# INVESTIGATING THE "OUTSIDE IN" MECHANISM OF GERMINATION IN CLOSTRIDIA

A Dissertation

by

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# ABSTRACT

The vegetative cells of Gram-positive, endospore-forming bacteria form dipicolinic acid (DPA)-rich, metabolically dormant spores upon exposure to nutrient or environmental stressors. These spores identify environmental signals (germinants) using germinant receptor proteins in the spore to return to the vegetative state. For the pathogenic bacterium *Clostridioides difficile*, the germination process is an attractive target for the rational design of novel therapeutics. Prior research on germination by *C. difficile* spores has shown that there are significant differences, when compared to what is observed in the model organism, *Bacillus subtilis*, in the germinant receptors used to initiate the germination process. Moreover, there are significant differences in the order of events that occur post-germinant recognition. Here, I describe a newly identified function for the *C. difficile* spore bile acid germinant receptor, CspC. Using semi-quantitative western blot analyses on several *C. difficile* strains, I show that this receptor likely has an inhibitor role for *C. difficile* spore germination. Building upon this work, I also demonstrate that the mechanism of germination by *C. difficile* spores is conserved in another organism, *Paraclostridium bifermentans*. Finally, I developed a reliable and reproducible protocol for transformation *C. difficile* R20291 using electroporation. This protocol also works in another commonly utilized strain, *C. difficile* CD630.

# DEDICATION

Dedicated to family and friends

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## Contributors

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Work in all sections was performed under the direction of Dr. Sorg. All other work described in the dissertation was completed by the student independently.

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# NOMENCLATURE

| | |
|---|---|
| ABE | Acetone-butanol-ethanol |
| ACE | Allele-coupled exchange |
| AF | L-alanine (A), L-phenylalanine (F) |
| AGFK | L-asparagine, D-glucose, D-fructose, and potassium ions |
| ANOVA | One-Way Analysis of Variance |
| AR | L-alanine (A), L-arginine (R) |
| ARF | L-alanine (A), L-arginine (R), L-phenylalanine (F) |
| BHIS | Brain Heart Infusion with 5 g / L yeast extract and 0.1% L-cysteine |
| CA | Cholic acid |
| CaDPA | Ca2+-dipicolinic acid |
| CDCA | Chenodeoxycholic acid |
| CDI | *Clostridium difficile* infection |
| CRISPR | Clustered Regularly Interspaced Short Palindromic Repeats |
| DAP | Diaminopimelic acid |
| DCA | Deoxycholic acid |
| DNA | Deoxyribonucleic acid |
| DPA | Dipicolinic acid (with or without $Ca^{2+}$) |
| DSM | Difco Sporultaion Medium |
| $EC_{50,TA}$ | Taurocholate binding coefficient |
| FN | Functional CspB fused to non-functional CspA |
| KEGG | Kyoto Encyclopedia of Genes and Genomes |

| | |
|---|---|
| LB | Luria Bertani Broth |
| MAL | Muramic-δ-lactam |
| NAG | N-acetylglucosamine |
| NAM | N-acetylmuramic acid |
| PCR | Polymerase chain reaction |
| PVDF | Polyvinylidene difluoride |
| RCM | Reinforced Clostridial Medium |
| SASP | Small acid-soluble protein |
| SCLE | Spore cortex lytic enzyme |
| SDS-PAGE | SDS polyacrylamide |
| SMG | 0.5 M D-sorbitol, 0.5 M mannitol, and 15% glycerol |
| SMP | 270 mM sucrose, 0.938 mM $MgCl_2.6H_2O$, 7 mM sodium phosphate dibasic, 15 % glycerol at pH 7.4 |
| TA | Taurocholic acid |
| TBS | Tris-buffered saline |
| TBST | Tris-buffered saline with 0.1% Tween 20 |
| TY | Tryptone-Yeast extract |
| UV | Ultraviolet |

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

# 1. INTRODUCTION TO CLOSTRIDIAL SPORES: GERMINANTS AND THEIR RECEPTORS IN CLOSTRIDIA*

## 1.1. Introduction

Historically, bacteria found in the genus *Clostridium* were classified by their bacilli shape, anaerobic growth requirements and their ability to form spores (1). However, with the advent of more sophisticated methods for taxonomy (*e.g.*, multi-locus sequence analysis), clostridia have recently undergone a diversification in genus. Though the names of several clostridia 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 and 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 propan-di-ol (6).

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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 food-borne 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 food-borne and nonfood-borne gastrointestinal illnesses in both humans and animals (16). Finally, the Centers for Disease Control and Prevention listed *Clostridium difficile* [recently renamed *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).

Though 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 IV 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*, 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. Though there are certainly differences between *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 is matured 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 (Figure 1). The germ cell wall is surrounded by a peptidoglycan-like cortex layer. 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 (Figure 1). In some spore-forming bacteria, the coat is surrounded by an exosporium layer (31, 32).

During sporulation, the factors required to initiate germination, the transition from a metabolically dormant to a metabolically active state, 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, a 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.

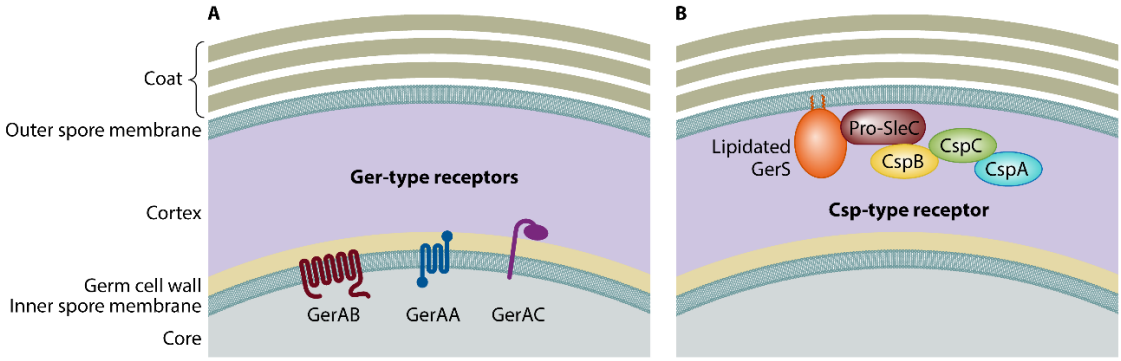



**Figure 1:** Representation of Ger- and Csp-type receptors in the spore (reprinted with permission from (41)). (A) GerAA, GerAB, and GerAC are located within or on the inner spore membrane, and their location in clostridia is based mostly on what is observed in *B. subtilis*. The topology of Ger-type germinant receptors for clostridia is unknown. (B) The *C. difficile* germinant receptor complex contains the bile acid germinant receptor CspC, the CspB protease CspA.

### 1.2. 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 (42-47). Though 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 context of their potential uses in germinated organisms.

**Table 1:** List of clostridial species with their identified germinants and their respective inhibitors (reprinted with permission from (41))

| | Germinants | | | |
|---|---|---|---|---|
| Organism | Amino acids | Minerals | Organic biomolecules | Inhibition |
| *C. bifermentans* | L-alanine, L-arginine, L-phenylalanine [48-51] | $K^+$, $Na^+$ [48] | L-lactate, pyruvate [50] | - |
| *C. botulinum* proteolytic | L-alanine [52] | - | Sodium bicarbonate, exogenous DPA [52-54] | D-alanine, D-serine, D-cysteine, D-phenylalanine, D, methionine, sorbate [55, 56] |
| *C. botulinum* non-proteolytic | L-alanine, L-cysteine, L-serine [54] | - | L-lactate [52, 54] | - |
| *C. botulinum* Group IV type G | L-cysteine [53] | - | Sodium thioglycolate, sodium bicarbonate[53] | - |

**Table 1 continued**

| | Germinants | | | |
|---|---|---|---|---|
| Organism | Amino acids | Minerals | Organic biomolecules | Inhibition |
| *C. butyricum* | L-cysteine [57] | - | Sodium bicarbonate, glucose [57] | - |
| *C. difficile* | Glycine, L-alanine, L-cysteine, L-norvaline, L-2-aminobutyric acid, L-phenylalanine, L-arginine [44, 58] | - | Cholic acid and related bile acids [44, 59, 60] | CDCA and related bile acids, Progesterone and related derivatives [44, 46, 61, 62] |
| *C. frigidicarnis* | L-valine, L-cysteine, L-norvaline, L-threonine, glycine, L-serine, L-alanine [63] | $NaHPO_4$ [63] | L-lactate, sodium bicarbonate [63] | - |

**Table 1 continued**

| | Germinants | | | |
|---|---|---|---|---|
| Organism | Amino acids | Minerals | Organic biomolecules | Inhibition |
| *C. pasteurianum* | - | | exogenous DPA (64) | - |
| *C. perfringens* FP isolates | L-asparagine, L-glutamine, L-cysteine, L-threonine, L-serine (65-67) | KCl, $Na^+$, Pi (65, 66) | exogenous DPA (68) | - |
| *C. perfringens* NFB isolates | L-alanine, L-valine, L-asparagine, L-cysteine, L-threonine, L-serine (65, 67) | KCl (65) | - | - |
| *C. roseum* | L-alanine, L-arginine, L-phenylalanine (69) | - | - | - |

**Table 1 continued**

| | Germinants | | | |
|---|---|---|---|---|
| Organism | Amino acids | Minerals | Organic biomolecules | Inhibition |
| *C. sordellii* | L-alanine, L-arginine, L-phenylalanine [70] | - | Sodium bicarbonate, Progesterone and related derivatives [46, 70] | - |
| *C. sporogenes* | L-alanine [53, 55] | - | L-lactate, sodium bicarbonate [53, 55] | D-alanine [55] |
| *C. tetani* | Methionine [71] | $Na^+$ [71] | nicotinamide, lactate [71] | - |

### 1.2.1. 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 (72). 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 crosslinked to

the 4th amino acid (D-alanine) of the neighboring stem peptide resulting in the cleavage of the terminal D-alanine (72). 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 non-food-borne isolates germinate in response to L-alanine (commonly in combination with other factors, see 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 (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 (73, 74).

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 above).

Finally, glycine can act as a germinant for *C. frigidicarnis* and co-germinant for *C. difficile* spores [and is most-widely used as a co-germinant to stimulate *C. difficile* spore germination in combination with taurocholate, a bile salt] (Table 1). Glycine is not normally found in the cell walls of bacteria. However, Pelteir *et al.* (2011) demonstrated that the *C. difficile* cell wall has several uncommon features (the structure of the *C. frigidicarnis* peptidoglycan is not known) (75). Significantly, the authors found that, in

some stem peptides, glycine was present in the 4$^{th}$ position (in substitution for alanine) (75). Thus, though 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).

### 1.2.2. 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) (73, 74). 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* peptidoglycan, glycine also is important for optimal growth of the organism (75, 76). *C. difficile* can oxidatively deaminate and decarboxylate amino acids in Stickland metabolism to generate ATP and NADH (77). 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 (77). In this reductive branch of Stickland metabolism, glycine reductase regenerates $NAD^+$ and generates acetate, ammonium ion and ATP thus providing important biomolecules for growth (77). Lawley and colleagues (2009) 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) (78, 79).

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 byproduct (which could be used for peptidoglycan synthesis) (80). The sulfur is then incorporated into Fe-S clusters which are required for *C. botulinum* growth (80, 81).

During growth, cells must elongate in order to preserve cell size after division and this process requires membrane (lipid) synthesis. Towards this requirement, *C. frigidicarnis* and spores derived from non-food-borne *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*) (58, 63, 65). 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 can participate in multiple 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-CoA while L-methionine is important for S-

adenylmethione synthesis. Finally, nicotinamide is important for $NAD^+$ synthesis and L-glutamine / L-asparagine are important for ammonia generation (Table 1) (73, 74).

### 1.2.3. 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) (44, 59, 60). Bile acids are cholesterol-derived, small, amphipathic molecules synthesized by the liver (82). 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 (TA) or taurochenodeoxycholic acid). During passage through the intestinal tract, these bile acids are absorbed and recycled back to the liver (82). However, a small portion escapes this enterohepatic recirculation and enters the large intestine where they become substrate for modification by the normal intestinal microbiota. Bile acids are rapidly deconjugated to CA and CDCA and then modified further through 7α-dehydroxylation (82). This enzymatic reaction converts primary bile acids (*e.g.* CA) to secondary bile acids [*e.g.* deoxycholic acid (DCA)]. *C difficile* spore germination is activated by CA-derivatives including deoxycholic acid, a molecule that is growth inhibitory to *C. difficile* vegetative cells (44, 82).

Though *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 (46, 70). This pathogen causes serious infection in post-partum women or in medically-induced abortions using

mifepristone (83). The hormonal changes that occur in post-partum women, or mifepristone itself, are hypothesized to prime or enhance germination by *C. sordellii* spores and thus initiate colonization of the host (46).

### 1.2.4. 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 (84). 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 (55). 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 (58). Germination by some strains of *C. botulinum* is strongly inhibited by sorbate (56). Finally, *C. difficile* germination is competitively inhibited by CDCA and its derivatives (61, 62). Progesterone, and its analogs, also had an inhibitory effect on *C. difficile* germination (46). 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 (85).

### 1.3. Clostridia 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 isolates germinate in response to DPA. The most widely used germinant receptors are of the Ger-type receptors, 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 (86-88). Figure 1 depicts the two classes of germinant receptors and their hypothesized location within the spore (86, 88-91).

#### 1.3.1. 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-AB-AC 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-BB-BC) and GerK (composed of GerKA-KB-KC) 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, 90-92).

In almost all Clostridia, *ger*-receptors are conserved. *C. botulinum* encodes an operon homologous to the *B. subtilis gerA* operon which is essential for *C. botulinum* spore germination (53). The *C. botulinum* GerAB ortholog is located in the inner spore membrane, and the *C. botulinum* GerAA- and GerAC-homologous proteins are suggested to localize to the same region (53, 93). *C. acetobutylicum* also encodes orthologs of *gerAA*, *gerAB* and *gerAC* (53). 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, 53). *C. asparagiforme, C. beijerinckii, C. butyricum, C. hathewayi, C. nexile, C. leptum, C. thermocellum, C. novyi, C. tetani* and *C. scindens* also encode *gerA* orthologs 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* (53). *C. sporogenes* strain ATCC15579 encodes three tri-cistronic germinant receptor operons and one tetracistronic germinant receptor operon (*gerXA1*, *gerXA2*, *gerXA3*, *gerXA4*) (55). 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 (2014) found that *C. sporogenes gerXA1* to be important for germination in TY medium supplemented with lactate but less important for germination in buffer supplemented with certain amino acids

(55). However, *gerXA3* was important for germination in all tested conditions and *gerXA3* alone permitted germination in a strain where the three other germinant receptors were mutated (55). In *C. botulinum*, *gerXA1* and *gerXA3* were required for spore germination but *gerXA2* was not (55). 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 activates downstream germination events (65, 94). 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 amongst all studied strains vary and contribute to germinant recognition.

### 1.3.2. 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, 88).

In *C. difficile*, the germination-specific protease locus (*csp*) is a bicistronic operon encoding *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 (95). CspC is encoded downstream of *cspBA* and is the receptor for the bile acid germinants (86). 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 (96, 97). Much work has been done on the subtilase-family of proteases. 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 (98, 99). Subtilases are commonly produced as a pro-enzyme in which the prodomain is autocatalytically removed to activate the protease (98, 100-103). In *C. perfringens*, these three proteins are predicted to 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 (103). Adams *et al.* (2013) demonstrated that CspB cleaves the pro-SleC zymogen to an active form (103). 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 (86) and CspA controls the levels of CspC in the spore (95). Recently, mutations in *cspA* caused *C. difficile* to germinate without co-germinants, indicating that CspA may be involved in germination with co-germinant, amino acids (104).

Interestingly, it appears that only members of Peptostreptococcaceae (*e.g., C. difficile*) use catalytically-dead versions of the Csp proteins for spore germination (95). Recently, Kevorkian and colleagues (2016) 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 (95). 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 (95).

Though the signals that stimulate germination by *C. difficile* spores is not as variable as what is observed for germination by *C. perfringens* spores, there are differences between strains / ribotypes (105-107). Some *C. difficile* strains are more primed to germinate in response to the taurocholic acid germinant than others (107). 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 (107).

Recently, another germination regulator was identified (GerS) and is hypothesized to regulate germination by an unknown mechanism (89). 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) (89). 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 (89).

Though 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*.

However, the “outside-in” mechanism of germination has been to be conserved in another Clostridia. It is possible that other Clostridial species that do not encode orthologues of the *ger* receptors could initiate spore germination using a similar mechanism or use both the Csp-type germinant receptor in combination with the Ger-type germinant receptor, as *C. perfringens* might (65). In this dissertation, I will attempt to shed new light on how the *C. difficile* bile acid germinant receptor, CspC, functions. In Section 2, I demonstrate that, apart from functioning as the bile acid germinant receptor, *C. difficile* CspC may function to inhibit *C. difficile* spore germination until the bile acid signal is received. In Section 3, I show that the mechanism by which *C. difficile* spores germinate is also conserved in a related organism, *Paraclostridium bifermentans*.

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. In Section 4, I describe the development of a protocol to transform *C. difficile* strains using electroporation.

# 2. RE-EXAMINING THE GERMINATION PHENOTYPES OF SEVERAL *Clostridium difficile* STRAINS SUGGESTS ANOTHER ROLE FOR THE CSPC GERMINANT RECEPTOR*

In Section 2, I find that strains previously observed to germinate in the absence of taurocholic acid demonstrate more potent $EC_{50,TA}$ values towards the germinant and are still inhibited by CDCA. By comparing the germination kinetics and the abundance of proteins in the germinant receptor complex, we revise our original model for CspC-mediated activation of spore germination and propose that CspC may activate spore germination and then inhibit downstream processes.

## 2.1. Introduction

Spore formation and germination by *Peptoclostridium difficile* spores (17) (referred herein as *Clostridium difficile* for simplicity) are significant hurdles for overcoming the transmission of this pathogen within the hospital environment. Due to the strict anaerobic nature of *C. difficile* vegetative cells, spores are thought to be the main reservoir for transmission within the healthcare setting (26, 108). Prior antibiotic treatment is the greatest risk factor for *C. difficile* infection (109). Broad-spectrum antibiotics alter the balance of the colonic microbiota allowing *C. difficile* an opportunity to colonize the newly generated niche (110-112).

Once in a host, *C. difficile* spores germinate to form the toxin-producing vegetative cells that colonize a host's colonic environment. In susceptible hosts, *C. difficile* vegetative cells secrete two toxins that damage the colonic epithelium and lead to the primary symptoms of disease (77, 113). *C. difficile* infections are commonly treated with more antibiotics (*e.g.*, metronidazole, vancomycin or fidaxomicin) (114). After disease symptoms are alleviated and the antibiotics are discontinued, patients frequently relapse with recurring disease due to germination of spores that remain in the colon or that re-infect the host from the surrounding environment (114). Because germination by the spore form is required for pathogenesis, compounds that prevent spore germination could be an attractive way to prevent the primary or recurring infections (62, 85, 86).

Endospores are dormant forms of bacteria and are formed by vegetative cells in response to environmental stress (27, 32). In *Bacillus subtilis*, sporulation is a tightly regulated process and involves the formation of a forespore within a mother cell (29). The forespore and mother cell communicate through a cascade of sigma factor activation and waves of transcription/translation (29). The end result of this developmental program is a dormant spore consisting of a DNA-containing core where much of the water has been replaced by DPA as a calcium chelate (32). Surrounding the spore core is an inner spore membrane, a thin germ-cell peptidoglycan layer, a thick cortex peptidoglycan layer, an outer spore membrane and layers of coat protein (32). The coat proteins, cortex and the contents of the desiccated core help protect the spore from environmental stressors (*e.g.*, heat, radiation, chemicals and antibiotics) and keep the spore in a metabolically dormant state (30, 32).

Endospore germination has been extensively studied in *B. subtilis* (32). In *B. subtilis*, spore germination can be initiated when L-alanine (a germinant) interacts with the GerAA-AB-AC germinant receptor which is embedded within the inner spore membrane (32). This interaction triggers the release of the large cytoplasmic store of CaDPA, presumably through the SpoVA membrane channel (32). This allows the partial hydration of the spore's core. As CaDPA passes through the cortex layer, CaDPA activates the CwlJ cortex hydrolase and the actions of the CwlJ and SleB hydrolases degrade the spore cortex resulting in core expansion and full rehydration of the spore core (32).

The receptors with which spore germinants interact are conserved between most of the studied spore-forming bacteria (*e.g.*, *B. subtilis*, *B. anthracis* and *C. perfringens*) while the germinants which activate spore germination vary between organisms (but germinants are generally amino acids, nucleotides, ions or sugars) (35). *C. difficile* spore germination is initiated by certain bile acids (presumably a host signal) and glycine (presumably a nutrient signal) (27, 42, 44, 60). Bile acids are small, steroid acids that are released by the gall bladder into the digestive tract to aid in the absorption of fats and cholesterol (82). Two families of bile acids are synthesized by the liver: cholic acid-derivatives and chenodeoxycholic acid-derivatives (CDCA). Each of these families is conjugated with either a taurine (TA or taurochenodeoxycholic acid) or a glycine (glycocholic acid or glycochenodeoxycholic acid) (82). *C. difficile* spore germination is stimulated by cholic acid while CDCA-derivatives inhibit cholic acid-mediated germination (44, 58, 61, 62). Based on previous studies, *C. difficile* spores have an $EC_{50}$ value (the concentration that achieves half-maximum germination rate) in the low millimolar range for TA and the high micromolar range for CDCA, suggesting that *C. difficile* spores may have a tighter

interaction with inhibitors of germination than with the activators of germination (58, 61, 62, 86).

Though the signals that stimulate spore germination have been studied for some time, only recently was the receptor with which the bile acids interact identified (86). *C. difficile* does not encode the classical, membrane-embedded, *ger*-type, germinant receptor (88). Instead, *C. difficile* spore germination proceeds through a novel germination pathway involving direct stimulation of cortex hydrolysis and subsequent release of CaDPA from the spore core (opposite to what is observed for germinant receptor-mediated germination in *B. subtilis* or *Clostridium perfringens*) (27, 32, 86, 87, 115). In our working model, *C. difficile* spore germination is initiated when the germination-specific, pseudoprotease, CspC, interacts with the cholic acid-class of bile acids (86). Activated CspC transmits the bile acid activating signal to the CspB protease which then activates the spore cortex lytic enzyme SleC (deposited in the spore as a zymogen) (86, 103). Activated SleC then begins to degrade the spore cortex and CaDPA is subsequently released from the spore core (87, 115).

Based on the model described above, all *C. difficile* isolates should have the requirement for bile acids to stimulate germination. Indeed, spores derived from most clinical isolates require taurocholic acid (TA) to initiate spore germination in rich medium (105, 106). However, a few isolates have been reported not to require TA as a spore germinant or not to be inhibited the CDCA anti-germinant (105). If these strains have no requirement for TA or are not inhibited by CDCA, by what mechanism would they be activated for germination (*i.e.*, would the mechanisms for germination in these strains be different from what has been described previously)?

