Trends in Microbiology

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Review

A Revised Understanding of *Clostridioides difficile* Spore Germination

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The dormant resistant spores of *Clostridioides difficile* are transformed into metabolically active cells through the process of germination. Spore germination in *C. difficile* is regulated by the detection of bile salt germinants and amino acid cogerminants by pseudoproteases CspC and CspA, respectively. The germinant signal is transduced to the serine protease CspB, which processes the cortex lytic enzyme SleC, leading to degradation of the spore cortex peptidoglycan and subsequent reactivation of the spore. Divergent *C. difficile* germination models have been proposed to explain interactions between key regulators and transduction of germinant and cogerminant signals. This review summarises advances in understanding *C. difficile* germination and outlines current models of germination regulation.

Highlights

The *cspBAC* locus is a major regulator of *C. difficile* germination. The subtilisin-like pseudoprotease CspC is the likely receptor for bile salt germinants, whereas the related pseudoprotease CspA is thought to detect amino acid cogerminants. Ligand binding to both is thought to be necessary to initiate germination. Catalytically active CspB transduces the germination signal and is essential for completion of germination.

C. difficile spore germination is influenced by calcium: exogenous calcium functions as an alternative cogerminant that coordinates with bile salts, whereas endogenous calcium released from the spore core enhances germination in the presence of sub-inducing concentrations of amino acid cogerminants.

Two *C. difficile* germination regulation models are proposed. In the lock and key model, bile salt receptor activation on the inner membrane unlocks the transport of cogerminants through the outer membrane. In the germinosome complex model, germinant and cogerminant receptors are colocated on the outer membrane, where proximity facilitates transduction of the germinant signal.

Clostridioides difficile Infection (CDI)

Disruption of the normal intestinal microbiota by broad-spectrum antibiotics is the key risk factor for the development of CDI [1,2]. Susceptibility to CDI increases with age, and the majority of cases occur in those aged >65 years [3,4]. Other risk factors include underlying disease, the duration of hospital stay, and the duration of treatment with broad-spectrum antibiotics [3]. Rates of CDI in the UK have declined dramatically since the introduction of a multicomponent care bundle to prevent healthcare-associated infections (HAIs), but CDI remains a global health burden and a leading source of morbidity and mortality; it is reported to be the most common cause of HAIs in US hospitals [5–7]. Symptoms of CDI range from asymptomatic carriage or mild watery diarrhoea to antibiotic-associated colitis and life-threatening pseudomembranous colitis, which is characterised by visible adherent plaques (pseudomembranes) [1,8]. The clinical presentation of CDI results from the mucosal damage, malabsorption, and inflammatory events caused by the production of the *C. difficile* toxins TcdA (toxin A) and TcdB (toxin B), and in some strains the binary toxin [9,10]. Despite antibiotic treatment, recurrence of CDI, defined as an episode occurring within 8 weeks of a previous episode because of relapse or reinfection, occurs in ~25% of cases, although reported rates of recurrence vary considerably (from 4% to >50%) [11–14].

Spore formation by *C. difficile* is crucial for the survival and dissemination of the bacterium in the environment. The dormant aerotolerant and highly resistant spore facilitates efficient transmission and persistence in the host [9,15,16]. Germination of *C. difficile* spores is activated by defined environmental signals that trigger the return of metabolic activity and outgrowth of the vegetative cell [4,17]. Despite the clinical significance of CDI, the signalling pathway that leads to the transition from dormant spore to metabolically active vegetative cell has not yet been fully elucidated. This review summarises recent developments in understanding *C. difficile* germination and the proposed germination signal transduction models.

Clostridioides difficile Spore Structure

Spore formation by *C. difficile* is crucial for survival in an aerobic atmosphere, resistance to extreme environmental conditions, and for the dissemination and persistence of CDI [15,16].

