ANNUAL REVIEWS

Annual Review of Microbiology

Clostridioides difficile Spore Formation and Germination: New Insights and Opportunities for Intervention

Aimee Shen

Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, Massachusetts 02111, USA; email: aimee.shen@tufts.edu

Annu. Rev. Microbiol. 2020. 74:545–66

The *Annual Review of Microbiology* is online at micro.annualreviews.org

https://doi.org/10.1146/annurev-micro-011320-011321

Keywords

C. difficile, spore, sporulation, germination, pseudoprotease

Abstract

Spore formation and germination are essential for the bacterial pathogen *Clostridioides difficile* to transmit infection. Despite the importance of these developmental processes to the infection cycle of *C. difficile*, the molecular mechanisms underlying how this obligate anaerobe forms infectious spores and how these spores germinate to initiate infection were largely unknown until recently. Work in the last decade has revealed that *C. difficile* uses a distinct mechanism for sensing and transducing germinant signals relative to previously characterized spore formers. The *C. difficile* spore assembly pathway also exhibits notable differences relative to *Bacillus* spp., where spore formation has been more extensively studied. For both these processes, factors that are conserved only in *C. difficile* or the related *Peptostreptococcaceae* family are employed, and even highly conserved spore proteins can have differential functions or requirements in *C. difficile* compared to other spore formers. This review summarizes our current understanding of the mechanisms controlling *C. difficile* spore formation and germination and describes strategies for inhibiting these processes to prevent *C. difficile* infection and disease recurrence.

Contents

INTRODUCTION

Clostridioides (formerly *Clostridium*) *difficile* is a gram-positive, spore-forming obligate anaerobe that is the leading cause of health care–associated infections in many developed countries (17, 62). In 2017, *C. difficile* caused ~225,000 infections in the United States alone that led to ~13,000 deaths (17). *C. difficile* infections (CDIs) range in severity from mild diarrhea to fulminant disease such as pseudomembranous colitis, but they are generally limited to individuals with gut dysbiosis (3) who have lost microbiota species that normally suppress *C. difficile* growth (72).

Antibiotic usage is the most common cause of decreased colonization resistance against CDI (72). While antibiotics are still the first-line therapy for treating CDI (72), their collateral effects on the gut microbiota cause high rates of disease recurrence (~15–30%) (75). *C. difficile* disease recurrence results in longer hospital stays, increased disease severity and mortality, approximately threefold-higher treatment costs, and decreased quality of life (20). Thus, the most effective therapeutic strategies prevent *C. difficile* growth or disease without diminishing the gut microbiota. For example, regenerating healthy microbiota through fecal microbiota transplants (FMTs)

effectively resolves and prevents *C. difficile* disease recurrence (141). However, FMTs can also cause unintended side effects and even death in rare instances (26). Since understanding the mechanisms that control *C. difficile*'s infection cycle could reveal strategies for specifically preventing CDI or reinfection, this review discusses how *C. difficile* forms infectious spores, how these spores germinate to initiate infection, and how this information is being used to prevent CDI or disease recurrence.

THE ROLE OF SPORES IN *C. DIFFICILE*'S INFECTION CYCLE

The metabolically dormant spore form of *C. difficile* is its major infectious particle (25), since *C. difficile* is an obligate anaerobe. *C. difficile* spores are found in a wide range of environments, such as livestock, food, and asymptomatic individuals (61). When *C. difficile* spores are ingested, they sense specific bile acid germinants in the small intestine (51, 128) and induce a signaling cascade that culminates in spores transforming back into vegetative cells. The toxins produced by these vegetative cells are responsible for the symptoms associated with *C. difficile* disease (3).

C. difficile spore germination is most strongly activated in the ileum, where bile acids that induce germination are present at high levels and the pH is favorable to germination (67) (**Figure 1*a***). Vegetative *C. difficile* growth primarily occurs in the colon (and cecum in mice). Analyses of the dynamics of CDI in mice have revealed that (*a*) spores germinate and outgrow to form vegetative

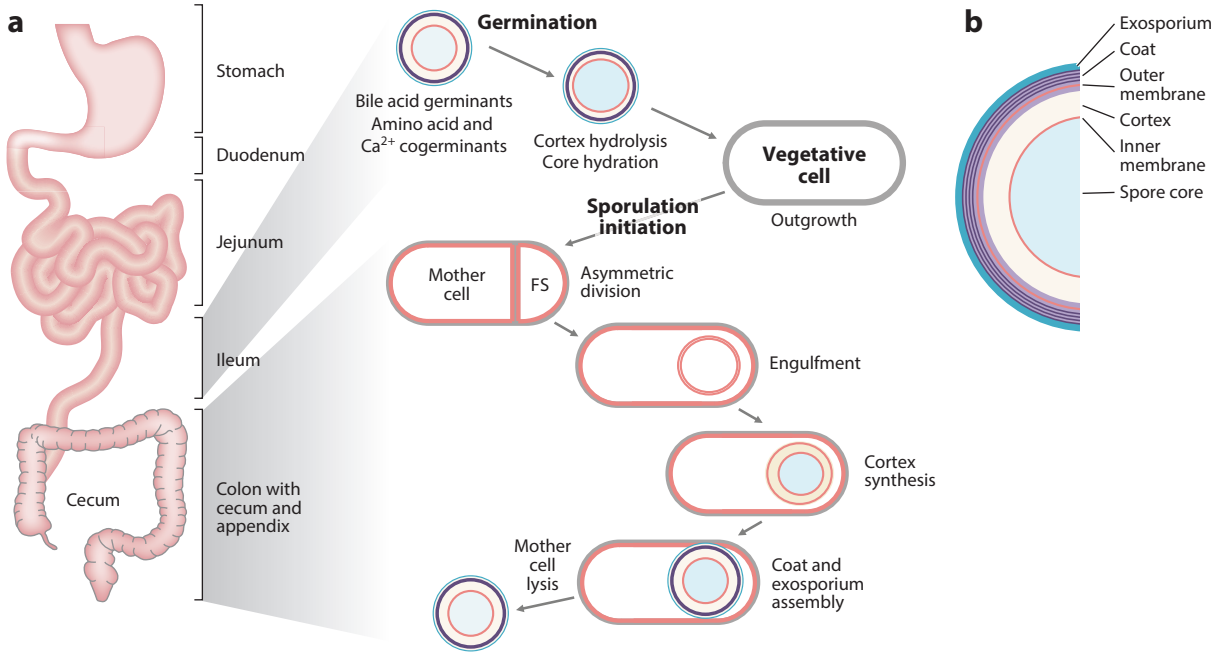


Figure 1

Spores and *Clostridioides difficile*'s infection cycle. (*a*) *C. difficile* infections begin when its dormant spores sense cholate-derived bile acid germinants and amino acid and Ca^{2+} cogerminants in the ileum and activate degradation of the cortex. Calcium dipicolinic acid release allows for core hydration, which allows metabolism to resume and the spore to outgrow into a vegetative cell. During growth in the colon, a subset of *C. difficile* vegetative cells initiates sporulation in response to nutrient deprivation. (*b*) Structural layers of *C. difficile* spores. The outermost exosporium layer contains glycoproteins, while the coat is a series of concentric proteinaceous shells. The cortex is a thick layer of modified peptidoglycan that surrounds the spore core, which is the partially dehydrated cytosol of the forespore. Abbreviation: FS, forespore.

cells within 6 h of inoculation; (*b*) vegetative cells reach maximal levels and glucosylating toxins reach their highest concentrations in the colon and ceca after 24 h; and (*c*) spores are first detected at 24 h and represent ~20% of all *C. difficile* detected in the colon, cecum, and stool (69).

REGULATION OF *C. DIFFICILE* SPORULATION

Overview of Sporulation

C. difficile spore formation follows a developmental pathway generally conserved across endospore formers (**Figure 1*a***), which occurs exclusively in the *Firmicutes* phylum and has been extensively characterized in the model organism *Bacillus subtilis* (135). Since the mechanisms underlying spore formation in *B. subtilis* were originally thought to be conserved across spore formers, this review highlights areas where *C. difficile* spore assembly differs from *B. subtilis*.

The first morphological stage of sporulation is asymmetric division, which generates a larger mother cell and a smaller forespore (119). The mother cell then phagocytoses the forespore in a process known as engulfment, leaving the forespore suspended within the mother cell cytosol and surrounded by two membranes. The mother cell nurtures the forespore, helping it generate a thick layer of modified peptidoglycan known as the cortex, and encases it in a series of proteinaceous shells known as the coat (**Figure 1*b***). The cortex helps spores maintain metabolic dormancy and confers protection against heat and ethanol (98), while the coat acts as a molecular sieve to protect the spore's inner layers against enzymatic and chemical insults (35). The final layer of *C. difficile* spores is the exosporium, which modulates the interaction of spores with their environment (119).

The mother cell also synthesizes large amounts of calcium dipicolinic acid (Ca-DPA), a spore-specific small molecule that is concentrated in the spore cytosol (core) during spore formation. Ca-DPA is exchanged for water, leading to partial dehydration of the core, which reduces metabolism and contributes to heat resistance (119, 135). Following maturation of the forespore into a spore, the mother cell lyses and releases the dormant spore into the environment. These spores resist commonly used disinfectants like ethanol and quaternary amines, facilitating *C. difficile*'s survival and spread in health care settings (71).

Sporulation Initiation

While *C. difficile* must form a spore to survive exit from the host, only a subpopulation of *C. difficile* exits the host in the spore form (69). *C. difficile* sporulates in response to environmental (146) and nutritional signals (37) that converge to control the activity of the master transcriptional regulator, Spo0A (37). As a response regulator, Spo0A's activity is controlled by phosphorylation, which is modulated by three orphan histidine kinases in *C. difficile*. CD1579 and CD2492 can both phosphorylate Spo0A (140), while CD1492 likely dephosphorylates Spo0A (119).

The mechanism by which these orphan histidine kinases integrate environmental signals remains unclear, but two additional modulators of *C. difficile* Spo0A activity have been identified. The RRNPP family member RstA (regulator of sporulation and toxins) promotes Spo0A phosphorylation through an unknown mechanism, although its N-terminal helix-turn-helix (HTH) DNA-binding motif is dispensable for this function (38). Interestingly, in *Bacillus* spp. RRNPP family orthologs (the Rap phosphatases) the HTH domain has been replaced by phosphatase domains. The Rap phosphatases repress sporulation by indirectly dephosphorylating Spo0A, and their activity is antagonized by quorum-sensing peptides (30, 91).