Here we aimed to re-examine the germination of spores derived from *C. difficile* strains that were reported previously to germinate in rich medium alone or germinate in the presence of a known inhibitor of *C. difficile* spore germination, CDCA (105), and compare this germination to that of spores that clearly require TA for germination (62, 86, 87, 116). Upon reinvestigation of these strains, we find that strains previously thought not to be inhibited by CDCA are actually inhibited by CDCA and interact with CDCA with similar inhibitor constant values as other strains. However, these strains do exhibit greater $EC_{50,TA}$ values, possibly explaining previous observations (105). We further characterize these strains by determining the abundance of the germinant receptor complex and show that the levels of CspC, CspB and SleC differ between strains. By comparing the generated kinetic data with the abundance of spore proteins, we revise our model for the mechanism of initiating *C. difficile* spore germination. Based on our prior genetic data and the data herein, we propose that CspC acts to initiate spore germination in the presence of TA but then to inhibit downstream processes until another signal is received.

## 2.2. Materials and Methods

### 2.2.1. Strains and growth conditions

*C. difficile* strains (Table S1) were grown in a Model B, Coy Laboratories anaerobic chamber (10% $H_2$, 5% $CO_2$, 85% $N_2$) at 37 °C in Brain Heart Infusion supplemented with 5 g / L yeast extract and 0.1% L-cysteine (BHIS), as described previously (61, 86, 87, 116). *Escherichia coli* routinely was grown at 37 °C in LB medium or in 2xTY medium (5 g NaCl, 10 g yeast extract, 16 g tryptone / L) for protein expression (see below). Antibiotics were supplemented as needed (100 μg / mL ampicillin).

### 2.2.2. Molecular Biology

The *C. difficile* UK1 *sleC* gene was amplified with Phusion DNA polymerase (New England Biolabs, Beverly, MA) using primers 5'pET_SleC (TTTTGTTTAACTTTAAGAAGGAGATATACATATGCAAGATGGTTTCTTAACAG TAAGC) and 3'pET_SleC (ATCTCAGTGGTGGTGGTGGTGGTGCTCGAGAATTAAAGGATTTAAAGAAGCT ATT). The resulting PCR product was inserted into pET22b between the XhoI and NdeI restriction sites using Gibson Assembly (117) and transformed into *E. coli* DH5α to generate pKS08. To construct a strain producing CspB for recombinant protein expression, the *cspB* gene was amplified with Phusion DNA polymerase from *C. difficile* UK1 genomic DNA with primers 5'CO_cspB (GTTTAACTTTAAGAAGGAGATATACATATGATTATCATTAATTACGAACTGAT TGTGAAG) and 3'CO_cspB_CPD (TTCCATCCGCGAGCTCGCTTTTATTAATGCTGCG). To aid in solubility and allow for the generation of an un-tagged CspB protein, the cysteine protease domain from the *Vibrio cholerae* MARTX protease (CPD) was fused to the 3'end of *cspB* (118). *cpd* was amplified from the p*cspC-CO-Δ50CPD* plasmid using primers 5'CPD_cspB (CGCAGCATTAATAAAAGCGAGCTCGCGGATGGAA) and 3'CPD_pET (ATCTCAGTGGTGGTGGTGGTGGTGCTCGAGACCTTGCGCGTCCCA). The resulting *cspB* and *cpd* PCR products were inserted into pET22b between restriction sites XhoI and NdeI using Gibson Assembly (117) and the resulting plasmid was named pKS02. The *C. difficile cspC* gene was codon optimized for expression in *E. coli* (Eurofins

Genomics, Ebersberg, Germany). The resulting gene was sub-cloned into pET22b-CPD (118). The sequences of all inserts were verified.

### 2.2.3. Spore preparation

Spores were generated and purified as described previously (86, 87, 116). Briefly, *C. difficile* strains were streaked onto 10 – 15 plates of BHIS agar medium. After 4 days, the growth from each plate was scraped into 1 mL sterile water and incubated overnight at 4 °C. The following day, the growth suspension was washed 5 times in sterile water. During each wash step, the layer of white cell debris was removed. After washing, the spores/cell debris were suspended in 25% (w/v) HistoDenz (Sigma Aldrich, St. Louis, MO.) and layered onto a 50% (w/v) HistoDenz solution. The resulting gradient was centrifuged at 18,900 x g for 30 minutes. During centrifugation, spores migrate through the 50% HistoDenz while the less-dense vegetative cells and cell debris remain at the interface. After centrifugation, the solution is removed and the pellet, containing the purified spores, was washed 5 times in sterile water before being resuspended in a final volume of 1 mL. Purified spores were examined microscopically and were found to be >99.9% pure and phase-bright (dormant).

### 2.2.4. Germination

To quantify the interaction between the bile acids and *C. difficile* spores, purified spores were heat-activated at 65 °C for 30 min and then placed on ice. Ten microliters of the heat-activated spores were added to a final $OD_{600}$ of 0.5 in 990 μL BHIS medium alone or medium supplemented with the indicated concentrations of bile acids and germination

was monitored at 600 nm for 15 minutes in a PerkinElmer (Waltham, MA) Lambda25 UV/Vis Spectrophotometer. The data points at $OD_{600}$ ($T_x$) were normalized to the starting $OD_{600}$ value ($T_0$). Germination rates and $EC_{50}$ values were calculated using the slopes of the linear portions of the germination plots, as described previously (62, 86). The data shown in Figures 1 and 2 are a representative of three independent experiments (the data cannot be averaged between experiments due to differences in time points generated by the PerkinElmer spectrophotometer. For transparency, all germination plots are included Figures S1 – S3.). Rates and $EC_{50}$ values were individually calculated from each germination experiment and are reported as averages with the standard error of the mean.

The release of CaDPA from germinating *C. difficile* spores was monitored in real time using terbium fluorescence (119). An opaque, 96-well plate was prepared with 125 μL of 10 mM Tris (pH 7.5), 150 mM NaCl, 800 μM $TbCl_3$ and 100 mM glycine alone or supplemented with 10 mM TA, as described previously (87). Heat-activated spores were then sedimented for 1 min at 14,000 x g and resuspended in an equal volume of water to remove any CaDPA that may have been released due to auto germinating spores. A 5-μL sample of a spore suspension ($OD_{600}$ = 60) was added to each well, and the CaDPA release was monitored in a Molecular Devices (Sunnydale, CA) Spectramax M3 fluorescence plate reader. CaDPA release was monitored with the following settings: excitation = 270 nm; emission = 545 nm; cutoff = 420 nm. The CaDPA release data are reported as the average of 3 independent experiments and error bars represent the standard error of the mean.

Total DPA content of the spores was determined by suspending $1x10^5$ spores in buffer and incubating at 95 °C for 30 minutes. The spores were cooled and sedimented for

2 minutes at 14,000 x g. The amount of released DPA was determined as above using terbium fluorescence.

### 2.2.5. Protein purification

Overnight cultures of the appropriate BL21 (DE3) strains were diluted 1:200 in 2xTY medium and incubated at 200 rpm and 37 ºC. When an $OD_{600}$ of 0.6-0.8 was reached, IPTG was added to 250 µM final concentration, and cultures were grown at 16 ºC for 12-14 hr. Subsequently, the cultures were pelleted at 6,000 rpm and 4 °C for 30 min and then frozen at -80 °C. Cell pellets were resuspended in LIB1 (50 mM Tris, pH 7.5, 500 mM NaCl, 15 mM imidazole and 10% glycerol) and lysed by sonication. HisPur Ni-NTA resin (Thermo Scientific) was added to the clarified supernatants and washed with LIB2 (50 mM Tris, pH 7.5, 300 mM sodium chloride, 30 mM imidazole and 10% glycerol). For $SleC_{6His}$, the resulting protein was eluted from the Ni-resin in LIB1 supplemented with 500 mM imidazole. For CspB-$CPD_{6His}$ and CspC-$CPD_{6His}$, all proteins were cleaved from the CPD tag in LIB1 supplemented with 75 µM (final concentration) phytic acid at room temperature for 20 minutes with shaking (4 cleavage reactions were sufficient to cleave the respective proteins from the CPD tag). The volume of the phytic acid cleavings was equal to the Ni-beads bed volume. The resulting proteins were analyzed for their purity SDS-PAGE and Coomassie staining and quantified by NanoDrop.

### 2.2.6. Protein extraction and Western Blotting

NuPAGE soluble proteins (which include CspB, CspC and SleC) were extracted from $2x10^9$/mL purified spores as described previously (78). A separate spore extract from

$2x10^9$ *C. difficile* JSC11 (*cspBA::ermB*) spores or *C. difficile* CAA5 (*sleC::ermB*) spores was also generated. Standard amounts of NuPAGE-solubilized recombinant proteins (generated above) were added to a 10% SDS polyacrylamide gel (a standard curve was generated on every gel run). Equal volume of extract from mutant spores was added to the wells containing the recombinant protein standard to allow for equal transfer efficiencies between recombinant protein-containing wells and extract wells. Proteins were separated by SDS-PAGE. After separation, the gels were transferred overnight at 30v to a low fluorescence polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Subsequently, the membrane was blocked for 1 hour at room temperature with 10% dried, skimmed milk in tris-buffered saline (TBS). Each membrane was washed three times for 15 min each with TBS containing 0.1 % (v/v) Tween 20 (TBST). The membrane was then incubated at room temperature with either rabbit anti-CspC, anti-CspB or anti-SleC antibodies. After incubation with the primary antibody, membranes were washed as above in TBST. The membranes were then labeled with Alexa Fluor 555®-labeled donkey anti-rabbit IgG (Life Technologies) in the dark for 2 hours at room temperature. The membranes were again washed as above but in the dark. Finally, the fluorescent signal on the membranes were detected by scanning on a GE Typhoon Scanner using the Cy3 setting (appropriate wavelength for Alexa Fluor 555®). The resulting scans were quantified using ImageQuantTL 7.0, image analysis software provided by GE.

### 2.2.7. Statistical Analyses

Statistical analysis between the calculated $EC_{50,TA}$ or inhibitor constant (CDCA) values was accomplished with a One-Way Analysis of Variance (ANOVA) with Tukey's

test for multiple comparisons. A 99% confidence interval was set for significance (p-val < 0.01)

## 2.3. Results

### 2.3.1. Taurocholic acid-induced germination by several *C. difficile* isolates.

There have been recent reports describing heterogeneity of spore germination between *C. difficile* strains (105, 106). Some of these strains appeared to germinate in rich medium in the absence of the TA germinant. Other strains were not inhibited by CDCA (105). This calls into question the impact bile acids may have on *C. difficile* infection and the utility of germination/antigermination-based therapies. While there are undoubtedly differences in germination responses between *C. difficile* strains, the lack of inhibition by CDCA and germination in the absence of TA warranted further investigation.

Germination by *C. difficile* strains UK1 and M68 have been described previously by our lab and spore germination by these strains is activated by TA and inhibited by CDCA (86). We also analyzed germination by *C. difficile* strains 5108111, DH1834, DH1858, CD2315 (generously provided by Dr. Nigel Minton) and M120. Of these, spores derived from *C. difficile* strains 5108111, DH1834 and CD2315 were reported not to be inhibited by CDCA (spore germination by DH1858 was inhibited by CDCA) (105). To quantitate the differences in germination responses between isolates, we determined the $EC_{50}$ values for taurocholic acid and spores from different *C. difficile* strains. These types of assays have been useful to determine the potency of activators and inhibitors of germination (43, 46, 58, 62, 86, 120). Though not traditional enzyme kinetics (spore germination is a multi-enzyme process), these studies can provide a quantitative measure

for the interaction of the bile acids and *C. difficile* spores. *C. difficile* spores were suspended in BHIS medium alone or in medium supplemented with increasing TA concentrations (2 mM, 5 mM, 10 mM, 20 mM or 50 mM) and germination was monitored at $OD_{600}$. These TA concentrations are routinely used by our laboratory to assign $EC_{50}$ values for bile acids. As shown in Figure 2 and Figure S1, BHIS medium alone did not stimulate germination of the 7 strains tested, suggesting a requirement for TA to initiate spore germination in rich medium. However, when supplemented with increasing concentrations of TA, *C. difficile* UK1, M68 and M120 (Figure 2A, 2F and 2G, respectively) exhibited a concentration-dependent increase in the rate of germination. Interestingly, *C. difficile* strains 5108111, CD2315, DH1834 and DH1858 achieved near maximum germination rates at the lowest tested TA concentration (BHIS + 2 mM TA) (Figure 2B – 2E).

Because these strains germinated at near maximum rates in the lowest tested TA concentration, we modified our germination conditions to determine if these strains are capable of germinating at even lower TA concentrations (Figure S2). When germination by *C. difficile* strains 5108111, CD2315, DH1834 and DH1858 were tested at lower TA concentrations, we observed dose-dependent increases in germination rates. From the newly generated germination plots, we generated rate curves and $EC_{50,TA}$ values (Table 2). Spores from *C. difficile* UK1 and *C. difficile* M68 generated $EC_{50,TA}$ values similar to those described previously (86). *C. difficile* DH1834, DH1858 and M120 had similar $EC_{50,TA}$ values (3.0, 3.03 and 2.37 respectively) and these values are similar to UK1 and M68. Interestingly, compared to the other tested strains, *C. difficile* 5108111 and CD2315 had a

statistically significant increased $EC_{50}$ values for TA (Table 2) suggesting that spores derived from these strains require less TA to activate spore germination.

**Table 2:** Quantifying the interaction of *C. difficile* spores with bile acids (reprinted with permission from (121)).

| Strain | Apparent $EC_{50,TA}$ (mM) | Inhibitor Constant (mM)[a] | Inhibition ratio[b] |
|---|---|---|---|
| UK1 | 2.95 ± 0.23 | 0.21 ± 0.07 | 14.0 |
| 5108111 | 0.76 ± 0.14 * | 0.52 ± 0.03 * | 1.5 |
| CD2315 | 0.42 ± 0.07 * | 0.18 ± 0.03 | 2.3 |
| DH1834 | 3.00 ± 0.45 | 0.21 ± 0.08 | 14.3 |
| DH1858 | 3.03 ± 0.36 | 0.29 ± 0.07 | 10.4 |
| M68 | 3.35 ± 0.39 | 0.12 ± 0.02 | 27.9 |
| M120 | 2.37 ± 0.14 | 0.17 ± 0.02 | 13.9 |

The apparent $EC_{50,TA}$ and inhibitor constant values were calculated from the germination curves as described previously (62, 86, 116).

[a]Inhibitor Constant= [inhibitor]/[($EC_{50,TA}$ with inhibitor)/(($EC_{50,TA}$ without inhibitor) – 1)]

[b]Inhibition ratio = Inhibitor Constant / $EC_{50,TA}$

* For statistical comparisons, the $EC_{50,TA}$ values were compared to each other and the inhibitor constant values were compared to each other as described in the materials and methods. p-val < 0.01

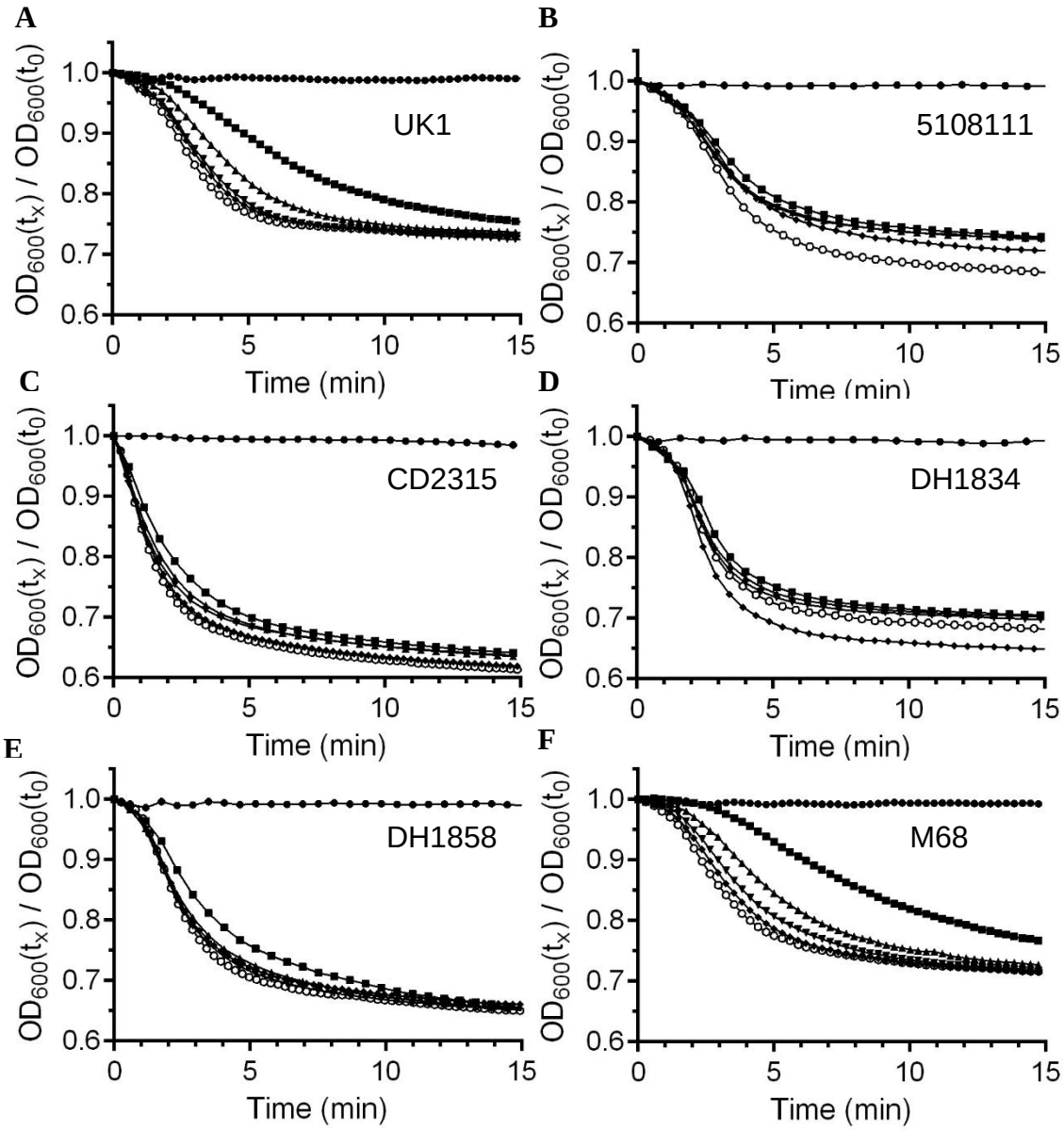
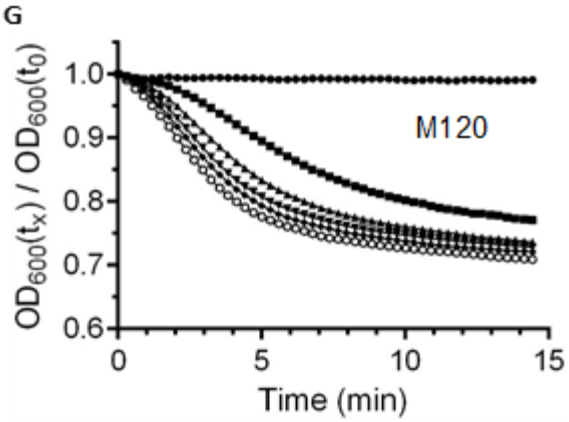



**Figure 2:** Bile acid-mediated spore germination of several *C. difficile* strains (reprinted with permission from (121)). Purified *C. difficile* spores were suspended in BHIS medium alone (●) or medium supplemented with 2 mM (■), 5 mM (▲), 10 mM (▼), 20 mM (♦) or 50 mM (○) TA. Germination was monitored at $OD_{600}$ as described previously. (A) *C. difficile* UK1, (B) *C. difficile* 5108111, (C) *C. difficile* CD2315, (D) *C. difficile* DH1834, (E) *C. difficile* DH1858, (F) *C. difficile* M68, (G) *C. difficile* M120. A representative sample from 3 independent experiments is shown. For transparency, all plots are shown in Figure S1.

**Figure 2 continued**




### 2.3.2. CDCA-dependent inhibition of *C. difficile* spore germination.

Because we observed that some *C. difficile* strains (5108111, CD2315) exhibited a more potent interaction with TA, we tested if there were differences in in the interaction with CDCA. *C. difficile* spores were suspended in BHIS medium or BHIS medium supplemented with 1 mM CDCA and increasing concentrations of TA (2 mM, 5 mM, 10 mM, 20 mM or 50 mM) and germination was monitored at $OD_{600}$. CDCA completely inhibited germination of the 2 mM TA sample for spores derived from all *C. difficile* strains except *C. difficile* strain CD2315. Interestingly, CDCA had less of an effect on germination by *C. difficile* CD2315 spores (Figure 2C). However, CDCA still affected its ability to respond to TA as a germinant. By comparing Figure 1C with Figure 2C, germination by *C. difficile* CD2315 spores clearly is affected by CDCA.

Inhibition constants were generated from the curves in Figure 2/S3 and the calculated $EC_{50,TA}$ (Table 2). *C. difficile* UK1 spores and *C. difficile* M68 spores generated similar inhibitor constant values as previously described (Table 1) (86) and all strains generated values near or below 0.5 mM. Spores derived from *C. difficile* 5108111 had the

weakest interaction with CDCA (0.52 mM; p-val < 0.01) when compared to the other strains. These results suggest that CDCA inhibits germination by spores of *C. difficile* strains previously thought not to be affected by the inhibitor.

Though germination of all strains was inhibited by CDCA, the effectiveness of the inhibitor varied. When the inhibition ratio was calculated (the ratio of the $EC_{50,TA}$ and $EC_{50,CDCA}$), most strains exhibited a ratio greater than 10, suggesting that germination is strongly inhibited by CDCA in these strains (Table 1). Germination by *C. difficile* 5108111 and CD2315 spores was not strongly inhibited by CDCA (the same strains with an increased $EC_{50,TA}$) (Table 1). Taken together, the results suggest that, though there are differences in the interactions of the spores with the bile acids, all tested strains are inhibited by CDCA and are activated by TA.

To determine if the observed differences in the interaction of the bile acids with the *C. difficile* spores correlated with specific alterations in the CspB, CspA or CspC protein sequences, we sequenced the *cspBAC* locus from each strain. (Figure S4 – S6). All ribotype 027 isolates (UK1, 5108111, DH1834 and DH1858) had identical CspC sequences, while ribotypes 078 (CD2315 and M120) and 017 (M68) had 26 substitutions between them (Figure S4). *C. difficile* CD2315 had a P317L substitution that was not present in any of the other tested strains (Figure S4). The CspB sequence only varied in ribotype 078 strains (Figure S5) with 13 substitutions. The CspA proteins varied most between the 3 Csp proteins with a total of 42 substitutions (Figure S6). With the exception of the CD2315 $CspC_{P317L}$ substitution, all substitutions were consistent between ribotypes. Because substitutions were ribotype-specific but the $EC_{50,TA}$ and inhibitor constants were

not, these results suggest that the observed differences are not due to specific changes in the CspB, CspA or CspC sequence.

