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MINIREVIEW

Germinants and Their Receptors in Clostridia

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Many anaerobic spore-forming clostridial species are pathogenic, and some are industrially useful. Although many are strict anaerobes, the bacteria persist under aerobic and growth-limiting conditions as multilayered metabolically dormant spores. For many pathogens, the spore form is what most commonly transmits the organism between hosts. After the spores are introduced into the host, certain proteins (germinant receptors) recognize specific signals (germinants), inducing spores to germinate and subsequently grow into metabolically active cells. Upon germination of the spore into the metabolically active vegetative form, the resulting bacteria can colonize the host and cause disease due to the secretion of toxins from the cell. Spores are resistant to many environmental stressors, which make them challenging to remove from clinical environments. Identifying the conditions and the mechanisms of germination in toxin-producing species could help develop affordable remedies for some infections by inhibiting germination of the spore form. Unrelated to infectious disease, spore formation in species used in the industrial production of chemicals hinders the optimum production of the chemicals due to the depletion of the vegetative cells from the population. Understanding spore germination in acetone-butanol-ethanol-producing species can help boost the production of chemicals, leading to cheaper ethanol-based fuels. Until recently, clostridial spore germination is assumed to be similar to that of *Bacillus subtilis*. However, recent studies in *Clostridium difficile* shed light on a mechanism of spore germination that has not been observed in any endospore-forming organisms to date. In this review, we focus on the germinants and the receptors recognizing these germinants in various clostridial species.

Historically, bacteria found in the genus *Clostridium* were classified by their bacillus-like shape, anaerobic growth requirements, and ability to form spores (1). However, with the advent of more sophisticated methods for taxonomy (e.g., multilocus sequence analysis), clostridia have recently undergone a diversification in genus. Although the names of several clostridial species have changed, the fact that these organisms cause public health threats or are industrially important has not.

Many clostridia generate industrially relevant organic compounds, and they play a crucial role in biodegradation and industrial production of a large set of metabolites (e.g., acetone, butanol, ethanol, butanediol, propanol, acetoin, butyrate, and acetate) (2). As examples, *Clostridium acetobutylicum* and *Clostridium butyricum* are well known to produce acetone-butanol-ethanol (ABE) products during industrial fermentation (3). *Clostridium beijerinckii* can be used to produce butanol (4), while *Clostridium cellulolyticum* can use cellulose as a carbon source and generate lactate, acetate, and ethanol as valuable end products (5). Finally, *Clostridium pasteurianum* converts algal biomass to commercially useful butanol, ethanol, and propanediol (6).

Apart from their value in chemical production, other clostridial species are known to cause major human, animal, and economic losses in a variety of industries (7, 8). *Clostridium perfringens* is well known to cause foodborne illnesses as a result of contamination of food sources (9, 10), and several other clostridia have been shown to spoil food (11–13). Other clostridia also are known for their roles as pathogens of humans and animals. For example, *Clostridium botulinum* produces the acutely lethal botulinum toxin and is considered a potential bioterror agent due to the potent activity of the neurotoxin, which causes a fatal neuroparalytic illness (14). Moreover, *Clostridium tetani* secretes the potent tetanospasmin neurotoxin that elicits the primary symptoms of tetanus disease, leading to an estimated 500,000 worldwide fatalities per year (15). Apart from its ability to cause food spoilage, *C. perfringens* causes a range of diseases from myonecrosis (gas gangrene) to foodborne and nonfoodborne gastrointestinal illnesses in both humans and animals (16). Finally, the Centers for Disease Control and Prevention listed *Clostridium difficile* (recently proposed to be renamed as *Peptoclostridium difficile* [17]) as an immediate and urgent threat to the public, due to it causing ~500,000 infections/year, approximately 29,000 deaths, and nearly $4.8 billion in treatment-associated costs (18).

Although many clostridia are important human pathogens, some may have potential for treating or controlling human diseases. *Clostridium novyi*, a soil-dwelling organism incriminated in wound associated gangrene and infections in intravenous (i.v.) drug users (19), was shown to be important in treating tumors (20–23). Moreover, *Clostridium bifermentans* subsp. *malaysia* is active against a host of mosquito genera, especially *Anopheles*, a carrier of malarial parasites (24, 25).

Many clostridia survive in the environment in the dormant spore form. Spores are metabolically dormant forms of bacteria and, for many spore-forming pathogens, the spore is the infectious form (26). The majority of the sporulation process is conserved across species of endospore-forming bacteria. *Bacillus subtilis* has served as a model spore former for decades, and most of the processes of spore formation and germination were elucidated in this organism. Although there are certainly differences between

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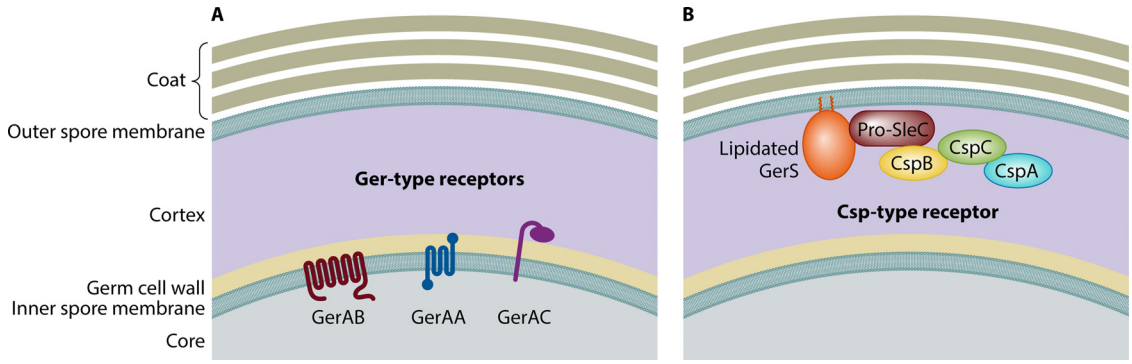

FIG 1 Representation of Ger- and Csp-type receptors in the spore. (A) GerAA, GerAB, and GerAC are located within or on the inner spore membrane, and their location in clostridia is based mostly on what is observed in *B. subtilis*. The topology of Ger-type germinant receptors for clostridia is unknown. (B) The *C. difficile* germinant receptor complex contains the bile acid germinant receptor CspC and the CspB protease CspA and may be localized to the outer membrane by lipidated GerS.

Bacillus and *Clostridium* spore formation and germination, the spore form itself is largely conserved (27). Sporulation begins with the phosphorylation of the master sporulation transcription regulator Spo0A. Subsequently, the vegetative cell then divides asymmetrically into a smaller forespore and larger mother cell. Through coordinated gene expression between the forespore and the mother cell, the mother cell engulfs the forespore, and the smaller compartment matures into a metabolically dormant spore (28, 29). The spore form is composed of a partially dehydrated DNA-containing core surrounded by an inner cell membrane. In the core, much of the water is replaced with Ca^{2+}-dipicolinic acid (DPA), which helps maintain spore dormancy and protect the spore from UV radiation and heat (30). The core is also packed with small acid-soluble proteins (SASPs) that bind and protect the DNA from radiation, heat, and genotoxic chemicals (30, 31). Surrounding the inner membrane is a germ cell wall composed of the typical *N*-acetylglucosamine (NAG)- and *N*-acetylmuramic acid (NAM)-containing peptidoglycan (Fig. 1). The germ cell wall is surrounded by a peptidoglycan-like cortex layer. The cortex is a specialized peptidoglycan, where much of the NAM residues are converted to muramic-δ-lactam (MAL) residues that are recognized by spore cortex lytic enzymes (SCLEs) during the process of germination. Surrounding the cortex layer is an outer membrane and a thick proteinaceous spore coat (Fig. 1). In some spore-forming bacteria, the coat is surrounded by an exosporium layer (31, 32).