While RstA contains a similar domain that could bind quorum-sensing peptides, the role of these signaling molecules during *C. difficile* sporulation remains to be studied. Peptides nevertheless regulate *C. difficile* sporulation, since loss of the Opp and App peptide transporters increases sporulation, likely because the peptides they transport are used as nutrients. Nutritional

cues regulate *C. difficile* sporulation because two global regulators, CcpA and CodY, repress sporulation. Carbon catabolite protein A (CcpA) directly represses sporulation in response to sugars by binding the promoters of *spo0A* and *sigF*, the latter of which encodes the earliest-acting sporulation-specific sigma factor (8). The CodY transcriptional repressor indirectly inhibits sporulation, likely by binding the *opp* and *sinR* operons in response to sensing GTP or branched-chain amino acids (82). *sinR* encodes an operon that enhances *spo0A* transcription (54), which downregulates sporulation, unlike its *B. subtilis* homolog.

Strain-Specific Differences in Sporulation Levels

Although sporulation has primarily been studied in laboratory-adapted strains of 630 (22, 84), a ribotype 012 strain isolated from a symptomatic patient (112), *C. difficile* strains are highly genetically diverse [its core genome represents ~20% of its pangenome (70, 86)], and they vary widely in their sporulation frequency under laboratory conditions (36). The mechanisms underlying these differences in sporulation level are unknown, but some studies have correlated strains with higher sporulation levels in vitro with a higher likelihood of recurrent disease (55, 78). However, others have failed to observe this correlation (16, 85). While it is difficult to compare these studies due to differences in media conditions and sporulation assay methods, strain-specific differences in the roles of certain sporulation regulators have been observed (53). For example, a *codY* mutant in UK1 (RT027) exhibits an ~50-fold-more severe defect in sporulation than an equivalent mutant in 630Δ*erm* (82).

Sporulation-Specific Sigma Factor Regulation

The transcriptional program downstream of Spo0A activation, however, is likely well conserved across *C. difficile* strains (27). Spo0A activation ultimately leads to the activation of four sporulation-specific sigma factors, σ^E, σ^F, σ^G, and σ^K, whose regulons are required for sporulation (41, 92, 111). Genome-wide transcriptional profiling has defined these gene subsets (41, 111), and visualizable transcriptional reporters have confirmed that these sigma factors exhibit compartment-specific activity (92). Similar to *B. subtilis*, *C. difficile* σ^F and σ^G are active in the forespore, and σ^E and σ^K are active in the mother cell.

Despite these similarities, the activation order and dependencies of these sigma factors differ markedly between *C. difficile* and *B. subtilis*. Since these have been reviewed extensively elsewhere (44, 110), they are briefly summarized here. Early-acting sigma factor (σ^F and σ^E) regulation appears to follow the model defined in *B. subtilis*, although σ^E activation does not fully depend on σ^F activation in *C. difficile*. This is because production of the SpoIIR signaling protein that induces pro-σ^E processing can be mediated by both Spo0A and σ^F in *C. difficile* (111), whereas *spoIIR* transcription is strictly dependent on σ^F in *B. subtilis* (142).

σ^G and σ^K activation mechanisms diverge considerably between *C. difficile* and *B. subtilis*. Whereas engulfment completion and the SpoIIQ-SpoIIIAH transmembrane complex are critical for *B. subtilis* σ^G activation (15, 32, 105), these processes are dispensable for *C. difficile* σ^G activation (43, 113). While σ^K requires proteolytic activation in a σ^G-dependent manner in *B. subtilis*, *C. difficile* σ^K is active upon synthesis (95). Transcription of *C. difficile sigK* nevertheless depends on excision of the prophage-like element interrupting *sigK*, similar to the case of *B. subtilis* (114).

Epigenetic Regulation of *C. difficile* Sporulation

While Spo0A activation licenses sporulation in spore-forming organisms, *C. difficile* sporulation is additionally regulated at the level of σ^F activation. Loss of the orphan DNA methyltransferase

CamA (CD2758) reduces spore production by approximately twofold and sixfold in 630 and R20291 (RT027) strains, respectively (27, 86). CamA impacts σ^F activation rather than Spo0A activation, since Spo0A activity is similar between Δ*camA* and wild type, but σ^F activation and events downstream are decreased two- to threefold (86). Furthermore, while similar numbers of Δ*camA* cells induce sporulation, fewer complete engulfment relative to wild type. While CamA's methyltransferase activity is necessary to promote spore formation and is the first example of sporulation being epigenetically regulated to our knowledge (86), the specific CAAAA**A** sites methylated by CamA that modulate σ^F activation remain unknown.

SPORE ASSEMBLY

Sporulation-specific sigma factors induce the expression of genes whose products control spore assembly, namely, engulfment, cortex synthesis, coat assembly, exosporium assembly, and Ca-DPA transport into spores. Recent studies have identified specific regulators of these processes, some of which are unique to *C. difficile* or its closest relatives in the *Peptostreptococcaceae* family, while others are conserved in *B. subtilis*. However, gene conservation in *C. difficile* does not always predict protein function.

Engulfment

Engulfment, the process that allows the mother cell to surround the forespore and create a cell within a cell, involves the coordinated degradation and synthesis of peptidoglycan. In *C. difficile*, peptidoglycan degradation is mediated by the SpoIIP amidase and endopeptidase (28, 106) and the SpoIID transglycosylase (28). These enzyme activities are identical to those defined in *B. subtilis*, although the two organisms differ in their requirement for the conserved protein SpoIIM. In *B. subtilis*, SpoIIM coordinates SpoIIP and SpoIID activity and thus spore formation (18, 127), whereas SpoIIM is largely dispensable for spore formation in *C. difficile* (28, 106). This differential requirement likely reflects differences in localization and interactions between SpoIIP and SpoIID (reviewed in 63).

Cortex Assembly and Modification

Following engulfment, the cortex layer is synthesized on top of the germ (vegetative) cell wall between the two membranes surrounding the forespore (**Figure 1*b***). The cortex is much thicker than the vegetative cell wall and functions to maintain dormancy and confer heat and ethanol resistance (98). The cortex differs from the vegetative cell wall because it contains muramic-δ-lactam (MAL), which is a spore-specific modification essential for cortex lytic enzymes to recognize the cortex during germination (98). The cortex also contains fewer peptide cross-links, which may help the spore expand and contract with changing environmental conditions (135). The unique properties of the cortex are shared between *B. subtilis*, *Clostridium perfringens*, and *C. difficile*, although the percentages of cross-links and MAL residues vary between these organisms (23, 87, 99). MAL residues are approximately twofold-more frequent in *B. subtilis* and *C. perfringens* than in *C. difficile*, while free *N*-acetylmuramic acid (NAM) residues and reduced peptide cross-linking are observed in *C. difficile*.

MAL is produced by the coordinated activities of the CwlD amidase and PdaA deacetylase, which remove the peptide side chain from NAM and deacetylate the muramic acid, respectively (98). PdaA also cyclizes the resulting sugar into MAL. Loss of these enzymes in *C. difficile* results in ~100-fold decreases in germination, slower germination kinetics, and decreased heat resistance relative to wild type (23, 31). Δ*cwlD* and Δ*pdaA1* (*CD1430*) mutants also exhibit abnormal

forespore shapes and a mislocalized coat (23, 31). While CwlD and PdaA have identical activities in *C. difficile* and *B. subtilis*, in *B. subtilis* they are essential for germination but do not impact spore heat resistance, shape, or coat localization (48).

However, the biggest difference in cortex modification between *C. difficile* and *B. subtilis* is that *C. difficile* CwlD amidase function depends on the GerS lipoprotein (31, 42), which is conserved exclusively in the *Peptostreptococcaceae* family (42). *gerS* and *cwlD* mutant spores have identical muropeptide profiles and germination and heat-resistance defects. Since GerS and CwlD directly interact (31), binding between these two proteins is likely required for CwlD to deamidate NAM. However, it is unclear why *C. difficile* CwlD's function depends on GerS, since *B. subtilis* CwlD independently has amidase activity (52). *C. difficile* GerS may help CwlD localize properly or act as a cofactor for CwlD amidase activity. *C. difficile*'s use of GerS may also result in the reduced levels of MAL observed in *C. difficile* relative to *B. subtilis* and *C. perfringens* (23).

Comparatively less is known about how *C. difficile* synthesizes the cortex, although factors required for this process in *B. subtilis* are conserved in *C. difficile*. In *B. subtilis* the spore-specific machinery, namely SpoVB, SpoVE, and SpoVD, synthesizes the large amounts of peptidoglycan that comprise the cortex (135). SpoVB is a lipid II flippase that flips the lipid intermediates across the cell membrane (77). SpoVE likely assembles the glycan strands through its glycosyltransferase activity as part of the SEDS (shape, elongation, division, sporulation) family (60, 76). SpoVD is a penicillin-binding protein (PBP) transpeptidase that binds SpoVE and cross-links the peptidoglycan strands together (24, 40).

SpoVB, SpoVD, and SpoVE are all essential for cortex synthesis and thus spore formation in *B. subtilis* (135). Despite the conservation of these proteins across all spore formers, *C. difficile spoVB* transcription is not sporulation regulated, while *C. difficile spoVD* and *spoVE* expression is activated by Spo0A rather than σ^E in *B. subtilis* (24, 41). Furthermore, *C. difficile* SpoVB is dispensable for spore formation based on transposon mutagenesis, whereas SpoVD and SpoVE are required for this process (27). SpoVD was recently confirmed to be essential for cortex synthesis and spore formation in different strain backgrounds (5, 131).

Coat Assembly

The coat consists of proteinaceous shells that surround spores and protect them from chemical and enzymatic insults (35). The composition of this layer varies widely between bacterial spore formers, with only ~25% of coat proteins in *C. difficile* having homologs in *B. subtilis* (56). While we are only starting to understand how these coat proteins assemble around the *C. difficile* forespore, several proteins required for this process, i.e., coat morphogenetic proteins, have been identified.

SpoIVA and SipL (SpoIVA-interacting protein L) (CD3567) were the first coat morphogenetic proteins identified in *C. difficile* (100). They comprise the innermost layer of the coat, because loss of either leads to the coat sloughing off the forespore or mislocalizing to the cytosol. SpoIVA is likely an ATPase that self-polymerizes around the forespore based on analyses in *B. subtilis* (103); it recruits SipL to the forespore by binding SipL's C-terminal LysM domain (100). This interaction stabilizes SpoIVA and promotes SpoIVA's encasement of the forespore (139).