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Sporulation of *C. difficile* is under the control of the master regulator Spo0A and is believed to be triggered by nutrient depletion and quorum sensing [18–20]. The conserved protective bacterial spore structure has features and constituents that are not associated with the vegetative cell and contribute to its resistance and durability (Figure 1) [21,22]. At the centre of the spore is the dehydrated spore core containing DNA, tRNA, ribosomes, and enzymes that are essential for return to metabolic activity during germination [22]. Low water content is a primary factor in spore enzymatic dormancy and resistance to wet heat, making up only 25–55% spore core wet weight, depending on the species [23]. The core is saturated with dipicolinic acid (DPA) chelated 1:1 with the divalent cation Ca^{2+}, and Ca-DPA contributes to the reduced core water content [22,24]. DNA within the spore core is bound to protective small acid-soluble proteins (SASPs) that alter the properties of DNA, are found exclusively in the spore core, and are responsible for spore resistance to desiccation and damage by heat, chemicals, and UV [22,25]. The spore core is surrounded by the inner membrane that acts as a permeability barrier and contributes to spore resistance by preventing entry of DNA-damaging compounds [26]. The inner membrane is further surrounded by a peptidoglycan germ cell wall that becomes the outgrowing cell wall upon germination [16,22]. Next, the large cortex layer is composed of spore-specific modified crosslinked peptidoglycans that are selectively hydrolysed by cortex lytic enzymes during germination [16,27]. The cortex is enclosed by the outer membrane, which in contrast to the surrounding spore coat is not a significant permeability barrier and does not have a role in spore resistance [26]. The proteins of the spore coat protect against attack from enzymes, chemicals, and biocides [25]. Finally, the multilayered protein exosporium, that demonstrates variability in its morphology between and within strains of *C. difficile*, is not present in all spore-forming bacteria but is believed to contribute to *C. difficile* spore resistance: spores lacking the *C. difficile* exosporium cysteine-rich protein (CdeC) demonstrate reduced resistance to lysozyme, ethanol, and heat treatment [28,29].

Spore Germinants, Germination Inhibitors, and Cogerminants of *C. difficile*

To colonise the host, spores of *C. difficile* reactivate in the presence of specific environmental signals (germinants) associated with the small intestine that lead to return to full metabolic activity and vegetative cell growth [4,30]. Although it has long been recognised that addition of the primary bile salt taurocholate to culture media enhances the recovery of *C. difficile* spores in the laboratory, Sorg *et al.* demonstrated that the bile salt cholate as well as the conjugated bile salts glycocholate and taurocholate induce the germination of *C. difficile* spores [31,32]. The abundance of primary bile acids and secondary bile salts during passage through the gastrointestinal tract is the crucial signal that modulates germination. The primary bile acids cholic acid