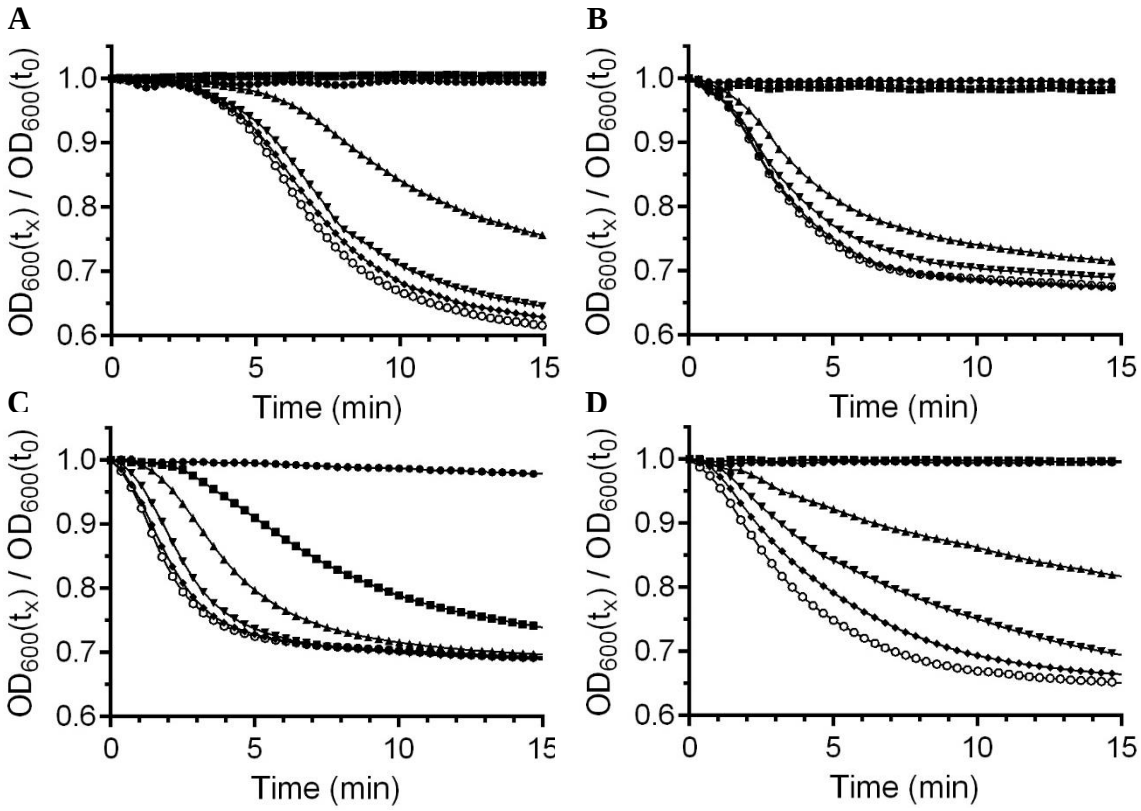
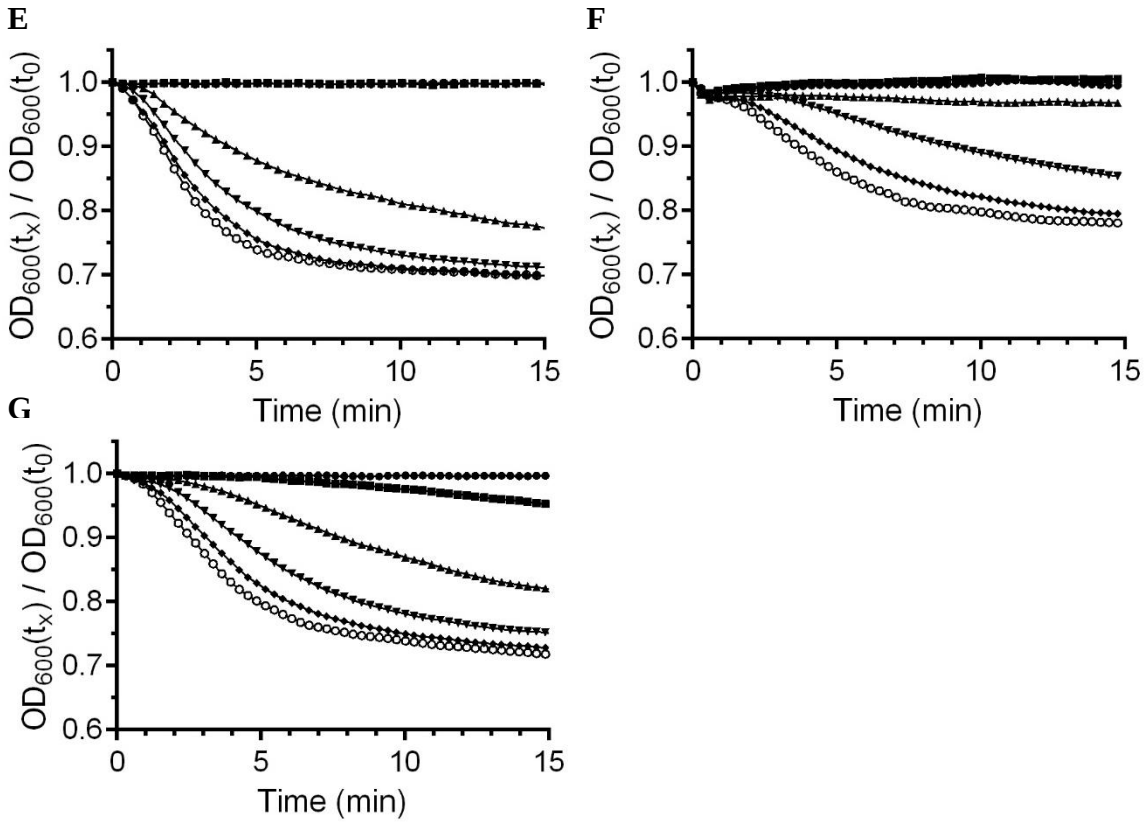



**Figure 3:** Chenodeoxycholic acid inhibits *C. difficile* spore germination (reprinted with permission from (121)). Purified *C. difficile* spores were suspended in BHIS medium supplemented with 1 mM CDCA (●) or medium supplemented with 2 mM (■), 5 mM (▲), 10 mM (▼), 20 mM (♦) or 50 mM (○) TA and 1 mM CDCA. Germination was monitored at OD600 as described previously. (A) *C. difficile* UK1, (B) *C. difficile* 5108111, (C) *C. difficile* CD2315, (D) *C. difficile* DH1834, (E) *C. difficile* DH1858, (F) *C. difficile* M68, (G) *C. difficile* M120. A representative sample from 3 independent experiments is shown. For transparency, all plots are shown in Figure S3.

**Figure 3 continued**




### 2.3.3. Taurocholic acid is required for *C. difficile* spore germination

Previously, germination by *C. difficile* strains 5108111, DH1834, DH1858 and CD2315 occurred slowly but in the absence of the TA germinant (105). To further investigate the phenomenon, we utilized an assay which measures the release of CaDPA from the spore core during germination (CaDPA release is a requirement for spore germination). Because this assay is (i) based on the detection of fluorescent CaDPA-$Tb^{3+}$ complexes and not OD and (ii) each well is mixed prior to analysis, this assay eliminates any chance of spores settling/clumping/localizing to the center of a well during the 4 hour incubation. *C. difficile* spores were suspended in germination buffer supplemented with

glycine only (non-germinating conditions) or TA and glycine (germination-inducing conditions) and $Tb^{3+}$ fluorescence was monitored for 4 hours (Figure S7). Spores from all *C. difficile* strains tested released CaDPA which resulted in $Tb^{3+}$ fluorescence when suspended in both TA and glycine. Spores suspended in glycine alone did not release CaDPA during the 4 hour assay. These results suggest that these strains require TA to induce germination in combination with glycine and have a low frequency of autogermination.

The data presented in Figure S7 suggests that all tested strains have a requirement for TA to stimulate germination in combination with the glycine co-germinant, but not if these strains require glycine to germinate. Therefore we tested CaDPA release in the absence of glycine (Figure 3). *C. difficile* strains UK1 (Figure 4A), 5108111 (Figure 4B), DH1834 (Figure 4D), DH1858 (Figure 4E), M68 (Figure 4F) and M120 (Figure 4G) all required the presence of both TA and glycine to germinate. Interestingly, *C. difficile* CD2315 (Figure 4C) slowly, but significantly, germinated in the presence of TA alone. However, germination by CD2315 spores was enhanced strongly by the presence of glycine (Figure 4C). These results suggest that glycine is a co-germinant for spores of the tested strains but that *C. difficile* CD2315 spores can release CaDPA slowly in the absence of glycine.

To further characterize the differences between these strains, we analyzed the amount of CaDPA present within the core of each strain and normalized this to the amount found in *C. difficile* UK1 (Figure 4H). For all strains, $1x10^5$ spores were boiled in buffer, sedimented and the amount of CaDPA determined using $Tb^{3+}$ fluorescence. As shown in Figure 4H, the amount of CaDPA present in *C. difficile* spores varied between strains

(though only by approximately 2-fold in the lowest DPA-containing strain, *C. difficile* DH1834).

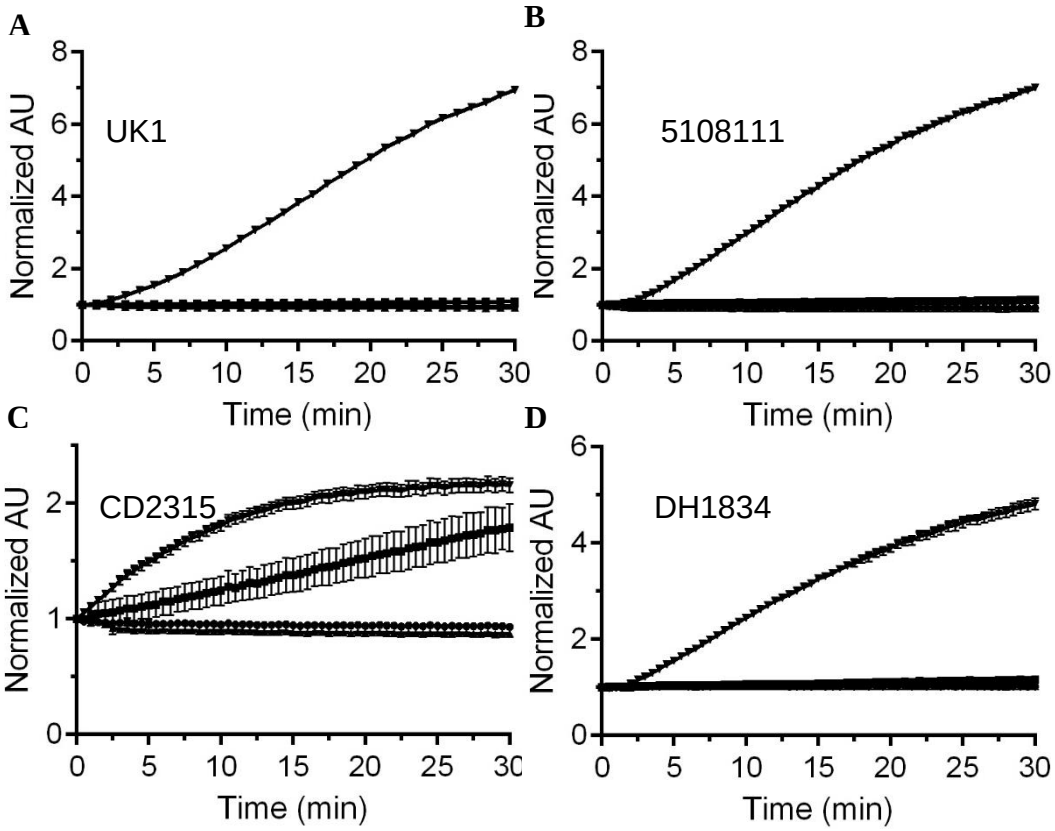
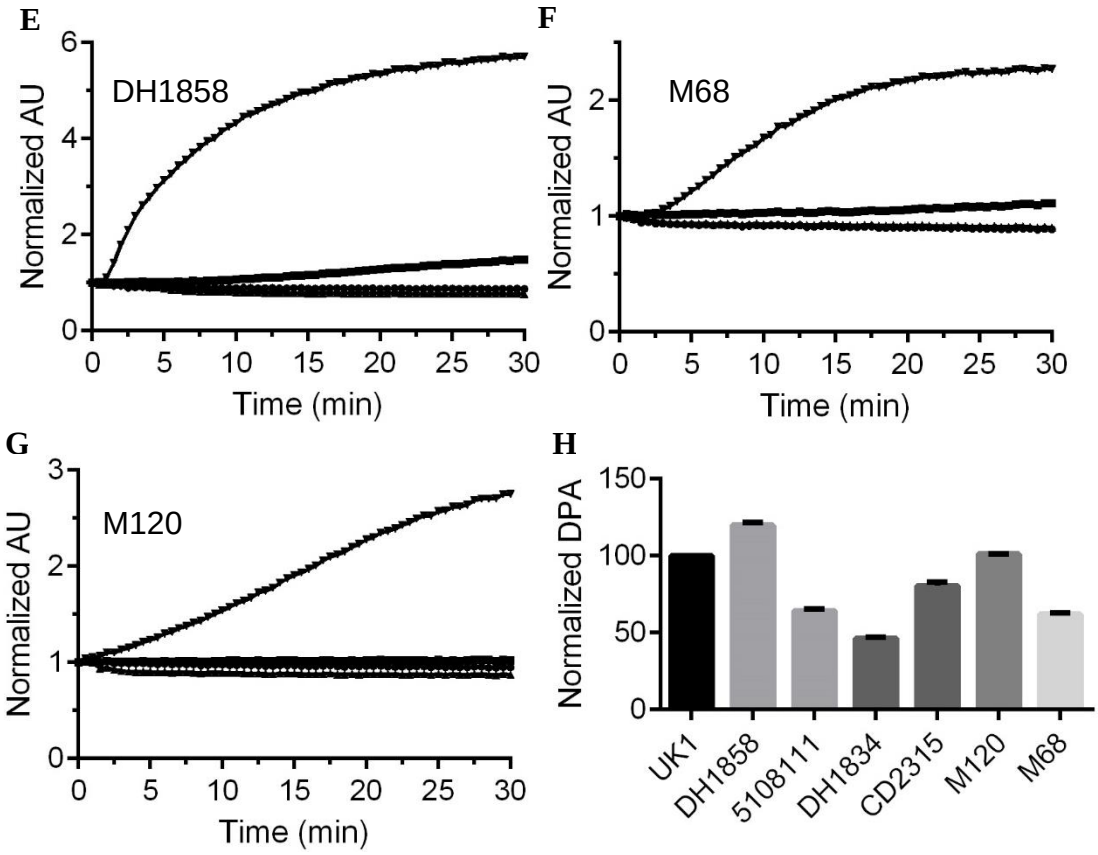



**Figure 4:** DPA release by several *C. difficile* strains (reprinted with permission from (121)). Purified *C. difficile* spores were suspended in HEPES buffer (●) or buffer supplemented with 10 mM TA (■), 100 mM glycine (▲) or 10 mM TA and 100 mM glycine (▼). DPA release during spore germination was monitored using Tb3+ fluorescence, as described previously. (A) *C. difficile* UK1, (B) *C. difficile* 5108111, (C) *C. difficile* CD2315, (D) *C. difficile* DH1834, (E) *C. difficile* DH1858, (F) *C. difficile* M68, (G) *C. difficile* M120. (H) Purified *C. difficile* spores were suspended in buffer and incubated at 100 °C for 30 minutes. Total DPA content was normalized to the amount of DPA found in *C. difficile* UK1. All data represent the average of three independent experiments and error bars represent the standard deviation from the mean.

**Figure 4 continued**




### 2.3.4. Germinant receptor levels vary between *C. difficile* strains.

To begin to understand how different *C. difficile* isolates display different $EC_{50}$ or inhibitor constant values for bile acids, we analyzed the abundance of CspB, CspC and SleC in purified *C. difficile* spores. Purified *C. difficile* spores were extracted with NuPAGE buffer, as described previously (78). We found that by boiling spores in NuPAGE buffer, the amount of remaining CspB, CspC or SleC in the NuPAGE insoluble fraction (containing unbroken spores) to be below the limit of detection. Known amounts of the recombinant CspC, CspB or SleC protein and spore extracts were separated by SDS-PAGE and detected using rabbit polyclonal antisera followed by Alexa Fluor® 555-

conjugated donkey anti-rabbit IgG. The standard curves generated from the signals on each SDS-PAGE were linear (Figure 5). From the signal intensities of the extracted CspB (Figure 5A), CspC (Figure 5B) and SleC (Figure 5C), the volume loaded onto the SDS-PAGE, the molecular weights of each protein and the amount of spores extracted, we were able to determine the average abundance of each protein in a *C. difficile* spore (Table 3).

The abundance of these proteins varied between *C. difficile* isolates. For example, *C. difficile* UK1 had approximately 2,000 CspC molecules per spore while *C. difficile* M68 had over 3,800. Interestingly, *C. difficile* CD2315 and *C. difficile* M120 had a very low abundance of CspB (435 and 633 molecules per spore, respectively). As described previously for *C. perfringens,* the average amount of SleC per spore was greater than that of both CspC and CspB but also varied between strains (Table 3) (122).

Prior work has suggested that much of the OD change observed during spore germination is due to the release of CaDPA from the spore core (123). During *C. difficile* spore germination, this event is dependent on the hydrolysis of the spore cortex by SleC (87). Because SleC is deposited into the spore as a zymogen, SleC must be activated by CspB in order to stimulate germination. In our original model for bile acid mediated spore germination, CspB is activated by the CspC germinant receptor. Because the abundance of CspC varied between isolates, we determined if the maximum rate of spore germination correlated with the abundance of CspC. Thus, for each strain we calculated the maximum rate of germination from the germination curves used to calculate the $EC_{50,TA}$ (Table 4). Surprisingly, there was a slight trend for an inverse correlation of the abundance of CspC and rate ($R^2$ = 0.32; Figure 6A), suggesting that CspC may have an inhibitory effect on spore germination.

Because SleC-mediated cortex hydrolysis is required for CaDPA release during *C. difficile* spore germination, if the above trend is true we should observe a positive correlation between the ratio of SleC to CspC and germination rate (more SleC molecules to CspC molecules should result in an increased rate of germination). As shown in Figure 6B, we observed a strong correlation ($R^2 = 0.81$) between SleC/CspC and germination rate, again suggesting that CspC has an inhibitory effect on spore germination.

**Table 3:** Number of Csp molecules per *C. difficile* spore (reprinted with permission from (121))

| Strain | Average CspB | Average CspC | Average SleC | Ratio SleC/CspC |
|---|---|---|---|---|
| UK1 | 1,394 ± 426 | 2,141 ± 164 | 46,090 ± 5,089 | 21.5 |
| 5108111 | 3081 ± 154 | 2,304 ± 209 | 31,614 ± 3,497 | 13.7 |
| CD2315 | 435 ± 86 | 1,689 ± 137 | 41,239 ± 1,832 | 24.4 |
| DH1834 | 1,178 ± 120 | 3,155 ± 139 | 37,876 ± 2,027 | 12.0 |
| DH1858 | 1,572 ± 60 | 1,435 ± 154 | 37,557 ± 827 | 26.2 |
| M68 | 1,284 ± 160 | 3,836 ± 541 | 46,685 ± 3,507 | 12.2 |
| M120 | 633 ± 36 | 2,423 ± 203 | 29,473 ± 1,724 | 12.2 |

The average number of molecules CspB, CspC and SleC were calculated as described in Figure 5. The numbers represent the averages of three independent extractions ± the standard deviation from the mean.

**Table 4:** Rates of *C. difficile* spore germination (reprinted with permission from (121))

| Strain | Germination rate (OD x $10^{-3}$ / sec) |
|---|---|
| UK1 | 1.47 ± 0.092 |
| 5108111 | 1.30 ± 0.082 |
| CD2315 | 1.67 ± 0.047 |
| DH1834 | 1.23 ± 0.047 |
| DH1858 | 1.62 ± 0.11 |
| M68 | 1.35 ± 0.054 |
| M120 | 1.04 ± 0.043 |

The rates of spore germination were calculated using the linear portion of the germination curves, as described previously (62, 86, 116). Values represent the mean from three independent experiments ± the standard deviation from the mean.

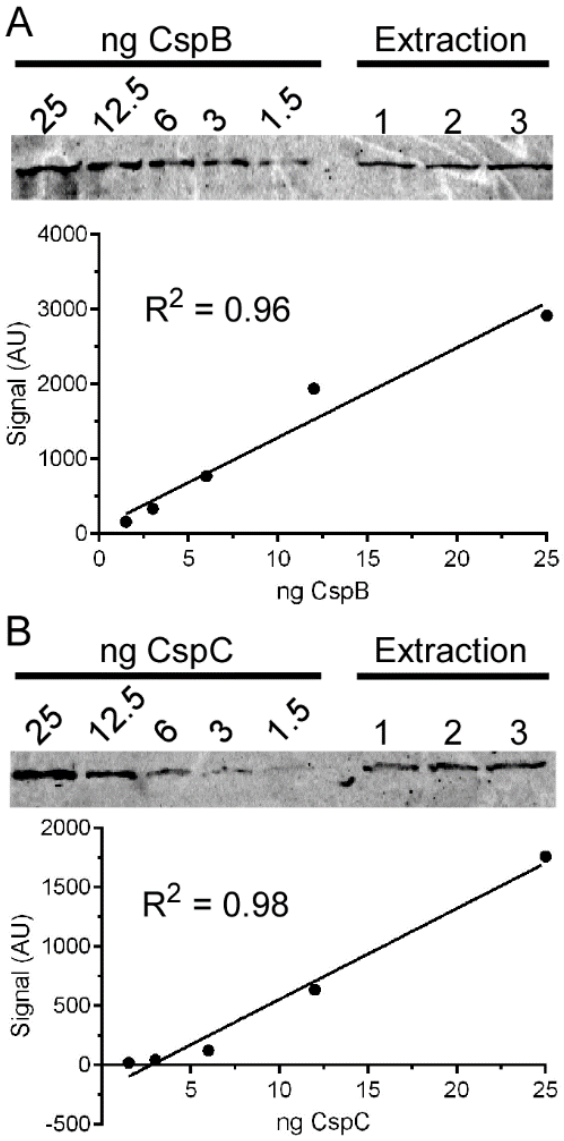
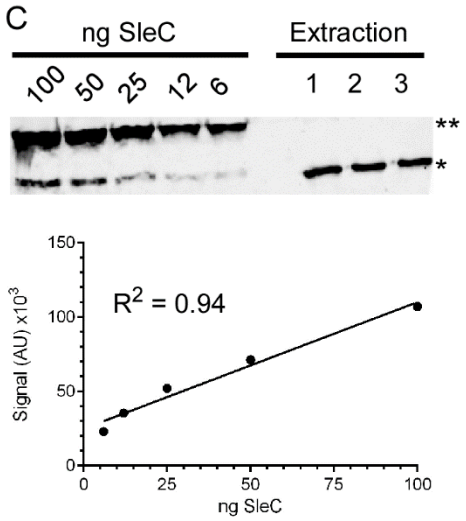



**Figure 5: Quantifying the abundance of CspB, CspC and SleC in *C. difficil*e spores** (reprinted with permission from (121)). 1x109 C. difficile UK1 spores were extracted with NuPAGE buffer, as described previously. NuPAGE-soluble protein from three independent extracts was loaded onto a 10% SDS polyacrylamide gel along with recombinantly expressed and purified protein, as a protein standard. Samples were separated and detected as described in the materials and methods. Signal intensities were quantified and used to generate a standard curve for (A) CspB, (B) CspC and (C) SleC. Signal intensities in the spore extract samples were quantified and the generated standard curve was used to quantify the abundance of the specified proteins in the spore extracts. Independent standard curves were generated for each strain and the results of the quantification can be found in Table 2. ** full length pre-pro-SleC. * pro- SleC.