While SpoIVA is conserved across all spore formers (1, 49), SipL is only found in the *Clostridiales* (100), implying that the *Bacillales* and *Clostridiales* use distinct pathways to assemble coat proteins around the forespore. Consistent with this notion, the highly conserved protein SpoVM is largely dispensable for *C. difficile* spore formation (107) despite being essential for *B. subtilis* spore formation (90). The differential requirement for SpoVM is likely due to a quality control pathway in the *Bacillales* that senses SpoVM or SpoIVA encasement defects and induces lysis of the mother cell (136). However, since *C. difficile spoIVA*, *sipL*, and *spoVM* mutants still exhibit irregularities in

cortex thickness (107; J.W. Ribis, A. Shen, unpublished data), all these proteins likely modulate *C. difficile* cortex synthesis through an unknown mechanism.

A third morphogenetic protein, CotL, was recently shown to regulate *C. difficile* coat, cortex, and exosporium assembly (7). Loss of CotL, a small lysine-rich protein conserved only in the *Peptostreptococcaceae* family, leads to spores that lack visible coat layers, produce thinner cortex, and exhibit germination defects (7). *cotL* mutant spores also carry reduced levels of coat, cortex, and exosporium proteins. This decrease could be caused by impaired SpoIVA encasement, although mislocalized coat is not visible in the *cotL* mutant in phase-contrast microscopy analyses (7), in contrast with *spoIVA* and *sipL* mutants (100). Alternatively, CotL may be required to retain coat and cortex proteins. Analyzing sporulating cultures of the *cotL* mutant by transmission electron microscopy (TEM) and determining the mutual localization dependencies of CotL, SpoIVA, and SipL using functional fusions would distinguish between these models.

CotL's impact on SleC levels in spores is particularly intriguing because nothing is known about how SleC is incorporated into the cortex layer (79). For example, it is unclear whether SpoIVA is required for packaging SleC into spores, so analyzing the localization of SleC fluorescent protein fusions in *cotL* and *spoIVA* mutants would provide insight into the mechanisms controlling SleC incorporation.

Of the ~50 coat proteins identified in proteomic analyses of *C. difficile* coat fractions (2), the localization dependencies and specific functions of most of these proteins are unclear. Enzymatic activities have been identified for (*a*) the alanine racemase Alr2, which alters the sensitivity of spores to D-alanine cogerminant (123), and (*b*) the CotE mucinase (93), which facilitates spore binding to intestinal epithelial cells (57). Interestingly, CotE enhances *C. difficile* colonization levels and disease severity, indicating that *C. difficile* spores actively contribute to infection despite their metabolically dormant state (93).

Exosporium Assembly

As the outermost layer of *C. difficile* spores, the exosporium determines how spores interact with their environment. Notably, this layer varies between different *C. difficile* strains, with some producing a tightly-associated exosporium and others producing a bag-like structure (73, 101); most also produce hair-like extensions that are likely composed of glycosylated BclA family proteins (94, 97), similar to those of *Bacillus anthracis* (56). Different exosporium morphotypes are even observed within the same strain, with spores producing thin versus thick exosporia (96) and others generating polar extensions of the spore, termed appendages (9). This phenotypic heterogeneity likely serves as a bet-hedging strategy to enhance *C. difficile* colonization and infection as described below.

The cysteine-rich proteins CdeC and CdeM modulate *C. difficile* exosporium assembly (14). These proteins form higher-order multimers that likely resemble the 2D arrays (14) observed in the *B. anthracis* exosporium (132). CdeC impacts spore resistance properties by affecting both coat and exosporium assembly (14). CdeC is conserved in the *Peptostreptococcaceae* family and also modulates coat and exosporium formation in *Paeniclostridium sordellii* (101). CdeM is found exclusively in *C. difficile* and regulates exosporium protein content, since *cdeM* mutant spores make thinner exosporia and smaller appendages (9, 14). Spores lacking either of these morphogenetic factors exhibit adherence defects to colonic mucosa and surprisingly exhibit increased virulence in mouse and hamster models of infection (9, 14).

Ca-DPA Transport

Following engulfment, Ca-DPA is synthesized in the mother cell and transported across two membranes into the forespore via the mechanosensitive channel, SpoVAC (33, 47); the putative

ATPase, CD3298 (68); and likely the SpoVV homolog (104), CD1168. Ca-DPA is concentrated in the spore core (5–15% of spore dry weight) in exchange for water (116). The resulting partial dehydration of the core reduces metabolism and increases *C. difficile* spore heat resistance. *C. difficile* spores lacking Ca-DPA are more hydrated and heat sensitive than wild type (33), but it is unclear whether this decreased resistance impacts infection.

SPORE GERMINATION

Overview of Spore Germination

When bacterial spores sense small-molecule signals known as germinants, they initiate a signaling cascade that culminates in cortex hydrolysis and core hydration. Removal of the cortex layer is necessary for core hydration, since the cortex prevents water uptake by constraining the size of the core. Core hydration results in loss of spore resistance properties, since it allows metabolism to resume and the spore to outgrow into a vegetative cell (116). While *C. difficile* spore germination follows these general steps, it differs from that of previously studied spore formers in (*a*) the germinants it senses and (*b*) the mechanism it uses to sense these germinants (66, 147) (**Figure 2**).

C. difficile Germinants and Cogerminants

While almost all spore formers sense nutrient germinants like amino acids and sugars (116), *C. difficile* senses cholate-derived bile acids (128), which are found exclusively in the vertebrate gut (108). This mechanism allows *C. difficile* to control the anatomical location of its germination, which occurs optimally in the ileum (small intestine) (**Figure 1*a***), where bile acid levels are

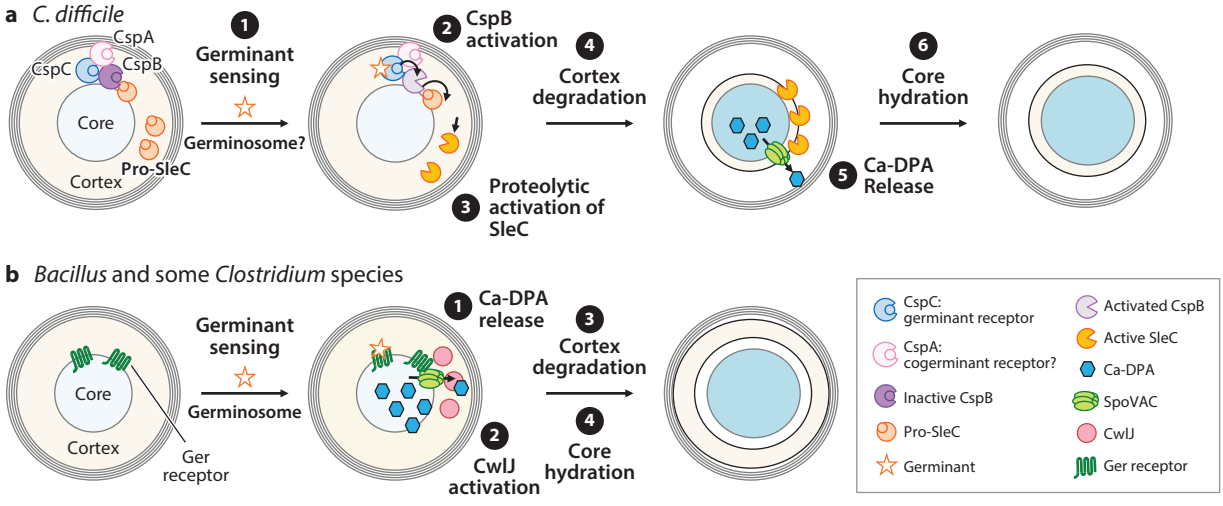


Figure 2

(*a*) A unique signaling pathway regulates *Clostridioides difficile* spore germination. Ger receptors are conserved in all spore formers except *C. difficile*. (❶) They sense nutritional germinants like sugars and amino acids (*orange stars*) in the inner spore membrane. (❷) In contrast, *C. difficile* lacks Ger receptors and germinates in response to nonnutritional bile acid germinants, namely taurocholate, using two soluble pseudoproteases. (❸) (Co)germinant signaling allows CspC and CspA to activate the CspB protease through an unknown mechanism, although it is hypothesized that all three Csp proteins and SleC are complexed together like a germinosome (115). CspB then proteolytically activates the cortex lytic enzyme SleC. (❹) Cortex hydrolysis then permits (❺) Ca-DPA release by SpoVAC, which (❻) leads to core hydration and permits metabolism. (*b*) In *Bacillus* spp. and those lacking a Csp system, (❶) released Ca-DPA activates (❷) the CwlJ cortex lytic enzyme, which then (❸) degrades the cortex and permits (❹) core hydration. Abbreviation: Ca-DPA, calcium dipicolinic acid.

BILE ACID TRANSFORMATIONS IN THE GUT

Primary bile acids are made in the liver and consist of cholate and chenodeoxycholate. These bile acids can be conjugated to either taurine or glycine to generate conjugated primary bile acids (technically salts) (108). The liver releases these molecules into the duodenum to facilitate digestion; most are reabsorbed by the terminal ileum and returned to the liver via enterohepatic circulation. However, the remaining 5% enter the colon and are transformed by the resident microbiota into secondary bile acids via (*a*) deconjugation, which removes the conjugated amino acids, and (*b*) dehydroxylation, which converts cholate into deoxycholate and chenodeoxycholate into lithocholate.

high and the pH has increased sufficiently to permit germination. The most potent germinant for *C. difficile* is the conjugated cholate derivative, taurocholate.

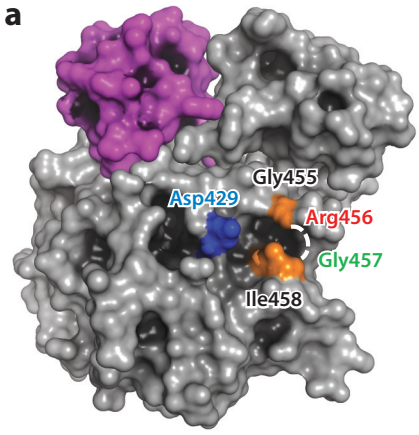
Since bile acid metabolism is complex, and since there is confusion in the literature regarding the role of primary versus secondary bile acids during *C. difficile* spore germination (118), we have summarized bile acid metabolism in the sidebar titled Bile Acid Transformations in the Gut. Notably, while primary bile acids are often described as activators of *C. difficile* spore germination, the primary bile acid chenodeoxycholate is a potent inhibitor of taurocholate-induced spore germination (129), and the secondary bile acid deoxycholate induces germination (albeit less efficiently than taurocholate) (128, 143).