During sporulation, the factors required to initiate the transition from a metabolically dormant to a metabolically active state, germination, are built into the spore (28). Germination is stimulated when the spore responds to an environmental signal (germinant) (33). The signals that stimulate germination can be a variety of small molecules, and these germinants activate germination by binding to specific germinant receptors (33). In most spore-forming organisms studied to date, the activated germinant receptor then propagates a signal to a channel that is important for releasing the DPA from the core (27, 33, 34). Subsequently, an SCLE is activated, which then degrades the cortex. Cortex degradation leads to swelling and further hydration of the core. Finally, a vegetative cell grows out from the germinated spore (35).

Germination has been studied, largely, in bacilli. With the development of new genetic tools, the mechanisms of germination in clostridia are being examined in detail (36–40). Here, we review the germinants and receptors that have been identified in clostridia.

GERMINANTS

Although dormant, spores respond to germinants that stimulate the return to vegetative growth using proteins specific to the signal (germinant receptors). Commonly, germinants are low-molecular-weight biomolecules found in the environment where growth of the organism is favored. Germinants, most commonly, are amino acids, but other molecules (e.g., cholesterol-based compounds, organic acids, nucleosides, peptidoglycan fragments, etc.) have been identified (41–46). Although germination of some spores can be triggered by a simple germinant, some spores require the presence of more than one type of molecule to stimulate germination (35). Table 1 summarizes the known germinants, the combinations required to stimulate germination, and inhibitors of germination of the discussed clostridia. Below, we discuss the germinants and inhibitors of germination in the context of potential uses for the germinated organisms.

CELL WALL PRECURSORS

Similar to what is observed in bacilli, the most common germinant in clostridia is L-alanine (32). The reason(s) behind L-alanine functioning as the favored germinant across many spore-forming organisms is not known but may be linked to the conservation of peptidoglycan structure (47). Peptidoglycan is composed of alternating NAG and NAM residues, and each NAM includes a stem peptide that is composed of 5 amino acids. In many organisms, this structure is NAM–L-alanine–D-glutamic acid–diaminopimelic acid (DAP)–D-alanine–D-alanine. During transpeptidation, the DAP is cross-linked to the 4th amino acid (D-alanine) of the neighboring stem peptide, resulting in the cleavage of the terminal D-alanine (47). Thus, 3 alanines are required to synthesize each cell wall stem peptide (2 of the alanyl residues are converted to D-alanine by an alanine racemase). *C. botulinum* (except group IV type G isolates), *C. sporogenes*, *C. frigidicarnis*, *C. bifermentans*, *C. roseum*, *C. sordellii*, *C. difficile*, and *C. perfringens* spores derived from nonfoodborne isolates germinate in response to L-alanine (commonly in combination with other factors) (Table 1). Additionally, some strains of *C. botulinum*, *C. sporogenes*, *C. bifermentans*, and *C. frigidicarnis* germinate in response to L-lactate (Table 1). Even though L-lactate is not normally found in peptidoglycan

TABLE 1 List of clostridial species with their identified germinants and their respective inhibitors

Organism(s)[a]	Germinant(s) (reference[s])			Inhibitor(s) (reference[s])
	Amino acid(s)	Mineral(s)	Organic biomolecule(s)	
C. bifermentans	L-Alanine, L-arginine, L-phenylalanine (44, 93–95)	K^+, Na^y (93)	L-Lactate, pyruvate (44)	
C. botulinum, proteolytic	L-Alanine (96)		Sodium bicarbonate, exogenous DPA (77, 96, 97)	D-Alanine, D-serine, D-cysteine, D-phenylalanine, D-methionine, sorbate (65, 66)
C. botulinum, nonproteolytic	L-Alanine, L-cysteine, L-serine (97)		L-Lactate (96, 97)	
C. botulinum group IV type G	L-Cysteine (77)		Sodium thioglycolate, sodium bicarbonate (77)	
C. butyricum	L-Cysteine (98)		Sodium bicarbonate, glucose (98)	
C. difficile	Glycine, L-alanine, L-cysteine, L-norvaline, L-2-aminobutyric acid, L-phenylalanine, L-arginine (43, 58)		Cholic acid and related bile acids (43, 59, 60)	CDCA and related bile acids, progesterone, and related derivatives (43, 45, 67, 68)
C. frigidicarnis	L-Valine, L-cysteine, L-norvaline, L-threonine, glycine, L-serine, L-alanine (57)	$NaHPO_4$ (57)	L-Lactate, sodium bicarbonate (57)	
C. pasteurianum			Exogenous DPA (99)	
C. perfringens FP isolates	L-Asparagine, L-glutamine, L-cysteine, L-threonine, L-serine (34, 100, 101)	KCl, Na^+, P_i (34, 102)[b]	Exogenous DPA (100)	
C. perfringens NFB isolates	L-Alanine, L-valine, L-asparagine, L-cysteine, L-threonine, L-serine (34, 101)	KCl (34)		
C. roseum	L-Alanine, L-arginine, L-phenylalanine (103)			
C. sordellii	L-Alanine, L-arginine, L-phenylalanine (62)		Sodium bicarbonate, progesterone, and related derivatives (45, 62)	
C. sporogenes	L-Alanine (65, 77)		L-Lactate, sodium bicarbonate (65, 77)	D-Alanine (65)
C. tetani	Methionine (104)	Na^+ (104)	Nicotinamide, lactate (104)	

[a] FP, food poisoning; NFB, nonfoodborne.
[b] P_i, inorganic phosphate.

(D-lactate is found in some vancomycin resistance mechanisms), L-lactate can be converted to L-alanine in two enzymatic steps: lactate dehydrogenase converts L-lactate to pyruvate, which is then transaminated to L-alanine (48, 49).

In addition to L-alanine, *C. botulinum*, *C. frigidicarnis*, and *C. perfringens* type A isolate spores germinate in response to L-serine (Table 1). Again, and similar to L-lactate, L-serine is not normally found in peptidoglycan, except in certain vancomycin resistance mechanisms. However, L-serine dehydratase converts L-serine to pyruvate. From this precursor, L-alanine can be synthesized in one step (as described above).

Finally, glycine can act as a germinant for *C. frigidicarnis* and a cogerminant for *C. difficile* spores (and is most widely used as a cogerminant to stimulate *C. difficile* spore germination in combination with taurocholate, a bile salt [see below]) (Table 1). Glycine is not normally found in the cell walls of bacteria. However, Peltier et al. (50) demonstrated that the *C. difficile* cell wall has several uncommon features (the structure of the *C. frigidicarnis* peptidoglycan is not known) (50). Significantly, the authors found that in some stem peptides, glycine was present in the 4th position (in substitution for alanine) (50). Thus, although glycine is not normally found as a germinant among spore-forming organisms, this correlates with glycine being found as a component of the *C. difficile* peptidoglycan (in addition to its potential role as a nutrient; see below).