**Figure 5 continued**



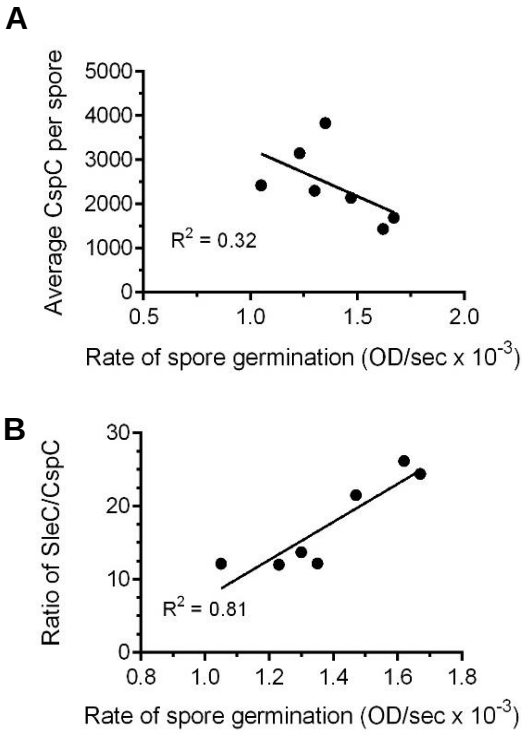




**Figure 6:** Correlating the abundance of *C. difficile* proteins with the kinetics of spore germination (reprinted with permission from (121)). (A) The calculated average per spore abundance of CspC was plotted vs. the calculated rates of spore germination. (B) The ratio of SleC to CspC was plotted vs. the calculated rates of spore germination. For all plots, GraphPad Prism was used to generate the linear fit to the curves and the listed $R^2$ values represent the fit of the curve to the data.

## 2.4. Discussion

Spore germination is an important step in the lifecycle of any spore-forming organism. The metabolically dormant spores must sense when conditions again become favorable for growth and quickly respond to the germinants used by that bacterium. Germination in an environment unfavorable for growth could prevent the outgrowth of a vegetative cell from the germinated spore or prevent the vegetative growth of that bacterium. Thus the signals that stimulate germination (germinants) can vary between organisms depending on their growth niche. For example, *C. difficile* vegetative cells are not normally found growing outside of a host, thus it would be logical for *C. difficile* spores to respond to a molecule that is host-specific. In fact, *C. difficile* spore germination is activated by cholic acid-class bile acids (*e.g.*, TA) and is inhibited by chenodeoxycholic acid-class bile acids (*e.g.*, CDCA) (27, 44, 61, 62). Though essential, TA is not sufficient to stimulate *C. difficile* spore germination. Glycine is also required, presumably as a measure of the nutrient status of the surrounding environment (44, 76, 79).

Until recently, most of the studies on *C. difficile* spore germination have relied on identifying germinants and their interactions with bile acids, bile acid analogs, amino acids and amino acid analogs (44, 58, 61, 62, 86, 120, 124, 125). Only recently have the mechanisms of *C. difficile* spore germination been studied, largely due to breakthroughs in genetics (36-40). These breakthroughs allowed us to determine that the germination-specific, pseudoprotease, CspC, is the bile acid germinant receptor.

The germination-specific proteases have largely been studied in *C. perfringens* (96, 97, 102, 122). In *C. perfringens* the CspA, CspB and CspC proteins are all catalytically active proteases that can cleave the cortex hydrolase, SleC, to an active form. In *C.*

*difficile*, the *cspB* and *cspA* genes have been translationally fused and yield CspBA upon translation (88, 103). The CspBA protein undergoes interdomain processing by the YabG protease to generate CspB and CspA (126). We proposed a model whereby activated CspC transmits the signal to the CspB protease which then activates SleC. In support of this model, we found that CaDPA release from the core is dependent on hydrolysis of the spore cortex (87).

If SleC activation occurs prior to CaDPA release from the spore core and if SleC-mediated cortex hydrolysis is essential for germination to begin, all *C. difficile* strains should have the requirement for bile acids to initiate germination (unless some *C. difficile* strains germinate using yet another novel mechanism for spore germination). However, some *C. difficile* strains were reported to have no apparent requirement for TA-mediated germination or whose germination was not inhibited by CDCA (105). If germination in these strains was not influenced by bile acids, by what mechanism(s) were they germinating?

To characterize the germination phenotypes in these strains, we determined the the $EC_{50,TA}$ and inhibitor constant values for these strains and strains previously shown to be activated by TA and inhibited by CDCA. As suggested previously, the germination characteristics of these strains varied and did not correlate with ribotype (105). Importantly, though, we did observe that some strains have increased $EC_{50,TA}$ values while another had a reduced inhibitor constant for CDCA (Table 2). These strains' increased potency towards TA could possibly explain previous observations (105). Spores derived from strains 5108111, DH1834, DH1858 and CD2315 were found previously to lose $OD_{600}$ in rich medium in the absence of apparent TA (bile acids can be found in blood and,

thus, animal products). Moreover, the authors found that germination of spores derived from strains 5108111, DH1834 and CD2315 was not inhibited by the CDCA anti-germinant. Importantly, those strains whose germination was not inhibited by CDCA had increased $EC_{50,TA}$ values. Because the prior work used only one concentration of TA and CDCA [0.1% (1.8 mM) and 2 mM, respectively], to test the effects of CDCA as an inhibitor, it is likely that these strains' increased $EC_{50,TA}$ overcame the inhibitory effect of CDCA (105). By quantifying the interactions between spores derived from these strains and TA /CDCA, we re-establish the importance of bile acids in promoting and inhibiting germination in these strains.

The differences in $EC_{50,TA}$ and inhibitor constants were not consistent between ribotypes. A growing body of evidence suggests that toxin production, sporulation and germination are not correlated with ribotype (105, 106, 127). In support of this observation, the calculated $EC_{50,TA}$ and inhibitor constant values were not attributed to specific substitutions within the CspB, CspA or CspC protein sequences and substitutions were ribotype-specific. Of the three proteins, CspB had the fewest substitutions. Because CspB is required to cleave pro-SleC, CspB is likely under evolutionary pressure to perform this function. Interestingly, CspC and CspA had 26 and 42 substitutions, respectively. The many substitutions in CspA could suggest that of the Csp proteins, it is under the least amount of selective pressure. Though the role of CspA is unknown, the presence of CspA is important for controlling the levels of the germinant receptor, CspC (126). Only one strain, *C. difficile* CD2315, encoded an extra substitution in the CspC protein sequence that was not present in the other 078 ribotype, P317L. Curiously, *C. difficile* CD2315 is the only strain that germinated in buffered TA without the need for the glycine co-germinant

(though glycine still enhanced germination in this strain suggesting the spore still responds to glycine). Clearly, more studies are needed to characterize the effects of these substitutions on *C. difficile* spore germination.

We further characterized these strains by determining the average per spore abundance of CspB, CspC and SleC (we could not generate a standard curve for CspA – it is insoluble when recombinantly expressed). As expected, the number of molecules per spore varied between isolates and did not correlate with ribotype (Table 3). The abundance of each of these proteins is greater than the amount of germinant receptors found in *B. subtilis* (128). However, the increased abundance of CspB and SleC found in the *C. difficile* spore, compared to the *B. subtilis* germinant receptors, is consistent with a recent publication describing the amount of these proteins in *C. perfringens* (122). And, as seen in *C. perfringens*, the amount of SleC was greater than the amount of CspB. The amount of CspC in the *C. difficile* spore was similar to CspB but varied (Table 3).

By determining the kinetics of spore germination for several *C. difficile* strains, we were positioned to determine if the kinetic properties of germination could be attributed to differences in the abundance of germination-specific proteins (CspB, CspC and SleC). In *B. subtilis*, overexpression of the GerAA germinant receptor results in an increase in the germination rate (as measured by the decrease in $OD_{600}$ and by the release of CaDPA) (129). Prior work has shown that much of the $OD_{600}$ change that occurs during germination is due to the release of CaDPA from the spore core (123). In *C. difficile*, CaDPA release is dependent on SleC-mediated cortex hydrolysis (87, 115). Thus, we hypothesized that an increased abundance of SleC would correlate with an increased rate of germination. Also, because SleC activity is dependent on activation by CspB, an increased abundance of

CspB could result in more active SleC during germination and, thus, an increased rate of germination. However, we did not observe any correlation between the rate of spore germination and the abundance of the SleC hydrolase or CspB protease. One possible explanation for this could be that the abundance of both SleC and CspB in the *C. difficile* spores are far past saturating levels and any alteration in the observed levels would have minimal effects on the rate of germination. Surprisingly, we observed a trend for an inversed correlation between the rate of germination and CspC abundance [the more CspC molecules/spore the slower the rate of germination (Figure 6A)].

If this correlation is true, we predicted that more SleC molecules would be needed to overcome the potential inhibitory effect CspC would have on *C. difficile* spore germination. Indeed, we observed a strong correlation between SleC/CspC and the rate of germination (Figure 6B; $R^2 = 0.81$). These results suggest that, instead of the activator function we originally hypothesized, that CspC may be acting to inhibit *C. difficile* spore germination.

If true, how would CspC function during germination? Our prior genetic data suggests that CspC transmits the bile acid signal to begin the germination process, though the mechanism is unclear (86). Recently, GerS was identified and recognized to play a role during germination (89). GerS is a protein that is anchored to the inner leaflet of the outer spore membrane (though the lipidation of GerS is not required for it to function during germination). Interestingly, *C. difficile gerS*$^-$ strains have a defect in germination but still cleave SleC to the active hydrolase form. This suggested that SleC activity, somehow, is inhibited in this strain (89). Based on the correlation between CspC and SleC abundance and our kinetic data, we hypothesize that CspC activates CspB but inhibits SleC activity in

the absence of GerS. Investigating the biochemistry of this germinant receptor complex could lead to a greater understanding of the mechanism of initiating *C. difficile* spore germination.

# 3. CONSERVATION OF THE 'OUTSIDE-IN' GERMINATION PATHWAY IN *Paraclostridium bifermentans*[*]

In Section 3, I analyzed the germination of *Paraclostridium bifermentans* spores to understand if the mechanism *C. difficile* spore germination is unique or if spores from other organisms germinate in a similar fashion. I found that *P. bifermentans* spores release cortex fragments prior to DPA during germination and the DPA release from the *P. bifermentans* spore core can be blocked by high concentrations of osmolytes. Moreover, I found that *P. bifermentans* spores do not respond to steroid-like compounds (unlike the related *C. difficile* and *P. sordellii* organisms), indicating that the mere presence of the Csp proteins does not permit germination in response to steroid compounds. The findings, here, indicate that the 'outside in' mechanism of spore germination observed in *C. difficile* can be found in other bacteria suggesting that this mechanism is a novel pathway for endospore germination.

## 3.1. Introduction

The endospore-forming *Paraclostridium bifermentans* belongs to the Clostridia family and *P. bifermentans subsp Malaysia* is the only known anaerobic larvicidal toxin producer whose toxins target *Anopheles* and *Aedes* mosquitoes (25).

*Reprinted with permission from "Conservation of the "Outside-in" Germination Pathway in *Paraclostridium bifermentans*" by Bhattacharjee, D. and Sorg, J.A., 2018. Frontiers in microbiology, Volume 9, 1-10, Copyright [2108] by Frontiers in Microbiology.

Due to the anaerobic nature of *P. bifermentans* vegetative cells, the organism likely survives between hosts in the form of a dormant spore. Endospore formation is conserved in many Bacilli and Clostridia, though the sporulation pathway exhibits some differences between organisms (130). Despite these differences, the overall architecture of the metabolically dormant spore is conserved.

Located in the center of the spore, the core contains DNA, RNA, ribosomes and protein, and the core has a low water content with high amounts of 2,4-dipicolinic acid (DPA), which provides resistance against heat (31, 32). Surrounding the spore core is a thin germ cell wall, which becomes the cell wall peptidoglycan of the vegetative cell upon germination (32), and a thick cortex peptidoglycan layer composed of N-acetylglucosamine (NAG), N-acetylmuramic acid (NAM) and muramic-δ-lactam residues. Finally, layers of coat proteins surround the cortex layer and protect the spore from environmental insults.

During spore development, receptors that identify suitable environmental conditions for metabolism and growth are incorporated into the spores by the mother cell or the forespore (28, 41, 86). Upon binding to small molecule germinants, these receptors trigger the irreversible germination process (41). The germination process has been described best in the model spore-forming bacterium, *Bacillus subtilis*. *B. subtilis* spores germinate in response to L-alanine or a mixture of L-asparagine, glucose, fructose and potassium ions (AGFK) (32). These germinants are thought to interact with their respective germinant receptors embedded within the inner spore membrane (32, 91). The GerBA-BB-BC and GerKA-KB-KC germinant receptor responds to AGFK, while the GerAA-AB-AC germinant receptor responds to L-alanine (32). Though the signals that activate spore

germination in other organisms vary, nearly all endospore-forming organisms studied to date encode orthologues of the transmembrane Ger-type germinant receptor (35). Germinant receptor activation leads to the release of monovalent cations and the large depot of DPA, from the channel composed of the SpoVA proteins, resulting in rehydration of the spore core (32). Subsequently, two redundant spore cortex lytic enzymes (SCLE), CwlJ and SleB, are activated and, through their combined actions, the cortex is degraded (32). Cortex degradation allows for full core rehydration, loss of dormancy, restoration of metabolism and, finally, outgrowth of a vegetative cell from the germinated spore.

Unlike what is found in *B. subtilis* and other spore-forming bacteria, *Clostridium difficile* [also *Clostridioides difficile* (131, 132)] does not encode orthologues of the known *ger*-type germinant receptors, suggesting that *C. difficile* spore germination occurs through a novel mechanism or uses novel signals (88). *C. difficile* initiates germination in response to cholic acid derivatives (bile acids) and amino acids (*e.g.*, glycine or alanine), while chenodeoxycholic acid derivatives are competitive inhibitors of cholic acid-mediated germination (27, 44, 61, 62, 121). Though necessary for *C. difficile* spore germination, bile acids are not sufficient (133). A second, amino acid-based signal is required to activate the germination process. Glycine is the best co-germinant, but most other amino acids can substitute with varying efficiencies (44, 134, 135). Recently, calcium was reporting to function as an enhancer of *C. difficile* spore germination, however, it is unclear if it is acting in the role of a *bona fide* germinant or if it functions as a co-factor / essential component of germination proteins (136).

Previously, our lab determined that the bile acid germinant receptor is the germination-specific, subtilisin-like, pseudoprotease, CspC (27, 86, 121). The Csp

proteases were originally studied in *C. perfringens* (96, 97, 102). In *C. perfringens,* the CspA, CspB and CspC proteases cleave the inactive pro-SleC, to its active form resulting in cortex degradation (97). The *C. perfringens* CspB, CspA and CspC proteins can be extracted from spore coats (97). But, in the article that describes this, no control was given for cortex-localized proteins suggesting that these proteins could be coat- or cortex-localized (97). In *C. difficile*, the *cspB* and *cspA* sequences are fused, translationally, and *cspC* is encoded downstream of *cspBA* (103). Though CspB is produced as a fusion with CspA, the timing for interdomain cleavage and the fate of CspA after cleavage is still unknown. However, the loss of CspA leads to a significant decrease in spore germination, and CspA has been shown to control CspC levels in the spore (86, 95). CspB is capable of processing pro-SleC to its active form. However, the catalytic triads that are characteristic of subtilisin-like proteases are absent in both CspA and CspC, and loss of *cspA* or *cspC* negatively affects spore germination, suggesting that CspA and CspC function in a regulatory role and not a catalytic role (86, 103).

Based upon the predicted location of CspB, CspA, CspC and SleC (near the cortex layer and not in / on the inner spore membrane) (137), we hypothesized that *C. difficile* spore germination may be initiated differently than what had been described in other endospore-forming bacteria (32, 87, 123). Specifically, we hypothesized that bile acids and glycine would stimulate cortex degradation prior to the release of DPA from the spore core (a process opposite to what is observed during *B. subtilis* spore germination). Indeed, cortex degradation precedes release of DPA from the spore core and the release of DPA is dependent on the osmotic changes that occur at the inner membrane when cortex is degraded (87, 138). This suggests that *C. difficile* spore germination proceeds through a

novel spore germination pathway where the germinants stimulate cortex degradation. In order to understand if this mechanism of germination is unique to *C. difficile* or if other organisms share this pathway of spore germination, we analyzed germination in *P. bifermentans*. *P. bifermentans* encodes a *csp* locus that is similar to what is observed in *C. difficile* where the *cspB* and *cspA* sequences are translationally fused and *cspA* and *cspC* do not encode proteins with complete catalytic triads. Moreover, *P. bifermentans* encodes a peptidoglycan binding protein that is 56% similar to the peptidoglycan degrading protein SleC from *C. difficile*. Herein, we find that *P. bifermentans* cortex degradation precedes the release of DPA from the spore core and the release of DPA can be delayed by high concentrations of osmolytes. Our data suggest that, like *C. difficile*, *P. bifermentans* spores germinate through an 'outside-in' mechanism and add to the list of organisms that germinate through this novel pathway of spore germination.

## 3.2. Materials and Methods

### 3.2.1. Bacterial Strains

Wild type *P. bifermentans* ATCC 19299 was purchased from the American Type Culture Collection (ATCC) and grown in an anaerobic atmosphere (10% $H_2$, 5% $CO_2$, 85%$N_2$) at 37°C on Difco Reinforced Clostridial Medium agar (RCM) medium, as recommended by ATCC. *B. subtilis* PS533 was grown on Difco Sporulation Medium (DSM) medium and LB medium.

### 3.2.2. Sporulation

*P. bifermentans* cells were streaked onto pre-reduced Duncan-Strong Sporulation Media (DSSM) agar plates (139) under anaerobic conditions at 37°C. The cells were allowed to grow for 3-4 days before harvesting by scraping the growth into sterile water. *B. subtilis* cells were streaked onto DSM agar medium and allowed to grow for 4 days at 30°C, as described previously (87), and harvested as described previously(87).

### 3.2.3. Spore purification

*S*pores, vegetative cells, debris and any agar that contaminated the harvested preparation (*P. bifermentans* and *B. subtilis* grew into the agar surface) were stored overnight at 4°C. Agar was removed from the scraped spores by incubating the suspension at 75°C for 1 hour, as described previously (87). The resulting suspension was washed 5 times in sterile water and purified on 60% (w/v) sucrose solution as described previously (121). Purified spores were again washed 5 times and stored in 1 mL sterile water. Purified spores appeared phase bright and did not contain observable vegetative cells.

### 3.2.4. Spore Germination

Purified *P. bifermentans* spores were heated for 30 min at 75°C (heat activation was required for *P. bifermentans* spore germination; Figure S9) and *B. subtilis* spores at 80°C prior to germination (87, 140). Spores were added to Falcon clear 96 well plates containing germination buffer (50 mM HEPES pH 7.5, 100 mM NaCl) alone or in buffer supplemented with germinants (50 mM L-Alanine, 5 mM L-Phenylalanine, 5 mM L-Arginine for *P. bifermentans*; 100 mM L-valine for *B. subtilis*). Sorbitol was added where

indicated. Germination of the $OD_{600}$ = 0.5 – 0.7 spore suspension was measured over time at $OD_{600}$ nm in SpectraMax M3 plate reader at 37°C.

The release of DPA from $OD_{600}$ = 0.25 – 0.3 spores was measured by adding 250 µM terbium chloride (final concentration) to germination buffer (above) with or without added germinants and / or osmolyte, as described previously (121). Terbium ($Tb^{3+}$) fluorescence was monitored using a SpectraMax M3 plate reader with excitation at 270 nm and emission at 545 nm with a 420 nm cut-off as previously described (121).

### 3.2.5. Cortex hydrolysis

Cortex hydrolysis assay was performed for *P. bifermentans* spores, as previously published (87). Spores were suspended in germination buffer at $OD_{600}$ ~3. However, the germinant concentrations were increased to 100 mM each of L-Alanine, L-Phenylalanine and L-Arginine to increase germination of the spores.

### 3.2.6. Sequence analysis

The accession number of the *P. bifermentans* ATCC 19299 genome is NZ_AVNB01000000. The CspBA protein sequence was identified by protein BLAST. The protein *C. difficile* CspBA had 68% identity to Accession EQK47151.1. This protein, in turn, was encoded between 84673 and 86385 of the whole genome shotgun sequence contig 15 (Accession AVNB01000015.1). Similarly, *P. bifermentans* CspC protein sequence was identified by protein BLAST as EQK47087.1, encoded between 86404 and 88077 of the whole genome shotgun sequence contig 15 (Accession AVNB01000015.1).

### 3.2.7. Statistical Analyses

Data points represent the average from three, independent experiments and error bars represent the standard error of the mean. Statistical significance was determined using a 2-way ANOVA with Sidak's multiple comparisons test.

## 3.3. Results

### 3.3.1. Identifying potential *P. bifermentans* germination receptors

Prior research from our laboratory demonstrated that there are differences between mechanisms for the initiation of spore germination observed in *B. subtilis* and *C. difficile* (87, 138). The primary difference is the presence of *csp*-type germinant receptors and the absence of *ger*-type receptors in *C. difficile* (35, 86). *P. bifermentans* also encodes homologues to *C. difficile cspBA* and *cspC* (Figure 7A). In the unannotated *P. bifermentans* whole genome sequence, the *cspBA* gene is predicted to encode a truncated protein. Upon sequencing the *P. bifermentans cspBA* gene, we found that there is a sequencing error in the *cspBA* gene in the deposited NCBI sequence in the form of a deletion of an adenine within a stretch of consecutive adenine residues (Supplementary Fig S8). This indicates that the *P. bifermentans* CspBA protein sequence is intact and has the potential to function similarly to what is observed in *C. difficile.*

To determine if *P. bifermentans* encodes a *gerAA* orthologue, we used BLAST to search the *P. bifermentans* genome for the orthologs of the *B. subtilis* GerAA protein. Interestingly there is an homolog of *gerA* sequence in *P. bifermentans*. Using this protein sequence as a query, we found that the *P. bifermentans* sequence most-closely matches the *B. subtilis* SpoVAF spore protein and not GerA. Taken together, these results suggest that

*P. bifermentans* does not encode *ger*-type germinant receptors but encodes a *csp* locus that is similar to that of *C. difficile*.

### 3.3.2. Germination of *P. bifermentans* spores in response to amino acids

Previously, the germinants for *P. bifermentans* spores were identified (48, 141). In order to dissect the mechanism of *P. bifermentans* spore germination, we monitored germination using both change in $OD_{600}$ (which measures the sum of events during loss of dormancy) and release of DPA (as measured by $Tb^{3+}$ fluorescence). Purified spores were suspended in HEPES-buffer alone or supplemented with L-alanine (A), L-arginine (R), L-phenylalanine (F) or all three amino acids (ARF). *P. bifermentans* spores rapidly germinated in the presence of ARF but not when exposed to the amino acids individually (Figure 7B). When DPA release was assayed, ARF stimulated the rapid release of DPA from the germinating *P. bifermentans* spores (Figure 7C). Next, we tested whether binary combinations of the amino acids could stimulate *P. bifermentans* spore germination. Though ARF was the best activator of spore germination, AF and AR could also stimulate germination as measured by OD change (Figure 7D) and DPA release (Figure 7E). As determined by the rate of OD change and the rate of DPA release, AF was a weaker activator of spore germination than ARF, AR was weaker than AF and AL was weaker than AR (ARF > AF > AR > AL). These results suggest that *P. bifermentans* spore germination requires at least L-alanine and another amino acid (L-arginine, L-phenylalanine or L-leucine) and that L-alanine is an essential, but not sufficient, germinant for *P. bifermentans* spores. We then tested other amino acids to understand if other amino acids can synergize with L-alanine to stimulate *P. bifermentans* spore germination. In

doing so, we found that L-leucine can function as a germinant with L-alanine as measured by both germination at $OD_{600}$ (Figure 7F) and DPA release (Figure 7G).