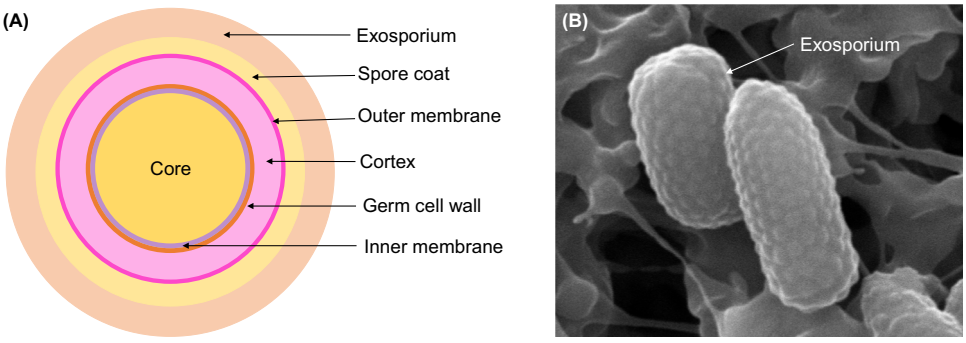
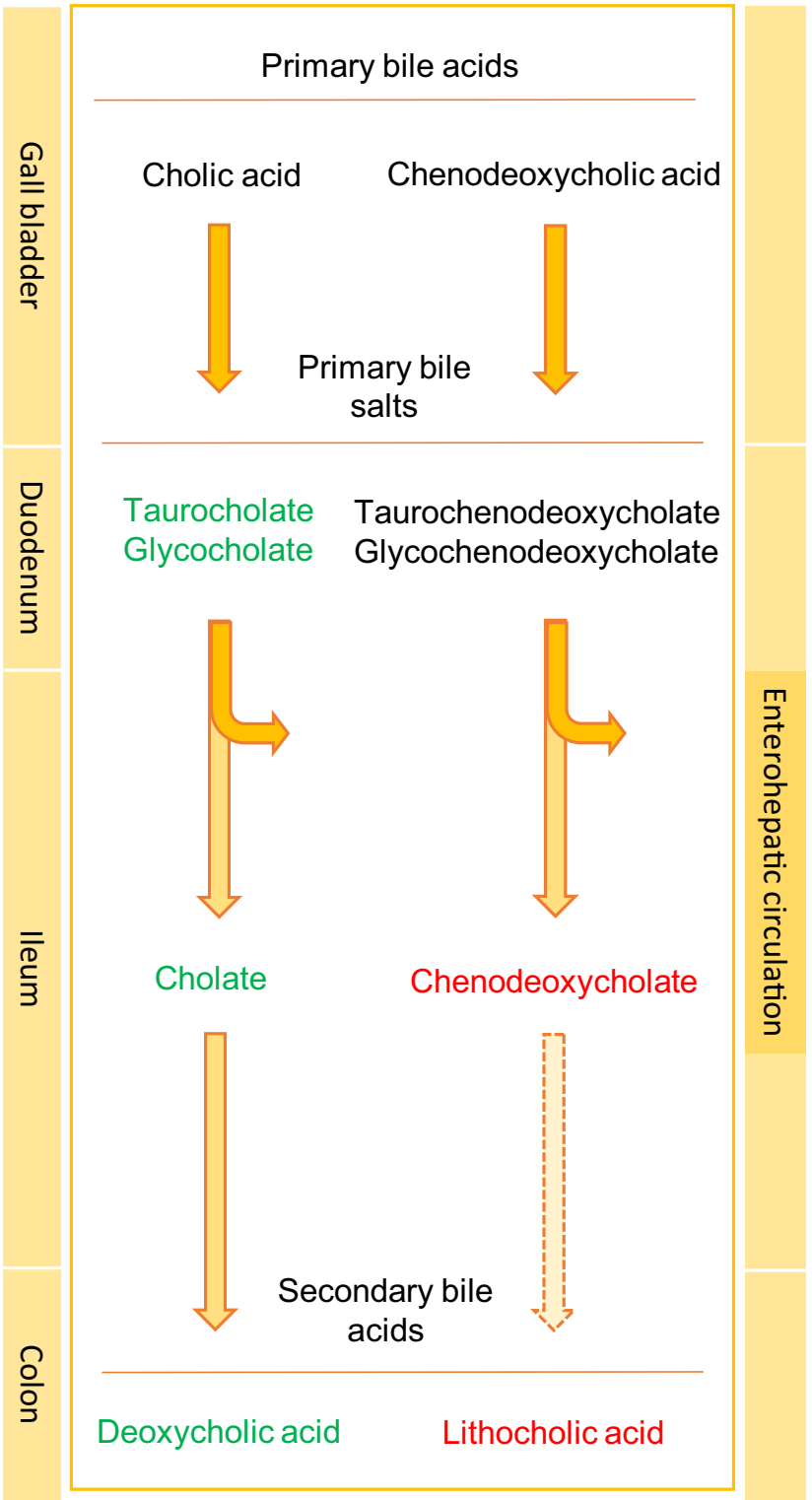


Figure 1. *Clostridioides difficile* Spore Structure. (A) Schematic cross-sectional representation of spore layer structure (not to scale). (B) Scanning electron microscopy image of the *C. difficile* strain NCTC 11204 spore surface showing the exosporium layer (image provided by Rachel Sammons, University of Birmingham).

