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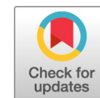
MINIREVIEW



Updates to *Clostridium difficile* Spore Germination

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ABSTRACT Germination of *Clostridium difficile* spores is a crucial early requirement for colonization of the gastrointestinal tract. Likewise, *C. difficile* cannot cause disease pathologies unless its spores germinate into metabolically active, toxin-producing cells. Recent advances in our understanding of *C. difficile* spore germination mechanisms indicate that this process is both complex and unique. This review defines unique aspects of the germination pathways of *C. difficile* and compares them to those of two other well-studied organisms, *Bacillus anthracis* and *Clostridium perfringens*. *C. difficile* germination is unique, as *C. difficile* does not contain any orthologs of the traditional GerA-type germinant receptor complexes and is the only known sporeformer to require bile salts in order to germinate. While recent advances describing *C. difficile* germination mechanisms have been made on several fronts, major gaps in our understanding of *C. difficile* germination signaling remain. This review provides an updated, in-depth summary of advances in understanding of *C. difficile* germination and potential avenues for the development of therapeutics, and discusses the major discrepancies between current models of germination and areas of ongoing investigation.

KEYWORDS *Clostridium difficile*, germination, spores

Clostridium difficile is an anaerobic, Gram-positive, spore-forming bacterium that is the leading cause of nosocomial infectious diarrhea worldwide (1, 2). *C. difficile* infection (CDI), the symptoms of which include severe diarrhea and pseudomembranous colitis, results in half a million cases and ~29,000 deaths in the United States annually (1). Although several factors influencing an individual's susceptibility to infection are known, the primary risk factor for CDI is broad-spectrum antibiotic use that disrupts the composition and function of the normal intestinal microbiota. The resulting ecological changes in the gut reduce a person's intrinsic ability to resist the colonization of several pathogens, including *C. difficile* (3–7). However, it has been reported that preexposure to antibiotics is not a requirement for *C. difficile* spore germination but that germination and outgrowth in mouse ileal contents can be enhanced with antibiotic treatment (8–10).

Clostridium difficile, being an obligate anaerobe, is highly sensitive to oxygen, so the production of aerotolerant spores allows this organism to survive in the external environment until it infects a new host. In general, all bacterial spores are produced during a complex process known as sporulation, most often in response to nutrient deprivation ("starvation"). Spores are metabolically dormant and resistant to numerous environmental stresses, including oxygen, radiation, desiccation, ethanol, extremes of temperature, and low pH (11). Dormant spores can remain viable for hundreds of years (or longer) and return to a metabolically active state, a process known as germination, within minutes upon sensing specific nutrients that signal that the external environment may be favorable for growth (12).

The unique architectural design and structures present within bacterial spores contribute to their dormancy and extreme resistance properties and are, to a large

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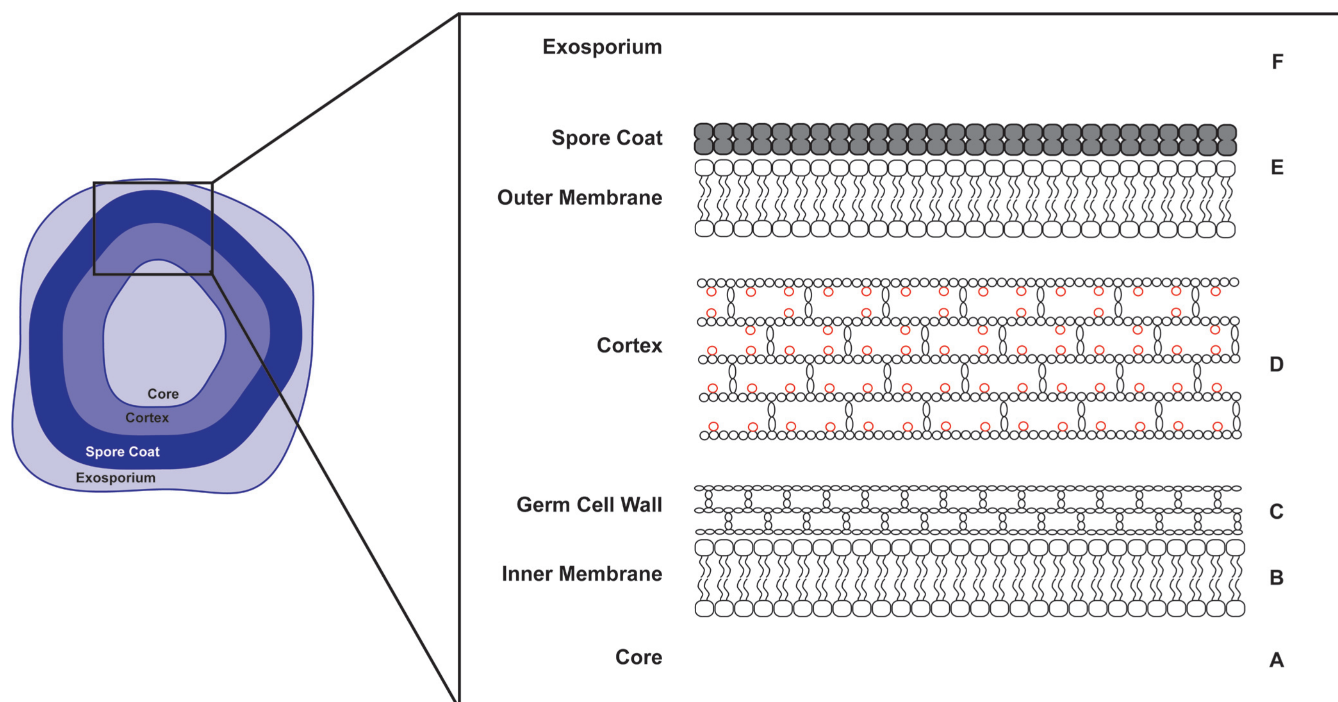