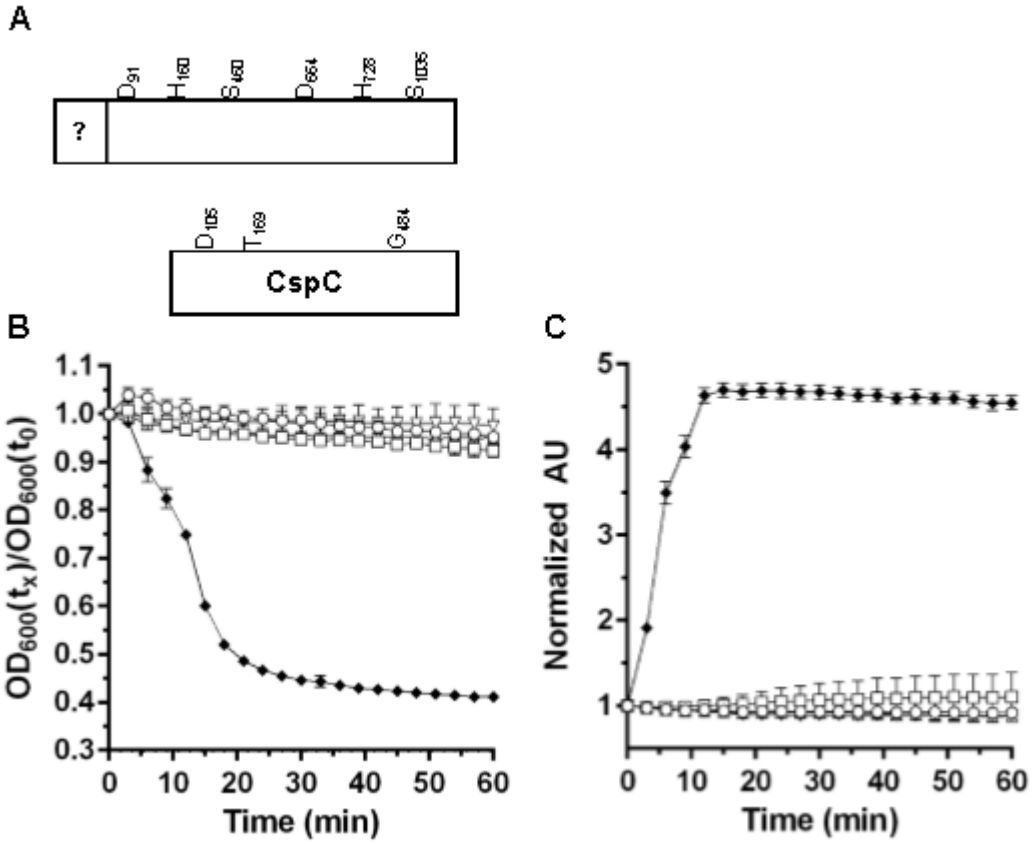
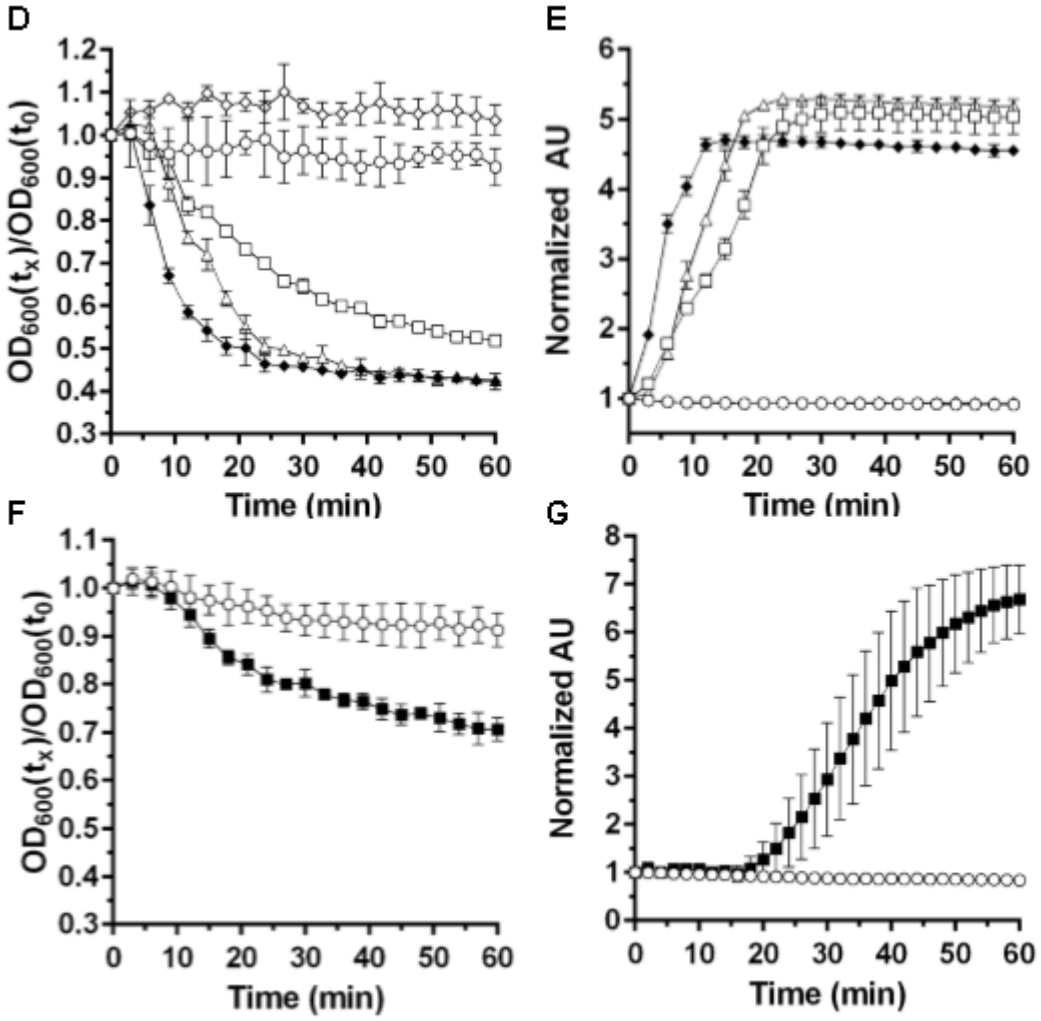



**Figure 7:** Characterizing the germinants of *P. bifermentans* spores (reprinted with permission from (142)). (A) Illustration of the *P. bifermentans* CspBA and CspC proteins and the location of the predicted catalytic residues. Germination of *P. bifermentans* spores was analyzed by changes in $OD_{600}$ (B, D and F) and Tb3+ fluorescence upon complexing with DPA (C, E, G). Germination assays were conducted in 50 mM HEPES, 100 mM NaCl at pH 7.5 buffer supplemented amino acids. (B and C) Spores alone, ○; 50 mM L-alanine □; 50 mM L-arginine, Δ; 50 mM L-phenylalanine, open inverted triangle ; 50 mM L-alanine, 5 mM L-phenylalanine, 5 mM L-arginine, ♦; (D and E). Spores alone, ○; 50 mM L-alanine, 5 mM L-arginine, □; 50 mM L-alanine, 5 mM L-phenylalanine, Δ; 5 mM L-phenylalanine, 5 mM L-arginine, ◊; 50 mM L-alanine, 5 mM L-phenylalanine, 5 mM L-arginine, ♦; (F and G) Spores alone, ○; 50 mM L-alanine, 5 mM L-leucine, ■. (■). Data points represent the average from three independent experiments and error bars represent the standard deviation from the mean.

**Figure 7 continued**




### 3.3.3. Bile acids do not influence *P. bifermentans* spore germination

*C. difficile* germination is activated in response to a combination of cholic acid-class bile acids and an amino acid (*e.g.*, glycine) (44, 58, 121). In *Clostridium sordellii*, a related organism, which also encodes orthologs of *cspBA* and *cspC*, ARF-mediated spore germination is enhanced by steroid-like compounds, including bile acids(46). Therefore, we hypothesize that steroid / bile acid recognition may be a property of organisms that encode the *cspBAC* locus. To determine whether bile acids can be recognized in *P.*

*bifermentans*, we germinated *P. bifermentans* spores in presence of various bile acids [taurocholic acid (TA), deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA)] (Figure 8). *P. bifermentans* spores did not germinate in response to bile acids alone and still required ARF to activate spore germination. Interestingly, the OD change during *P. bifermentans* spore germination was faster in the presence of 2 mM TA (Figure 8A), similar to what was observed in *C. sordellii*, though DPA release by *P. bifermentans* spores was unaffected (Figure 8B). CDCA, normally an inhibitor of germination in *C. difficile*, did not affect germination by *P. bifermentans* spores (Figures 8C and 8D). However, similar to TA, DCA increased the rate of germination measured by OD (Figure 8E) but did not influence the release of DPA (Figure 8F). To confirm that these observations are not due to a detergent-like effect of TA or DCA, spores were germinated in the presence of 2 mM Triton X-100. Triton X-100 did not increase the rate of germination by *P. bifermentans* spores either by OD (Figure 8G) or DPA release (Figure 8H). These results suggest that, though TA and DCA increase the rate of OD change during germination, DPA release is unaffected by bile acids suggesting that they do not influence *P. bifermentans* spore germination and the observed effects on OD are likely an artifact.

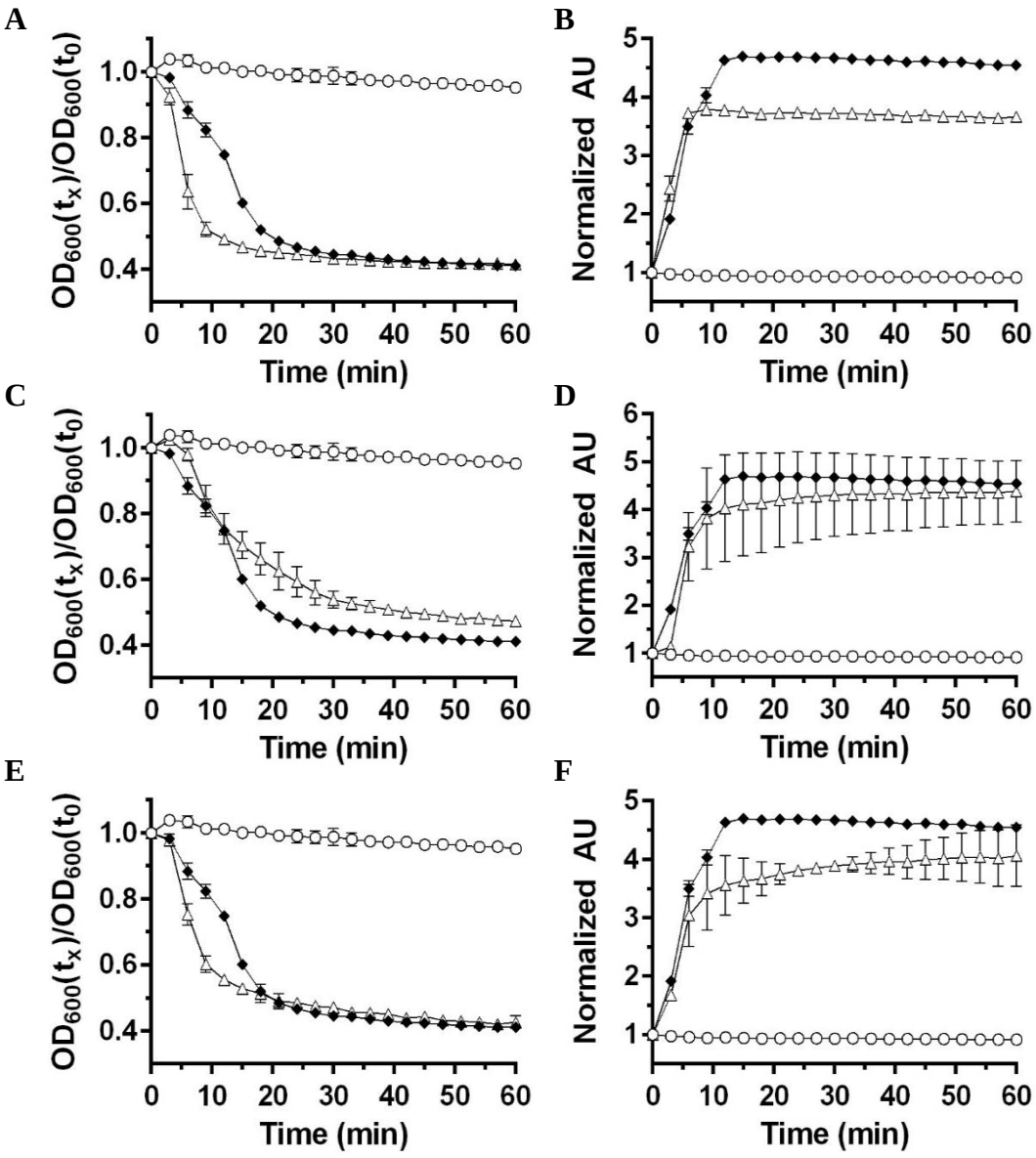
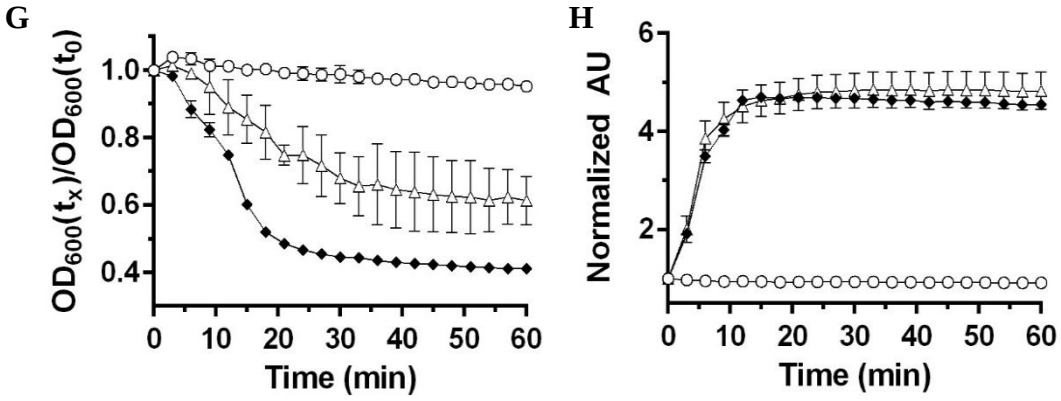



**Figure 8:** Analyzing the influence of bile acids on *P. bifermentans* spore germination (reprinted with permission from (142)). Germination of *P. bifermentans* spores was analyzed by changes in $OD_{600}$ (A, C, E and G) and Tb3+ fluorescence upon complexing with DPA (B, D, F, H). Spores, ○; 50 mM L-alanine, 5 mM L-phenylalanine, 5 mM L-arginine, ♦; 50 mM L-alanine, 5 mM L-phenylalanine, 5 mM L-arginine, indicated compound, Δ. (A and B). 2 mM taurocholic acid; (C and D) 2 mM chenodeoxycholic acid; (E and F) 2 mM deoxycholic acid; (G and H) 2 mM triton X-100. Data points represent the average from three independent experiments and error bars represent the standard deviation from the mean.

**Figure 8 continued**




### 3.3.4. Cortex degradation precedes DPA release during *P. bifermentans* spore germination

A major difference between *C. difficile* and *B. subtilis* spore germination is the timing / order of the release of DPA and cortex fragments (87). To understand if *P. bifermentans* spores release cortex fragments before DPA (similar to *C. difficile* spore germination) or vice versa (similar to *B. subtilis* spore germination), we utilized an assay that detects the presence of reducing sugars formed by cortex degradation during germination, as described previously (87). Spores were suspended in germination buffer supplemented with 100 mM ARF (the concentration of ARF was increased to 100 mM each in order to achieve higher frequencies of spore germination in this assay) and germination was monitored over time. At the indicated time points, samples were removed and processed for the presence of reducing sugars and the presence of DPA. Within 2 minutes after the induction of germination, we observed a statistically significant difference between the amount of released reducing sugar and DPA (Figure 9). This difference was also present at 5 minutes. However, by 10 minutes post-germinant

addition, the fraction of released cortex fragments and DPA were indistinguishable. Because we observed the presence of reducing sugars in the germination medium before we observed the presence of DPA, these results suggest that cortex degradation occurs prior to DPA release during *P. bifermentans* spore germination.

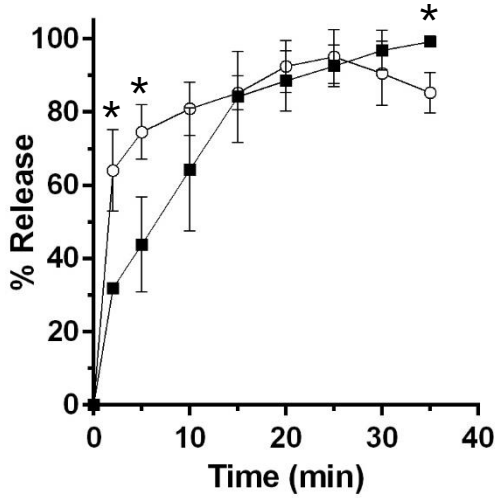



**Figure 9:** Cortex degradation precedes DPA release during *P. bifermentans* spore germination (reprinted with permission from (142)). *P. bifermentans* spores were germinated in the presence of L-alanine, L-arginine and L-phenylalanine. At the indicated times, samples were taken to analyze cortex fragment release (o) and for DPA release (■). Data points represent the average from three independent experiments and error bars represent the standard deviation from the mean. Statistical significant was determined using a 2-way ANOVA with Sidak's multiple comparisons test. The asterisk marked points indicate statistical significance (p-value <0.05).

### 3.3.5. Analyzing DPA release in the presence of high concentrations of osmolytes

DPA release by germinating *C. difficile* spores can be delayed by high osmolyte concentrations (*e.g.*, sorbitol) (138). We hypothesized that if cortex degradation precedes DPA release during *P. bifermentans* spore germination, the release of DPA may be dependent on the osmotic changes that occur at the inner spore membrane. To test this hypothesis, we added increasing amounts of sorbitol to the germination buffer and

monitored DPA release during germination by both *P. bifermentans* spores and *B. subtilis* spores (Figure 10). We could not simultaneously measure cortex degradation and DPA release during *P. bifermentans* spore germination. Due to unknown reasons, the ARF amino acids in the sorbitol-containing germination buffer reacted with the components of the cortex hydrolysis assay and did not yield a colorimetric signal. When *P. bifermentans* spores were suspended in germination buffer supplemented with 10% sorbitol (Figure 10A), we observed a short, but non-significant, delay in the release of DPA compared to spores suspended in germination buffer alone. There also was no significant delay in DPA release for germinating *B. subtilis* spores in 10% sorbitol (though sorbitol increased the total signal during *B. subtilis* spore germination; Figure 10B). When the amount of sorbitol was increased to 20% (Figure 10C), 30% (Figure 10E) and 38% (Figure 10G), the delay in DPA release by germinating *P. bifermentans* spores increased by nearly 15 minutes (Figure 10G). For *B. subtilis* spore germination, the presence of increasing concentrations of sorbitol slightly delayed DPA release but did not appear to be dose-dependent as observed for *P. bifermentans* (Figure 10B, 10D, 10F and 10H).

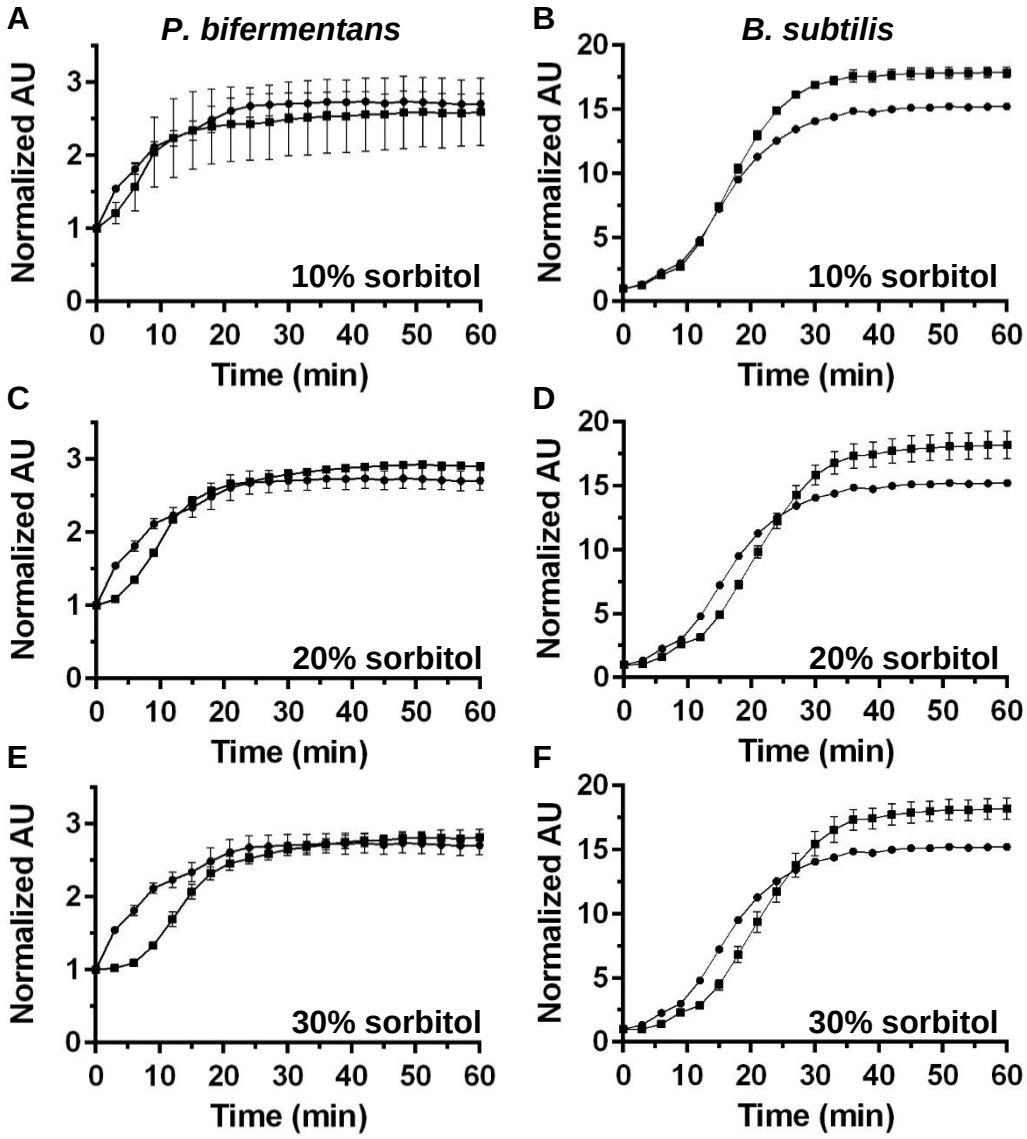
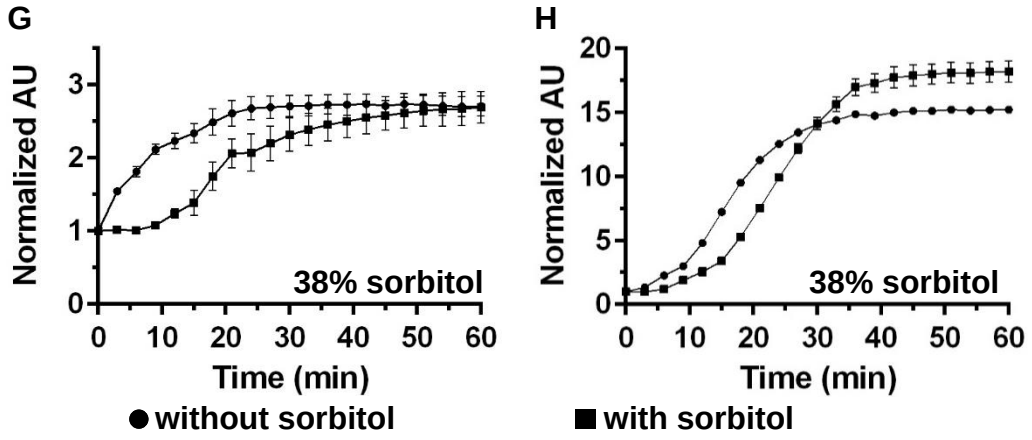



**Figure 10:** Analyzing DPA release in the presence of high concentrations of sorbitol (reprinted with permission from (142)). DPA release from germinating *P. bifermentans* spores (A, C, E and G) or from germinating *B. subtilis* spores (B, D, F and H) was analyzed germinated in presence of (■) and in absence of (●) sorbitol. (A and B). 10% sorbitol; (C and D). 20% sorbitol; (E and F) 30% sorbitol; (G and H) 38% sorbitol. Data points represent the average from three independent experiments and error bars represent the standard deviation from the mean.