and chenodeoxycholic acid, that are secreted into the small intestine to assist the digestion of lipids, are conjugated with taurine or glycine to produce primary bile salt derivatives (Figure 2) [33]. Although active transport of primary bile salts ensures that the majority are recycled back to the liver via the enterohepatic circulation, a low concentration enters the large intestine and is deconjugated by species of the intestinal microbiota to generate cholate and chenodeoxycholate [33,34]. In the colon there is disproportionate passive reabsorption of chenodeoxycholate, leading to a higher ratio of remaining cholate [35]. In healthy individuals 7α-dehydroxylation of primary bile salts by selective microbiota species generates the secondary bile aids deoxycholate and lithocholate (Figures 2,3). However, in the absence of these microbiota species, for example, following their elimination by broad-spectrum antibiotics such as β-lactams, conversion to secondary bile acids is reduced, resulting in an increased concentration of *C. difficile* spore germinants [2,33]. Correspondingly, secondary bile salts are associated with colonisation resistance, and inhibit *C. difficile* germination and outgrowth [2]. Responding only in the presence of cholate-derived germinants ensures that *C. difficile* spores germinate specifically in the ileum, preventing germination until spores receive the germination-positive signals that are indicative of the small intestine environment [36,37]. Sorg *et al.* also established that the primary bile acid chenodeoxycholate inhibits taurocholate-mediated germination in a competitive manner because *C. difficile* has a higher affinity for chenodeoxycholate, thereby suppressing germination until the concentration of germinant overcomes the inhibitory action of chenodeoxycholate [32,38,39]. The secondary bile salt lithocholate is also an inhibitor of germination, whereas deoxycholate is a weak spore germinant but inhibits the growth of *C. difficile* [32,38,40]. With the exception of structural alterations of conjugated bile salt derivatives, bile salt germinants and inhibitors of *C. difficile* are structurally similar, and the presence or absence of the 12α-hydroxyl group appears to determine promotion or suppression of germination (Figure 3) [38]. Variation in germinant specificity, however, has been recognised across clinically relevant strains of *C. difficile* [41]. Heeg *et al*. demonstrated variation in the rate and extent of germination in response to taurocholate; furthermore, taurocholate-mediated germination was not inhibited by chenodeoxycholate in some clinical isolates [41]. Disparity in the germination response to bile salt germinants and inhibitors was also demonstrated across a broad range of *C. difficile* ribotypes [40]. A selected mutation of the identified bile salt receptor CspC modified germinant specificity: chenodeoxycholate induced germination of *C. difficile* spores instead of having inhibitory action in an optical density-based germination assay [42]. By contrast, through assessment of germination on solid media (pretreated with potential germinants), Rohlfing *et al*. recently demonstrated that the selected CspC mutation resulted in heightened sensitivity to germinants and cogerminants, but did not germinate in response to chenodeoxycholate [43].

C. difficile germination does not occur in the presence of a single germinant, and a cogerminant is also required. Sorg and Sonenshein identified the amino acid glycine as a *C. difficile* spore cogerminant and the component of complex media that provides the additional signal required for germination [32]. Other amino acids have also been established as cogerminants, but their activity varies considerably: although glycine has the greatest cogermination activity, L-alanine, taurine, and L-glutamine display further activity. Although this is strain-dependent, amino acids with branched side chains typically demonstrate the least cogerminant activity [32,37,44,45]. Histidine was found to significantly enhance taurocholate- and glycine-mediated germination, whereas a specific combination of five amino acids in addition to taurocholate demonstrated similar germination activity to that of taurocholate and complex media. The combination of the amino acid cogerminants is believed to be an additive effect rather than synergistic [37,44,46]. The activity of amino-acid co-germinants is highly temperature-dependent; lysine and serine, previously not considered to be cogerminants and D-forms of amino acids including D-alanine and D-serine, demonstrate cogerminant activity at the physiologically relevant temperature of



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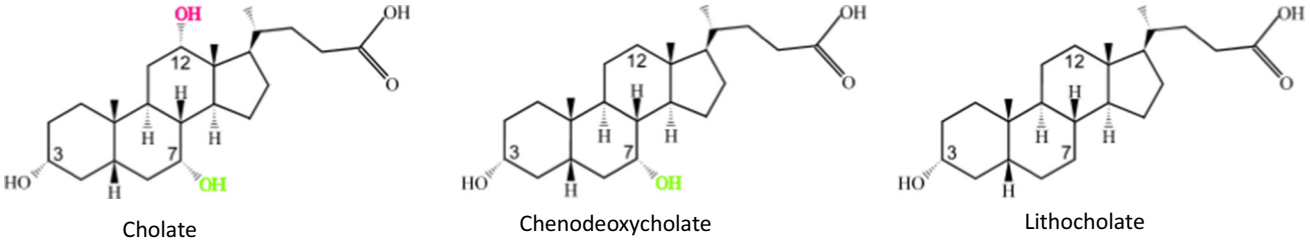