Part of the confusion likely arises because primary and secondary bile acid levels change after antibiotic treatment, and these changes correlate with increased susceptibility to CDI (72, 138). Since secondary bile acids are generated from microbiota-mediated metabolism of primary bile acids, secondary bile acid levels decrease after antibiotic treatment, while primary bile acid levels increase (108, 138). Coincident with these changes, *C. difficile* spore germination levels increase in small intestinal and cecal extracts of antibiotic-treated mice relative to untreated mice (51, 69). However, the extent to which increased spore germination during gut dysbiosis contributes to infection is unclear because secondary bile acids, which potently inhibit *C. difficile* growth (137), also decrease (138).

Although taurocholate potently activates *C. difficile* spore germination, it requires cogerminants to potentiate germination (66). Two classes of cogerminants can induce germination when coupled with taurocholate: amino acids and divalent cations (68, 128). Glycine is the most potent amino acid cogerminant (124), while calcium is the most potent and physiologically relevant divalent cation cogerminant (68, 125). The two cogerminant classes function additively (at a minimum) such that spore germination is induced at physiologically relevant concentrations of taurocholate (67). While cogerminant synergy has yet to be mathematically determined (21), these results imply that the two cogerminant classes bind different sites on the same receptor or different receptors entirely.

Mechanism of Germinant and Cogerminant Sensing

The second major difference between spore germination in *C. difficile* relative to other spore formers is that *C. difficile* does not encode the Ger family transmembrane germinant receptors conserved in almost all spore formers (89). Instead, *C. difficile* appears to use a soluble receptor, the CspC pseudoprotease, to sense bile acid germinants. CspC was identified in an elegant genetic selection for altered germinant specificity mutants (45) that gained the ability to germinate in response to the potent germination inhibitor chenodeoxycholate (129). Mutation of a single CspC residue, G457R, was sufficient to permit germination in response to chenodeoxycholate.



	TA	Amino acids	Ca^{2+}
G457R	↑	↑	↑
R456G	↑	↑	
D429K			↑

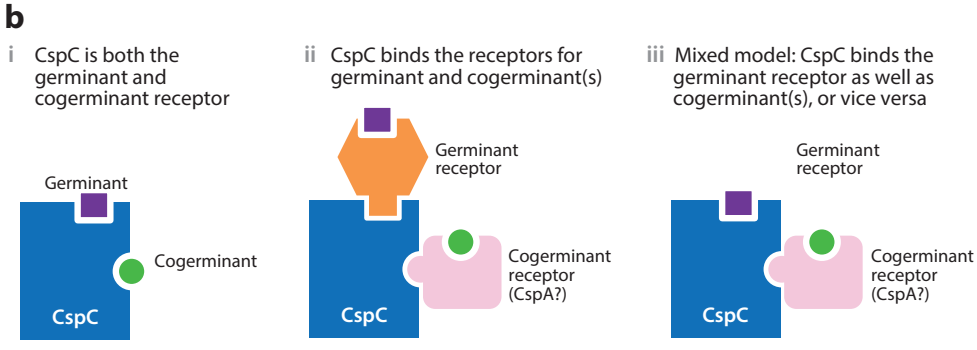


Figure 3

Models for *Clostridioides difficile* spore germinant sensitivity. (*a*) CspC point mutations that confer bile acid–independent germination and enhanced sensitivity to the indicated germinant and cogerminants (109). (*b*) CspC binds either (co)germinants directly or the direct receptors of these molecules, with CspA having been proposed to be the cogerminant receptor. Abbreviation: TA, taurocholate.

However, we recently showed that *G457R* mutant spores germinate when plated on rich media alone, independent of bile acids (109), and thus do not specifically respond to chenodeoxycholate. We also identified two additional alleles that permit bile acid–independent germination, *R456G* and *D429K*, using structure-guided mutagenesis (109). Since the *G457R*, *R456G*, and *D429K* alleles differentially impact the sensitivity of *C. difficile* spores to germinants and cogerminants (**Figure 3*a***), CspC integrates multiple environmental signals (i.e., bile acid germinants and cogerminants) to induce germination.

Notably, the residues altered in these mutants cluster to the same surface-exposed region, suggesting that this region could directly bind the three classes of germinant and cogerminants (bile acids, amino acids, and Ca^{2+}). However, there is currently no biochemical evidence for CspC binding to any of these small molecules, and no Ca^{2+} was detected in the CspC structure despite related subtilisin-like serine protease family members using Ca^{2+} as a cofactor (121). Alternatively, CspC could function as a signaling node by interacting with the direct sensors of these small molecules, or use a mixture of these two models (**Figure 3*b***).

Consistent with this latter proposal, the related CspA pseudoprotease was recently implicated in either directly sensing cogerminants or integrating signals from both classes of cogerminants (**Figure 3*b***). In a genetic screen for altered glycine cogerminant specificity, Shrestha et al. (122)

instead identified mutations that surprisingly allowed for germination in the presence of taurocholate alone (i.e., independent of cogerminants). These mutations alter interdomain processing of the CspA pseudoprotease, which is initially produced as a fusion to the CspB protease during sporulation but undergoes interdomain processing during spore maturation (65).

Proteolytic Regulation of *C. difficile* Cortex Hydrolysis

Unlike CspA and CspC, which are pseudoproteases, CspB is an active subtilisin-like serine protease that functions to proteolytically activate the pro-SleC cortex lytic enzyme (4, 120). Active SleC degrades the cortex layer to facilitate Ca-DPA release and core hydration (46) (**Figure 2**). Thus, two pseudoproteases, CspA and CspC, modulate germinant and cogerminant signaling in *C. difficile* by ultimately activating the CspB protease.

This mechanism of regulation is similar to how some pseudoenyzmes function as signal integrators to regulate the activity of their cognate enzymes (81). Ligand binding by the pseudoenzyme induces conformational changes that relieve the pseudoenzyme's suppressive interaction with its cognate enzyme. This mechanism has been suggested by the Sorg group (122) and is consistent with the observation that some subtilisin-like serine proteases form dimers (88). Interestingly, our structure of *C. perfringens* CspB protease revealed that its protease activity is constrained by its inhibitory N-terminal prodomain at least with recombinant CspB (4). Thus, it is tempting to speculate that CspC and CspA alleviate this inhibition by binding (co)germinants in some combination to displace the prodomain and activate CspB.

According to the germinosome model, the Csps and SleC are present as a complex (12, 122); sensing of germinant and cogerminant signals by CspC and CspA, possibly respectively, allows them to activate the CspB protease. CspB would then be poised to access its substrate, the SleC cortex lytic enzyme. Since *C. perfringens* SleC localizes to the cortex region of *C. perfringens* spores by immuno–electron microscopy (79), CspB likely resides in the cortex region in complex with SleC, along with CspA and CspC (and possibly GerG; see below).

Consistent with this model, CspC incorporation (or stability) in mature spores depends on CspA; incorporation of all three Csps into spores depends on a *C. difficile*–specific protein, GerG (34); and loss of SleC decreases the levels of all three Csps (approximately twofold) (64). Notably, while all these proteins are made in the mother cell, none of them carry signal sequences for facilitating their entry into the cortex layer (34, 41). Whether these proteins are actively transported across the outer forespore membrane and whether the outer forespore membrane is even intact in mature spores remain open questions, especially since it is unclear how bile acids might traverse this membrane to access the soluble Csp proteins.

An alternative model has been suggested by Kochan et al. (66) where CspB activity requires Ca^{2+} as a cofactor. This model is based on their finding that *CD3298* mutant spores fail to germinate in response to glycine cogerminant because they lack internal Ca-DPA and thus a source of Ca^{2+} cogerminant (68). However, our data indicate that this mutant and several other mutants lacking Ca-DPA simply require higher glycine concentrations to induce germination (A.R. Rohlfing, A. Shen, unpublished data). Thus, it seems unlikely that CspB activity requires Ca^{2+} as a cofactor. Regardless, elucidating the signaling mechanism that allows *C. difficile* spores to respond to germinant and cogerminant signals will require biochemical analyses of (*a*) Csp protein-protein interactions during germination and (*b*) ligand binding by Csps.

Cortex Hydrolysis and DPA Release

In *C. difficile*, SleC-mediated cortex degradation is required for the mechanosensitive channel, SpoVAC, to release internal Ca-DPA stores (46, 47). Cortex hydrolysis appears to occur from

the outside in based on TEM analyses (42), suggesting that active SleC and possibly the Csps are localized to the outer perimeter of the *C. difficile* cortex. This mechanism is likely conserved in clostridial organisms that encode Csps and SleC (89). In the *Peptostreptococcaceae* family member *Paraclostridium bifermentans* (13), cortex hydrolysis also precedes Ca-DPA release even though *P. bifermentans* likely uses Ger receptors to activate the Csp proteases (89). In contrast, the order of events is reversed in *B. subtilis* (inside out), where inner forespore membrane-localized Ger receptors induce SpoVAC to release some Ca-DPA, which then posttranslationally activates the cortex lytic enzyme CwlJ to degrade the cortex and potentiate further Ca-DPA release by SpoVAC (116).

Spore Revival: Ripening and Outgrowth

Metabolism in the spore resumes when the core hydrates following cortex hydrolysis and Ca-DPA release. Transcription has been detected as early as 15 min after germination has been induced in *C. difficile* (29). However, beyond this analysis, little is known regarding how *C. difficile* spores revive. Based on studies in *B. subtilis* (126), *C. difficile* spores likely undergo a ripening period when no morphological changes are apparent but macromolecular synthesis prepares the spore for outgrowth. During outgrowth, the spore remodels its peptidoglycan and elongates to form a vegetative cell. Interestingly, in *B. subtilis*, cell wall modifications that confer resistance to lysozyme and antimicrobial peptides are not produced until late stages of outgrowth (126). Since *C. difficile* produces similar cell wall modifications (74), studying the outgrowth properties of *C. difficile* spores may reveal a time period when they are more vulnerable to antimicrobial factors or antibiotics (see below).