**Figure 10 continued**




### 3.3.6. Quantifying the effects of sorbitol on spore germination

The data in Figure 10 suggest that *P. bifermentans* spores may be more susceptible to a sorbitol-mediated delay in DPA release than *B. subtilis* spores. A 2-way ANOVA analysis of each curve found that a significant delay in the initial DPA release can be observed during *P. bifermentans* and *B. subtilis* spore germination. To quantify this effect we determined the time at which the maximum rate of DPA release occurred by taking the first order derivative of the germination plots used to derive the data in Figure 11. Plotted in Figure 11A and 11B are the raw data from the derivative and show that *P. bifermentans* spores delay DPA release, in a dose dependent manner, upon sorbitol addition (Figure 11A). However, *B. subtilis* spores appear to be not as influenced as *P. bifermentans* spores (Figure 11B). To provide a clearer understanding of what is occurring in Figures 11A and 11B, we took a rolling average of the surrounding 8 data points for every data point in 11A and 11B to smooth the plots (Figures 11C and 11D). As shown in Figure 11C and quantified in Table 5, the time at which the maximum rate of DPA release occurs during *P. bifermentans* spore germination is delayed in a step-wise manner with increasing

concentrations of sorbitol. However, this same step-wise delay is not nearly as dramatic during *B. subtilis* spore germination (Figure 11D, Table 5). These results suggest that: (i) high osmolyte concentrations prevent the release of DPA during *P. bifermentans* spore germination similar to prior observations during *C. difficile* spore germination; (ii) that germination by *P. bifermentans* spores occurs more similar to *C. difficile* than *B. subtilis*; and (iii) that, though mechanosensing may play a role during *B. subtilis* spore germination (143), other factors are influencing DPA release by *B. subtilis* spores (*e.g.*, germinant receptors or GerD).

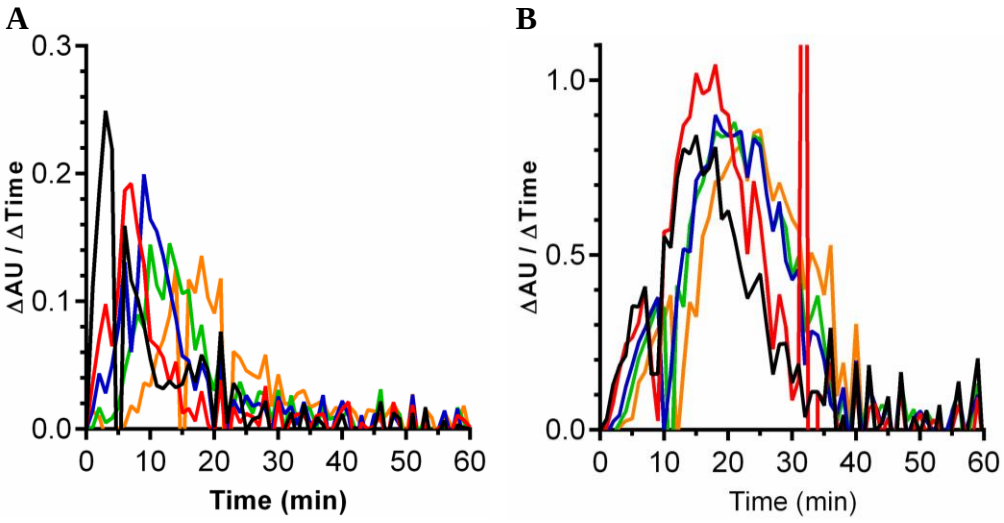



**Figure 11:** Quantifying the effects of sorbitol on spore germination (reprinted with permission from (142)). The maximum rates of spore germination in the presence or absence of sorbitol was determined by applying a first order derivative to the germination curves found in Figure 10 for both *P. bifermentans* (A and C) and *B. subtilis* (B and D). The raw data from the first order derivative (A and B) was smoothed using a rolling average of 8 surrounding data points for every data point in the plot (C and D). Experiments were performed in triplicate and the plots are a representative of one of the replicates. The average maximum rate of DPA release from the triplicate samples are tabulated in Table 5.

**Figure 11 continued**

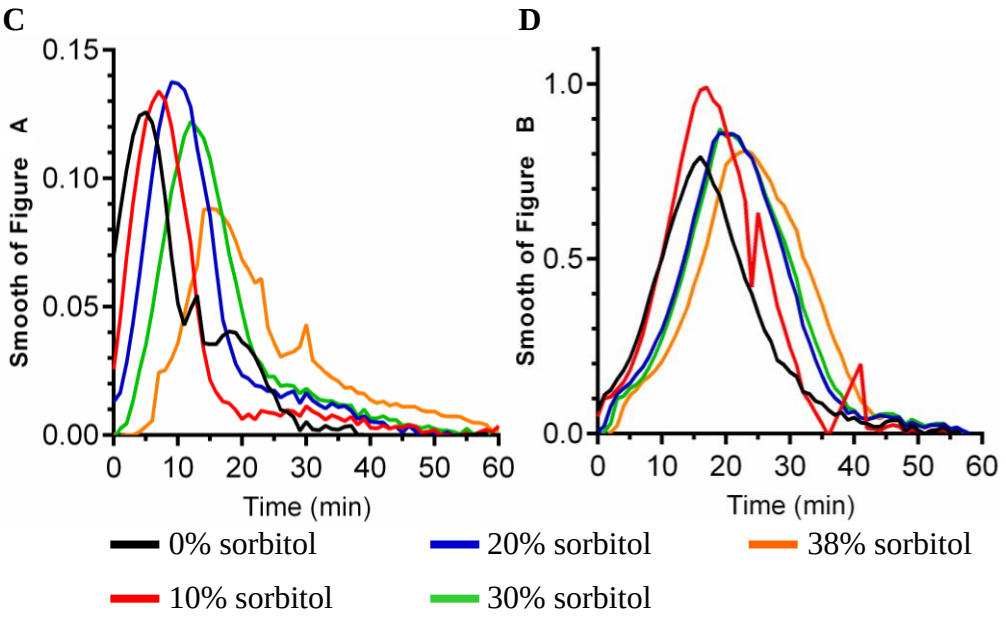



**Table 5:** Quantifying the sorbitol-dependent delay in DPA release (min) (reprinted with permission from (142)).

| | 0% Sorbitol | 10% Sorbitol | 20% Sorbitol | 30% Sorbitol | 38% Sorbitol |
|---|---|---|---|---|---|
| *P. bifermentans* | 5 ± 0.6 | 7.3 ± 0.3 (2.3) | 9 ± 0 (4.0) | 12.3 ± 0.3 (7.3) | 17.7 ± 2.7 (12.7) |
| *B. subtilis* | 16 ± 0 | 16.7 ± 0.3 (0.7) | 20 ± 0.6 (4.0) | 19.7 ± 0.7 (3.7) | 22.3 ± 0.7 (6.3) |

The time at which the maximum rate of DPA release under the conditions in Figure 11C and 11D (smoothed data) occurred was tabulated. The values reported are the averages from three independent experiments ± SEM. Values in parentheses highlight the delay and are tabulated by subtracting the value at 0% sorbitol from the other values.

### 3.4. Discussion

Most spore-forming bacteria studied to date germinate using a mechanism similar to what has been described for *B. subtilis*. In *B. subtilis*, germinant recognition by the Ger-type germinant receptor results in the release of DPA from the core, likely through a channel composed of the SpoVA proteins. This event triggers the degradation of the spore cortex layer and the irreversible loss of dormancy. *C. difficile* spore germination is triggered by the combinatorial actions of certain bile acids and glycine (44, 121). Though the hypothesized amino acid germinant receptor has not been identified, the bile acid germinant receptor is the subtilisin-like, pseudoprotease, CspC (86). Because this model differs from other models of spore germination, we hypothesized that other organisms whose *csp* locus is similar to that of *C. difficile* may initiate germination through this alternate pathway.

Using NCBI BLAST to search for *cspBAC* loci similar to *C. difficile* yielded a couple of Clostridial species with high genetic similarity, *P. bifermentans* and *P. sordellii* (95). *P. sordellii* is a virulent organism which causes a range of health issues such as hemorrhagic enteritis in animals, infections due to penetrating trauma (*e.g.*, black tar heroin use), and gynecological procedures in humans (70, 144-149). *P. bifermentans*, is rarely associated with disease directly, with only 13 cases reported (150). Recently, a subspecies, *P. bifermentans* subsp. *Malaysia*, was discovered that produces toxins that are active against a host of mosquito genera, especially *Anopheles*, carrier of malarial parasite (24). The genetic similarity between *P. bifermentans* and *P. sordellii* is high and, at one point, the two species had been classified as one organism, suggesting that their mechanisms of germination are likely shared (151, 152).

Similar to what is found in *C. difficile*, *P. bifermentans* encodes a translational fusion between *cspB* and *cspA*. Encoded downstream and, likely, part of the same transcriptional unit is *cspC*. Of the three encoded proteins, only CspB is predicted to have catalytic activity (153, 154). In CspB, the catalytic Asp, His and Ser [and the residues surrounding the triads common for Peptidase S8 family of proteases (subtilisin)] are found between amino acids 96 – 98, 160 – 163, and 458 – 464, respectively (154). For CspA, only the catalytic Asp is positioned correctly within the surrounding amino acid motif characteristic of S8 family members (amino acids 664 – 666 of CspBA) (154). Though the His and Ser residues are present, the surrounding amino acid motifs that are characteristic of the S8 family of peptidases are not in tact [the amino acid motifs surrounding the catalytic His and Ser are one amino acid and three amino acids off, respectively (Figure S8, highlighted residues)] (95, 154). In *P. bifermentans* CspC, the would-be catalytic His and Ser are absent (amino acids 167 – 170 and 482 – 487, respectively) but the Asp is present (amino acids 105 – 108) (154). Thus, similar to *C. difficile*, only CspB has predicted catalytic activity. Though CspC is a pseudoprotease, CspA may have no catalytic activity or, more likely, may have reduced activity due to the motif for the triad being off by one amino acid for two of the residues.

In the NCBI database, *P. bifermentans* is predicted to encode a *gerA* homologue. GerA belongs to the Ger-type family of germinant receptors. Because we were interested in understanding the mechanism of *P. bifermentans* spore germination, we searched for homologs of the *P. bifermentans* GerA spore germination protein to identify other organisms with mechanisms of spore germination that might resemble *P. bifermentans*. Interestingly, though the *P. bifermentans* gene is annotated as *gerA* in NCBI and UniProt

databases, when used as a BLAST query to *B. subtilis*, the most closely-related protein identified was not GerA, but rather, SpoVAF. Thus, it is probable that, similar to *C. difficile*, *P. bifermentans* does not encode orthologues of the *ger*-type germinant receptor.

Germination of *P. bifermentans* spores is initiated in response to ARF, though other combinations can stimulate spore germination (*i.e.*, AR, AF) (Figure 1). L-alanine is the most common germinant among all, studied, spore-forming bacteria (41). Thus, it is not surprising that *P. bifermentans* spores initiate germination in response to L-alanine. Waites and Wyatt (48) previously characterized the germinants for *P. bifermentans* spores and described lactate and pyruvate as germinants. Since we observe rapid and efficient germination in response to ARF, as measured both by OD change and DPA release (Figure 7C and 7D), we did not test pyruvate or lactate with the amino acid mixture. Because both *C. difficile* and *P. sordellii* encode *csp*-type receptors and both respond to steroid-based compounds (bile acids) as cues for germination, we tested if the presence of these proteins results in a spore that responds to bile acids as germinants (46, 133). As shown in Figure 8, the rate of *P. bifermentans* spore germination, measured by changes in $OD_{600}$ nm, was enhanced by both taurocholic acid and deoxycholic acid. However, this effect on germination was not apparent when the release of DPA was analyzed. Potentially, taurocholic acid and deoxycholic acid help germinants gain access to the germinant receptors or help cortex degradation. However, the reason for the impact of the bile acids on germination, measured by changes in $OD_{600}$ nm, is unknown. Thus, bile acids do not appear to impact the *P. bifermentans* spore germination and, therefore, the mere presence of the Csp proteins does not indicate that a spore germinates in response to steroid molecules. This is similar to other germinant receptors. Despite homology among the

Ger-family of germinant receptors, there are differences in the signals that stimulate germination. For example, the *B. subtilis* GerAA protein is 43% identical (62% similar) to the GerBA protein. These two germinant receptors respond, at least in part, to very different germinants (L-alanine or AGFK, respectively). The *C. difficile* CspBA protein is 59% identical and 75% similar to the *P. bifermentans* CspBA protein (Figure S8) suggesting that, should the CspBAC proteins from *P. bifermentans* function similarly to that of *C. difficile*, these proteins could respond to different germinants (95).

In prior studies, we found that the release of cortex fragments by germinating *C. difficile* spores precedes the release of DPA from the core and that high concentrations of osmolytes (*i.e.*, sorbitol, trehalose or sucrose) could delay the release of DPA from the core of germinating *C. difficile* spores (87, 138). Similar to what is observed during *C. difficile* spore germination, we found that cortex fragments appear in the germination solution before DPA during *P. bifermentans* spore germination. Moreover, the release of DPA by germinating *P. bifermentans* spores could be delayed by sorbitol in a dose-dependent manner. Because cortex degradation preceded DPA release during *P. bifermentans* spore germination, these results suggest that DPA release by germinating *P. bifermentans* spores occurs in a mechanosensing fashion. Because the rate of DPA release *B. subtilis* was not as affected by high concentrations of osmolyte, mechanosensing is likely not important for DPA release during nutrient-mediated germination; DPA-mediated germination likely would stimulate cortex degradation and thus release of DPA from the core in a mechanosensing fashion (143, 155).

Prior work done by Kevorkian *et al.* revealed that the Peptostreptococcaceae family members conserve a catalytically-dead CspC protein but the CspBA proteins vary in their

hypothesized protease activities (95). For example, *C. difficile* encodes a functional CspB protein (F) fused to a non-functional CspA protein (N). This FN arrangement is conserved across all *C. difficile* isolates, but is not universally conserved in all Peptostreptococcaceae. For *P. bifermentans*, the authors found an arrangement of a non-functional CspB but a potentially functional CspA. Based upon our findings here, CspB is likely to be catalytically active (due to the sequencing error in the published genome) and CspA may be inactive or exhibit reduced activity (see above). But, pseudoprotease regulation of spore germination may be a common feature of the Peptostreptococcaceae family. Moreover, in the absence of Ger-family germinant receptors, organisms that encode Csp pseudoproteases may germinate in an 'outside-in' mechanism.

In summary, we found that *P. bifermentans* spore germination occurs most similarly to that observed in *C. difficile*. Unfortunately, due to the lack of a genetic system in *P. bifermentans*, we could not directly test the effects of mutations introduced into the *cspBA* or *cspC* coding regions. However, based on the appearance of cortex fragments before DPA during germination of *P. bifermentans* spores and that high osmolyte concentrations can delay the release of DPA, we predict that *P. bifermentans* spore germination proceeds through the same pathway as observed during *C. difficile* spore germination. These findings build upon the hypothesis that Csp-type germinant receptor activation stimulates spore germination though an 'outside-in' direction and represents a novel germination pathway involving pseudoproteases.

# 4. FACTORS AND CONDITIONS THAT IMPACT ELECTROPORATION IN *C. difficile* STRAINS

In Section 4, I describe a protocol that I developed for transforming plasmids into *C. difficile* strains using electroporation. I also test the factors that contribute to higher transformation efficiencies (*e.g.*, osmoprotectants, DNA concentration & recovery time post-electroporation). The reported method was successful in transforming two different *C. difficile* strains: the RT027 *C. difficile* R20291 strain and the RT012 *C. difficile* CD630 strains.

## 4.1. Introduction

*Clostridioides* (*Clostridium*) *difficile* (132), a Gram-positive spore forming bacterium that is the cause of antibiotic-associated diarrhea in the hospital setting, affects approximately half a million people in the USA and was associated with 29,000 fatalities in 2011 (18). Additionally, treatment and management of patients that have *C. difficile* infections (CDI) costs nearly $6.3 billion annually to the United States healthcare system (156). Antibiotic use is the greatest risk factor for the on-set and the recurrence of CDI (157). Antibiotics disrupt the normally-protective colonic microbiome and lead to a dysbiotic environment that is susceptible to *C. difficile* invasion. The clinical severity of the disease are caused by the action of two, large, secreted cytotoxins and range from self-limiting, non-recurring diarrhea to more serious conditions like recurring CDI disease and pseudomembranous colitis (113, 158, 159).

The development of novel therapeutics to counter threats from any bacterial pathogen requires a detailed knowledge of molecular basis of virulence. Studying virulence

and related pathways requisite advanced tools of genetic manipulation. Historically, Clostridial species, especially *C. difficile*, are notoriously difficult to genetically manipulate in comparison to model organisms (*e.g. Escherichia coli* or *Bacillus subtilis*) (160, 161). To date, several *C. difficile* genetic tools available are: i) gene inactivation using a segregationally unstable pIP404-based *E.coli-Clostridium perfringens* shuttle vector (162, 163); ii) TargeTron, used widely for insertion of mobilizable group II introns into genes to produce stable insertion mutants (38, 164); iii) mariner transposition (165); iv) Allele-Coupled Exchange (ACE), using either *codA*-based or *pyrE*-based counter-selection markers (36, 105, 166) and v) CRISPR-Cas9 mediated genome editing (167). Despite their utilities, these tools require plasmids to transfer genetic elements into the *C. difficile* cell. Currently, the only way to transfer plasmids into *C. difficile* is by conjugation with either *E.coli* (162) or *B. subtilis* (168) as conjugal donors.

Plasmid introduction using conjugation requires counter-selection against the donor strain (169). Conjugation-based DNA transfer occurs at lower frequencies for plasmids with segregationally unstable origins of replication (*e.g.*, ACE systems). Moreover, "suicide" plasmids (*i.e.*, replication-deficient plasmids) are not feasible for delivery of heterologous sequences to *C. difficile* chromosome using conjugation due to extremely low numbers of transconjugants (36). To transform such plasmids, electroporation is often used. First utilized for introducing genes into mouse lyoma cells in 1982 (170), electroporation has since been used for transforming both Gram-positive and Gram-negative bacteria (171-173), yeast (174), plant protoplasts (175), and a host of mammalian tissues (176). Electroporation has an additional advantage that it can be used

in other recombination-based genetic systems (*e.g.*, the lambda RED system that requires the transformation of linear DNA fragments) (177).

In prior work, *B. subtilis* was shown to be transformed by plasmids during electroporation (178), in addition to being naturally competent. *B. subtilis* can naturally transform linear DNA and multimeric plasmids (179) but only in certain strains (180). Moreover, electroporation has drastically improved genetics in *Bacillus cereus* (181) and many Clostridia (182-191). In *C. difficile*, Ackermann *et al.* reported an electroporation protocol for the clinical strain P-881 (192). Unfortunately, for unknown reasons, this method has not been reproduced by other laboratories (162). Another attempt to electroporate *C. difficile* strains CD3, CD6, CD630 using a protocol developed for using in *C. beijerinckii* NCIMB 8052, also was not successful (162).

Here, we report a method to introduce plasmid DNA into *C. difficile* using electroporation. We also test the factors that contribute to higher transformation efficiencies (*e.g.*, osmoprotectants, DNA concentration & recovery time post-electroporation). The reported method was successful in transforming two different *C. difficile* strains: the RT027 *C. difficile* R20291 strain, and the RT012 *C. difficile* CD630 strain.

## 4.2. Materials and methods

### 4.2.1. Bacterial strains and growth conditions

*C. difficile* strains R20291 and *C. difficile* CD630 were grown in an anaerobic chamber maintained at 37 °C under anaerobic conditions (85% nitrogen, 5% carbon dioxide, >3.6% hydrogen) on BHIS [(Brain Heart Infusion (Difco) supplemented with 5 g /

L yeast extract and 0.1% L-cysteine] medium or, where indicated, BHIS supplemented with either 500 mM D-sorbitol (VWR) or 270 mM sucrose (Alfa Aesar) at pH 7.4. Antibiotics were used as needed (10 µg / mL thiamphenicol). *Escherichia coli* DH5α was grown on LB medium at 37 °C and antibiotics were added as required (20 µg / mL chloramphenicol).

### 4.2.2. Plasmid construction

Plasmid pJS116, a pCD6 origin plasmid modified to encode *tn916*, has been used for majority of the study as an empty-vector control (193). Plasmid pMTL-YN4 (an ACE vector) has been described previously (166). To introduce the $cspC_{G457R}$ allele (86) into pMTL-YN4, the *C. difficile* $cspC_{G457R}$ allele was amplified using primers 1377 and 1379 (Supplementary table S2) from the genome of the EMS mutant isolated by Francis *et al.* (86). The upstream 1 kb homology region was amplified from *C. difficile* R20291 genome using primers 916 and 1378 and the downstream 1 kb region from the mutation was already included in the *cspC*-amplified region. Both these inserts were Gibson assembled into pMTL-YN4 cut with NdeI and XhoI and transformed in *E. coli* DH5α to generate pDB29. The plasmids pKM197 and pKM126 are used to introduce CRISPR mutations in *C. difficile* R20291. The previously published CRISPR-Cas9 *pyrE* targeting plasmid, pJK02 (167), was modified by replacing *traJ* with oriT *tn*916 for *B. subtilis* conjugation by amplification from pJS116 using primers 5'Tn916ori and 3'Tn916ori. The resulting fragment was introduced into pJK02 by Gibson assembly at the *Apa*I site and transformed into *E. coli* DH5α to generate pKM126. To replace the tetracycline inducible system, the xylose inducible promoter (194) was PCR amplified from pIA33 using primers

5'pyrE_HR_xylR 2 and 3'cas9_Pxyl 2 and inserted by Gibson assembly into pKM126 at the *Xho*I and *Pac*I restriction sites and transformed into *E. coli* DH5α to generate pKM197.