Figure 3. Structure of the Primary Bile Salts Cholate and Chenodeoxycholate, and of the Secondary Bile Acid Lithocholate. The 7α-hydroxyl group (highlighted in green) is dehydroxylated during conversion of primary bile salts to secondary bile acids. The 12α-hydroxyl group (highlighted in pink) is a structural determinant that differentiates between germinants and inhibitors of *Clostridioides difficile* germination.

37°C [45,47]. The role of glycine as a *C. difficile* cogerminant is thought to be linked to its requirement for optimal growth through its incorporation into peptidoglycan of the vegetative cell wall [48,49].

Recently, *C. difficile* spore germination was demonstrated in response to the germinant taurocholate in the presence of calcium [50]. Calcium functions as a cogerminant, inducing germination in conjunction with the bile salt germinant taurocholate, and is also presumed to be involved in transmission of the germination signal by activating cortex degradation during taurocholate- and glycine-derived germination, through an unidentified calcium-dependent enzyme [50]. Germination in response to calcium as a cogerminant can be induced by either exogenous (derived from the host) or endogenous calcium released during rehydration of the spore core as Ca-DPA [50]. Calcium demonstrates synergistic activity with amino acid cogerminants, and enhances the rate of germination in the presence of taurocholate and low concentrations of amino acid [37]. Crucially, this synergistic activity together with amino acid cogerminants can overcome the inhibitory activity of chenodeoxycholate [37].

Regulation of *Clostridioides difficile* Spore Germination

Universally across spore-forming bacteria, the binding of germinants and in some cases cogerminants to their appropriate receptors results in transformation of a dormant spore into a metabolically active vegetative cell. Although there is commonality with other *Bacillus* species and members of the Clostridia, the *C. difficile* spore germination regulation pathway is unique amongst spore formers [17,48]. Typically the *Ger* receptors are responsible for detecting germinants and are highly conserved across *Bacillus* and *Clostridioides* species; however, distinctively, *C. difficile* does not possess Ger-type receptors but instead senses germinants and cogerminants via the Csp subtilisin-like serine proteases of the *cspBAC* locus [42,48,51]. The products of this locus, CspB, CspA, and CspC, are key regulators of germination signal transduction, and interactions between the Csp proteins during spore formation are likely to be important for effective germination (Figure 4). Specific to *C. difficile,* GerG controls the incorporation of CspA, CspB, and CspC into the mature spore and is required for *C. difficile* spores to complete germination [52]. In the absence of functional GerG, the decreased levels of CspA, CspB, and CspC alter the detection of germinants and inhibit downstream cortex hydrolysis [52]. The catalytic triad of the Csp proteases, consisting of aspartate, histidine, and serine residues, is functional in only CspB [42,53]. Mutations in the catalytic residues of CspA and CspC eliminate the ability to process the cortex lytic enzyme to its active form and transduce the germination signal [42].

Figure 2. Modification of Primary Bile Acids during Transit through the Small and Large Intestines. Green text indicates a bile salt or bile acid that has *C. difficile* spore germination activity; red text indicates an inhibitor [32,38,39].