Variation in Spore Phenotypes Between Strains and Spore Preparations

Spore germination mechanisms have primarily been studied in the 630 (RT012) and UK1 (RT027) strain backgrounds. However, strains vary widely in their responsiveness to bile acid germinants (137), but it is unclear whether this variation is clinically relevant. Strains with lower levels of taurocholate-only germination have been correlated with higher disease severity (16), whereas other studies have correlated increased germinant sensitivity with higher disease recurrence (80, 85). Since these studies used different strain backgrounds, spore preparation methods, and germination methods, it is difficult to resolve the apparent differences.

Spore preparation (i.e., growth media) and isolation methods nevertheless modulate spore germination responses and adherence properties (39, 73), likely because they alter the frequency of spore morphotypes within a population and the stability of the exosporium (9, 96). Variation in spore morphotypes within the same strain (9) likely contributes to the germination variation observed between spore preparations of the same strain (64, 109). While standardizing spore preparation methods and assay conditions would facilitate direct comparisons between studies, a critical question that needs to be addressed is whether *C. difficile* spores generated during infection have distinct properties from those isolated under specific laboratory conditions.

THERAPEUTIC STRATEGIES TARGETING *C. DIFFICILE* SPORES

The antibiotics vancomycin and fidaxomicin are the current standard of care for CDI (75). However, these antibiotics are still associated with relatively high disease recurrence rates (~10–15%) (20, 75), so strategies that specifically inhibit *C. difficile* without disrupting the native gut microflora are desired. Given the critical role that spores play in *C. difficile*'s infection cycle, strategies that target their formation or germination are currently being developed.

Germination Inhibitors

The natural bile acid inhibitor of *C. difficile* spore germination (129), ursodeoxycholic acid (UDCA), can prevent recurrent CDI in patients with ileal pouchitis (144, 145). These patients have their colons removed due to ulcerative colitis, so reabsorption of UDCA is not an issue in these patients. While this treatment is limited to a small subset of patients, its success highlights the potential of developing antigermination inhibitors, which could also have the added benefit of suppressing vegetative *C. difficile* growth (137).

Since the utility of natural bile acid germinant inhibitors (130) is limited by their low solubility, metabolism by the gut microflora, and ability to stimulate host signaling, synthetic bile acid analogs are currently in development (58, 108, 117, 130, 133). The taurocholate analog CamSA inhibits spore germination and delays *C. difficile* infection in hamsters (59), which are acutely sensitive to CDI. Since combining CamSA with suboptimal concentrations of vancomycin effectively prevented CDI in this model (59), CamSA does not completely prevent spore germination in vivo. An ~200-fold more potent derivative of CamSA with low micromolar efficacy was recently developed, and it inhibits germination in a broader range of strains (117). However, its efficacy and metabolism in animal models of CDI, along with additional bile acid analogs recently developed (130, 133), remain to be tested in vivo.

Germination Activators

Since *C. difficile* spores are highly resistant and actively released from infected patients (25, 71), decontaminating hospital environments is critical to preventing the spread of CDI. Although bleach is effective at killing *C. difficile* spores, this agent can corrode surfaces. To avoid this issue, investigators have explored triggering *C. difficile* spore germination in the presence of UV light and ethanol to kill germinating spores (83). While this strategy shows promise, it may not be cost-effective.

Spore-Specific Vaccines

One study to our knowledge has tested the efficacy of developing vaccines against immunogenic components of *C. difficile* spores. Vaccinating mice and hamsters against the exosporium morphogenetic proteins CdeC and CdeM conferred protection against infection (50), suggesting that antispore vaccines can prevent CDI. It will be important to test whether such a vaccine would provide broad protection against different *C. difficile* strains, since the exosporium varies morphologically between strains (102).

Sporulation Inhibitors

The first-line antibiotic therapy, fidaxomicin, is associated with lower recurrence rates. While fidaxomicin's efficacy is related to its relatively low impact on the gut microbiota compared to metronidazole (75) (a former first-line therapy) and vancomycin, fidaxomicin can also prevent sporulation at sub–minimum inhibitory concentration (MIC) (6, 11) and spore outgrowth, apparently by adhering to *C. difficile* spore exteriors (19), which may contribute to decreased recurrence levels.

A recent study demonstrated that the broad-spectrum β-lactams, cephamycins, potently inhibit *C. difficile* sporulation at concentrations that do not affect vegetative growth (131). This work revealed that cephamycins primarily target SpoVD (131), the PBP essential for cortex synthesis (5, 131). Administration of cephamycins at sub-MIC in combination with vancomycin prevented

C. difficile disease recurrence in a mouse model of infection (131), suggesting that antisporulation inhibitors could be effective at preventing disease recurrence and transmission in hospital settings. Since cephamycin administration can be a risk factor for CDI in humans (134), this proof-of-principle work illustrates the utility in developing inhibitors that specifically target sporulation factors rather than essential factors required for growth (57). Ideally, these optimized inhibitors would minimize cross-reactivity against other clostridial spore formers, which have been associated with gut health (10).

The identification of spore proteins specific to *C. difficile* and its closest relatives that are required for spore formation or germination has revealed possible targets for therapeutic interventions. For example, inhibiting the functions of *Peptostreptococcaceae*-specific spore proteins, CotL (7), CotE (57), GerS (31), or the *C. difficile*–specific spore protein GerG (34) could be a strategy to selectively prevent spore formation or germination in *C. difficile*. Indeed, loss of GerS or PdaA1, which disrupts cortex modification, prevents or delays *C. difficile* infection in hamsters (23, 42).

SUMMARY—CHALLENGES AND KEY UNANSWERED QUESTIONS

Over the last decade, our understanding of the molecular mechanisms that allow *C. difficile* to form infectious spores and germinate those spores has increased dramatically. The transcriptional program that underlies *C. difficile* sporulation has been broadly defined, and spore morphogenetic factors that control the assembly of the cortex, coat, and exosporium have been identified. These analyses have implicated specific spore coat and exosporium proteins in regulating the interaction of spores with intestinal epithelial cells and modulating disease recurrence. They further highlight the importance of determining whether and how additional *C. difficile* spore proteins contribute to infection and transmission, especially since only a fraction of these proteins have been studied.

Major factors required for *C. difficile* spore germination have also been identified (CspA, CspB, CspC, GerG, SleC), as have the germinant and cogerminant molecules used to stimulate this process. Crystal structures of critical signaling components along with genetic screens have identified key residues required for germinant signaling, although the precise mechanism by which (co)germinant signals are transduced remains to be determined. Regardless, these analyses have revealed that the mechanism by which *C. difficile* induces germination differs markedly from that of previously studied organisms and represents a unique opportunity for specifically inhibiting this critical process to prevent CDI.

Despite these advancements, there are critical gaps in our knowledge of spore germination and outgrowth. Biochemical evidence for germinant and cogerminant binding to their receptors would provide key insight into the mechanism by which these small molecules are sensed. The location and interactions between the different germination-signaling components need to be determined. These analyses will require the development of methods that can reliably separate coat versus cortex proteins, since the current method for decoating spores removes known cortex proteins, like SleC. In addition, virtually nothing is known about how *C. difficile* outgrows into vegetative cells. Analyses of this stage could identify new therapeutic targets that would be easier to access with small molecules or protein-based inhibitors due to the loss of spore-resistance properties.

Important advances in our understanding of *C. difficile*'s infection cycle have also been made, with the anatomical location of *C. difficile* spore germination (the ileum) and the molecular signals sensed having been established. While the location of spore formation has been identified, the precise environmental cues sensed by *C. difficile* that induce sporulation remain unknown. Whether there are specific microenvironments within the colon that stimulate *C. difficile* sporulation also remains unclear. Since growth conditions impact spore assembly and their adherence and germination properties, the extent to which spores isolated in the laboratory resemble those isolated

from an infected host should be determined. Addressing these general questions in the next decade of research will undoubtedly lead to exciting new insights into the mechanisms underlying these intriguing developmental processes.

DISCLOSURE STATEMENT

The author is a paid consultant for BioVector, a diagnostic start-up company.

ACKNOWLEDGMENTS

Research in this manuscript was funded by award number R01AI22232 from the National Institute of Allergy and Infectious Diseases (NIAID) and award number R01GM108684 from the National Institute of General Medical Sciences. A.S. is a Burroughs Wellcome Investigator in the Pathogenesis of Infectious Disease, supported by the Burroughs Wellcome Fund.

LITERATURE CITED

1. Abecasis AB, Serrano M, Alves R, Quintais L, Pereira-Leal JB, Henriques AO. 2013. A genomic signature and the identification of new sporulation genes. *J. Bacteriol.* 195:2101–15
2. Abhyankar W, Hossain AH, Djajasaputra A, Permpoonpattana P, Ter Beek A, et al. 2013. In pursuit of protein targets: proteomic characterization of bacterial spore outer layers. *J. Proteome Res.* 12:4507–21
3. Abt MC, McKenney PT, Pamer EG. 2016. *Clostridium difficile* colitis: pathogenesis and host defence. *Nat. Rev. Microbiol.* 14(10):609–20
4. Adams CM, Eckenroth BE, Putnam EE, Doublie S, Shen A. 2013. Structural and functional analysis of the CspB protease required for *Clostridium* spore germination. *PLOS Pathog*. 9:e1003165
5. Alabdali YAJ, Oatley P, Kirk JA, Fagan RP. 2019. A cortex-specific PBP contributes to cephalosporin resistance in *Clostridium difficile*. bioRxiv 715458
6. Aldape MJ, Packham AE, Heeney DD, Rice SN, Bryant AE, Stevens DL. 2017. Fidaxomicin reduces early toxin A and B production and sporulation in *Clostridium difficile* in vitro. *J. Med. Microbiol.* 66:1393–99
7. Alves Feliciano C, Douche T, Giai Gianetto Q, Matondo M, Martin-Verstraete I, Dupuy B. 2019. CotL, a new morphogenetic spore coat protein of *Clostridium difficile*. *Environ. Microbiol*. 21:984–1003
8. Antunes A, Camiade E, Monot M, Courtois E, Barbut F, et al. 2012. Global transcriptional control by glucose and carbon regulator CcpA in *Clostridium difficile*. *Nucleic Acids Res*. 40:10701–18
9. Antunes W, Pereira FC, Feliciano CA, Saujet L, dos Vultos T, et al. 2018. Structure and assembly of a *Clostridioides difficile* spore polar appendage. bioRXiv 468637
10. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, et al. 2011. Induction of colonic regulatory T cells by indigenous *Clostridium* species. *Science* 331:337–41
11. Babakhani F, Bouillaut L, Gomez A, Sears P, Nguyen L, Sonenshein AL. 2012. Fidaxomicin inhibits spore production in *Clostridium difficile*. *Clin. Infect. Dis*. 55(Suppl. 2):S162–69
12. Bhattacharjee D, McAllister KN, Sorg JA. 2016. Germinants and their receptors in clostridia. *J. Bacteriol.* 198(20):2767–75
13. Bhattacharjee D, Sorg JA. 2018. Conservation of the "outside-in" germination pathway in *Paraclostridium bifermentans*. *Front. Microbiol*. 9:2487
14. Calderon-Romero P, Castro-Cordova P, Reyes-Ramirez R, Milano-Cespedes M, Guerrero-Araya E, et al. 2018. *Clostridium difficile* exosporium cysteine-rich proteins are essential for the morphogenesis of the exosporium layer, spore resistance, and affect *C. difficile* pathogenesis. *PLOS Pathog*. 14:e1007199
15. Camp AH, Losick R. 2009. A feeding tube model for activation of a cell-specific transcription factor during sporulation in *Bacillus subtilis*. *Genes Dev*. 23:1014–24
16. Carlson PE Jr., Walk ST, Bourgis AE, Liu MW, Kopliku F, et al. 2013. The relationship between phenotype, ribotype, and clinical disease in human *Clostridium difficile* isolates. *Anaerobe* 24:109–16

