catalytic site mutations within CspA and CspC, which are largely conserved across Peptostreptococcaceae family members (235), would appear to confer unique functions to these proteins.

Recent studies have identified two unique proteins that modulate *C. difficile* spore germination. GerG (CD0311) is a *C. difficile*-specific gel-forming protein that is necessary for incorporating CspA, CspB, and CspC into mature spores, while GerS (CD3464) is a Peptostreptococcaceae family-specific lipoprotein that is necessary for cortex hydrolysis (248) because it is necessary for generating the muramic- γ -lactam required for SleC to recognize its cortex substrate (Diaz & Shen, unpublished data).

While the Csp pseudoproteases, GerG, and GerS are distinguishing features of the *C. difficile* spore germination pathway, it is likely that additional novel regulators specific to certain clostridial families will be identified as this fascinating developmental process is studied using the genetic tools recently developed for studying clostridial pathogens (140, 277–279).

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Figure 1. Lifecycle of Endospore Formers.

(A) Sporulation: Upon sensing certain environmental conditions, endospore formers activate Spo0A and initiate sporulation. The first morphological event is the formation of a polar septum, which creates a larger mother cell and smaller forespore. The mother cell engulfs the forespore, and the two cells work together to assemble the dormant spore. Calcium dipicolinic acid (Ca-DPA) is synthesized in the mother cell and transported into the forespore in exchange for water. The cortex is formed between the two membranes, and coat proteins polymerize on the surface of the mother cell-derived membrane. Once the spore is mature, the mother cell lyses and releases the dormant spore into the environment. (B) Germination: Upon sensing the appropriate small molecule germinants, the spore initiates a signaling cascade that leads to activation of cortex hydrolases and core hydration, which is necessary for metabolism to resume in the germinating spore.

Figure 2. Sporulation Initiation via Spo0A Phosphorylation.

Sigma factors are shown as circles, histidine kinases and phosphatases as hexagons (adapted from Al-Hinai *et al.* (85)). Positive regulators are shown in green (with the exception of σ^K , which is shown in purple), while negative regulators are shown in red. In *B. subtilis*, the KinA-E orphan histidine kinases phosphorylate the phosphotransfer protein Spo0F, the first component in the phosphorelay (25). The Rap phosphatases remove phosphates from phosphorylated Spo0F. The phosphate is transferred from Spo0F to Spo0B to Spo0A. In *C. difficile*, the orphan histidine kinases CD1579 and CD2492 appear to phosphorylate Spo0A (32), while CD1492 likely dephosphorylates Spo0A (47). A more detailed description of *C. difficile* Spo0A regulation is shown in Figure 3. Although all the putative orphan histidine kinases with the potential to phosphorylate Spo0A in *C. perfringens* and *C. botulinum* are shown, whether they act as positive or negative regulators remains unstudied. The stationary factor σ^H activates expression of *spo0A* in *B. subtilis* and *C. difficile* (101), while σ^K activates *spo0A* transcription in *C. botulinum* (74) and possibly *C. perfringens*, the latter of which induces sporulation during log-phase growth (65).

Figure 3. Regulatory pathway controlling Spo0A activation in *C. difficile*.

Early sporulation factors experimentally determined to function as positive regulators of Spo0A are highlighted in green, while those that inhibit Spo0A are highlighted in red (32, 47, 51, 55, 56, 59, 61, 62, 101). Hexagons indicate histidine kinase/phosphatases; rounded rectangles demarcate transcription factors; and circles highlight sigma factors. Red lines indicate negative regulation, and black lines indicate positive regulation. SinR and SinR', *C. difficile* orthologs for regulatory proteins characterized in *B. subtilis* (grey), were recently shown to promote sporulation (60). Solid lines indicate defined regulatory interactions, and dashed lines suggest proposed, and potentially indirect, regulatory effects. Branched-chain amino acids (BCAAs) are a CodY cofactor (59), and their precursors are likely imported primarily through the Opp and App oligopeptide transporters (55, 61). CcpA-independent carbon-specific regulation is not shown (56). The reciprocal transcriptional regulation of early sporulation factors by Spo0A has also been omitted for simplicity (100).

Figure 4. Diversity in the regulation of the transcriptional programs controlling sporulation in the Firmicutes.