FIG 1 Anatomy of bacterial spores. Bacterial spores are composed of several layers, including a dehydrated spore core (A), an inner membrane (B), a germ cell wall (C), a spore cortex (D), a spore coat/outer membrane (E), and an exosporium (F). The spore cortex is a thick layer of modified peptidoglycan where ~50% of *N*-acetylmuramic acid side chains are removed to produce muramic acid delta-lactam rings, which are depicted in red.

extent, remarkably similar between the various bacterial species. For example, all bacterial spores have a partially dehydrated spore core containing up to 1 M calcium-dipicolinic acid (Ca-DPA), a major contributor to a spore's heat resistance properties (Fig. 1A) (13–15). Within the spore core, dormancy is maintained due to very low core water content. The DNA is supercoiled and bound with small, acid-soluble proteins (SASPs) that are believed to block transcription and protect against DNA damage over long periods of time (16, 17). The spore core is surrounded by the inner membrane, which serves as a permeability barrier against damaging chemicals (18–20) (Fig. 1B). Protecting the inner membrane is the germ cell wall, which will become the cell wall for the emerging *Bacillus* during outgrowth (Fig. 1C) (21). Surrounding this is the spore cortex, a thick layer of modified peptidoglycan where peptide side chains are removed from *N*-acetylmuramic acid and a lactam ring is formed to make muramic acid-delta lactam (Fig. 1D) (21, 22). This modification is essential for specific hydrolysis by cortex lytic enzymes (CLEs), a strategy that ensures that CLEs degrade the cortex and not the germ cell wall during germination. The cortex layers are enclosed by the outer membrane/spore coat region, which contains the enzymes involved in cortex hydrolysis (Fig. 1E) (23–25). The outermost layer is a highly permeable layer of carbohydrates known as the exosporium, which likely plays a major role in host-pathogen interactions and spore persistence during recurrent CDIs (Fig. 1F) (26–28). Collectively, these structures provide resistance to environmental stresses that this pathogen encounters during its unique life cycle (11).

When a patient ingests *C. difficile* spores, they spores are able to endure the acidic stomach and then transition to a metabolically active state (germinate) in the small intestine (29, 30). As *C. difficile* spores germinate, they lose resistance properties, outgrow in vegetative cells that produce toxins, and colonize the large intestine, where the vast majority of all pathologies occur (31–34). Therefore, germination is an essential step that occurs prior to outgrowth, colonization, toxin production, and the development of CDIs (35, 36). In this review, we compare and contrast well-examined *Bacillus* and *Clostridia* germination pathways and discuss the recent advances in understanding

TABLE 1 Germination pathways of *Clostridium difficile* and *Bacillus anthracis*

Species and pathway	Germinant(s)	Receptors
Bacillus anthracis		
Alanine	L-Alanine, >30 mM	GerK, GerL
Alanine-proline	L-Alanine, L-proline	GerK, GerL
Aromatic amino acid enhanced alanine	L-Alanine and L-tyrosine L-histidine, or L-tryptophan	GerS, GerL
Amino acid inosine dependent 1	Inosine and L-alanine, L-valine, L-serine, L-proline, or L-methionine	GerH, GerL, GerK
Amino acid inosine dependent 2	Inosine and L-tyrosine, L-histidine, L-phenylalanine, or L-tryptophan	GerS, GerH
Clostridium difficile		
Bile salt-amino acid	Taurocholate and glycine, L-histidine, L-serine, or L-alanine	CspC, ?
Bile salt-divalent cation	Taurocholate and calcium or magnesium	CspC, ?
Bile salt-alanine racemase-dependent D-amino acid	Taurocholate and D-alanine or D-serine	CspC, ?

of *C. difficile* spore germination, including novel germination proteins, clinical significance of germination, discrepancies between current germination models, and the need for future investigation.

SENSING THE ENVIRONMENT: GERMINANTS AND RECEPTORS

Bacterial spore germination occurs within minutes in response to specific environmental cues, named germinants, that are believed to serve as indicators of conditions favorable for vegetative growth. Nutrient germinants are small molecules, such as sugars, amino acids, ions, and nucleotides, that induce the irreversible reactivation of spores into metabolically active bacilli via interaction with specific germination receptors (37, 38). Typically, germinant receptors are found on the spore inner membrane, and the most extensively studied are the tricistronic GerA family of germination receptors (22, 30, 37, 39–41). The GerA family of receptors is highly conserved among sporeformers, including clostridia; e.g., the *Clostridium perfringens* genome encodes two GerA-type receptors, GerA and GerK (39, 42, 43). The *C. difficile* genome, however, encodes no GerA family ortholog. Instead, *C. difficile* senses the external environment with a unique pseudoprotease known as CspC (44). One model organism of the pathogenic *Bacillus* spp., *B. anthracis*, carries up to seven *gerA*-type loci: *gerA*, *gerH*, *gerK*, *gerL*, *gerS*, *gerX*, and *gerY* (45).