17. Cent. Dis. Control Prev. 2019. *Antibiotic resistance threats in the United States*. AR Threats Rep., Cent. Dis. Control Prev., Atlanta, GA. **https://www.cdc.gov/drugresistance/biggest-threats.html**
18. Chastanet A, Losick R. 2007. Engulfment during sporulation in *Bacillus subtilis* is governed by a multiprotein complex containing tandemly acting autolysins. *Mol. Microbiol.* 64:139–52
19. Chilton CH, Crowther GS, Ashwin H, Longshaw CM, Wilcox MH. 2016. Association of fidaxomicin with *C. difficile* spores: effects of persistence on subsequent spore recovery, outgrowth and toxin production. *PLOS ONE* 11:e0161200
20. Chilton CH, Pickering DS, Freeman J. 2018. Microbiologic factors affecting *Clostridium difficile* recurrence. *Clin. Microbiol. Infect.* 24:476–82
21. Cokol M, Kuru N, Bicak E, Larkins-Ford J, Aldridge BB. 2017. Efficient measurement and factorization of high-order drug interactions in *Mycobacterium tuberculosis*. *Sci. Adv.* 3:e1701881
22. Collery MM, Kuehne SA, McBride SM, Kelly ML, Monot M, et al. 2017. What's a SNP between friends: the influence of single nucleotide polymorphisms on virulence and phenotypes of *Clostridium difficile* strain 630 and derivatives. *Virulence* 8:767–81
23. Coullon H, Rifflet A, Wheeler R, Janoir C, Boneca IG, Candela T. 2018. *N*-Deacetylases required for muramic-δ-lactam production are involved in *Clostridium difficile* sporulation, germination, and heat resistance. *J. Biol. Chem.* 293:18040–54
24. Daniel RA, Drake S, Buchanan CE, Scholle R, Errington J. 1994. The *Bacillus subtilis spoVD* gene encodes a mother-cell-specific penicillin-binding protein required for spore morphogenesis. *J. Mol. Biol.* 235:209–20
25. Deakin LJ, Clare S, Fagan RP, Dawson LF, Pickard DJ, et al. 2012. The *Clostridium difficile spo0A* gene is a persistence and transmission factor. *Infect. Immun.* 80:2704–11
26. DeFilipp Z, Bloom PP, Torres Soto M, Mansour MK, Sater MRA, et al. 2019. Drug-resistant *E. coli* bacteremia transmitted by fecal microbiota transplant. *N. Engl. J. Med.* 381:2043–50
27. Dembek M, Barquist L, Boinett CJ, Cain AK, Mayho M, et al. 2015. High-throughput analysis of gene essentiality and sporulation in *Clostridium difficile*. *mBio* 6:e02383
28. Dembek M, Kelly A, Barwinska-Sendra A, Tarrant E, Stanley WA, et al. 2018. Peptidoglycan degradation machinery in *Clostridium difficile* forespore engulfment. *Mol. Microbiol.* 110:390–410
29. Dembek M, Stabler RA, Witney AA, Wren BW, Fairweather NF. 2013. Transcriptional analysis of temporal gene expression in germinating *Clostridium difficile* 630 endospores. *PLOS ONE* 8:e64011
30. Diaz AR, Core LJ, Jiang M, Morelli M, Chiang CH, et al. 2012. *Bacillus subtilis* RapA phosphatase domain interaction with its substrate, phosphorylated Spo0F, and its inhibitor, the PhrA peptide. *J. Bacteriol.* 194:1378–88
31. Diaz OR, Sayer CV, Popham DL, Shen A. 2018. *Clostridium difficile* lipoprotein GerS is required for cortex modification and thus spore germination. *mSphere* 3:e00205-18
32. Doan T, Morlot C, Meisner J, Serrano M, Henriques A, et al. 2009. Novel secretion apparatus maintains spore integrity and developmental gene expression in *Bacillus subtilis*. *PLOS Genet.* 5:e1000566
33. Donnelly ML, Fimlaid KA, Shen A. 2016. Characterization of *Clostridium difficile* spores lacking either SpoVAC or dipicolinic acid synthetase. *J. Bacteriol.* 198:1694–707
34. Donnelly ML, Li W, Li YQ, Hinkel L, Setlow P, Shen A. 2017. A *Clostridium difficile*-specific, gel-forming protein required for optimal spore germination. *mBio* 8:e02085-16
35. Driks A, Eichenberger P. 2016. The spore coat. *Microbiol. Spectr.* 4(2). **https://doi.org/10.1128/microbiolspec.TBS-0023-2016**
36. Edwards AN, Krall EG, McBride SM. 2020. Strain-dependent RstA regulation of *Clostridioides difficile* toxin production and sporulation. *J. Bacteriol.* 202(2):e00586-19
37. Edwards AN, McBride SM. 2014. Initiation of sporulation in *Clostridium difficile*: a twist on the classic model. *FEMS Microbiol. Lett.* 358:110–18
38. Edwards AN, Tamayo R, McBride SM. 2016. A novel regulator controls *Clostridium difficile* sporulation, motility and toxin production. *Mol. Microbiol.* 100:954–71
39. Escobar-Cortes K, Barra-Carrasco J, Paredes-Sabja D. 2013. Proteases and sonication specifically remove the exosporium layer of spores of *Clostridium difficile* strain 630. *J. Microbiol. Methods* 93:25–31
40. Fay A, Meyer P, Dworkin J. 2010. Interactions between late-acting proteins required for peptidoglycan synthesis during sporulation. *J. Mol. Biol.* 399:547–61

41. Fimlaid KA, Bond JP, Schutz KC, Putnam EE, Leung JM, et al. 2013. Global analysis of the sporulation pathway of *Clostridium difficile*. *PLOS Genet*. 9:e1003660
42. Fimlaid KA, Jensen O, Donnelly ML, Francis MB, Sorg JA, Shen A. 2015. Identification of a novel lipoprotein regulator of *Clostridium difficile* spore germination. *PLOS Pathog*. 11(10):e1005239
43. Fimlaid KA, Jensen O, Donnelly ML, Siegrist MS, Shen A. 2015. Regulation of *Clostridium difficile* spore formation by the SpoIIQ and SpoIIIA proteins. *PLOS Genet*. 11:e1005562
44. Fimlaid KA, Shen A. 2015. Diverse mechanisms regulate sporulation sigma factor activity in the Firmicutes. *Curr. Opin. Microbiol.* 24:88–95
45. Francis MB, Allen CA, Shrestha R, Sorg JA. 2013. Bile acid recognition by the *Clostridium difficile* germinant receptor, CspC, is important for establishing infection. *PLOS Pathog*. 9:e1003356
46. Francis MB, Allen CA, Sorg JA. 2015. Spore cortex hydrolysis precedes DPA release during *Clostridium difficile* spore germination. *J. Bacteriol.* 197(14):2276–83
47. Francis MB, Sorg JA. 2016. Dipicolinic acid release by germinating *Clostridium difficile* spores occurs through a mechanosensing mechanism. *mSphere* 1:e00306-16
48. Fukushima T, Yamamoto H, Atrih A, Foster SJ, Sekiguchi J. 2002. A polysaccharide deacetylase gene (*pdaA*) is required for germination and for production of muramic δ-lactam residues in the spore cortex of *Bacillus subtilis*. *J. Bacteriol*. 184:6007–15
49. Galperin MY, Mekhedov SL, Puigbo P, Smirnov S, Wolf YI, Rigden DJ. 2012. Genomic determinants of sporulation in *Bacilli* and *Clostridia*: towards the minimal set of sporulation-specific genes. *Environ. Microbiol.* 14:2870–90
50. Ghose C, Eugenis I, Edwards AN, Sun X, McBride SM, Ho DD. 2016. Immunogenicity and protective efficacy of *Clostridium difficile* spore proteins. *Anaerobe* 37:85–95
51. Giel JL, Sorg JA, Sonenshein AL, Zhu J. 2010. Metabolism of bile salts in mice influences spore germination in *Clostridium difficile*. *PLOS ONE* 5:e8740
52. Gilmore ME, Bandyopadhyay D, Dean AM, Linnstaedt SD, Popham DL. 2004. Production of muramic δ-lactam in *Bacillus subtilis* spore peptidoglycan. *J. Bacteriol.* 186:80–89
53. Girinathan BP, Monot M, Boyle D, McAllister KN, Sorg JA, et al. 2017. Effect of tcdR mutation on sporulation in the epidemic *Clostridium difficile* strain R20291. *mSphere* 2(1):e00383-16
54. Girinathan BP, Ou J, Dupuy B, Govind R. 2018. Pleiotropic roles of *Clostridium difficile sin* locus. *PLOS Pathog*. 14:e1006940
55. Gomez S, Chaves F, Orellana MA. 2017. Clinical, epidemiological and microbiological characteristics of relapse and re-infection in *Clostridium difficile* infection. *Anaerobe* 48:147–51
56. Henriques AO, Moran CP Jr. 2007. Structure, assembly, and function of the spore surface layers. *Annu. Rev. Microbiol.* 61:555–88
57. Hong HA, Ferreira WT, Hosseini S, Anwar S, Hitri K, et al. 2017. The spore coat protein CotE facilitates host colonisation by *Clostridium difficile*. *J. Infect. Dis*. 216(11):1452–59
58. Howerton A, Patra M, Abel-Santos E. 2013. A new strategy for the prevention of *Clostridium difficile* infection. *J. Infect. Dis.* 207:1498–504
59. Howerton A, Seymour CO, Murugapiran SK, Liao Z, Phan JR, et al. 2018. Effect of the synthetic bile salt analog CamSA on the hamster model of *Clostridium difficile* infection. *Antimicrob. Agents Chemother.* 62:e02251-17
60. Ikeda M, Sato T, Wachi M, Jung HK, Ishino F, et al. 1989. Structural similarity among *Escherichia coli* FtsW and RodA proteins and *Bacillus subtilis* SpoVE protein, which function in cell division, cell elongation, and spore formation, respectively. *J. Bacteriol.* 171:6375–78
61. Janezic S, Mlakar S, Rupnik M. 2018. Dissemination of *Clostridium difficile* spores between environment and households: dog paws and shoes. *Zoonoses Public Health* 65(6):669–74
62. Jones AM, Kuijper EJ, Wilcox MH. 2013. *Clostridium difficile*: a European perspective. *J. Infect.* 66:115–28
63. Kelly A, Salgado PS. 2019. The engulfasome in *C. difficile*: variations on protein machineries. *Anaerobe* 60:102091
64. Kevorkian Y, Shen A. 2017. Revisiting the role of Csp family proteins in regulating *Clostridium difficile* spore germination. *J. Bacteriol.* 199(22):e00266-17
65. Kevorkian Y, Shirley DJ, Shen A. 2016. Regulation of *Clostridium difficile* spore germination by the CspA pseudoprotease domain. *Biochimie* 122:243–54