The temporal progression of sporulation is shown from top to bottom. Transcription factors and sigma factors are shown in bold, and proteins enclosed in boxes directly participate in signaling between the mother cell and forespore (dashed boxes indicate that trans-septum signaling has not been tested yet). Text color denotes whether the factor has been detected at both the transcript and protein level (black), at either the transcript or protein level (purple), or has not been tested yet at the transcript or protein level (blue). Black arrows delineate transcriptional control of gene expression; red arrows indicate signaling pathways; dashed

lines indicate that the regulatory relationship remains unknown; and thick arrows demarcate notable points of divergence from the pathway defined in *B. subtilis*. AND gates are indicated. Figure is adapted from Fimlaid *et al.* (137) under Creative Commons BY 4.0.

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Figure 5. Spore coat and exosporium structure in *C. difficile*.

(A and B) Transmission electron microscopy sections of *C. difficile* spores highlighting (from outside to inside) the bumpy, outermost exosporium (Ex) layer with its hair-like projections (HPs), outer coat (OC), inner coat (IC), cortex layer (Cx), germ cell wall (GCW), inner forespore membrane (IM), and spore core (cytosol). (C) Scanning electron microscopy of *C. difficile* spores reveals the bumpy surface created by the exosporium. Images used without modification from Rabi *et al.* (280) (under Creative Commons BY 4.0). (D) Schematic of spore coat layers highlighting morphogenetic factors identified as being important for the assembly of specific layers. Assembly of the outermost exosporium depends on the BclA collagen-like proteins, which likely create hair-like projections on the spore surface (186, 187), CdeC (185), and CdeM (D. Paredes-Saja, unpublished data). The proteins that make up the outer and inner coat layers are unknown, but CotA and the mucinase, CotE, have been shown to be surface accessible (180, 182). SpoIVA (IVA) and SipL are interacting coat morphogenetic proteins that are essential for recruiting coat proteins to the forespore and forming heat-resistant spores (173, 174). The specific proteins recruited by SpoIVA, SipL, CdeC, and CdeM remain unknown.

Figure 6. Putative Locations of Germination Regulators in *C. difficile* and *C. perfringens*.

Germinant signaling proteins, CspC (pseudoprotease and germinant receptor) and its downstream effectors, the CspB protease, and cortex hydrolase, SleC, are all produced in the mother cell under the control of either σ^E or σ^K (137–139). CspB is produced as a fusion to the pseudoprotease, CspA, which is critical for CspC incorporation into mature spores (233–235); all three Csp proteins are incorporated into mature spores. GerG is required for optimal incorporation of CspC, CspB, and CspA into mature spores (253). The GerS lipoprotein (248) is produced in the mother cell and does not directly participate in spore germination (Diaz & Shen, unpublished data), even though it is required for spore germination to proceed. The ATP/GTP binding protein CD3298 presumably localizes to the cytosolic face of the outer forespore membrane and regulates calcium release and possibly internalization (205). Germinant sensing induces the proteolytic activation of SleC by CspB in both organisms, but CspA and/or CspC can cleave SleC in *C. perfringens* (marked in brackets) (218, 242, 245), since they are active proteases unlike their cognate partners in *C. difficile* (233). *C. perfringens* also produces inner membrane bound germinant receptors, similar to most spore-forming organisms, in the forespore, in contrast with the soluble CspC protein used by *C. difficile* to sense germinant. The locations of all proteins in mature spores is putative, with the exception of SleC, which has been shown to localize to the *C. perfringens* cortex region by immunoEM (251).

Figure 7. Schematic of Spore Germination Signaling Pathways.

Germinants that are sensed by *B. subtilis*, *C. botulinum*, *C. perfringens*, and *C. difficile* are shown in dark blue; *C. botulinum* Group I and III germinants are shown in the brackets (and includes L-Ala). Amino acid and calcium ion co-germinants are not pictured for *C. difficile* (199, 204, 205). Germinant receptors are shown in green. The signaling pathway between *B. subtilis* and *C. botulinum* Groups I and III (far left) differs from the other clostridial organisms mainly with respect to cortex hydrolase (shown in orange) activation mechanisms, with SleC being activated by proteolytic cleavage by Csp proteases, and the CwlJ and SleB cortex hydrolases being activated by directly or indirectly by DPA release. Accordingly, the order of cortex hydrolysis and DPA release via SpoVAC differs between these two types of mechanisms. *C. botulinum* Group II and IV encode germinant receptors with variable numbers of A and B components. Adapted from (183).