Multiple distinct germination pathways have been identified in both *B. anthracis* and *Clostridium difficile*, each requiring specific germinant-receptor interactions. For example, there are five distinct germination pathways for *B. anthracis* (Table 1) (45, 46). The alanine (Ala) pathway requires extremely high (nonphysiological) concentrations of L-alanine (>30 mM) and the combination of GerK and GerL receptors (Fig. 2) (47, 48). At physiologically relevant concentrations, L-alanine can coordinate with L-proline (AP pathway) or aromatic amino acids such as L-histidine (AEA pathway) (47). In addition, several amino acids can cooperate with the purine ribonucleoside inosine (the most potent cogerminant) to make up the amino acid inosine-dependent pathways (AAID-1 and -2 pathways) (Table 1) (47). For all *B. anthracis* pathways, germinants pass through the outer layers of the spore using dedicated channels that consist of multimeric proteins from the GerP family (GerPABCDEF) (Fig. 2A) (49–51). Receptor complexes on the spore inner membrane then bind to their cognate germinants and initiate the release of monovalent cations and Ca-DPA (Fig. 2B to D) (14, 18, 52, 53). Ca-DPA is released from the spore core and binds to the cortex lytic enzyme CwlJ, activating this enzyme and initiating hydrolysis of the cortex layer. Enzymatic degradation of the cortex is believed to lead to full core rehydration and initiation of outgrowth (Fig. 2E and F) (54).

Unlike *B. anthracis*, which germinates when exposed to the nutrient germinants associated with phagocytes, *C. difficile* germinates in the gut in response to a combi-