GROWTH-PROMOTING GERMINANTS

The germinants listed above were within 2 biochemical steps of alanine or could be directly incorporated into the peptidoglycan. Importantly, though, that is not to ignore the fact that the amino acids listed above are clearly involved in protein synthesis and important from that perspective as well. Some clostridial spores germinate in response to certain amino acids that are not closely tied to cell wall synthesis, and thus, we have categorized them as growth promoting. In this section, we discuss the potential use(s) for the germinants using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (48, 49). If an organism's genome was not present in KEGG, a closely related organism was used as a surrogate (e.g., *C. difficile* was substituted for *C. sordellii*).

Despite the fact that glycine is found in the *C. difficile* pepti-

doglycan, glycine also is important for optimal growth of the organism (50, 51). *C. difficile* can oxidatively deaminate and decarboxylate amino acids in Stickland metabolism to generate ATP and NADH (52). In this manner, NADH accumulates and must be reduced to NAD^+. This reduction is accomplished using proline or glycine and proline reductase or glycine reductase, respectively (52). In this reductive branch of Stickland metabolism, glycine reductase regenerates NAD^+ and generates acetate, ammonium ion, and ATP, thus providing important biomolecules for growth (52). Lawley and colleagues (53) have shown that both proline reductase (PrdB) and glycine reductase (GrdA) are present within *C. difficile* spores, suggesting that glycine (or proline) availability could be important for Stickland metabolism after the spores have germinated (either for outgrowth or for the vegetative cell) (53, 54).

Certain *C. botulinum* strains, *C. difficile*, *C. frigidicarnis*, certain *C. perfringens* isolates, and *C. butyricum* germinate in response to L-cysteine (Table 1). L-Cysteine is a known reducing agent and thus could be a good indicator of an anaerobic (reducing) growth-promoting environment. Importantly, though, *C. botulinum*, *C. difficile*, *C. perfringens*, and *C. butyricum* all encode cysteine desulfurase (*nifS*). NifS catalyzes the removal of sulfur from L-cysteine, generating L-alanine as a by-product (which could be used for peptidoglycan synthesis) (55). The sulfur is then incorporated into Fe-S clusters, which are required for *C. botulinum* growth (55, 56).

During growth, cells must elongate in order to preserve cell size after division, and this process requires membrane (lipid) synthesis. Toward this requirement, *C. frigidicarnis* and spores derived from nonfoodborne *C. perfringens* strains germinate in response to the branched-chain amino acid L-valine (norvaline could also be used by *C. frigidicarnis* and *C. difficile*) (34, 57, 58). L-Valine is a precursor to lipid biosynthesis, and thus, L-valine could be used as a metric to sense the ability to produce cell membrane from the surrounding environment.

Many other germinants in clostridia are not tied to particular metabolic pathways but are important for growth nonetheless. For example, L-arginine and L-phenylalanine are precursors for multiple compounds within the cell. Moreover, L-threonine can be metabolized to glycine and acetyl coenzyme A (acetyl-CoA), while L-methionine is important for *S*-adenosylmethionine synthesis. Finally, nicotinamide is important for NAD^+ synthesis and L-glutamine–L-asparagine are important for ammonia generation (Table 1) (48, 49).

HOST-DERIVED GERMINANTS

Some clostridial spores germinate in response to host-derived germinants. *C. difficile* spores germinate in response to a combination of certain bile acids and glycine (Table 1) (43, 59, 60). Bile acids are cholesterol-derived small amphipathic molecules synthesized by the liver (61). In humans, the liver synthesizes two primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA), which are then conjugated with glycine (glycocholic acid or glycochenodeoxycholic acid) or taurine (taurocholic acid or taurochenodeoxycholic acid). During passage through the intestinal tract, these bile acids are absorbed and recycled back to the liver (61). However, a small portion escapes this enterohepatic recirculation and enters the large intestine, where they become a substrate for modification by the normal intestinal microbiota. Bile acids are rapidly deconjugated to cholic acid and chenodeoxycholic acid and then modified further through 7α-dehydroxylation (61). This enzymatic reaction converts primary bile acids (e.g., cholic acid) to secondary bile acids (e.g., deoxycholic acid). *C. difficile* spore germination is activated by CA derivatives, including deoxycholic acid, a molecule that is growth inhibitory to *C. difficile* vegetative cells (43, 61).

Although *C. sordellii* germinates in response to growth-promoting germinants (Table 1), *C. sordellii* spore germination also is enhanced by progesterone and progesterone-based steroids (cholesterol derivatives), as well as certain bile acids (45, 62). This pathogen causes serious infection in postpartum women or in medically induced abortions using mifepristone (63). The hormonal changes that occur in postpartum women, or mifepristone itself, are hypothesized to prime or enhance germination by *C. sordellii* spores and thus initiate colonization of the host (45).

INHIBITORS OF SPORE GERMINATION

Not only can germination be stimulated by germinants, but other structurally related small molecules can inhibit spore germination. For example, D-alanine, the stereoisomer of L-alanine, was shown approximately 30 years ago to competitively inhibit *B. subtilis* spore germination (64). Similarly, D-alanine, D-serine, D-cysteine, D-phenylalanine, and D-methionine can inhibit spore germination of *C. sporogenes* and some strains of *C. botulinum* and seem to cross-inhibit germination by other amino acids (e.g., D-serine inhibits germination by L-cysteine, L-methionine, L-phenylalanine, and L-serine), possibly suggesting that the germinant receptors have a relaxed specificity for germinant recognition (65). Importantly, though, not all organisms whose spores are activated by L-alanine are inhibited by D-alanine. *C. sordellii* and *C. difficile* germination is activated by L-alanine, but D-alanine does not inhibit germination in these organisms (62). Germination by some strains of *C. botulinum* is strongly inhibited by sorbate (66). Finally, *C. difficile* germination is competitively inhibited by CDCA and its derivatives (67, 68). Progesterone and its analogs also had an inhibitory effect on *C. difficile* germination (45). Importantly, inhibiting germination by *C. difficile* spores has been used to prevent *C. difficile* infection in a mouse model of disease, suggesting that this strategy could be used for the treatment of *C. difficile* infection (69).

CLOSTRIDIAL GERMINANT RECEPTORS

During germination, germinant molecules interact with specific receptors, leading to the release of DPA and ions from the spore core and degradation of the spore cortex (33). After germinant recognition by the receptor, the spore becomes committed to the germination process. Generally, the first easily measured step in germination is the release of DPA from the core, followed by cortex degradation and, eventually, outgrowth (27, 35). In *B. subtilis*, DPA activates SCLEs, and germination can be directly stimulated by exogenous DPA. Similarly, *C. pasteurianum*, some strains of *C. botulinum*, and *C. perfringens* spores derived from food-poisoning-causing isolates germinate in response to DPA. The most widely used germinant receptors are the Ger-type receptors, the orthologues of which are found in most of the Gram-positive, endospore-forming bacteria identified to date (35). However, *C. difficile* does not encode orthologues of the Ger-type germinant receptors, and a novel mechanism was reported for germination by *C. difficile* spores (70–72). Figure 1 depicts the two classes of

germinant receptors and their hypothesized location within the spore (70, 72–75).

ger-TYPE GERMINANT RECEPTORS

The *ger* receptors have been most studied in *B. subtilis* (32). Here, L-alanine binds to the GerA germinant receptor (which is composed of the GerAA-GerAB-GerAC proteins) to initiate germination, while the mixture of L-asparagine, D-glucose, D-fructose, and potassium ions (AGFK) is recognized by the synergistic effects of GerB (composed of GerBA-GerBB-GerBC) and GerK (composed of GerKA-GerKB-GerKC) receptors (32). These Ger-type germinant receptors are thought to be located in or on the inner spore membrane, where they transmit the germinant signal to downstream proteins (35, 74–76).