66. Kochan TJ, Foley MH, Shoshiev MS, Somers MJ, Carlson PE, Hanna PC. 2018. Updates to *Clostridium difficile* spore germination. *J. Bacteriol.* 200(16):e00218-18
67. Kochan TJ, Shoshiev MS, Hastie JL, Somers MJ, Plotnick YM, et al. 2018. Germinant synergy facilitates *Clostridium difficile* spore germination under physiological conditions. *mSphere* 3(5):e00335-18
68. Kochan TJ, Somers MJ, Kaiser AM, Shoshiev MS, Hagan AK, et al. 2017. Intestinal calcium and bile salts facilitate germination of *Clostridium difficile* spores. *PLOS Pathog.* 13:e1006443
69. Koenigsknecht MJ, Theriot CM, Bergin IL, Schumacher CA, Schloss PD, Young VB. 2015. Dynamics and establishment of *Clostridium difficile* infection in the murine gastrointestinal tract. *Infect. Immun.* 83:934–41
70. Kumar N, Browne HP, Viciani E, Forster SC, Clare S, et al. 2019. Adaptation of host transmission cycle during *Clostridium difficile* speciation. *Nat. Genet.* 51:1315–20
71. Lawley TD, Clare S, Deakin LJ, Goulding D, Yen JL, et al. 2010. Use of purified *Clostridium difficile* spores to facilitate evaluation of health care disinfection regimens. *Appl. Environ. Microbiol.* 76:6895–900
72. Lewis BB, Pamer EG. 2017. Microbiota-based therapies for *Clostridium difficile* and antibiotic-resistant enteric infections. *Annu. Rev. Microbiol.* 71:157–78
73. Malyshev D, Baillie L. 2019. Surface morphology differences in *Clostridium difficile* spores, based on different strains and methods of purification. *Anaerobe* 61:102078
74. McBride SM, Sonenshein AL. 2011. The *dlt* operon confers resistance to cationic antimicrobial peptides in *Clostridium difficile*. *Microbiology* 157:1457–65
75. McDonald LC, Gerding DN, Johnson S, Bakken JS, Carroll KC, et al. 2018. Clinical practice guidelines for *Clostridium difficile* infection in adults and children: 2017 update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA). *Clin. Infect. Dis.* 66:987–94
76. Meeske AJ, Riley EP, Robins WP, Uehara T, Mekalanos JJ, et al. 2016. SEDS proteins are a widespread family of bacterial cell wall polymerases. *Nature* 537:634–38
77. Meeske AJ, Sham LT, Kimsey H, Koo BM, Gross CA, et al. 2015. MurJ and a novel lipid II flippase are required for cell wall biogenesis in *Bacillus subtilis*. *PNAS* 112:6437–42
78. Merrigan M, Venugopal A, Mallozzi M, Roxas B, Viswanathan VK, et al. 2010. Human hypervirulent *Clostridium difficile* strains exhibit increased sporulation as well as robust toxin production. *J. Bacteriol.* 192:4904–11
79. Miyata S, Kozuka S, Yasuda Y, Chen Y, Moriyama R, et al. 1997. Localization of germination-specific spore-lytic enzymes in *Clostridium perfringens* S40 spores detected by immunoelectron microscopy. *FEMS Microbiol. Lett.* 152:243–47
80. Moore P, Kyne L, Martin A, Solomon K. 2013. Germination efficiency of clinical *Clostridium difficile* spores and correlation with ribotype, disease severity and therapy failure. *J. Med. Microbiol.* 62:1405–13
81. Murphy JM, Mace PD, Eyers PA. 2017. Live and let die: insights into pseudoenzyme mechanisms from structure. *Curr. Opin. Struct. Biol.* 47:95–104
82. Nawrocki KL, Edwards AN, Daou N, Bouillaut L, McBride SM. 2016. CodY-dependent regulation of sporulation in *Clostridium difficile*. *J. Bacteriol.* 198:2113–30
83. Nerandzic MM, Donskey CJ. 2010. Triggering germination represents a novel strategy to enhance killing of *Clostridium difficile* spores. *PLOS ONE* 5:e12285
84. Ng YK, Ehsaan M, Philip S, Collery MM, Janoir C, et al. 2013. Expanding the repertoire of gene tools for precise manipulation of the *Clostridium difficile* genome: allelic exchange using *pyrE* alleles. *PLOS ONE* 8:e56051
85. Oka K, Osaki T, Hanawa T, Kurata S, Okazaki M, et al. 2012. Molecular and microbiological characterization of *Clostridium difficile* isolates from single, relapse, and reinfection cases. *J. Clin. Microbiol.* 50:915–21
86. Oliveira PH, Ribis JW, Garrett EM, Trzilova D, Kim A, et al. 2020. Epigenomic characterization of *Clostridioides difficile* finds a conserved DNA methyltransferase that mediates sporulation and pathogenesis. *Nat. Microbiol.* 5:166–80
87. Orsburn B, Melville SB, Popham DL. 2008. Factors contributing to heat resistance of *Clostridium perfringens* endospores. *Appl. Environ. Microbiol.* 74:3328–35

88. Ottmann C, Rose R, Huttenlocher F, Cedzich A, Hauske P, et al. 2009. Structural basis for Ca^{2+}-independence and activation by homodimerization of tomato subtilase 3. *PNAS* 106:17223–28
89. Paredes-Sabja D, Setlow P, Sarker M. 2011. Germination of spores of *Bacillales* and *Clostridiales* species: mechanisms and proteins involved. *Trends Microbiol.* 19:85–94
90. Peluso EA, Updegrove TB, Chen J, Shroff H, Ramamurthi KS. 2019. A 2-dimensional ratchet model describes assembly initiation of a specialized bacterial cell surface. *PNAS* 116:21789–99
91. Perchat S, Talagas A, Poncet S, Lazar N, Li de la Sierra-Gallay I, et al. 2016. How quorum sensing connects sporulation to necrotrophism in *Bacillus thuringiensis*. *PLOS Pathog.* 12:e1005779
92. Pereira FC, Saujet L, Tome AR, Serrano M, Monot M, et al. 2013. The spore differentiation pathway in the enteric pathogen *Clostridium difficile*. *PLOS Genet.* 9:e1003782
93. Permpoonpattana P, Phetcharaburanin J, Mikelsone A, Dembek M, Tan S, et al. 2013. Functional characterization of *Clostridium difficile* spore coat proteins. *J. Bacteriol.* 195:1492–503
94. Phetcharaburanin J, Hong HA, Colenutt C, Bianconi I, Sempere L, et al. 2014. The spore-associated protein BclA1 affects the susceptibility of animals to colonization and infection by *Clostridium difficile*. *Mol. Microbiol.* 92:1025–38
95. Pishdadian K, Fimlaid KA, Shen A. 2015. SpoIIID-mediated regulation of σ^K function during *Clostridium difficile* sporulation. *Mol. Microbiol.* 95:189–208
96. Pizarro-Guajardo M, Calderon-Romero P, Castro-Cordova P, Mora-Uribe P, Paredes-Sabja D. 2016. Ultrastructural variability of the exosporium layer of *Clostridium difficile* spores. *Appl. Environ. Microbiol.* 82:2202–9
97. Pizarro-Guajardo M, Olguin-Araneda V, Barra-Carrasco J, Brito-Silva C, Sarker MR, Paredes-Sabja D. 2014. Characterization of the collagen-like exosporium protein, BclA1, of *Clostridium difficile* spores. *Anaerobe* 25:18–30
98. Popham DL, Bernhards CB. 2015. Spore peptidoglycan. *Microbiol. Spectr.* 3(6). **https://doi.org/10.1128/microbiolspec.TBS-0005-2012**
99. Popham DL, Helin J, Costello CE, Setlow P. 1996. Muramic lactam in peptidoglycan of *Bacillus subtilis* spores is required for spore outgrowth but not for spore dehydration or heat resistance. *PNAS* 93:15405–10
100. Putnam EE, Nock AM, Lawley TD, Shen A. 2013. SpoIVA and SipL are *Clostridium difficile* spore morphogenetic proteins. *J. Bacteriol.* 195:1214–25
101. Rabi R, Larcombe S, Mathias R, McGowan S, Awad M, Lyras D. 2018. *Clostridium sordellii* outer spore proteins maintain spore structural integrity and promote bacterial clearance from the gastrointestinal tract. *PLOS Pathog.* 14:e1007004
102. Rabi R, Turnbull L, Whitchurch CB, Awad M, Lyras D. 2017. Structural characterization of *Clostridium sordellii* spores of diverse human, animal, and environmental origin and comparison to *Clostridium difficile* spores. *mSphere* 2(5):e00343-17
103. Ramamurthi KS, Losick R. 2008. ATP-driven self-assembly of a morphogenetic protein in *Bacillus subtilis*. *Mol. Cell* 31:406–14
104. Ramirez-Guadiana FH, Meeske AJ, Rodrigues CDA, Barajas-Ornelas RDC, Kruse AC, Rudner DZ. 2017. A two-step transport pathway allows the mother cell to nurture the developing spore in *Bacillus subtilis*. *PLOS Genet.* 13:e1007015
105. Regan G, Itaya M, Piggot PJ. 2012. Coupling of σ^G activation to completion of engulfment during sporulation of *Bacillus subtilis* survives large perturbations to DNA translocation and replication. *J. Bacteriol.* 194:6264–71
106. Ribis JW, Fimlaid KA, Shen A. 2018. Differential requirements for conserved peptidoglycan hydrolases during *Clostridium difficile* spore formation. *Mol. Microbiol.* 110:370–89
107. Ribis JW, Ravichandran P, Putnam EE, Pishdadian K, Shen A. 2017. The conserved spore coat protein SpoVM is largely dispensable in *Clostridium difficile* spore formation. *mSphere* 2(5):e00315-17
108. Ridlon JM, Kang DJ, Hylemon PB, Bajaj JS. 2014. Bile acids and the gut microbiome. *Curr. Opin. Gastroenterol.* 30:332–38
109. Rohlfing AE, Eckenroth BE, Forster ER, Kevorkian Y, Donnelly ML, et al. 2019. The CspC pseudoprotease regulates germination of *Clostridioides difficile* spores in response to multiple environmental signals. *PLOS Genet.* 15:e1008224