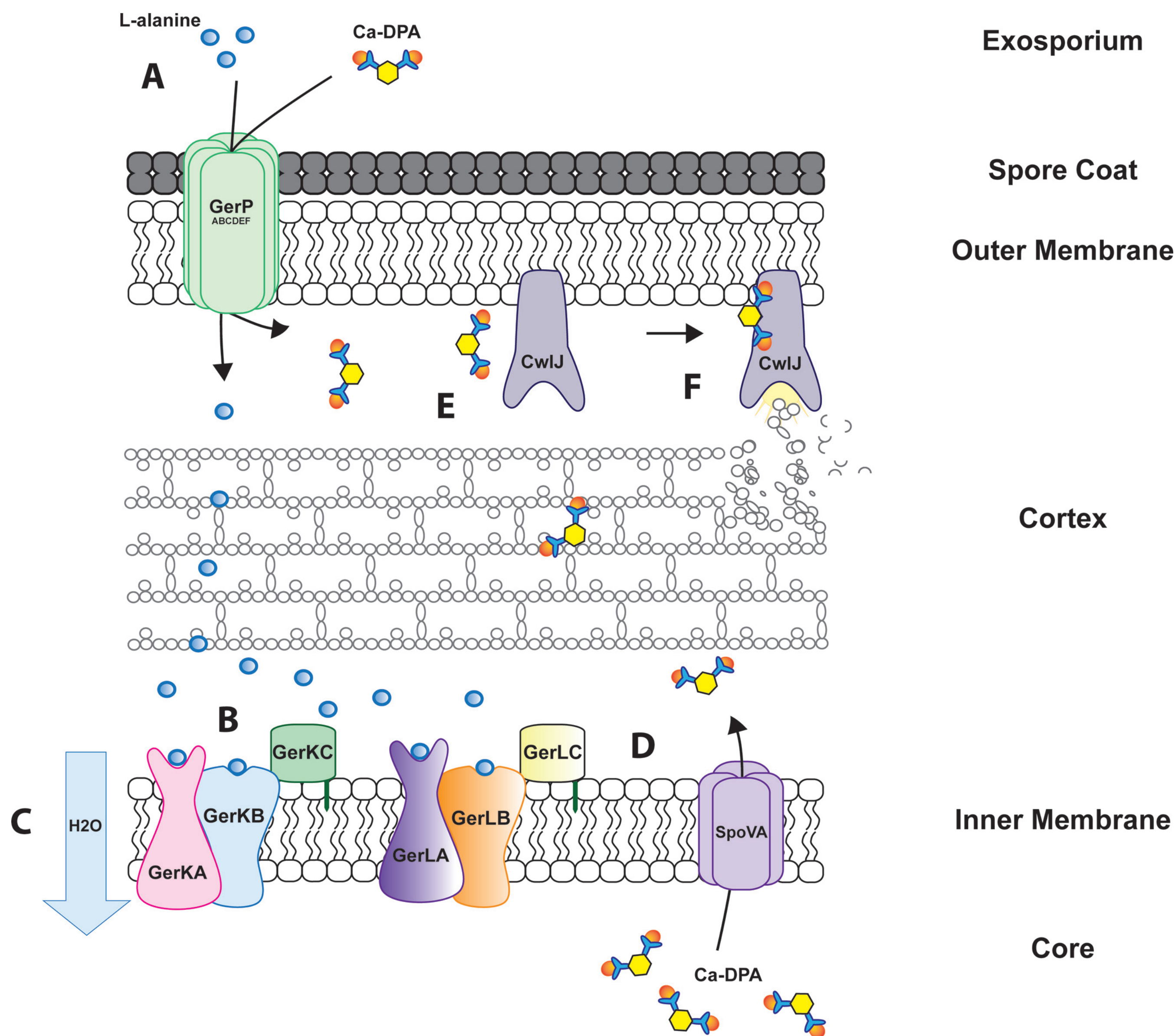

FIG 2 *Bacillus anthracis* L-alanine germination model. Nutrients enter the spore through $\mathrm{GerP_{ABCDEF}}$ complex (A), which facilitates movement of L-alanine through the spore coat and outer membrane to the spore inner membrane, where it binds to GerK and/or GerL germination receptors (B). These germinant-receptor interactions lead to slight core rehydration (C) and release of Ca-DPA from the spore core (D). Ca-DPA travels through the cortex (or can be added exogenously) and binds to CwlJ (E). This binding activates CwlJ, initiating hydrolysis of the cortex peptidoglycan (F). This leads to full core rehydration and spore outgrowth.

nation of nutrients and bile salts present in the gastrointestinal tract, specifically, the bile salt cholate (55–60). Most derivatives of cholate can trigger spore germination; however, taurocholate (Tc) is the most effective germinant *in vitro* (61–63). Bile salts are detected directly by a unique, noncanonical receptor, CspC, which is located in the spore coat/outer membrane (44). These bile salts are not sufficient for efficient *C. difficile* germination; a cogerminant, such as amino acids or divalent cations, is essential for the initiation of germination signaling (64–66). The receptors for cogerminant molecules remain unknown. Glycine is the most efficient amino acid cogerminant for *C. difficile* spores, but it can also be replaced by other amino acids, such as L-alanine, L-histidine, and L-serine (64, 67–69).

C. difficile can respond to a number of amino acids, although always in conjunction with bile salts, in what we describe as the bile salt-amino acid pathway (Table 1). In

addition, *C. difficile* maintains a unique germination pathway referred to here as the alanine racemase-dependent D-amino acid pathway (Table 1). Alanine racemases typically convert L-alanine to D-alanine (and vice versa). In *Bacillus*, D-alanine acts as a competitive inhibitor of L-alanine-dependent germination pathways. While D-alanine does not inhibit germination of *C. difficile* spores, a recent study found that the alanine racemase Alr2 is required for the epimerization of D-alanine and D-serine, which can facilitate germination along with taurocholate (69).

In addition to the amino acid-dependent germination pathways, the divalent cations calcium and magnesium can also function as cogerminants in what we define as the bile salt-divalent cation pathway (Table 1) (66). This pathway does not require any amino acids or other typical nutrient germinants. Instead, sufficient levels of calcium (along with bile salts) circumvent the need for any amino acid cogerminants. Interestingly, there is synergy between the calcium and glycine pathways, where 10-fold-lower concentrations of each can induce efficient spore germination when provided in combination (66). This is likely the most physiologically relevant germination pathway, as bile salts, calcium, and glycine are all present in the host gastrointestinal tract. In fact, a recent study from our group suggests that calcium in the gastrointestinal tract can play a major role in inducing germination *in vivo* (66).

While there are several environment-sensing pathways that can initiate spore germination, each initiates a signaling cascade that leads to activation of SleC, the spore CLE essential for germination. SleC activation initiates degradation of the cortex, resulting in full core rehydration and release of Ca-DPA from the spore core (15, 66, 69–71). The specific binding partners for amino acids or divalent cations during these early stages of germination, and any functional mechanisms linking them to initiation of cortex hydrolysis, remain to be elucidated, and this is an area of active investigation.

INITIATION OF CORTEX HYDROLYSIS: *cspBAC* OPERON

In order to maintain spore dormancy, CLEs remain inactive until they receive a specific signal downstream of germinant-receptor interactions. In *Bacillus*, after sensing that the environment is suitable for bacterial growth, Ca-DPA is released from the spore core, where it serves as a cofactor binding to and activating the CLE CwlJ (Fig. 2D and E) (54). *B. anthracis* maintains four cortex lytic enzymes that contribute to spore germination: CwlJ1, CwlJ2, SleL, and SleB (72). The *C. difficile* genome encodes a SleB/CwlJ-like ortholog, but it does not contribute to germination. Instead, cortex hydrolysis is facilitated by the CLE SleC, which has significant homology to CLEs from *C. perfringens* (73). In *C. perfringens*, activation of SleC occurs when the Csp family of subtilisin-like proteases (CspABC) (Fig. 3A) cleave the prodomain from SleC, initiating cortex hydrolysis (74, 75).