In almost all clostridia, *ger* receptors are conserved. *C. botulinum* contains an operon with homology to the *gerA* operon, which is essential for *C. botulinum* spore germination (77). The *C. botulinum* GerAB-homologous protein was shown to be located in the inner spore membrane, and the *C. botulinum* GerAA- and GerAC-homologous proteins are suggested to localize to the same region (77, 78). *C. acetobutylicum* also contains genes with homology to *gerAA*, *gerAB*, and *gerAC* (77). In *C. pasteurianum*, the *gerA*-homologous genes code for receptors that do not respond to common germinants (i.e., alanine or AGFK), by which many Ger-type receptors are activated, suggesting that homology alone is not sufficient to predict which germinants are recognized by germinant receptors (27, 32, 77). *Clostridium* strains *C. asparagiforme*, *C. beijerinckii*, *C. butyricum*, *C. hathewayi*, *C. nexile*, *C. leptum*, *C. thermocellum*, *C. novyi*, *C. tetani*, and *C. scindens* also encode *gerA*-homologous proteins, but the requirements for spore germination in many of these organisms are currently unknown (35).

C. sporogenes, a close relative of *C. botulinum* and whose spores respond to similar signals to trigger spore germination (Table 1), also encodes *ger*-type germinant receptors that resemble those found in *C. botulinum* (77). *C. sporogenes* strain ATCC 15579 encodes three tricistronic germinant receptor operons and one tetracistronic germinant receptor operon (*gerXA1*, *gerXA2*, *gerXA3*, and *gerXA4*) (65). Mutational analyses revealed differences in germination profiles between site-directed mutants in each of the *C. sporogenes* and *C. botulinum gerXA* germinant receptors. Brunt and colleagues found *C. sporogenes gerXA1* to be important for germination in tryptone yeast (TY) medium supplemented with lactate but less important for germination in buffer supplemented with certain amino acids (65). However, *gerXA3* was important for germination under all tested conditions, and *gerXA3* alone permitted germination in a strain where the three other germinant receptors were mutated (65). In *C. botulinum*, *gerXA1* and *gerXA3* were required for spore germination, but *gerXA2* was not (65). Thus, despite the close similarity between these two organisms, there are clear differences between their germinant receptor requirements.

C. perfringens does not encode a *gerA* orthologue, but other germinant receptors have been identified by homology searches and functional analyses. The *C. perfringens* GerKA and GerKC gene products are localized to the inner membrane of the spore and were shown to be necessary for activation of SCLEs and for DPA release, which then activate downstream germination events (34, 79). However, unlike what is observed in most spore-forming bacteria, germination by *C. perfringens* spores seems to vary considerably between strains/isolates. It will be interesting to determine how the different germinant receptors among all studied strains vary and contribute to germinant recognition.

csp-TYPE GERMINANT RECEPTORS

The *ger*-type receptors mostly respond to amino acid-based germinants. Typically, those clostridia that do not contain any genes with homology to known *ger*-type receptors do not respond to canonical germinants. For example, *C. difficile* and *Clostridium bartlettii* (recently renamed *Intestinibacter bartlettii*) do not encode orthologues of *ger*-type receptors (35, 72).

In *C. difficile*, the germination-specific protease locus (*csp*) is a bicistronic operon containing *cspBA* and *cspC* (35), where *cspBA* produces a fusion protein of CspB and CspA proteins. These proteins are cleaved to the CspB and CspA proteins, likely by the YabG protease (80). CspC is encoded downstream of *cspBA* and is the receptor for the bile acid germinants (70). Most of our knowledge of the Csp proteases has come from studies in *C. perfringens*. In *C. perfringens*, the subtilisin-like proteases CspA, CspB, and CspC are predicted to be catalytically active (81, 82). Much work has been done on the subtilase family of proteases, and prior work has led to the identification of the residues important for catalysis, and the identification of these residues is based on surrounding sequence motifs (83, 84). Subtilases are commonly produced as a proenzyme in which the prodomain is autocatalytically removed to activate the protease (83, 85–88). In *C. perfringens*, these three proteins are predicted activate the SCLE SleC. In *C. difficile*, the residues important for catalysis are mutated in CspA and CspC, but the catalytic triad characteristic of subtilisin-like proteases is maintained in CspB (88). Adams et al. demonstrated that CspB cleaves the pro-SleC zymogen to an active form (88). Since the catalytic triads are mutated in CspA and CspC, these proteins have alternative functions in *C. difficile*, where CspC is the bile acid germinant receptor (70) and CspA controls the levels of CspC in the spore (80).

Interestingly, it appears that only members of *Peptostreptococcaceae* (e.g., *C. difficile*) use catalytically dead versions of the Csp proteins for spore germination (80). Recently, Kevorkian and colleagues analyzed the Csp loci from several bacteria and found that *Clostridiaceae* (e.g., *C. perfringens*) and *Lachnospiraceae* (e.g., *C. phytofermentans*) maintained catalytically active CspA and CspC proteases (80). The authors suggest that *Peptostreptococcaceae* family members are under selective pressure to diversify their Csp loci, while *Clostridiaceae* and *Lachnospiraceae* are under selective pressure to maintain Csp enzymatic activity (80).

Although the signals that stimulate germination by *C. difficile* spores are not as variable as what is observed for germination by *C. perfringens* spores, there are differences between strains/ribotypes (89–91). Some *C. difficile* strains are more primed to germinate in response to the taurocholic acid germinant than others (91). However, these differences do not appear to be linked to differences in the CspBA or CspC protein sequences, because any differences in protein sequence were ribotype specific, but the observed differences in germination were not (91).

Recently, another germination regulator was identified (GerS) and is hypothesized to regulate germination by an unknown mechanism (73). Interestingly, a *gerS* mutation in *C. difficile* resulted in cleavage of pro-SleC to an active form in response to germinants; however, it appears that SleC was unable to hydrolyze cortex (presumably either by being held in an inactive state or due to modifications of the cortex that prevented SleC activity) (73). It

is possible that the *C. difficile* CspB, CspA, CspC, GerS, and pro-SleC are all part of a "germinosome" that functions to recognize germinants and transmit the germination signal to CspB to activate pro-SleC to initiate cortex degradation (73).

Although this new type of Csp germinant receptor has not been studied as well as the *ger* receptors, and many aspects of the mechanism of spore germination are not understood, the Csp proteins have not been shown to be a receptor in any species other than *C. difficile*. It is possible that other clostridial species that do not encode orthologues of the *ger* receptors initiate spore germination using a similar mechanism or use both the Csp-type germinant receptor in combination with the Ger-type germinant receptor.

CONCLUSIONS AND FUTURE PERSPECTIVES

Spores germinate in response to germinants, and this begins the transformation of a spore into a vegetative cell. These growing cells can produce toxins and cause physiological symptoms associated with certain diseases (92). The vegetative cells eventually sporulate to escape into the environment to either be taken up by other hosts or remain in the current host. If germination were to be blocked before the transformation into a vegetative cell and toxin production, hosts would not develop infections or diseases caused by these bacteria (69). From this perspective, germination is an attractive target for potential therapeutics.