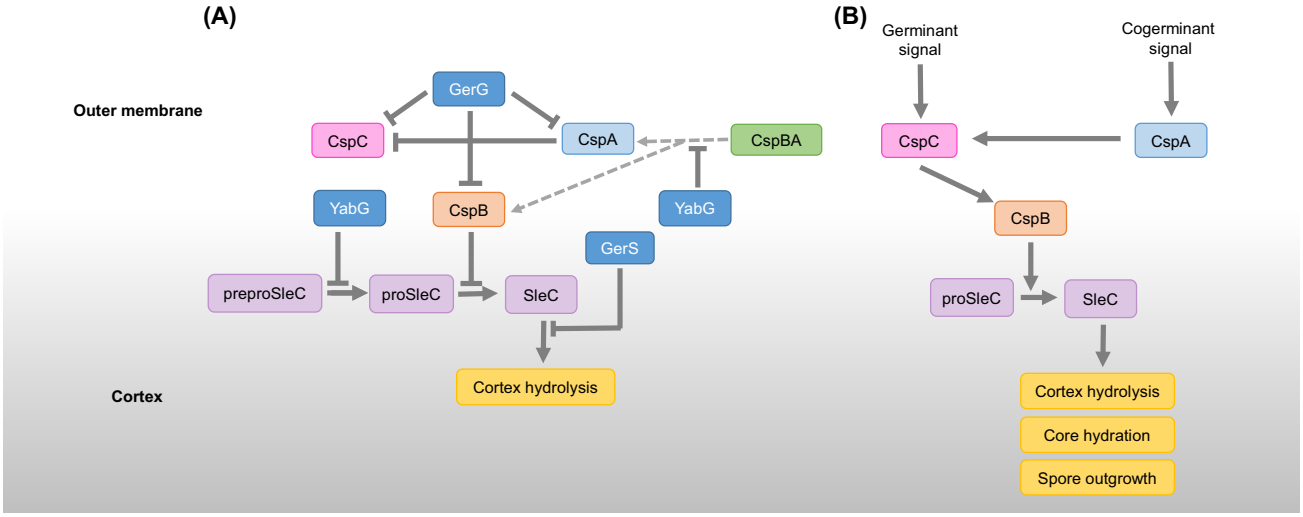

Figure 4. A Model of *C. difficile* Germination Regulation Pathways. (A) The role of key regulators of *Clostridioides difficile* germination, (B) and the sequence of the *C. difficile* germination signal cascade [42,51–60].

CspC has been identified as the bile salt germinant receptor and is responsible for transmitting the germination signal downstream to CspB, although the mechanism by which this occurs has yet to be fully determined [42,54]. However, a greater abundance of CspC protein in mature spores of clinically relevant *C. difficile* strains did not correlate with a rapid germination rate, suggesting that CspC has a more complex role in *C. difficile* germination signal transduction and may inhibit downstream processes [54]. Rohlfing *et al.* demonstrated that mutation of specific residues of CspC resulted in increased sensitivity to the germinant taurocholate and even negated the need for taurocholate to induce germination at low frequency [43]. CspA and CspB are encoded by *cspBA* as a fusion protein and undergo interdomain cleavage during spore formation [42,53]. CspBA is believed to stabilise CspC through sporulation and spore maturation, and thus regulates the level of CspC incorporated into mature spores [55]. Deletion of *cspA* results in mature spores that are germination defective and contain reduced levels of CspC protein [55]. Recently the role of CspA in *C. difficile* spore germination has been expanded through the work of Shresha *et al.* that implicates CspA in the recognition of amino acid and calcium cogerminants [51]. Site-directed deletions in *cspA* eliminated the requirement for cogerminants during taurocholate-mediated germination, although these mutants still responded to glycine as a cogerminant [51]. The interdomain processing of CspBA to CspB and CspA is dependent upon the protease YabG during sporulation [55]. Cleavage of CspBA regulates appropriate germination: mutation of YabG protease produces spores that are able to germinate in response to the germinant taurocholate alone. By contrast to the CspA mutant discussed previously, the YabG mutant no longer requires or responds to amino acid cogerminants [51].

CspB, the only subtilisin-like serine protease produced by *C. difficile* that has catalytic activity, is controlled by CspC through a putative protein–protein interaction and is required for specific cortex lytic enzyme activation [52,53,56]. Activation of CspB by CspC initiates cleavage of the N-terminal region of proSleC to produce the active cortex lytic enzyme, SleC [53,57]. Upon activation, SleC degrades the specialised peptidoglycan of the cortex, releasing stored Ca-DPA and rehydrating the spore core via a mechanosensing mechanism [57,58]. Eliminating the catalytic activity of CspB has a significant impact on *C. difficile* spore germination, and inhibits processing of the active protease SleC but does not eliminate germination completely, suggesting that other

enzymes are also involved in cortex hydrolysis [53]. YabG also regulates the cortex lytic enzyme and processes preproSleC to proSleC; elimination of YabG prevents incorporation of proSleC into the mature spore [51,55].