Subtilisin-like proteases contain a highly conserved catalytic triad consisting of a serine-histidine-aspartate motif (Fig. 3A) (76). The *C. difficile* genome carries an operon, *cspBAC*, encoding subtilisin-like proteases that are essential for germination and have high homology to those encoded by *C. perfringens* (Fig. 3B) (70). Expression of the *cspBAC* operon is controlled by SigE, and these proteins are found in the spore coat/outer membrane fraction of mature spores (70, 75, 77–79). The operon carries two genes, *cspBA* and *cspC*. CspBA is expressed as a fusion protein during sporulation but is ultimately processed by the YabG protease into two proteins, CspB and CspA, that are incorporated into mature spores (Fig. 3B) (78, 80). CspB cleaves the prodomain from pro-SleC, initiating cortex hydrolysis (70). While the signal that activates CspB has yet to be elucidated, a recent study showed that calcium is required for CspB-mediated cleavage of SleC (66). Since subtilisin-like proteases are calcium sensitive, one hypothesis is that CspB binds calcium and that this binding is required for CspB activity (66, 81–85). However, another study did not identify any calcium ions associated with the *C. perfringens* CspB (70). Since it is not clear whether calcium interacts directly with CspB, the identity of the specific signal that activates CspB and the proteins that calcium interacts with to facilitate *C. difficile* germination remain unknown.

The other portion of the CspBA fusion protein, CspA, is required for incorporation of

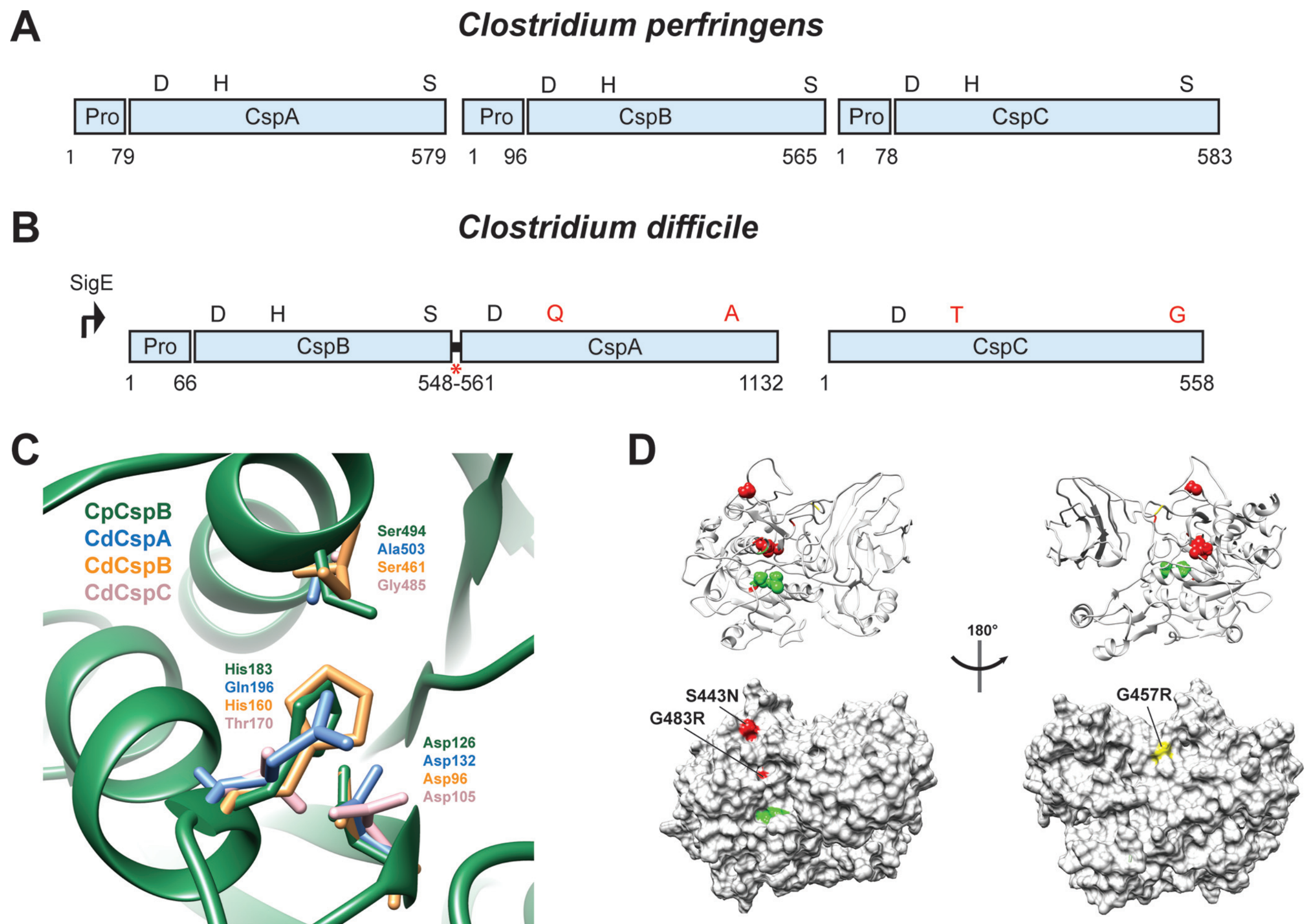

FIG 3 The *cspBAC* operon: structural map of the conserved catalytic triad. (A and B) Schematic of Csp proteases in *C. perfringens* (A) and *C. difficile* (B). Intact catalytic residues are in black, and mutated residues are in red. The asterisk indicates the YabG cleavage site. (C) Superposition of the *C. difficile* Csp mutated catalytic residues (model from I-TASSER structure prediction) with the *C. perfringens* CspB (PDB 4i0W) (70). (D) Map of the mutations reported by Francis et al. on CspC (I-TASSER structure prediction), showing both internal mutations in ribbon diagrams (top) and mutations on the protein surface (bottom) (44). Catalytic triad residues are highlighted in green, mutated residues that result in a loss of germination are highlighted in red, and the G457R mutation is highlighted in yellow.