Identifying the signals that trigger germination and the mechanisms by which germination occurs is necessary for understanding how pathogenic bacteria cause disease and how others can lead to food spoilage. Historically, due to the lack of genetic systems, our understanding of the mechanisms of spore germination in clostridia has lagged far behind that of bacilli. However, recent advances in genetics have made such studies feasible (36–40). In clostridia, the germinant signals are commonly amino acids, and their cognate receptors seem to be homologous to the Ger-type receptors identified in *B. subtilis*. Certain clostridia require more than mixtures of amino acids to germinate. As examples, *C. difficile* utilizes cholesterol derivatives (bile acids), and *C. sordellii* recognizes other cholesterol derivatives (progesterone and bile acids) as germinants (41, 43, 45, 59). Although the information on germination is not complete, and some pathogenic strains need to be studied further, genome sequences are becoming more and more available for many different clostridia (not just pathogenic clostridia). These genomes will yield important information about the modes of germination (i.e., whether the organism encodes the classical germinant receptors or potentially germinate using novel mechanisms or both).

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REFERENCES

1. **Finegold SM, Song Y, Liu C.** 2002. Taxonomy—general comments and update on taxonomy of clostridia and anaerobic cocci. Anaerobe **8:**283–285. http://dx.doi.org/10.1006/anae.2002.0436.
2. **Papoutsakis ET.** 2008. Engineering solventogenic clostridia. Curr Opin Biotechnol **19:**420–429. http://dx.doi.org/10.1016/j.copbio.2008.08.003.
3. **Schiel-Bengelsdorf B, Montoya J, Linder S, Durre P.** 2013. Butanol fermentation. Environ Technol **34:**1691–1710. http://dx.doi.org/10.1080/09593330.2013.827746.
4. **Dürre P.** 2014. Physiology and sporulation in *Clostridium*. Microbiol Spectr **2:**TBS-0010–2012.
5. **Desvaux M.** 2005. *Clostridium cellulolyticum*: model organism of mesophilic cellulolytic clostridia. FEMS Microbiol Rev **29:**741–764. http://dx.doi.org/10.1016/j.femsre.2004.11.003.
6. **Sabra W, Groeger C, Sharma PN, Zeng AP.** 2014. Improved *n*-butanol production by a non-acetone producing *Clostridium pasteurianum* DSMZ 525 in mixed substrate fermentation. Appl Microbiol Biotechnol **98:**4267–4276. http://dx.doi.org/10.1007/s00253-014-5588-8.
7. **Scharff RL.** 2012. Economic burden from health losses due to foodborne illness in the United States. J Food Prot **75:**123–131. http://dx.doi.org/10.4315/0362-028X.JFP-11-058.
8. **Willoughby DH, Bickford AA, Cooper GL, Charlton BR.** 1996. Periodic recurrence of gangrenous dermatitis associated with *Clostridium septicum* in a broiler chicken operation. J Vet Diagn Invest **8:**259–261. http://dx.doi.org/10.1177/104063879600800222.
9. **Van Immerseel F, De Buck J, Pasmans F, Huyghebaert G, Haesebrouck F, Ducatelle R.** 2004. *Clostridium perfringens* in poultry: an emerging threat for animal and public health. Avian Pathol **33:**537–549. http://dx.doi.org/10.1080/03079450400013162.
10. **Grass JE, Gould LH, Mahon BE.** 2013. Epidemiology of foodborne disease outbreaks caused by *Clostridium perfringens*, United States, 1998–2010. Foodborne pathogens and disease **10:**131–136. http://dx.doi.org/10.1089/fpd.2012.1316.
11. **Feng G, Churey JJ, Worobo RW.** 2010. Thermoaciduric *Clostridium pasteurianum* spoilage of shelf-stable apple juice. J Food Prot **73:**1886–1890.
12. **Brown KL.** 2000. Control of bacterial spores. Br Med Bull **56:**158–171. http://dx.doi.org/10.1258/0007142001902860.
13. **Yang X, Youssef MK, Gill CO, Badoni M, Lopez-Campos O.** 2014. Effects of meat pH on growth of 11 species of psychrotolerant clostridia on vacuum packaged beef and blown pack spoilage of the product. Food Microbiol **39:**13–18. http://dx.doi.org/10.1016/j.fm.2013.10.008.
14. **Dhaked RK, Singh MK, Singh P, Gupta P.** 2010. Botulinum toxin: bioweapon & magic drug. Indian J Med Res **132:**489–503.
15. **Afshar M, Raju M, Ansell D, Bleck TP.** 2011. Narrative review: tetanus—a health threat after natural disasters in developing countries. Ann Intern Med **154:**329–335. http://dx.doi.org/10.7326/0003-4819-154-5-201103010-00007.
16. **Smedley JG, III, Fisher DJ, Sayeed S, Chakrabarti G, McClane BA.** 2004. The enteric toxins of *Clostridium perfringens*. Rev Physiol Biochem Pharmacol **152:**183–204.
17. **Yutin N, Galperin MY.** 2013. A genomic update on clostridial phylogeny: Gram-negative spore formers and other misplaced clostridia. Environ Microbiol **15:**2631–2641.
18. **Lessa FC, Mu Y, Bamberg WM, Beldavs ZG, Dumyati GK, Dunn JR, Farley MM, Holzbauer SM, Meek JI, Phipps EC, Wilson LE, Winston LG, Cohen JA, Limbago BM, Fridkin SK, Gerding DN, McDonald LC.** 2015. Burden of *Clostridium difficile* infection in the United States. N Engl J Med **372:**825–834. http://dx.doi.org/10.1056/NEJMoa1408913.
19. **McGuigan CC, Penrice GM, Gruer L, Ahmed S, Goldberg D, Black M, Salmon JE, Hood J.** 2002. Lethal outbreak of infection with *Clostridium novyi* type A and other spore-forming organisms in Scottish injecting drug users. J Med Microbiol **51:**971–977. http://dx.doi.org/10.1099/0022-1317-51-11-971.
20. **Roberts NJ, Zhang L, Janku F, Collins A, Bai RY, Staedtke V, Rusk AW, Tung D, Miller M, Roix J, Khanna KV, Murthy R, Benjamin RS, Helgason T, Szvalb AD, Bird JE, Roy-Chowdhuri S, Zhang HH, Qiao Y, Karim B, McDaniel J, Elpiner A, Sahora A, Lachowicz J, Phillips B, Turner A, Klein MK, Post G, Diaz LA, Jr, Riggins GJ, Papadopoulos N, Kinzler KW, Vogelstein B, Bettegowda C, Huso DL, Varterasian M, Saha S, Zhou S.** 2014. Intratumoral injection of *Clostridium novyi*-NT