### 4.2.3. Plasmid purification

The plasmids were purified by either Miniprep (Thermo Scientific GeneJET Plasmid Miniprep kit) or Miraprep (195). Miniprep kit did not yield a high amount of plasmids, so approximately 5 miniprepped plasmid solutions were combined together and ethanol precipitated. One hundred microliters of autoclaved water was added to resuspend the pellet and stored at 4 °C until use. Miraprep plasmid yields were high, thus, was used in majority of the experiments with one modification. We noticed a lot of RNA when we ran the Miraprepped plasmid on an agarose gel, so we increased RNase A concentrations to 5-10 times the concentration listed in Pronobis, et al. (195).

### 4.2.4. Electroporation

*C. difficile* were grown from frozen stock on BHIS taurocholate (TA) plates or BHIS lysozyme plates in the anaerobic chamber kept at $H_2$ level between 3.6 – 4.2%. A single colony was streaked out on BHIS plates 17 – 24 hours before growing vegetative cells from a single colony in 5 ml BHIS liquid medium. After 8 – 12 hours of growth in BHIS liquid, the culture was diluted to a final $OD_{600}$ of 0.1 (~300 µL) in BHIS supplemented with 1% glycine and 270 mM sucrose (or 500 mM sorbitol). The resulting suspension was grown to an $OD_{600}$ of 0.5 – 0.8 (~12 – 16 hours). Subsequently, this culture was diluted to a final $OD_{600}$ of 0.1 – 0.2 (~5 mL) in 40 ml of BHIS supplemented with sucrose (or sorbitol) and 1% glycine. Cells were then grown to an $OD_{600}$ of 0.5 – 0.8. *C.*

*difficile* R20291 required ~2.5 – 3.5 hours to reach this density and *C. difficile* CD630 required 4 – 6 hours of growth. Once the $OD_{600}$ was reached, the ~45 ml cell culture was kept on ice for 10 min before pelleting the cells for 12 min at 4,000 x g and 4 °C. The supernatant was discarded. For *C. difficile* CD630, white scum may be found near the bottom, discarding the scum reduced transformation efficiency. Two electroporation buffers were used, SMG [0.5 M D-sorbitol, 0.5 M mannitol (BDH), and 15% glycerol (Fisher Scientific)] and SMP [270 mM sucrose, 0.938 mM $MgCl_2.6H_2O$ (VWR), 7 mM sodium phosphate dibasic (VWR), 15 % glycerol at pH 7.4]. For *C. difficile* CD630, the glycerol was omitted from SMP. The pelleted protoplasts were washed three times in 10 mL of electroporation buffer, with gentle mixing of the pellet using the 10 mL pipette. The cells were kept cold on ice at all times. After decanting the last wash, the pellet was gently mixed into 2 ml of electroporation buffer using a blunt 1 mL pipette tip (the end of the pipette tip was removed with scissors). The ice-cold plasmid solution (~4,000 ng in a total volume of 5-10 μL) was mixed into the protoplasts in 1.5 ml Eppendorf tubes that had been kept at -20 °C prior to mixing. The plasmid-cell mix is then added to ice-cold 0.2 cm electroporation cuvettes (Gene Pulser®/MicroPulser™ Electroporation Cuvettes, 0.2 cm gap, BioRad). The plasmid-cell mix was electroporated at 1,250 V, 25 uF, 200 ohm in Bio-rad Gene Pulser Xcell™. Immediately after electroporation, the electroporation cuvettes, still on ice, were introduced into the anaerobic chamber without using a vacuum manifold for gas exchange [the airlock was manually flushed (15 s purge gas and 15 s gas mix)]. The cells were immediately flooded with 1 mL of pre-reduced BHIS supplemented with either 500 mM D-sorbitol or 270 mM Sucrose (pH 7.4) and cultured in 5 mL total volume. After growing for the time illustrated in Figures 1 – 3, the 5 mL culture was centrifuged for

12 min at 4,000 xg at 4 °C and the entire pellet was resuspended and plated on BHIS medium supplemented with thiamphenicol. The colonies containing the plasmid appeared between 18 – 48 hours after plating, depending on the strain.

### 4.2.5. Statistics

Statistical analysis between the column values in Figures 1-5 was accomplished with a one-way ANOVA with Tukey's test for multiple comparisons. A 99% confidence interval was set for significance ($P<0.01$).

## 4.3. Results

### 4.3.1. Initial success transforming *C. difficile* R20291 by electroporation

Prior reports have suggested that *C. difficile* can be transformed by electroporation. However, these methods are not widely used to introduce DNA into *C. difficile* cells. As an initial foray into developing an electroporation protocol that might work between different *C. difficile* strains, we generated protoplasts of *C. difficile* vegetative cells. To accomplish this, *C. difficile* cells were grown in BHIS medium supplemented with 1% glycine (as a cell wall weakening agent) and 500 mM sorbitol (as an osmoprotectant). Even though other cell wall weakening agents such as D/L-threonine, ampicillin, Tween-80, and others, have been used for other Clostridia and Bacillus species (180, 188, 196, 197), only glycine was used as the cell wall weakening agent for this study. Glycine is incorporated into cell wall precursors in place of D-alanine, thereby, reducing peptidoglycan cross linkages (198). *C. difficile* R20291 cells were grown overnight in liquid BHIS medium. The next morning, the culture was back diluted to an $OD_{600}$ of 0.1 in

10 mL liquid BHIS medium supplemented with sorbitol and glycine. The cells were grown for approximately 12-16 hours to an $OD_{600}$ = 0.5. The culture was again back diluted into 40 mL BHIS medium supplemented with sorbitol and glycine to an $OD_{600}$ of 0.1 and grown to an $OD_{600}$ of 0.5. Subsequently, the cell culture was washed three times with in SMG buffer (180). Approximately 2 μg of empty pCD6-origin of replication-containing shuttle vector, pJS116, was added to the competent cells (193). The cells were then added to 0.2 cm electroporation cuvettes and electroporated at 1,250 V, 25 μF and 200 Ω. The transformed cells were then introduced into the anaerobic chamber and recovery medium added. The next day, the cells were pelleted, resuspended, and the entire growth plated on BHIS medium supplemented with thiamphenicol. Surprisingly, several colonies grew (data not shown). A DNA only (no electroporation) negative control did not yield any transformants and neither did cells grown in the absence of glycine (data not shown).

### 4.3.2. Transformation efficiency is influenced by recovery time and DNA concentration

To determine the time required to recover the electroporated cells, we used the protocol described above to determine the number of hours post-electroporation required to yield the maximum number of colonies on selection plates. As shown in Figure 12A, approximately 100 – 200 colonies grew after 12 hours of growth post-transformation. This number increased up until 16 hours post-transformation where we observed approximately 400 colonies per microgram DNA transformed. After 16 hours growth, the number of transformed cells began to decrease (Figure 12A). Based on this data, we chose to use a 16 hour recovery time for subsequent *C. difficile* R20291 transformations.

Next, to determine the concentration of the pJS116 empty vector that could yield the best transformation, we electroporated different plasmid concentrations into *C. difficile* R20291 competent cells and recovered the transformed cells for 16 hours (Figure 12B). We found that between 2 – 4 μg of plasmid DNA yielded most consistent number of colonies, even though results were not significantly different between the plasmid concentrations. Transformations with plasmid concentrations below 2 μg did not consistently yield colonies (Figure 12B).

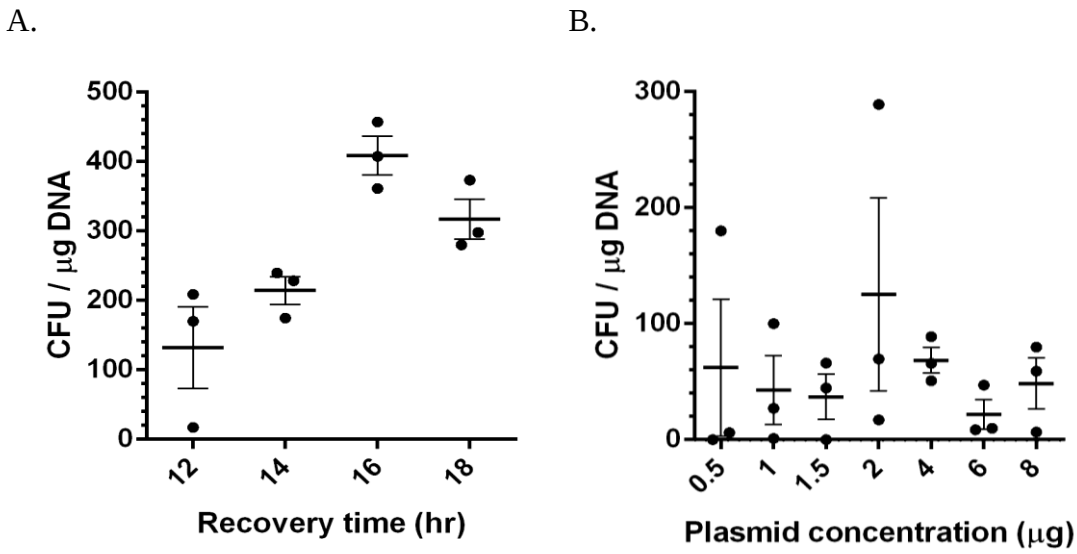



**Figure 12:** Electroporation of *C. difficile* R20291 competent cells grown in sorbitol-containing medium. A) *C. difficile* R20291 competent cells, from the same preparation, were recovered in BHIS and 500 mM sorbitol with time ranging from 12 – 18 hours. **, $p < 0.01$, significance between 12 and 16 h; *, $p < 0.05$, between 12h and 18h and 14h and 18h. B) *C. difficile* R20291 competent cells from the same stock were electroporated with different concentrations of pJS116 and recovered in BHIS-sorbitol (500mM) for 16 hours. No significance was observed between samples. Bars represent the average from three independent experiments (data points represent the individual experiments) and error bars represent the standard error of the mean.

### 4.3.3. Sucrose is superior to sorbitol when generating *C. difficile* electro-competent cells

In order to determine whether the above protocol was applicable to other *C. difficile* strains, the protocol was used on *C. difficile* CD630. Unfortunately, neither of the strains produced any colonies after transformation. Therefore, we tried another osmoprotectant, sucrose. Sucrose was previously used by Ackermann *et al.* (192). Because we found that *C. difficile* R20291 could be transformed by electroporation using sorbitol as an osmoprotectant, we chose first to test if sucrose could be used electroporate *C. difficile* R20291. *C. difficile* R20291 was grown for 8 – 10 hours in BHIS medium and then inoculated to an $OD_{600}$ = 0.1 in 10 mL BHIS supplemented with 270 mM sucrose and 1% glycine. Cells were growth for 14 – 16 hours to reach an $OD_{600}$ between 0.5 and 0.8. Subsequently, the 10 ml culture was used to inoculate 40 mL of BHIS medium supplemented with 270 mM sucrose and 1% glycine at an $OD_{600}$ = 0.1 and grown to an $OD_{600}$ = 0.5. The culture was then washed with SMP buffer and transformed as described above.

Because this new medium may influence the time required to recover post-electroporation and / or the concentration of the plasmid required to achieve the highest number of transformants, we again tested these variables (Figure 13). To determine the number of hours required to recover the transformed cells, we electroporated *C. difficile* R20291 with 4 μg of pJS116 and recovered the transformed cells for 6 – 18 hours (Figure 13A). We observed the highest number of colonies when the transformed cells were recovered for 11 hours to 14 hours. As was the case with the plasmid concentrations used Figure 12, we observed no statistical significance between different plasmid concentrations

in this new medium and buffer. However, at 4 μg, we observed more consistent numbers of colonies suggesting that 4 μg may yield the most consistent results going forward.

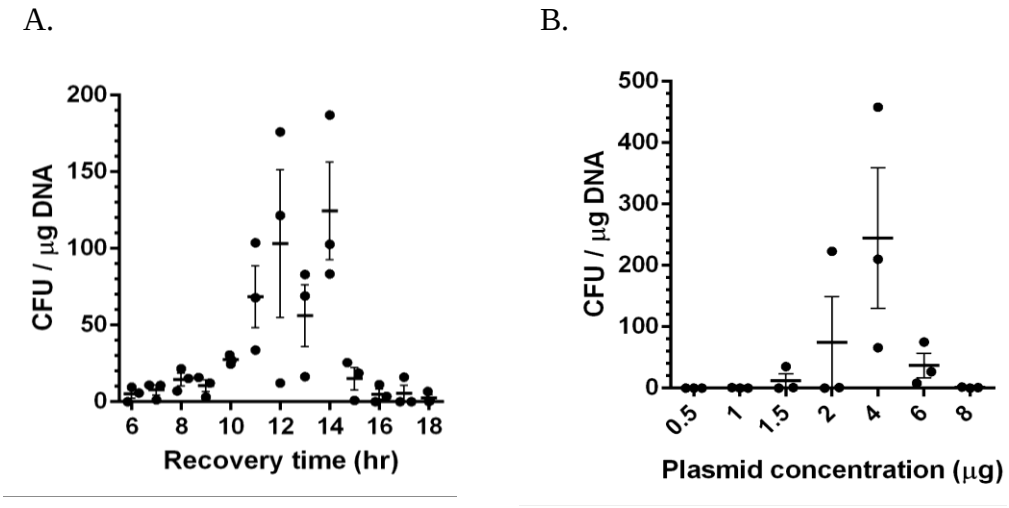



**Figure 13:** Electroporation of *C. difficile* R20291 competent cells grown in medium where sucrose is the osmoprotectant. A) *C. difficile* R20291 competent cells from the same preparation were recovered in BHIS and 270 mM sucrose for the indicated times. B) *C. difficile* R20291 competent cells from the same preparation were electroporated with different concentrations of pJS116 and recovered in BHIS 270 mM sucrose for 11h. No significance observed between samples. Bars represent the average from three independent experiments (data points represent the individual experiments) and error bars represent the standard error of the mean.

### 4.3.4. Growth in 1% glycine and subsequent transformation does not introduce mutations into the *C. difficile* genome

In order to understand if the electroporated cells were indeed *C. difficile* R20291, and not a thiamphenicol-resistant contaminant, genomic DNA from three biological replicates [2 from transformations with sorbitol as the osmoprotectant (Figure 12) and 1 from a transformation with sucrose as the osmoprotectant (Figure 13)] were prepared and sent for genome re-sequencing. As a control, the parental *C. difficile* R20291 strain was

also sent for genome re-sequencing. The sequences of the transformed isolates did not deviate from the parental strain, indicating that the electroporated cells were indeed the *C. difficile* R20291 strain and that the protocol to generate electrocompetent cells and subsequent transformation does not introduce mutations into the *C. difficile* genome.

### 4.3.5. Transformation of the *C. difficile* CD630

To determine if this protocol could be applied to other commonly utilized lab strain, *C. difficile* CD630 (ribotype 012) was electroporated. The electroporation SMP buffer for this strain omitted 15% glycerol as we did not recover any electroporated cells when there was glycerol in the background, thereby, requiring the production of fresh competent cells for every electroporation. *C. difficile* CD630 required 16 hours for recovery after electroporation with pJS116 (Figure 14), with nearly no colonies observed at any other time.

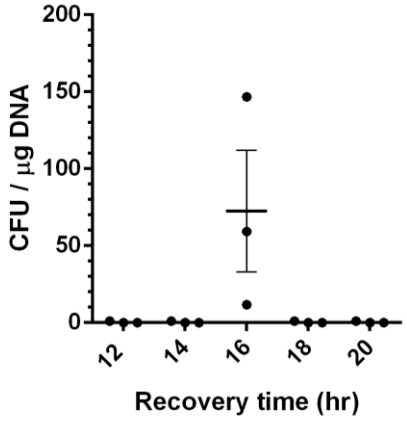



**Figure 14:** Electroporation of *C. difficile* CD630. *C. difficile* CD630 competent cells were electroporated with pJS116 and recovered for the indicated times. Bars represent the average from three independent experiments (data points represent the individual experiments) and error bars represent the standard error of the mean.

### 4.3.6. Freezing competent *C. difficile* R20291 cells reduces viability

It would be helpful if competent cells could be frozen for later use. To test if *C. difficile* R20291 competent cells can be safely stored at -80 °C, we added 15% glycerol to the SMP buffer. Competent *C. difficile* R20291 cells were frozen on dry ice, stored -80 °C and tested for their ability to be transformed and recovered over a four week period (Figure 15). Fresh, not frozen, competent cells yielded the highest number of colonies (Figure 15). Over a 4 week period, we observed that the frozen competent cells yielded fewer and fewer colonies when transformed with the pJS116 empty vector.

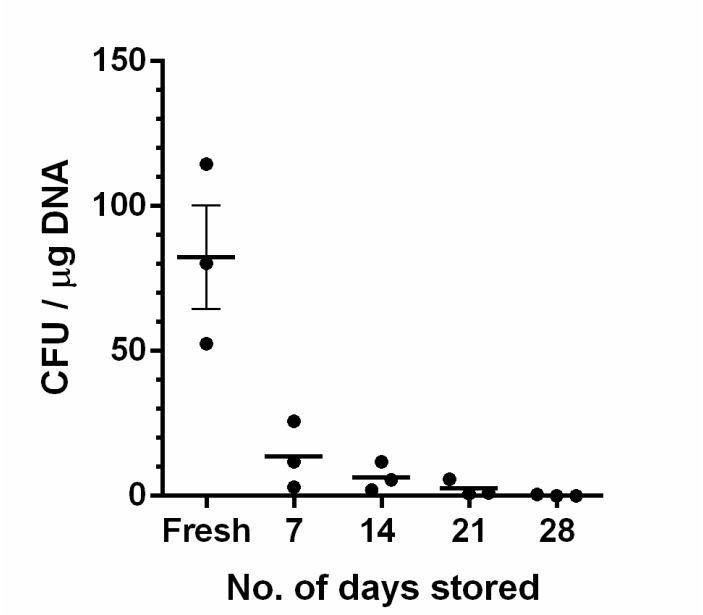



**Figure 15:** Testing the impact of -80 °C storage on eletrocompetence. Competent *C. difficile* R20291 cells frozen on dry ice and stored in –80 C for indicated number of days. Bars represent the average from three independent experiments (data points represent the individual experiments) and error bars represent the standard error of the mean.

### 4.3.7. The anaerobic chamber airlock does not significantly affect sucrose-prepared competent cells.

The protocol is setup such that a part of the procedure occurs in the aerobic, laboratory, environment (*i.e.*, washing and electroporation). However, in order to recover the transformed cell post-electroporation, the cells need to be passed into the anaerobic chamber. In this process, a series of gas exchanges is done with a vacuum cycle in between each exchange. Because the cells are in protoplast form (lack of or an incomplete cell wall), we hypothesized that the cells may be very sensitive to osmotic changes and vacuum applied to the cells may result in the protoplasts bursting. To test this hypothesis, we passed transformed cells into the anaerobic chamber by manually flushing the airlock with a 15 sec pulse of nitrogen gas and then a 15 sec pulse of gas mix flushing (without a vacuum cycle). We also passed transformed cells using the normal gas exchange procedure (involving vacuum). As shown in Figure 16, gas exchange using the vacuum results in a slight decrease in transformants, but this did not achieve statistical significance.

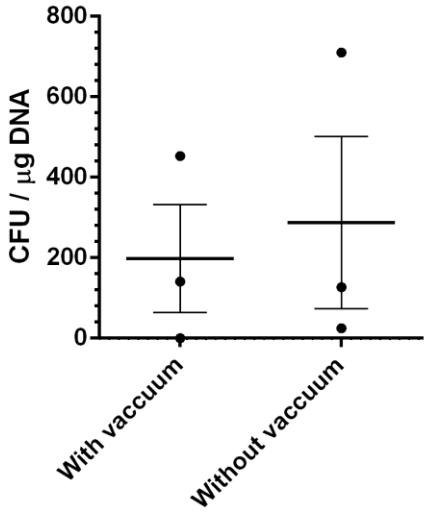



**Figure 16:** Competent *C. difficile* R20291 cells undergoing manual flushing vs normal airlock removal cycle. Bars represent the average from three independent experiments (data points represent the individual experiments) and error bars represent the standard error of the mean.

### 4.3.8. Transformation of other plasmids into the *C. difficile* R20291 strain.

Thus far, all the data generated in this manuscript used an empty plasmid pJS116 vector. The described protocol would be useful if other plasmids could be inserted. To demonstrate that other plasmids can be electroporated in *C. difficile* R20291 competent cells, 4 μg of 4 different plasmids (pMTL-YN4, pDB29, pKM197, and pKM126) were transformed by electroporation. The colonies grew on the BHIS agar medium in 24 – 48 hours (Figure 17).

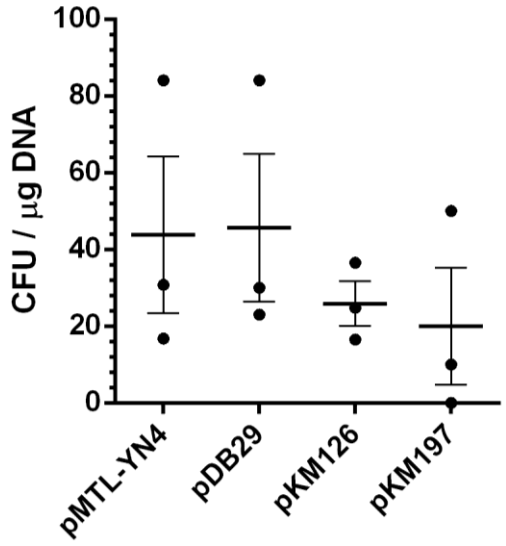



**Figure 17:** Competent *C. difficile* R20291 cells electroporated with different plasmids. Bars represent the average from three independent experiments (data points represent the individual experiments) and error bars represent the standard error of the mean.

### 4.4. Discussion

This study provides a reliable and reproducible electroporation protocol for use in *C. difficile*. Previous protocols by Ackermann *et al.* (192) and Purdy *et al.* (162) have not been widely adopted in the field. The electroporation protocol described in Ackermann *et al.* (192) was demonstrably successful only in one clinical strain, P-881. Additionally, their protocol utilized pBAD-TOPO and pCRII-TOPO plasmids with *tcdB* insertions. These plasmids encode the pBR322 origin of replication instead of a *C. difficile*-specific origin of replication, thus making the pBAD and pCRII plasmids replication defective in *C. difficile*.

An obstacle for transformation in bacteria is presence of the cell wall, which can be circumvented by weakening the walls with glycine, or penicillin. Fragility of the cell wall enhances transformation efficiency (199). Glycine is the most common additive used for

electroporating *B. subtilis* (180), *B. mycoides* (200) and *C. pasteurianum* (186). The degree of effect of the cell wall-weakening agent is directly related to the conditions required for pore formation. We observed that *C. difficile* requires glycine to generate transformable protoplasts; the absence of glycine did not produce colonies after electroporation (data not shown). We verified that 1% glycine produced misshapen cells / protoplasts using phase contrast microscopy (data not shown). Additionally we observed that increasing the glycine concentration (1.5% and 2%) delayed cell culture growth by 24 – 48 hours when growing in the 40 mL BHIS supplemented with sorbitol and glycine, whereas reducing glycine amount (0.75%) did not produce any colonies after electroporation (data not shown).