the germinant receptor, CspC, into the mature spore (78, 80). While CspA is required for *C. difficile* germination, the precise mechanism of action and its interactions with CspC remain to be elucidated. One confounding factor is that both CspA and CspC have mutations in their catalytic triad, rendering them catalytically inactive (44, 78, 80). To provide additional insight on the *C. difficile* Csp catalytic domains, CspBAC structures were predicted using I-TASSER and aligned with the *C. perfringens* CspB crystal structure (Fig. 3) (86). According to these predictions, the catalytic sites of CspBAC are structurally similar to that of *C. perfringens* CspB. However, only CspB has an intact D-H-S motif; CspA has a D-Q-A motif, and CspC has a D-T-G motif (Fig. 3C) (80). These mutations are expected to render the proteases catalytically null, which is likely since these "pseudo" proteases do not autoprocess their prodomains like CspB or other subtilisins (70, 78).

The final protein encoded by the *cspBAC* operon, CspC, is a pseudoprotease that is required for germination (44). CspC first was identified using a chemical mutagenesis screen to identify proteins required for germination (44). While this unbiased approach would be expected to identify several proteins involved in germination, the only reported mutations affecting germination were in *cspB* and *cspC*. Multiple point mutations in CspC that disrupt germination, presumably by destabilizing the protein as described below, were identified. One of these point mutations (G457R) was shown to alter receptor specificity, allowing germination in response to chenodeoxycholate, a

bile salt that typically inhibits *C. difficile* germination (56, 87). However, it is unclear whether chenodeoxycholate induces germination or whether these spores are now able to germinate in the absence of taurocholate. In any case, this observation suggests that CspC is directly involved in bile salt recognition and, therefore, can be characterized as a new class (non-GerA family) of germination receptor(s). In order to understand how these mutations may affect CspC function, we mapped the location of each mutated residue on the predicted protein structure (Fig. 3D) (88). The majority of the point mutations that disrupted germination were found on internal glycine residues that may have affected CspC protein folding, creating a nonfunctional protein and a mutant that behaves like a *cspC* deletion mutant. The G457R point mutation is located on the surface of the protein and could potentially alter a residue that interacts with bile salts. Since CspC has no proteolytic activity, the events that that lead to activation of CspB after bile salts interact with CspC remain unknown.

ROLE OF CALCIUM AND DIPICOLINIC ACID IN GERMINATION

Calcium-dipicolinic acid (Ca-DPA) is a major component of bacterial spores, making up approximately 15% of the dry weight of the spore (89). DPA is synthesized by the mother cell during sporulation, is transported across the outer membrane of the forespore by the nucleoside transporter SpoVV (recently characterized in *Bacillus*), and is then transported across the forespore inner membrane and into the core by SpoVA (13, 15, 90, 91). Inside the spore core, Ca-DPA displaces water, creating a dehydrated core and contributing to the heat resistance of the spore (13, 15, 91). In *Bacillus* spp., Ca-DPA is released after germinant-receptor interactions and acts as a signaling molecule, initiating cortex hydrolysis by binding to and activating CwlJ.

In *C. difficile*, the signaling events that trigger cortex hydrolysis remain unknown. In fact, Ca-DPA release does not occur until after cortex hydrolysis (92). Ca-DPA is eventually released by a mechanosensing mechanism whereby the core swells after cortex hydrolysis and DPA is released to relieve hypo-osmotic stress (71). *C. difficile* spores that are deficient for DPA biosynthesis retain the ability to germinate in rich media; however, recent work from our group has shown that DPA-deficient spores are also deficient for calcium and that germination of these spores in rich media is facilitated by the presence of exogenous calcium (15, 66). This study also showed that calcium packaged within the spore is required for germination through the bile salt-amino acid pathway. Germination of *C. difficile* spores was significantly diminished in the presence of the chelating agent egtazic acid, which has high affinity for calcium (66). Germination was blocked upstream of SleC activation, suggesting that calcium is required for CspB activity. Calcium is also transported from the spore core during taurocholate-glycine-induced germination (66). This has been shown through studies in which calcium from outer layers was depleted and using ion-channel inhibitors that presumably block calcium release. How calcium is released from the spore core independent of DPA is currently unknown. It is possible that this is the result of increased solubility of calcium salts. Given that calcium can be up to 100 times more soluble than DPA, only a slight core rehydration may be necessary to solubilize and release calcium independent of DPA. Determining specifically how calcium affects CspB enzymatic activity and whether calcium transport is an important part of the germination mechanism or an artifact of calcium chelation are subjects of ongoing investigation.