spores induces antitumor responses. Sci Transl Med **6:**249ra111. http://dx.doi.org/10.1126/scitranslmed.3008982.
21. **Staedtke V, Bai R, Sun W, Huang J, Kibler KK, Tyler BM, Gallia GL, Kinzler K, Vogelstein B, Zhou S, Riggins GJ.** 2015. *Clostridium novyi*-NT can cause regression of orthotopically implanted glioblastomas in rats. Oncotarget **6:**5536–5546. http://dx.doi.org/10.18632/oncotarget.3627.
22. **Zheng L, Zhang Z, Khazaie K, Saha S, Lewandowski RJ, Zhang G, Larson AC.** 2014. MRI-monitored intra-tumoral injection of iron-oxide labeled *Clostridium novyi*-NT anaerobes in pancreatic carcinoma mouse model. PLoS One **9:**e116204. http://dx.doi.org/10.1371/journal.pone.0116204.
23. **Zwagerman NT, Friedlander RM, Monaco EA, III.** 2014. Intratumoral *Clostridium novyi* as a potential treatment for solid necrotic brain tumors. Neurosurgery **75:**N17-18. http://dx.doi.org/10.1227/01.neu.0000457197.94533.68.
24. **Charles JF, Nicolas L, Sebald M, de Barjac H.** 1990. *Clostridium bifermentans* serovar *malaysia*: sporulation, biogenesis of inclusion bodies and larvicidal effect on mosquito. Res Microbiol **141:**721–733. http://dx.doi.org/10.1016/0923-2508(90)90066-Y.
25. **Qureshi N, Chawla S, Likitvivatanavong S, Lee HL, Gill SS.** 2014. The *cry* toxin operon of *Clostridium bifermentans* subsp. *malaysia* is highly toxic to *Aedes* larval mosquitoes. Appl Environ Microbiol **80:**5689–5697.
26. **Deakin LJ, Clare S, Fagan RP, Dawson LF, Pickard DJ, West MR, Wren BW, Fairweather NF, Dougan G, Lawley TD.** 2012. The *Clostridium difficile spo0A* gene is a persistence and transmission factor. Infect Immun **80:**2704–2711. http://dx.doi.org/10.1128/IAI.00147-12.
27. **Paredes-Sabja D, Shen A, Sorg JA.** 2014. *Clostridium difficile* spore biology: sporulation, germination, and spore structural proteins. Trends Microbiol **22:**406–416. http://dx.doi.org/10.1016/j.tim.2014.04.003.
28. **Errington J.** 2003. Regulation of endospore formation in *Bacillus subtilis*. Nat Rev Microbiol **1:**117–126. http://dx.doi.org/10.1038/nrmicro750.
29. **Tan IS, Ramamurthi KS.** 2014. Spore formation in *Bacillus subtilis*. Environ Microbiol Rep **6:**212–225. http://dx.doi.org/10.1111/1758-2229.12130.
30. **Setlow P.** 2016. Spore resistance properties, p 201–215. *In* Driks A, Eichenberger P (ed), The bacterial spore: from molecules to systems. ASM Press, Washington, DC. http://dx.doi.org/10.1128/microbiolspec.TBS-0003-2012.
31. **Permpoonpattana P, Tolls EH, Nadem R, Tan S, Brisson A, Cutting SM.** 2011. Surface layers of *Clostridium difficile* endospores. J Bacteriol **193:**6461–6470. http://dx.doi.org/10.1128/JB.05182-11.
32. **Setlow P.** 2014. Germination of spores of *Bacillus* species: what we know and do not know. J Bacteriol **196:**1297–1305. http://dx.doi.org/10.1128/JB.01455-13.
33. **Setlow P.** 2003. Spore germination. Curr Opin Microbiol **6:**550–556. http://dx.doi.org/10.1016/j.mib.2003.10.001.
34. **Paredes-Sabja D, Torres JA, Setlow P, Sarker MR.** 2008. *Clostridium perfringens* spore germination: characterization of germinants and their receptors. J Bacteriol **190:**1190–1201. http://dx.doi.org/10.1128/JB.01748-07.
35. **Paredes-Sabja D, Setlow P, Sarker MR.** 2011. Germination of spores of *Bacillales* and *Clostridiales* species: mechanisms and proteins involved. Trends Microbiol **19:**85–94. http://dx.doi.org/10.1016/j.tim.2010.10.004.
36. **Cartman ST, Kelly ML, Heeg D, Heap JT, Minton NP.** 2012. Precise manipulation of the *Clostridium difficile* chromosome reveals a lack of association between the *tcdC* genotype and toxin production. Appl Environ Microbiol **78:**4683–4690. http://dx.doi.org/10.1128/AEM.00249-12.
37. **Heap JT, Pennington OJ, Cartman ST, Minton NP.** 2009. A modular system for *Clostridium* shuttle plasmids. J Microbiol Methods **78:**79–85. http://dx.doi.org/10.1016/j.mimet.2009.05.004.
38. **Heap JT, Pennington OJ, Cartmant ST, Carter GP, Minton NP.** 2007. The ClosTron: a universal gene knock-out system for the genus *Clostridium*. J Microbiol Methods **79:**452–464.
39. **Ng YK, Ehsaan M, Philip S, Collery MM, Janoir C, Collignon A, Cartman ST, Minton NP.** 2013. Expanding the repertoire of gene tools for precise manipulation of the *Clostridium difficile* genome: allelic exchange using *pyrE* alleles. PLoS One **8:**e56051. http://dx.doi.org/10.1371/journal.pone.0056051.
40. **Dembek M, Barquist L, Boinett CJ, Cain AK, Mayho M, Lawley TD, Fairweather NF, Fagan RP.** 2015. High-throughput analysis of gene essentiality and sporulation in *Clostridium difficile*. mBio **6**(2):e02383-14. http://dx.doi.org/10.1128/mBio.02383-14.
41. **Wilson KH, Kennedy MJ, Fekety FR.** 1982. Use of sodium taurocholate to enhance spore recovery on a medium selective for *Clostridium difficile*. J Clin Microbiol **15:**443–446.
42. **Akoachere M, Squires RC, Nour AM, Angelov L, Brojatsch J, Abel-Santos E.** 2007. Identification of an *in vivo* inhibitor of *Bacillus anthracis* spore germination. J Biol Chem **282:**12112–12118. http://dx.doi.org/10.1074/jbc.M611432200.
43. **Sorg JA, Sonenshein AL.** 2008. Bile salts and glycine as cogerminants for *Clostridium difficile* spores. J Bacteriol **190:**2505–2512. http://dx.doi.org/10.1128/JB.01765-07.
44. **Gibbs PA.** 1967. The activation of spores of *Clostridium bifermentans*. J Gen Microbiol **46:**285–291. http://dx.doi.org/10.1099/00221287-46-2-285.
45. **Liggins M, Ramirez N, Magnuson N, Abel-Santos E.** 2011. Progesterone analogs influence germination of *Clostridium sordellii* and *Clostridium difficile* spores *in vitro*. J Bacteriol **193:**2776–2783. http://dx.doi.org/10.1128/JB.00058-11.
46. **Shah IM, Laaberki MH, Popham DL, Dworkin J.** 2008. A eukaryotic-like Ser/Thr kinase signals bacteria to exit dormancy in response to peptidoglycan fragments. Cell **135:**486–496. http://dx.doi.org/10.1016/j.cell.2008.08.039.
47. **Scheffers DJ, Pinho MG.** 2005. Bacterial cell wall synthesis: new insights from localization studies. Microbiol Mol Biol Rev **69:**585–607. http://dx.doi.org/10.1128/MMBR.69.4.585-607.2005.
48. **Kanehisa M, Goto S.** 2000. KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Res **28:**27–30. http://dx.doi.org/10.1093/nar/28.1.27.
49. **Kanehisa M, Sato Y, Kawashima M, Furumichi M, Tanabe M.** 2016. KEGG as a reference resource for gene and protein annotation. Nucleic Acids Res **44:**D457–D462. http://dx.doi.org/10.1093/nar/gkv1070.
50. **Peltier J, Courtin P, El Meouche I, Lemee L, Chapot-Chartier MP, Pons JL.** 2011. *Clostridium difficile* has an original peptidoglycan structure with a high level of *N*-acetylglucosamine deacetylation and mainly 3-3 cross-links. J Biol Chem **286:**29053–29062. http://dx.doi.org/10.1074/jbc.M111.259150.
51. **Karasawa T, Ikoma S, Yamakawa K, Nakamura S.** 1995. A defined growth medium for *Clostridium difficile*. Microbiology **141:**371–375.
52. **Bouillaut L, Dubois T, Sonenshein AL, Dupuy B.** 2015. Integration of metabolism and virulence in *Clostridium difficile*. Res Microbiol **166:**375–383. http://dx.doi.org/10.1016/j.resmic.2014.10.002.
53. **Lawley TD, Croucher NJ, Yu L, Clare S, Sebaihia M, Goulding D, Pickard DJ, Parkhill J, Choudhary J, Dougan G.** 2009. Proteomic and genomic characterization of highly infectious *Clostridium difficile* 630 spores. J Bacteriol **191:**5377–5386. http://dx.doi.org/10.1128/JB.00597-09.
54. **Bouillaut L, Self WT, Sonenshein AL.** 2013. Proline-dependent regulation of *Clostridium difficile* Stickland metabolism. J Bacteriol **195:**844–854. http://dx.doi.org/10.1128/JB.01492-12.
55. **Johnson DC, Dean DR, Smith AD, Johnson MK.** 2005. Structure, function, and formation of biological iron-sulfur clusters. Annu Rev Biochem **74:**247–281. http://dx.doi.org/10.1146/annurev.biochem.74.082803.133518.
56. **Reddy D, Lancaster JR, Jr, Cornforth DP.** 1983. Nitrite inhibition of *Clostridium botulinum*: electron spin resonance detection of iron-nitric oxide complexes. Science **221:**769–770. http://dx.doi.org/10.1126/science.6308761.
57. **Adam KH, Brunt J, Brightwell G, Flint SH, Peck MW.** 2011. Spore germination of the psychrotolerant, red meat spoiler, *Clostridium frigidicarnis*. Lett Appl Microbiol **53:**92–97. http://dx.doi.org/10.1111/j.1472-765X.2011.03071.x.
58. **Howerton A, Ramirez N, Abel-Santos E.** 2011. Mapping interactions between germinants and *Clostridium difficile* spores. J Bacteriol **193:**274–282. http://dx.doi.org/10.1128/JB.00980-10.
59. **Bliss DZ, Johnson S, Clabots CR, Savik K, Gerding DN.** 1997. Comparison of cycloserine-cefoxitin-fructose agar (CCFA) and taurocholate-CCFA for recovery of *Clostridium difficile* during surveillance of hospitalized patients. Diagn Microbiol Infect Dis **29:**1–4. http://dx.doi.org/10.1016/S0732-8893(97)00113-2.
60. **Wilson KH.** 1983. Efficiency of various bile salt preparations for stimulation of *Clostridium difficile* spore germination. J Clin Microbiol **18:**1017–1019.