GerS regulates germination through its modification of the peptidoglycan of the cortex, and thus regulates the activity of SleC [59]. Germination-deficient GerS mutants are unable to degrade the cortex and complete germination because these cortex-specific modifications are required for recognition by SleC [59,60]. Furthermore, inactivation of GerS results in an inability to cause disease in the *C. difficile* hamster infection model [60]. By contrast to other spore-forming bacteria, cortex degradation by cortex lytic enzymes occurs before the release of Ca-DPA during *C. difficile* germination [56]. Hydrolysis of cortex peptidoglycan relieves osmotic constraints inside the core, and this is detected by the mechanosensing protein, SpoVAC, that facilitates the release of Ca-DPA [57].

Clostridioides difficile Germination Models

In light of developments in the understanding *C. difficile* germination discussed above, models predicting the transduction of the germination signal have been hypothesised. Kochan *et al*. propose a model whereby bile salt germinants act as a 'chemical key' by binding to their 'lock', CspC [50,61]. Activation of CspC by germinants facilitates the transport of cogerminants including calcium and amino acids through the outer membrane via an unidentified mechanism. CspB is activated by binding to calcium, which is either exogenous or released from the spore core when amino acids bind to the cogerminant receptor. CspB processes proSleC to its active form SleC, which then degrades the peptidoglycan of the cortex, leading to core rehydration, DPA release, and spore outgrowth [50].

In the alternative 'germinosome' model, based on the findings of Adams *et al.*, Bhattacharjee *et al.*, Fimlaid *et al.*, and Francis *et al.*, and informed by the observed colocalisation of germinant receptors at the inner membrane of *Bacillus subtilis* spores, the products of the *cspBAC* operon together with proSleC are colocated in the cortex component of the outer membrane as a 'germinosome complex' [42,48,53,60–62]. Their proximity is hypothesised to facilitate direct interaction such that when taurocholate binds to the germinant receptor CspC, CspB is