OTHER PROTEINS INVOLVED IN *C. DIFFICILE* SPORE GERMINATION

A few novel sporulation/germination proteins have been identified in the course of studying *C. difficile* germination, including GerG, GerS, and Cd630_32980 (66, 93, 94). The expression of these genes is controlled by the mother cell sigma factor SigE (77). GerS is a novel lipoprotein regulator that is highly expressed during sporulation and localizes to the spore coat/outer membrane fraction of mature spores (94). Although SleC is activated in GerS mutants, these strains are unable to initiate cortex hydrolysis in response to any combination of cogerminants (66, 94). While the exact role of GerS

remains unclear, it is hypothesized that GerS regulates the activity of SleC by playing a role in modification of the cortex, allowing SleC to recognize it and initiate hydrolysis, similar to the role of CwlD cortex modification in *Bacillus* (95, 96).

GerG is a putative gel-forming protein that is found exclusively in *C. difficile* isolates and is required for *C. difficile* germination. GerG mutants generate spores that lack key Csp proteins, likely leading to defects in initiating cortex hydrolysis. It is hypothesized that GerG functions by facilitating transport of Csp proteins across the outer forespore membrane into the cortex region during sporulation (93). Interestingly, the central repeat of GerG that is predicted to be required for gel formation is not required for germination.

Cd630_32980 encodes a AAA+ ATPase that is highly induced during sporulation (77). Cd630_32980 mutants are deficient for both calcium and DPA and, as a result, are nonresponsive to Tc-Gly germination but can germinate in response to Tc-Ca (66). As an ATPase, this protein likely functions during sporulation, since ATP is not required for germination (97). Since spores lacking Cd630_32980 are deficient for DPA, it has been hypothesized that Cd630_32980 is involved in transport of Ca-DPA across the forespore outer membrane.

CLINICAL SIGNIFICANCE OF GERMINATION

Since spores are the infectious form of *C. difficile* and CDIs are mediated by toxin-producing vegetative cells, germination is necessary for the disease process to progress. In fact, many of the germination and sporulation proteins described above are required for *C. difficile* colonization and pathogenesis (44, 70, 73, 93, 94). Assessment of over 100 clinical isolates of *C. difficile* has also indicated a role for tight control of germination in human disease severity (98). Strains that were unable to germinate in response to bile salts alone, but rather required the presence of a cogerminant, were more likely to have caused severe disease in the individuals from whom they were isolated (98).

The importance of germination in the pathogenesis of many spore-forming bacterial species raises the possibility of generating novel therapeutics that target this pathway. The dependence of *C. difficile* germination on specific molecules, including bile salts, glycine, and calcium, has led to speculation that these molecules could be used to alter germination *in vivo*. In this context, many have focused on the idea of blocking germination in order to prevent outgrowth and the production of virulence factors. Indeed, if germination were completely inhibited, *C. difficile* spores would not convert to vegetative bacilli and, therefore, would not be produce toxins or cause disease. Since spores are inherently antibiotic resistant, this strategy could prevent disease in the short term, but since it does not specifically induce clearance of the spores from the body, it could increase the likelihood that the individual would experience a recurrence of CDI. Recurrence is a major problem in CDI, with 20 to 30% of individuals experiencing at least one bout of recurrent disease (99, 100). One recent study showed that this recurrence could be due to either reinfection of a susceptible individual or reactivation of latent spores within the individual's gastrointestinal tract (100). Knowledge of germination pathways and mechanisms could be exploited to activate spores within a host to prevent recurrence. If spores within the host are forced to germinate during antibiotic therapy, these would be killed by the antibiotic and unable to cause subsequent recurrence. Since bile salts are likely to be absorbed rapidly in the intestines, this strategy will likely require use of methods to target specific areas within the gastrointestinal tract. Increasing dietary calcium in individuals undergoing antibiotic therapy could have a similar effect, assuming the presence of sufficient levels of bile salt in the gut.

CONCLUDING REMARKS AND FUTURE PERSPECTIVE

While significant progress has been made in describing the germination mechanisms and identifying novel germination proteins, there are still major gaps that remain to be elucidated. The role of essential cogerminants and the downstream signaling