110. Saujet L, Pereira FC, Henriques AO, Martin-Verstraete I. 2014. The regulatory network controlling spore formation in *Clostridium difficile*. *FEMS Microbiol. Lett*. 358:1–10
111. Saujet L, Pereira FC, Serrano M, Soutourina O, Monot M, et al. 2013. Genome-wide analysis of cell type-specific gene transcription during spore formation in *Clostridium difficile*. *PLOS Genet*. 9:e1003756
112. Sebaihia M, Wren B, Mullany P, Fairweather N, Minton N, et al. 2006. The multidrug-resistant human pathogen *Clostridium difficile* has a highly mobile, mosaic genome. *Nat. Genet.* 38:779–86
113. Serrano M, Crawshaw AD, Dembek M, Monteiro JM, Pereira FC, et al. 2016. The SpoIIQ-SpoIIIAH complex of *Clostridium difficile* controls forespore engulfment and late stages of gene expression and spore morphogenesis. *Mol. Microbiol.* 100:204–28
114. Serrano M, Kint N, Pereira FC, Saujet L, Boudry P, et al. 2016. A recombination directionality factor controls the cell type-specific activation of σ^K and the fidelity of spore development in *Clostridium difficile*. *PLOS Genet*. 12:e1006312
115. Setlow P. 2014. Germination of spores of *Bacillus* species: what we know and do not know. *J. Bacteriol.* 196:1297–305
116. Setlow P, Wang S, Li YQ. 2017. Germination of spores of the orders *Bacillales* and *Clostridiales*. *Annu. Rev. Microbiol*. 71:459–77
117. Sharma SK, Yip C, Esposito EX, Sharma PV, Simon MP, et al. 2018. The design, synthesis, and characterizations of spore germination inhibitors effective against an epidemic strain of *Clostridium difficile*. *J. Med. Chem*. 61:6759–78
118. Shen A. 2015. A gut odyssey: the impact of the microbiota on *Clostridium difficile* spore formation and germination. *PLOS Pathog*. 11:e1005157
119. Shen A, Edwards AN, Sarker MR, Paredes-Sabja D. 2019. Sporulation and germination in clostridial pathogens. *Microbiol. Spectr.* 7(6). **https://doi.org/10.1128/microbiolspec.GPP3-0017-2018**
120. Shimamoto S, Moriyama R, Sugimoto K, Miyata S, Makino S. 2001. Partial characterization of an enzyme fraction with protease activity which converts the spore peptidoglycan hydrolase (SleC) precursor to an active enzyme during germination of *Clostridium perfringens* S40 spores and analysis of a gene cluster involved in the activity. *J. Bacteriol.* 183:3742–51
121. Shinde U, Thomas G. 2011. Insights from bacterial subtilases into the mechanisms of intramolecular chaperone-mediated activation of furin. *Methods Mol. Biol.* 768:59–106
122. Shrestha R, Cochran AM, Sorg JA. 2019. The requirement for co-germinants during *Clostridium difficile* spore germination is influenced by mutations in *yabG* and *cspA*. *PLOS Pathog*. 15:e1007681
123. Shrestha R, Lockless SW, Sorg JA. 2017. A *Clostridium difficile* alanine racemase affects spore germination and accommodates serine as a substrate. *J. Biol. Chem.* 292:10735–42
124. Shrestha R, Sorg JA. 2017. Hierarchical recognition of amino acid co-germinants during *Clostridioides difficile* spore germination. *Anaerobe* 49:41–47
125. Shrestha R, Sorg JA. 2019. Terbium chloride influences *Clostridium difficile* spore germination. *Anaerobe* 58:80–88
126. Sinai L, Rosenberg A, Smith Y, Segev E, Ben-Yehuda S. 2015. The molecular timeline of a reviving bacterial spore. *Mol. Cell* 57:695–707
127. Smith K, Bayer ME, Youngman P. 1993. Physical and functional characterization of the *Bacillus subtilis spoIIM* gene. *J. Bacteriol.* 175:3607–17
128. Sorg JA, Sonenshein AL. 2008. Bile salts and glycine as cogerminants for *Clostridium difficile* spores. *J. Bacteriol.* 190:2505–12
129. Sorg JA, Sonenshein AL. 2009. Chenodeoxycholate is an inhibitor of *Clostridium difficile* spore germination. *J. Bacteriol.* 191:1115–17
130. Sorg JA, Sonenshein AL. 2010. Inhibiting the initiation of *Clostridium difficile* spore germination using analogs of chenodeoxycholic acid, a bile acid. *J. Bacteriol.* 192:4983–90
131. Srikhanta YN, Hutton ML, Awad MM, Drinkwater N, Singleton J, et al. 2019. Cephamycins inhibit pathogen sporulation and effectively treat recurrent *Clostridioides difficile* infection. *Nat. Microbiol.* 4:2237–45
132. Stewart GC. 2015. The exosporium layer of bacterial spores: a connection to the environment and the infected host. *Microbiol. Mol. Biol. Rev.* 79:437–57

133. Stoltz KL, Erickson R, Staley C, Weingarden AR, Romens E, et al. 2017. Synthesis and biological evaluation of bile acid analogues inhibitory to *Clostridium difficile* spore germination. *J. Med. Chem.* 60:3451–71
134. Tabak YP, Srinivasan A, Yu KC, Kurtz SG, Gupta V, et al. 2019. Hospital-level high-risk antibiotic use in relation to hospital-associated *Clostridioides difficile* infections: retrospective analysis of 2016–2017 data from US hospitals. *Infect. Control Hosp. Epidemiol.* 40:1229–35
135. Tan IS, Ramamurthi KS. 2014. Spore formation in *Bacillus subtilis*. *Environ. Microbiol. Rep*. 6:212–25
136. Tan IS, Weiss CA, Popham DL, Ramamurthi KS. 2015. A quality-control mechanism removes unfit cells from a population of sporulating bacteria. *Dev. Cell* 34:682–93
137. Thanissery R, Winston JA, Theriot CM. 2017. Inhibition of spore germination, growth, and toxin activity of clinically relevant *C. difficile* strains by gut microbiota derived secondary bile acids. *Anaerobe* 45:86–100
138. Theriot CM, Young VB. 2015. Interactions between the gastrointestinal microbiome and *Clostridium difficile*. *Annu. Rev. Microbiol*. 69:445–61
139. Touchette MH, Benito de la Puebla H, Ravichandran P, Shen A. 2019. SpoIVA-SipL complex formation is essential for *Clostridioides difficile* spore assembly. *J. Bacteriol.* 201(8):e00042-19
140. Underwood S, Guan S, Vijayasubhash V, Baines S, Graham L, et al. 2009. Characterization of the sporulation initiation pathway of *Clostridium difficile* and its role in toxin production. *J. Bacteriol.* 191:7296–305
141. van Nood E, Vrieze A, Nieuwdorp M, Fuentes S, Zoetendal EG, et al. 2013. Duodenal infusion of donor feces for recurrent *Clostridium difficile*. *N. Engl. J. Med*. 368:407–15
142. Wang S, Setlow B, Conlon E, Lyon J, Imamura D, et al. 2006. The forespore line of gene expression in *Bacillus subtilis*. *J. Mol. Biol*. 358:16–37
143. Wang S, Shen A, Setlow P, Li YQ. 2015. Characterization of the dynamic germination of individual *Clostridium difficile* spores using Raman spectroscopy and differential interference contrast microscopy. *J. Bacteriol.* 197(14):2361–73
144. Webb BJ, Brunner A, Lewis J, Ford CD, Lopansri BK. 2019. Repurposing an old drug for a new epidemic: ursodeoxycholic acid to prevent recurrent *Clostridioides difficile* infection. *Clin. Infect. Dis.* 68:498–500
145. Weingarden AR, Chen C, Zhang N, Graiziger CT, Dosa PI, et al. 2016. Ursodeoxycholic acid inhibits *Clostridium difficile* spore germination and vegetative growth, and prevents the recurrence of ileal pouchitis associated with the infection. *J. Clin. Gastroenterol.* 50:624–30
146. Wetzel D, McBride SM. 2019. The impact of pH on *Clostridioides difficile* sporulation and physiology. *Appl. Environ. Microbiol.* 86(4):e02706-19
147. Zhu D, Sorg JA, Sun X. 2018. *Clostridioides difficile* biology: sporulation, germination, and corresponding therapies for *C. difficile* infection. *Front. Cell Infect. Microbiol.* 8:29