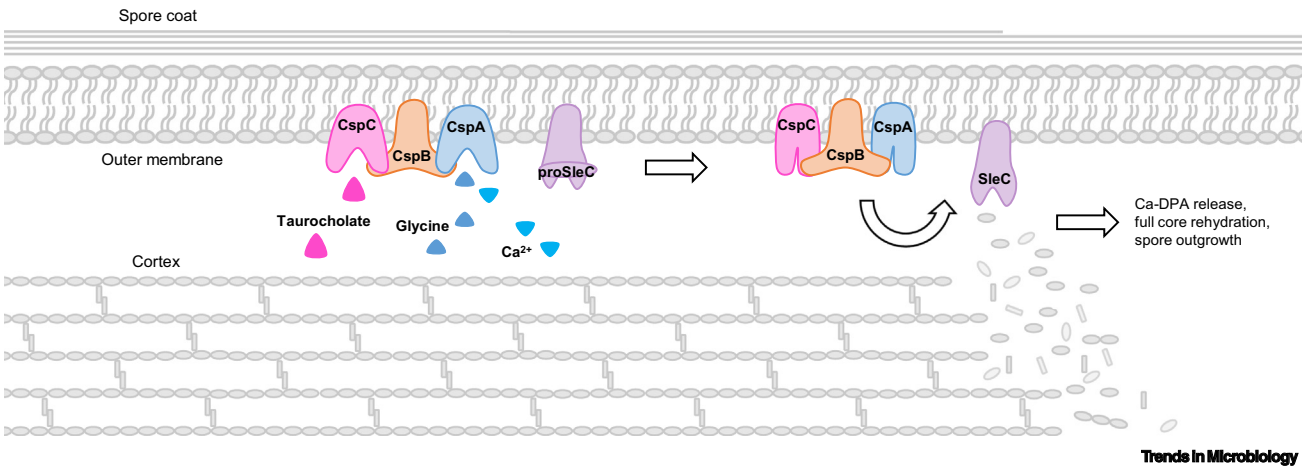

Figure 5. An Updated Germinosome Complex Model Demonstrating Germinant and Cogerminant Receptor Activation and Downstream Signalling Leading to Germination of *Clostridioides difficile* Spores. The model incorporates the recent identification of CspA as the cogerminant receptor and its interaction with CspB, which together with CspC activation results in processing of proSlec to SleC, subsequent cortex degradation, calcium dipicolinic acid (Ca-DPA) release, core rehydration, and spore outgrowth. Model modified from Kochan *et al*. and Shrestha *et al.* [51,61].

subsequently activated. The activation of CspB, together with simultaneous interaction of calcium and/or amino acid cogerminants with the germinosome, facilitates Csp-mediated processing of proSleC to SleC and ultimately cortex degradation [61]. Integrating the recent findings of Shrestha *et al*. that identify CspA as the proposed amino acid cogerminant receptor within the germinosome complex model, the proximity and physical interaction of CspC and CspA with CspB are hypothesised to inhibit its protease activity, thereby regulating germination (Figure 5). Upon binding of germinants and cogerminants to their receptors, CspC and CspA dissociate from CspB, allowing the processing of proSleC to SleC [51].

Recent characterisation of the crystal structure of CspC by Rohlfing *et al*. was unable to determine whether bile salt germinants interact directly or indirectly with CspC [43]. Mutational studies of CspC identified mutants that had increased sensitivity to the germinant taurocholate but also had increased sensitivity to amino acid cogerminants [43]. Although CspC plays a key role in germination signal transduction, it may not bind directly to bile salt germinants. The authors propose that it may interact with an unidentified bile salt germinant receptor and the cogerminant receptor to integrate and transduce the germination signal [43]. CspC activation may induce a conformational change that permits its interaction with CspB, resulting in induction of protease activity and subsequent interaction of CspA with CspB of the germinosome complex [43,51]. The authors also discuss an alternative role for CspC in the transport of germinants to their receptors located in the cortex by altering the permeability of the spore outer membrane [51]. This explanation of the role of CspC in germination regulation has similarities to the lock and key model, but also incorporates the colocation of the germinosome complex [50,61].

Concluding Remarks

The germination of *C. difficile* spores is a crucial step in the development of CDI. A comprehensive understanding of its regulation is therefore essential to improve upon existing strategies to control the transmission of spores and prevent disease in susceptible individuals. Advances in *C. difficile* research have enabled the identification of the germinant and cogerminant receptors CspC and CspA, as well as the role of CspB as the major cortex lytic enzyme. However, how these key regulators interact and the role of calcium during the sequence of germination initiation needs to be resolved (see Outstanding Questions). The germinosome complex and 'lock and key' germination models both provide convincing proposals for the regulation and transmission of the germination signal but deliver only a partial explanation, emphasising the current deficiency in knowledge. Further research into *C. difficile* germination and its regulation could provide vital information for the development of treatments to prevent the colonisation of patients and for designing sporicidal disinfection strategies for decontaminating clinical environments.

Outstanding Questions

How do bile salt germinants and amino acid cogerminants penetrate the exosporium and the proteinaceous spore coat to interact with their receptors and trigger germination? Are germinants and cogerminants transported via a specific transfer system?

What is the location of the germinant and cogerminant receptors and other key germination regulators within the spore ultrastructure? Are CspC and CspA located in the outer membrane, as proposed by the germinosome complex model, or are they located divergently on the outer and inner membranes?

What is the nature of the interaction of CspB with the germinant and cogerminant receptors? The germinosome complex model proposes a direct interaction, whereas the lock and key model suggests indirect communication involving calcium signalling.

What is the purpose of amino acid cogerminants given that exogenous calcium can act as alternative cogerminant? Is calcium another environmental signal indicating that *C. difficile* spores have reached the small intestine?

What role does calcium play in transduction of the germinant signal, and more specifically in relation to activation of CspB?

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