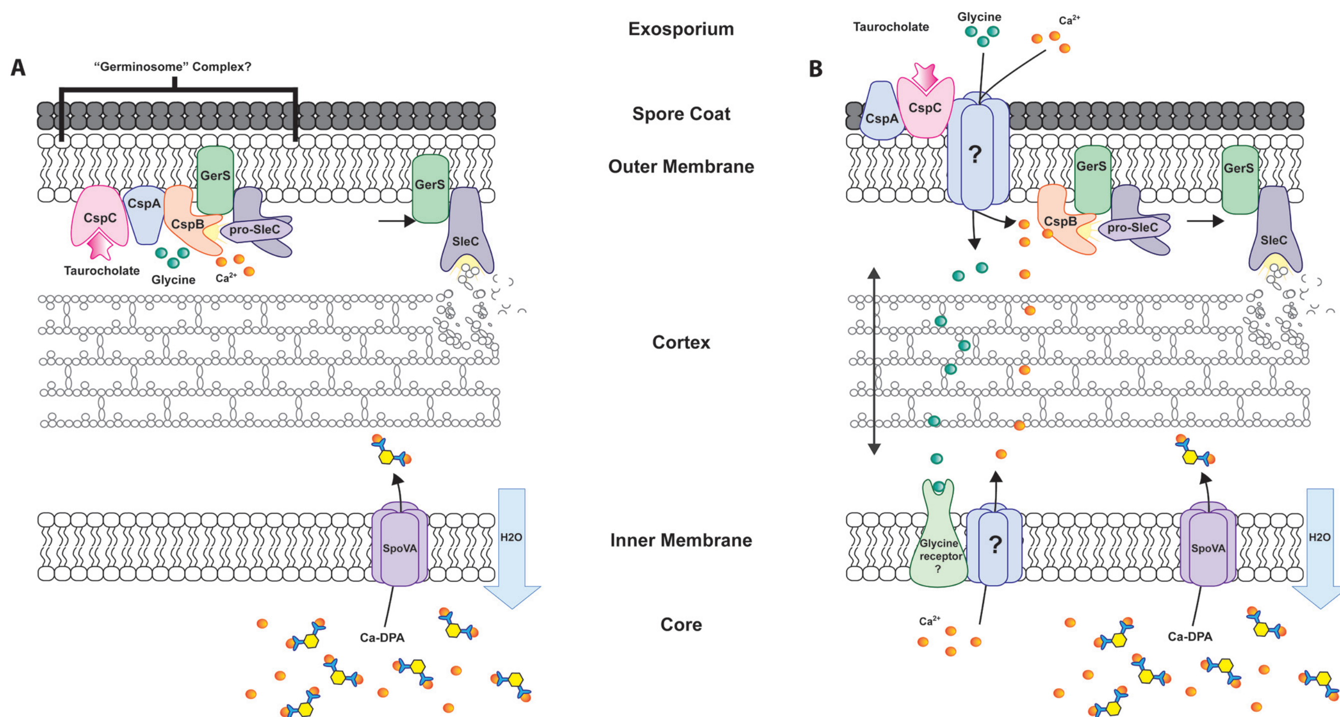

FIG 4 Comparison of proposed models of *Clostridium difficile* germination. (A) "Germinosome" model based on the findings from Francis et al., Adams et al., and Fimlaid et al., where proposed Csp protein-protein interactions lead to activation of SleC (44, 70, 94). (B) "Lock-and-key" model modified from that of Kochan et al., where CspC-taurocholate interactions facilitate cogerminant entry into the spore (66).

events that lead to CspB activation are two major gaps discussed above. There are two hypotheses for the downstream events occurring after CspC binds taurocholate, with some indirect data in support of each.

In the first model, CspC directly activates CspB through some yet-to-be characterized protein-protein interactions (Fig. 4A). CspA is hypothesized to function as a chaperone that helps to maximize CspC incorporation into the mature spore. CspA expression also seems to be controlled, in part, by CspB. Since the activity of each protein encoded by the *cspBAC* operon appears dependent the others, it is possible that they are forming a "germinosome complex," together with the cortex lytic enzyme SleC. In this model, after taurocholate binding to CspC and amino acids or calcium interacting with the germinosome, CspC activates CspB, and it cleaves the pro domain from pro-SleC, activating cortex hydrolysis.

In contrast, the "lock-and-key" model hypothesizes that taurocholate binding to CspC is a required event for facilitating passage of the other cogerminants, a chemical key provided by the host to open a path for glycine and calcium to access areas deeper in the spore (Fig. 4B). If so, CspC may serve a function for *C. difficile* similar to the one that the GerP complex serves for *B. anthracis*, with the addition of the key (GerP does not require bile salts to unlock its channel).

The latter model addresses two major questions: how do germinants gain access into the spore, and what role does calcium play during germination? In *Bacillus* spp., germinant access into the spore is facilitated by the GerP family of proteins (49); however, no orthologs have been identified in *C. difficile* genomes sequenced to date. Therefore, the relative permeability and mechanism by which germinants gain access to the spore's inner layers remain unknown. In this model, CspC is outward facing, and when taurocholate binds, it facilitates access of cogerminants into the spore, where they interact with their putative receptors. These receptors are likely found on the inner membrane, where they facilitate slight hydration of the core and release of Ca^{2+} ions. Calcium from the spore core (or the external environment) then binds to and activates CspB and subsequently SleC and cortex hydrolysis.

This new model raises some important questions that need to be investigated. First, the amino acid and calcium receptors need to be identified. The new "lock-and-key" model suggests that calcium and amino acids interact with different proteins to facilitate germination. It also is possible that calcium improves the effectiveness of a single amino acid germinant receptor. Next, the interactions between bile salts and CspC, and the downstream signaling that occurs, need to be characterized. Several groups have independently verified that CspC is essential for germination, but only a single piece of data exists to suggest that CspC is the taurocholate receptor, i.e., the G457R mutation described in Fig. 3 (44, 66, 70, 80). Finally, the localization of key germination proteins within the spore needs to be more precisely defined. Several groups have shown independently through fractionation and immune electron microscopy (EM) that these proteins reside in the spore coat/outer membrane; however, many of them lack transmembrane domains, and it is unclear how they could be localized to these outer layers while having activity within the cortex layer (75, 94, 101). Future research into *C. difficile* germination should focus on addressing these important gaps in the literature. In addition to advancing the field of spore germination, understanding the specific mechanisms involved in *C. difficile* germination has broader impacts and is essential for the development of novel, well-targeted therapeutics.

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