61. **Ridlon JM, Kang DJ, Hylemon PB.** 2006. Bile salt biotransformations by human intestinal bacteria. J Lipid Res **47:**241–259.
62. **Ramirez N, Abel-Santos E.** 2010. Requirements for germination of *Clostridium sordellii* spores *in vitro*. J Bacteriol **192:**418–425. http://dx.doi.org/10.1128/JB.01226-09.
63. **Vidor C, Awad M, Lyras D.** 2015. Antibiotic resistance, virulence factors and genetics of *Clostridium sordellii*. Res Microbiol **166:**368–374. http://dx.doi.org/10.1016/j.resmic.2014.09.003.
64. **Yasuda Y, Tochikubo K.** 1984. Relation between D-glucose and L- and D-alanine in the initiation of germination of *Bacillus subtilis* spore. Microbiol Immunol **28:**197–207. http://dx.doi.org/10.1111/j.1348-0421.1984.tb00671.x.
65. **Brunt J, Plowman J, Gaskin DJ, Itchner M, Carter AT, Peck MW.** 2014. Functional characterisation of germinant receptors in *Clostridium botulinum* and *Clostridium sporogenes* presents novel insights into spore germination systems. PLoS Pathog **10:**e1004382. http://dx.doi.org/10.1371/journal.ppat.1004382.
66. **Blocher JC, Busta FF.** 1985. Multiple modes of inhibition of spore germination and outgrowth by reduced pH and sorbate. J Appl Bacteriol **59:**469–478. http://dx.doi.org/10.1111/j.1365-2672.1985.tb03347.x.
67. **Sorg JA, Sonenshein AL.** 2010. Inhibiting the initiation of *Clostridium difficile* spore germination using analogs of chenodeoxycholic acid, a bile acid. J Bacteriol **192:**4983–4990. http://dx.doi.org/10.1128/JB.00610-10.
68. **Sorg JA, Sonenshein AL.** 2009. Chenodeoxycholate is an inhibitor of *Clostridium difficile* spore germination. J Bacteriol **191:**1115–1117. http://dx.doi.org/10.1128/JB.01260-08.
69. **Howerton A, Patra M, Abel-Santos E.** 2013. A new strategy for the prevention of *Clostridium difficile* infection. J Infect Dis **207:**1498–1504. http://dx.doi.org/10.1093/infdis/jit068.
70. **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. http://dx.doi.org/10.1371/journal.ppat.1003356.
71. **Francis MB, Allen CA, Sorg JA.** 2015. Spore cortex hydrolysis precedes dipicolinic acid release during *Clostridium difficile* spore germination. J Bacteriol **197:**2276–2283. http://dx.doi.org/10.1128/JB.02575-14.
72. **Sebaihia M, Wren BW, Mullany P, Fairweather NF, Minton N, Stabler R, Thomson NR, Roberts AP, Cerdeno-Tarraga AM, Wang H, Holden MT, Wright A, Churcher C, Quail MA, Baker S, Bason N, Brooks K, Chillingworth T, Cronin A, Davis P, Dowd L, Fraser A, Feltwell T, Hance Z, Holroyd S, Jagels K, Moule S, Mungall K, Price C, Rabbinowitsch E, Sharp S, Simmonds M, Stevens K, Unwin L, Whithead S, Dupuy B, Dougan G, Barrell B, Parkhill J.** 2006. The multidrug-resistant human pathogen *Clostridium difficile* has a highly mobile, mosaic genome. Nat Genet **38:**779–786. http://dx.doi.org/10.1038/ng1830.
73. **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:**e1005239. http://dx.doi.org/10.1371/journal.ppat.1005239.
74. **Hudson KD, Corfe BM, Kemp EH, Feavers IM, Coote PJ, Moir A.** 2001. Localization of GerAA and GerAC germination proteins in the *Bacillus subtilis* spore. J Bacteriol **183:**4317–4322. http://dx.doi.org/10.1128/JB.183.14.4317-4322.2001.
75. **Moir A, Corfe BM, Behravan J.** 2002. Spore germination. Cell Mol Life Sci **59:**403–409. http://dx.doi.org/10.1007/s00018-002-8432-8.
76. **Moir A, Smith DA.** 1990. The genetics of bacterial spore germination. Annu Rev Microbiol **44:**531–553. http://dx.doi.org/10.1146/annurev.mi.44.100190.002531.
77. **Broussolle V, Alberto F, Shearman CA, Mason DR, Botella L, Nguyen-The C, Peck MW, Carlin F.** 2002. Molecular and physiological characterisation of spore germination in *Clostridium botulinum* and *C. sporogenes*. Anaerobe **8:**89–100. http://dx.doi.org/10.1006/anae.2002.0424.
78. **Alberto F, Botella L, Carlin F, Nguyen-The C, Broussolle V.** 2005. The *Clostridium botulinum* GerAB germination protein is located in the inner membrane of spores. FEMS Microbiol Lett **253:**231–235. http://dx.doi.org/10.1016/j.femsle.2005.09.037.
79. **Banawas S, Paredes-Sabja D, Korza G, Li YF, Hao B, Setlow P, Sarker MR.** 2013. The *Clostridium perfringens* germinant receptor protein GerKC is located in the spore inner membrane and is crucial for spore germination. J Bacteriol **195:**5084–5091. http://dx.doi.org/10.1128/JB.00901-13.
80. **Kevorkian Y, Shirley DJ, Shen A.** 2016. Regulation of *Clostridium difficile* spore germination by the CspA pseudoprotease domain. Biochimie **122:**243–254. http://dx.doi.org/10.1016/j.biochi.2015.07.023.
81. **Masayama A, Hamasaki K, Urakami K, Shimamoto S, Kato S, Makino S, Yoshimura T, Moriyama M, Moriyama R.** 2006. Expression of germination-related enzymes, CspA, CspB, CspC, SleC, and SleM, of *Clostridium perfringens* S40 in the mother cell compartment of sporulating cells. Genes Genet Syst **81:**227–234. http://dx.doi.org/10.1266/ggs.81.227.
82. **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:**3742–3751. http://dx.doi.org/10.1128/JB.183.12.3742-3751.2001.
83. **Rawlings ND, Barrett AJ, Bateman A.** 2012. MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. Nucleic Acids Res **40:**D343–D350. http://dx.doi.org/10.1093/nar/gkr987.
84. **Siezen RJ, Leunissen JA.** 1997. Subtilases: the superfamily of subtilisin-like serine proteases. Protein Sci **6:**501–523.
85. **Shinde U, Inouye M.** 2000. Intramolecular chaperones: polypeptide extensions that modulate protein folding. Semin Cell Dev Biol **11:**35–44.
86. **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. http://dx.doi.org/10.1007/978-1-61779-204-5_4.
87. **Paredes-Sabja D, Setlow P, Sarker MR.** 2009. SleC is essential for cortex peptidoglycan hydrolysis during germination of spores of the pathogenic bacterium *Clostridium perfringens*. J Bacteriol **191:**2711–2720. http://dx.doi.org/10.1128/JB.01832-08.
88. **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. http://dx.doi.org/10.1371/journal.ppat.1003165.
89. **Heeg D, Burns DA, Cartman ST, Minton NP.** 2012. Spores of *Clostridium difficile* clinical isolates display a diverse germination response to bile salts. PLoS One **7:**e32381. http://dx.doi.org/10.1371/journal.pone.0032381.
90. **Carlson PE, Jr, Kaiser AM, McColm SA, Bauer JM, Young VB, Aronoff DM, Hanna PC.** 2015. Variation in germination of *Clostridium difficile* clinical isolates correlates to disease severity. Anaerobe **33:**64–70. http://dx.doi.org/10.1016/j.anaerobe.2015.02.003.
91. **Bhattacharjee D, Francis MB, Ding X, McAllister KN, Shrestha R, Sorg JA.** 2015. Reexamining the germination phenotypes of several *Clostridium difficile* strains suggests another role for the CspC germinant receptor. J Bacteriol **198:**777–786.
92. **Voth DE, Ballard JD.** 2005. *Clostridium difficile* toxins: mechanism of action and role in disease. Clin Microbiol Rev **18:**247–263. http://dx.doi.org/10.1128/CMR.18.2.247-263.2005.
93. **Waites WM, Wyatt LR.** 1971. Germination of spores of *Clostridium bifermentans* by certain amino acids, lactate and pyruvate in the presence of sodium or potassium ions. J Gen Microbiol **67:**215–222. http://dx.doi.org/10.1099/00221287-67-2-215.
94. **Gibbs PA.** 1964. Factors affecting the germination of spores of *Clostridium bifermentans*. J Gen Microbiol **37:**41–48. http://dx.doi.org/10.1099/00221287-37-1-41.
95. **Waites WM, Wyatt LR.** 1974. The effect of pH, germinants and temperature on the germination of spores of *Clostridium bifermentans*. J Gen Microbiol **80:**253–258. http://dx.doi.org/10.1099/00221287-80-1-253.
96. **Alberto F, Broussolle V, Mason DR, Carlin F, Peck MW.** 2003. Variability in spore germination response by strains of proteolytic *Clostridium botulinum* types A, B and F. Lett Appl Microbiol **36:**41–45. http://dx.doi.org/10.1046/j.1472-765X.2003.01260.x.
97. **Plowman J, Peck MW.** 2002. Use of a novel method to characterize the response of spores of non-proteolytic *Clostridium botulinum* types B, E and F to a wide range of germinants and conditions. J Appl Microbiol **92:**681–694. http://dx.doi.org/10.1046/j.1365-2672.2002.01569.x.
98. **Sarathchandra SU, Barker AN, Wolf J.** 1973. Studies on the germination of *Clostridium butyricum*, p 207–231. *In* Barker AN, Gould GW, Wolf J (ed), Spore research. Academic Press, London, United Kingdom.
99. **Mackey B, Morris J.** 1972. Calcium dipicolinate-provoked germination and outgrowth of spores of *Clostridium pasteurianum*. J Gen Microbiol **73:**315–324. http://dx.doi.org/10.1099/00221287-73-2-315.
100. **Paredes-Sabja D, Setlow P, Sarker MR.** 2009. Role of GerKB in germi-

nation and outgrowth of *Clostridium perfringens* spores. Appl Environ Microbiol **75:**3813–3817. http://dx.doi.org/10.1128/AEM.00048-09.

101. **Udompijitkul P, Alnoman M, Banawas S, Paredes-Sabja D, Sarker MR.** 2014. New amino acid germinants for spores of the enterotoxigenic *Clostridium perfringens* type A isolates. Food Microbiol **44:**24–33. http://dx.doi.org/10.1016/j.fm.2014.04.011.
102. **Paredes-Sabja D, Udompijitkul P, Sarker MR.** 2009. Inorganic phosphate and sodium ions are cogerminants for spores of *Clostridium perfringens* type A food poisoning-related isolates. Appl Environ Microbiol **75:**6299–6305. http://dx.doi.org/10.1128/AEM.00822-09.
103. **Hitzman DO, Halverson HO, Ukita T.** 1956. Requirements for production and germination of spores of anaerobic bacteria. J Bacteriol **74:**1–6.
104. **Shoesmith JG, Holland KT.** 1972. The germination of spores of *Clostridium tetani*. J Gen Microbiol **70:**253–261. http://dx.doi.org/10.1099/00221287-70-2-253.