To protect the cells from bursting, growth in medium designed to affect cell wall integrity requires an osmoprotectant. A cell with a deficient cell wall can succumb to the osmotic differences between the cytoplasm and the growth medium (199). Here, we used D-sorbitol or sucrose as osmoprotectants. D-sorbitol was previously used for electroporating *B. subtilis* (180, 201). Additionally, our lab used D-sorbitol as an osmoprotectant to show that *C. difficile* and *P. bifermentans* mechanosensing channels prevented release of dipicolinic acid from spore core (142). Though *C. difficile* R20291 grown in sorbitol containing medium could be transformed by electroporation, we could not obtain any transformants from the *C. difficile* CD630 strain (197). However, we could obtain transformants when 0.27 M sucrose was used as an osmoprotectant for both *C. difficile* R20291 and *C. difficile* CD630. It would be interesting to speculate whether 0.27 M sucrose could lead to successful transformation by electroporation for the other *C. difficile* strains.

One of the important factors that influence the transformation efficiency is the time after electroporation to recover in liquid medium (197, 199). Too short of a time, the electroporated cells would not have time to recuperate, rebuild cell wall and divide; recovering for a longer time could risk losing the plasmid from electroporated cells due to lack of selective pressure on the antibiotic resistance gene. We found that the two strains had different recovery times. Previous research on *C. pasteurianum* has shown that recovery time after electroporation is essential for high transformation efficiency. In this study the authors observe maximum transformation efficiency at 16 hours post-transformation (186). *C. difficile* R20291 had two different recovery times, depending on the osmoprotectant used. For sorbitol, the recovery time was 16 hours, similar to *C. pasteurianum*, but, with sucrose, recovery time reduced to 11 – 14 hours (197). Even though we observe the maximum number of colonies at 14 hours recovery, there is a sharp drop in efficiency by 15 hours. To ensure that we could reliably achieve transformation, we used 11 hours (where it starts to peak) for the rest of the experiments. *C. difficile* CD630 maximally recovered at 16 hours only, none of the other time frames produced colonies. A variety of reasons including growth conditions, chamber conditions, the strain itself, could contribute to maximum recovery times.

Higher plasmid concentrations increase transformation efficiency until saturation is achieved and is concentration is species dependent (199). We observed that the transformation efficiency with various plasmid concentrations depends on competent cell preparation. However, on an average, having higher plasmid concentrations ensured some colonies were observed on the selection plates regardless of the osmoprotectant used to generate or recover the transformed cells. For most experiments, we used ~4 µg of plasmid

DNA. Higher amounts of DNA led to diminishing returns on transformation, similar to what is observed in *Chromobacterium violaceum* (202).

The major concern we had regarding electroporation in an anaerobic organism is whether the cells would survive switching between anaerobic and aerobic. The oxygen removal from airlock uses 4 cycles of gas exchange (3 purge gasses and 1 anaerobic mix gas) with a vacuum cycle interspersed between each exchange. We did not observe a significant difference in the number of colonies per microgram plasmid DNA between the vacuum and non-vacuum flushing. However, for the rest of the experiments we used the non-vacuum flushing to ensure that we get transformation even when the competent cell stock is not made well.

In this study, we mostly used pJS116 for the experiments. So we tested if the ACE vectors and CRISPR-based vectors could be transformed using the described protocol. DNA molecules of different sizes may require different electroporation parameters in the same strain. We could electroporate the empty vector pMTL-YN4 (166), this vector with a $cspC_{G457R}$ mutant allele (pDB29), a CRISPR plasmid that targets the *pyrE* in *C. difficile* R20291 genome and is regulated by the TetR promoter (pKM126) (167), and pKM126 with xylose promoter, pKM197 using the same protocol in *C. difficile* R20291. These results indicate that the protocol described here can be used for the transformation of useful *C. difficile* plasmids. However, since many parameters may affect electrotransformation efficiency, special attention should be paid to the parameters discussed in this protocol and these may need adjusted depending on the strain used and conditions within the anaerobic chamber.

# 5. CONCLUSIONS AND FUTURE DIRECTIONS

Members of Clostridia and Bacillus form metabolically dormant, but highly environmentally-resistant spores that are very difficult to remove from the surroundings. 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. The toxins from *C. difficile* cause an antibiotic-associated diarrhea that can eventually lead to fatal conditions like toxic megacolon or pseudomembranous colitis. The vegetative cells eventually sporulate and escape into the environment to either be taken up by other hosts or remain in current host and, potentially, result in recurring CDI. Germination can act as an attractive target for potential therapeutics. Blocking germination before transformation into a vegetative cell and toxin production might protect hosts from infection. Alternatively, if germination were to be activated in the aerobic environment for an anaerobic organism like *C. difficile*, then the presence of vegetative cells and spores could potentially be reduced in community and hospital settings at higher risk for CDI. In spite of significant advantages of studying the field of *C. difficile* spore germination, the field has not progressed as rapidly as what is observed in model organisms. Part of the reason for this is the lack of genetic tools and difficulty in manipulating *C. difficile* genome. Below, I will summarize my findings in elucidating the mechanisms of *C. difficile* spore germination and the discovery of a novel genetic tool that may potentially help establish *C. difficile* as a model organism.

### 5.1. Additional role of CspC in spore germination

Most of the studies on Clostridial spore germination have been derived from studies in *B. subtilis* and *C. perfringens*. However, *B. subtilis* encodes canonical *ger*-type receptors which *C. difficile* does not (203). In prior work, Francis *et al.* (86) found that CspC, encoded with the *cspBAC* locus, is the bile acid germinant receptor. Prior to work done in *C. difficile*, the Csp proteins were mostly studied in *C. perfringens*, where CspA, CspB and CspC are all catalytically active proteases that can cleave the cortex hydrolase, SleC, to an active form (122, 204). On further examination of the *cspBAC* locus in *C. difficile*, CspB and CspA are translationally fused, which is a different arrangement from *C. perfringens*. CspB is the only catalytically active protease from the *cspBAC* locus – CspA and CspC have lost their catalytic residues. This suggests that CspB might be the only protein that interacts with and cleaves SleC. CspC, the bile acid germinant receptor, and CspA, the co-germinants' germinant receptor, are not catalytically active but perform functions critical for spore germination. After germinant signals are received by the germinant receptors, spore cortex hydrolase is activated and DPA is then released from the spore core. Research has demonstrated that in *B. subtilis*, DPA is released from the spore core before cortex can hydrolyzed (138).

As SleC activation occurs prior to DPA release, it indicates the importance of the initial step of bile acid binding with CspC in the process of germination (138). In a working model of *C. difficile* spore germination, wherein bile acid binding to CspC and co-germinants to CspA, the CspB protein then cleaves pro-SleC, activating the protein. Activated SleC hydrolyzes the cortex resulting in large changes in osmolarity on the inner spore membrane due to the large amount of DPA stored within the spore core. This

triggers the mechanosensing protein, SpoVAC, release DPA from the core and the rehydration of the core.

Even though numerous research articles show that *C. difficile* binds various bile acids as activators and inhibitors of germination, Heeg *et al.* (105) identified several *C. difficile* strains, from different ribotypes, that appeared to not require TA as an activator or CDCA as an inhibitor of germination. This new data could potentially modify the bile acid germination model in a novel direction and provide fresh insights into new germinants that could interact with *C. difficile* spores. In order to characterize the germination phenotype in these strains, we calculated $EC_{50}$ values for TA and inhibitor constant values ($K_i$) for CDCA (205). We observed certain strains had higher $EC_{50}$ values in response to TA, which was compared to values calculated using lab strains, indicating a lower requirement or a higher potency of TA to activate germination. We also observed that strains whose germination was not inhibited by CDCA anti-germinant had higher $EC_{50}$ values. By quantifying the interaction between bile acid and spores from various strains, we could reestablish the requirement of bile acid for initiating or prohibiting spore germination. A possible explanation for the ability of these strains to germinate in only BHIS medium is that very small amounts of bile acids can be found in the blood and tissues used to make the BHI medium in which the authors tested their *C. difficile* strains.

A growing body of evidence indicates that germination is not correlated with ribotype. By correlating $EC_{50}$ and $K_i$ values and specific protein substitutions, we observed that, even though the specific amino acid substitutions present within the CspB, CspA, and CspC primary sequences were ribotype specific, $EC_{50}$ and $K_i$ were not attributed to the substitutions. By comparing the kinetics of spore germination to the abundance of each

protein within the spore, we were positioned to predict if the kinetics of these strains that seemingly deviated a little from the lab strains, which could be attributed to variation in the quantity of the germinant receptor proteins. In *B. subtilis*, overexpression of the GerAA germinant receptor increases germination rate, indicating a positive correlation with the number of germinant receptors to faster germination rate (206). However, when we compared the levels of bile acid germinant receptor, CspC, against germination rate, we observed a surprising trend of inverse correlation between the two. Since germination in *C. difficile* germination essentially depends on SleC, we hypothesized that higher SleC levels are required to overcome the potential inhibitory effect of CspC. We observed a significant positive correlation between the numbers of CspC molecules / SleC molecules and rate of germination, indicating that a potentially inhibitory function of CspC on spore germination.

### 5.2. Novel germination mechanism of *C. difficile* can be observed in *P. bifermentans*

Prior to my work, the only organism described to germinate through this novel 'outside – in' germination mechanism was *C. difficile*. To identify other organisms whose spores might germinate similar to *C. difficile* spores, I used NCBI BLAST to search for *cspBAC* loci similar to *C. difficile*. *P. bifermentans*, also a Clostridia, was identified as one such organism.

The *cspBAC* locus in *P. bifermentans* is arranged similarly to *C. difficile cspBAC*. *P. bifermentans* also encodes a *gerA* homolog, making it a good target to investigate the germination scheme followed by a bacterium carrying both germinant receptor loci. I used the *gerA* homolog as a BLAST query to investigate its similarity to germinant receptors.

Interestingly, the protein was closer in similarity to SpoVAF instead of any of the germinant receptors, raising the possibility that *P. bifermentans* may only use the *cspBAC* locus to germinate spores.

In order to determine how germination occurs in *P. bifermentans*, I had to redesign the experiments that depicted germination of *P. bifermentans* spores by germinants described by Waites and Wyatt (48). I found that spores could be germinated by using a combination of L-alanine, L-arginine and L-phenylalanine alone. Because *C. difficile* CspC can respond to bile acids as germinants, we tested if CspC alleles in other organisms confer the ability to germinate with various bile acids. I observed that rate of germination was enhanced by both TA and DCA, but this effect wasn't observed in the DPA release. The reason for this is still unknown however, we speculated that the TA and DCA might help germinants gain access to receptors or help with cortex degradation. Despite homology between *ger* receptors, there is variability in the signals considered as germinants.

To identify the order DPA release and cortex degradation during *P. bifermentans* spore germination, I used the cortex hydrolysis assay to detect the release of hexosamine during spore germination. I observed that cortex fragments were in the solution before DPA was released, similar to what is seen in *C. difficile*. Moreover, we observed that the release of DPA can be delayed by sorbitol in a dose-dependent manner. On the contrary, increasing sorbitol concentrations could not delay DPA release in *B. subtilis*, indicating that mechanosensing is likely not important for DPA release during *B. subtilis* spore germination. This, in turn signifies that *P. bifermentans* spores germinate similarly to the 'outside – in' germination mechanism observed for *C. difficile* spores. Unfortunately, due

to the state of molecular genetics for *P. bifermentans*, I could not test if the *cspBAC* locus was involved *P. bifermentans* spore germination.

### 5.3. Electroporation in several commonly studied *C. difficile* strains

I developed a reliable and replicable protocol for electroporating *C. difficile* R20291 and *C. difficile* CD630. The protocol is fairly intuitive and was combined after studying electroporation of *B. subtilis* and *C. pasteurianum*. I identified some of the factors that may affect efficiency of electroporation. A major obstacle in electroporation is the resistance caused by cell walls. Previous studies used cell wall weakening agents such as glycine, D/L-threonine, penicillin and lysozyme. I observed that *C. difficile* requires glycine to generate protoplasts that can be transformed by electroporation. In absence of glycine, no colonies were observed after electrotransformation (data not shown). Additionally, I observed that increasing glycine concentration to 1.5% and 2% reduced the production of competent cells whereas reducing glycine concentration to 0.75% did not produce colonies on the selection plates after electroporation.

The cells in excess of glycine in the growth medium renders the cell wall impaired. Bacterial cells without an efficient cell wall require an outside osmotic pressure to maintain shape, which I provided by adding either 270 mM sucrose or 500 mM sorbitol. By using different osmoprotectants, I determined that sucrose works with multiple strains of *C. difficile*.

Another important factor that influences transformation efficiency is the time after electroporation for recovering in growth medium with osmoprotectant to rebuild cell wall and divide. When sorbitol is present in the growth medium, the time taken to recover by *C.*

*difficile* R20291 is around 16 hours. Unfortunately, *C. difficile* CD630 could not recover with sorbitol in the background. When sucrose is added to the growth medium, *C. difficile* R20291 could be recovered between 11 and 14 hours. *C. difficile* CD630 was recovered in 16 hours with sucrose as the osmoprotectant. The plasmid I added to test the recovery hour conditions was pJS116. To determine the plasmid concentration that would be required for electroporation, I ran a series of different concentrations starting at 0.5μg to 8 μg and found that 4 μg seemed optimal even though it was not significant.

Since the protocol described here included transferring competent cells to aerobic environment for electroporation but recovered in anaerobic chamber, a reason for concern is the oxygen removal system employed by the anaerobic chamber. I ran electroporated cells through the normal oxygen removal cycle and modified manual non-vacuuming cycle and observed no significant difference in the number of colonies in electroporated *C. difficile* R20291 cells.

The aim of developing this protocol was to introduce different genetic tools into different *C. difficile* strains easily. In order to fulfill this aim, I electroporated *pyrE* based ACE plasmids and CRISPR-Cas9 plasmids in *C. difficile* R20291. All of the plasmids were successfully electroporated in *C. difficile* R20291.

### 5.4. Future directions

Our discovery of CspC performing an unexpected function during germination should be thoroughly investigated. The binding of CspC to bile acids is an essential step in germination. Understanding how and where bile acids bind to CspC can potentially help identify other compounds that can interact with spore. Studying the binding can also

provide insights into the downstream processes that take place during germination. However, preliminary data suggests that recombinantly expressed and purified CspC does not interact with TA. Producing recombinant proteins from the *cspBAC* loci in *E. coli* has proved to be an arduous task. There is also a possibility that CspC might not be binding bile acids on its own and may need additional co-factors, as was demonstrated by Li *et al* with Ger proteins in *B. megaterium* (207). With a CspC crystal structure now available, producing an active protein might be in the realm of possibilities (208).

I have also demonstrated that *P. bifermentans* also follows the mechanism of germination as the recently discovered germination scheme in *C. difficile*. Studying the phenotypic effects of deleting or making mutations in the *cspBAC* locus of *P. bifermentans* would be ideal, which I have not been able to demonstrate due to a lack of genetic tools. Alternatively, this study could be done in an organism that has genetic tools available, for example in *P. sordellii*. This could help add to the repertoire of organisms that show the novel germination mechanism, thereby further solidifying the existence of such a mechanism. Knowing about the presence of the new germination mechanism associated with the type of germinant receptors encoded could lead to reclassifying spore formers according to the type of germinant receptors.

Our lab identified the bile acid germinant receptor involved in *C. difficile* germination because of an EMS screen. TargeTron deletion mutation made in *cspBAC* loci further verified the germination phenotype. More targeted genetic tools, like the allele-coupled exchange and the CRISPR-Cas9, have been developed over the years, giving a cleaner and more precise manipulation of the *C. difficile* genome. To date, all the genetic tools created can be inserted into *C. difficile* by conjugation. There has been no

development or use for tools that cannot be introduced without conjugation. In order to continue producing highly accurate genetic tools that might not be reliably conjugated in, I have developed a method of electroporation for 3 commonly utilized strains of *C. difficile*. The electroporation protocol can potentially be tried in clinical strains. Electroporation could potentially open new avenues for technologies that involve single or double stranded DNA or RNA to modify the genome, removing the need for plasmids.

Electroporation can ease development of new technologies to studying *C. difficile* and potentially convert *C. difficile* into a model organism. Studying about the field of spore germination in *C. difficile* will provide new insights into an interesting life form in a pathogenic bacterium. It can also provide potential new therapeutic targets not just for *C. difficile* but also for pathogenic bacteria with a similar germination system.

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APPENDIX A

SUPPLEMENTARY FIGURES

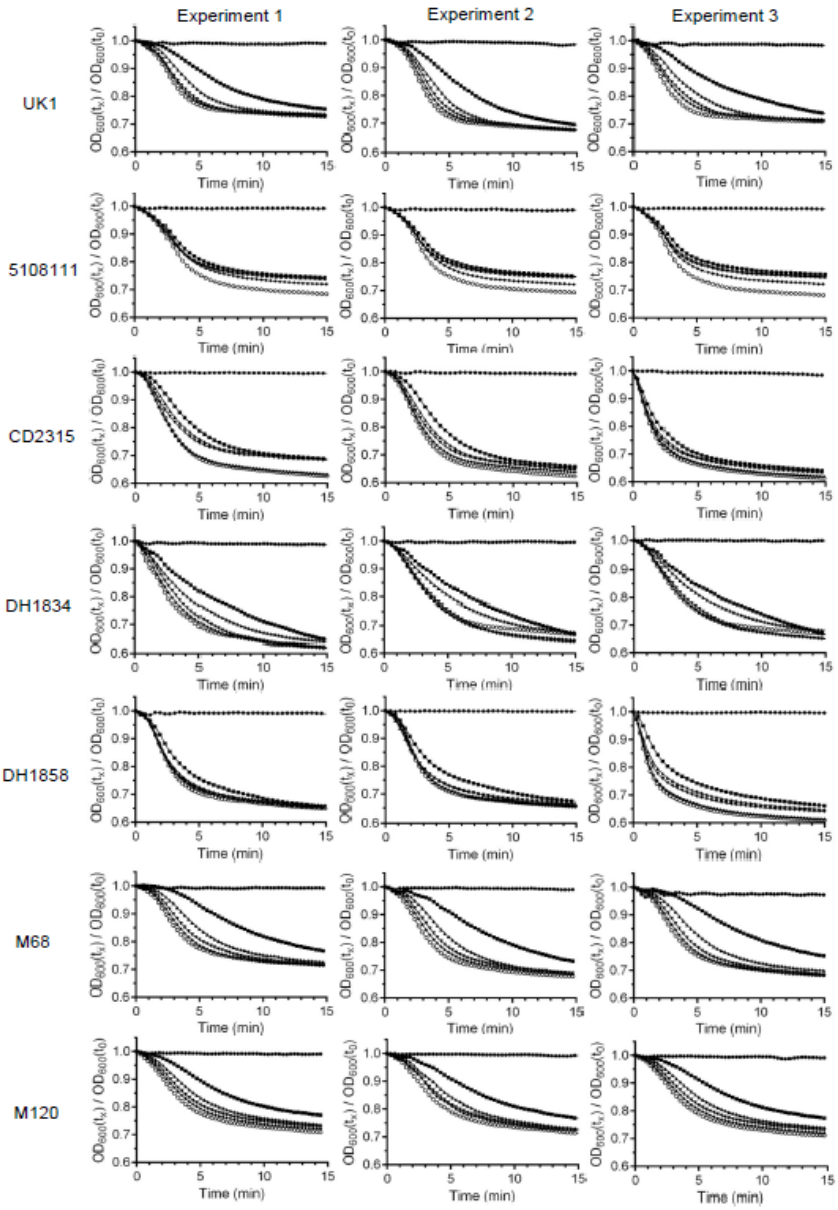


**Figure S 1:** Complete germination data for *C. difficile* strains (reprinted with permission from (121)). Purified *C. difficile* spores of the indicated strains were suspended in BHIS medium alone (●) or medium supplemented with 2 mM (■), 5 mM (▲), 10 mM (▼), 20 mM (♦) or 50 mM (○) TA. Germination was monitored at OD600 as described previously. Experiments were performed in triplicate and each experiment is shown. Data could not be displayed as the average due to inconsistent time points generated by the spectrophotometer between experiments.

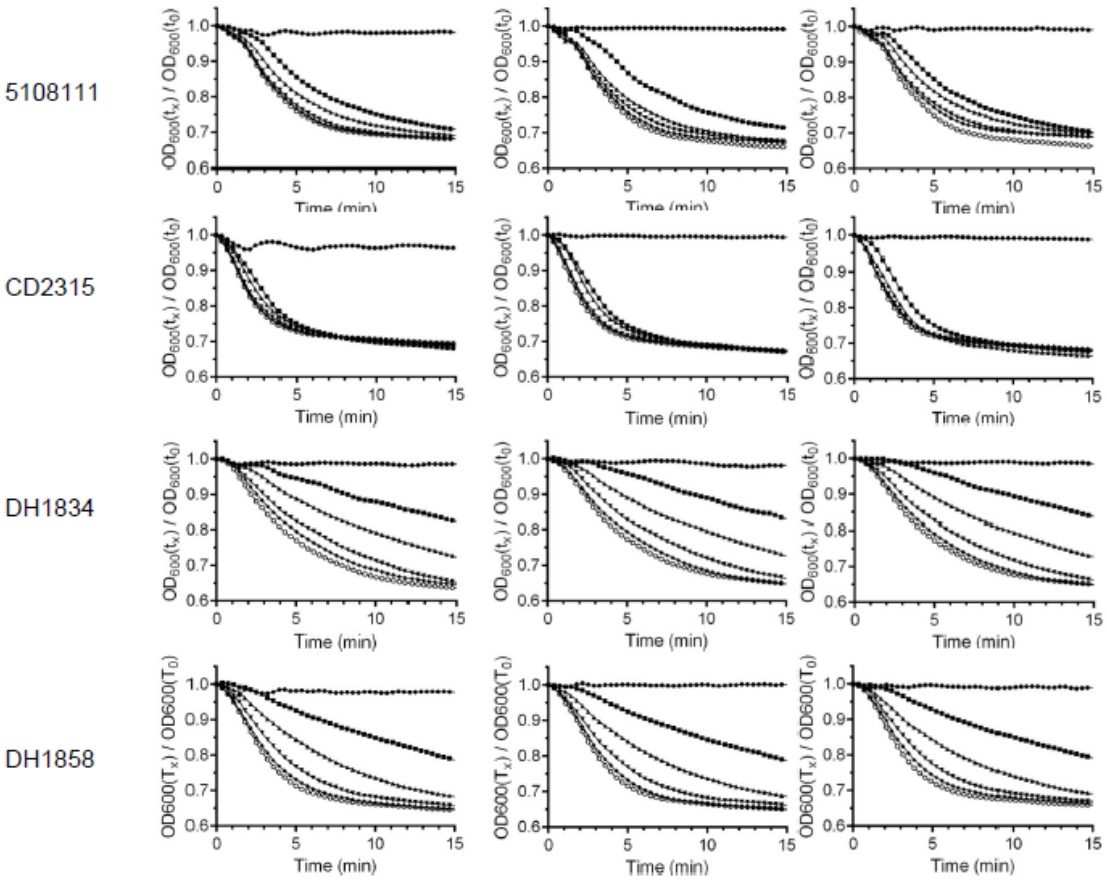


**Figure S 2:** Germination by certain *C. difficile* strains at lower TA concentrations (reprinted with permission from (121)). Purified *C. difficile* spores were suspended in BHIS medium alone or medium supplemented with the indicated TA concentrations. *C. difficile* 5108111, *C. difficile* CD2315, *C. difficile* DH1834, or *C. difficile* DH1858 spores suspended in BHIS medium alone (●) or medium supplemented with 1 mM (■), 2 mM (▲), 5 mM (▼), 10 mM (♦) or 20 mM (○) TA. Experiments were performed in triplicate and each experiment is shown. Data could not be displayed as the average due to inconsistent time points between experiments which is generated by the spectrophotometer.

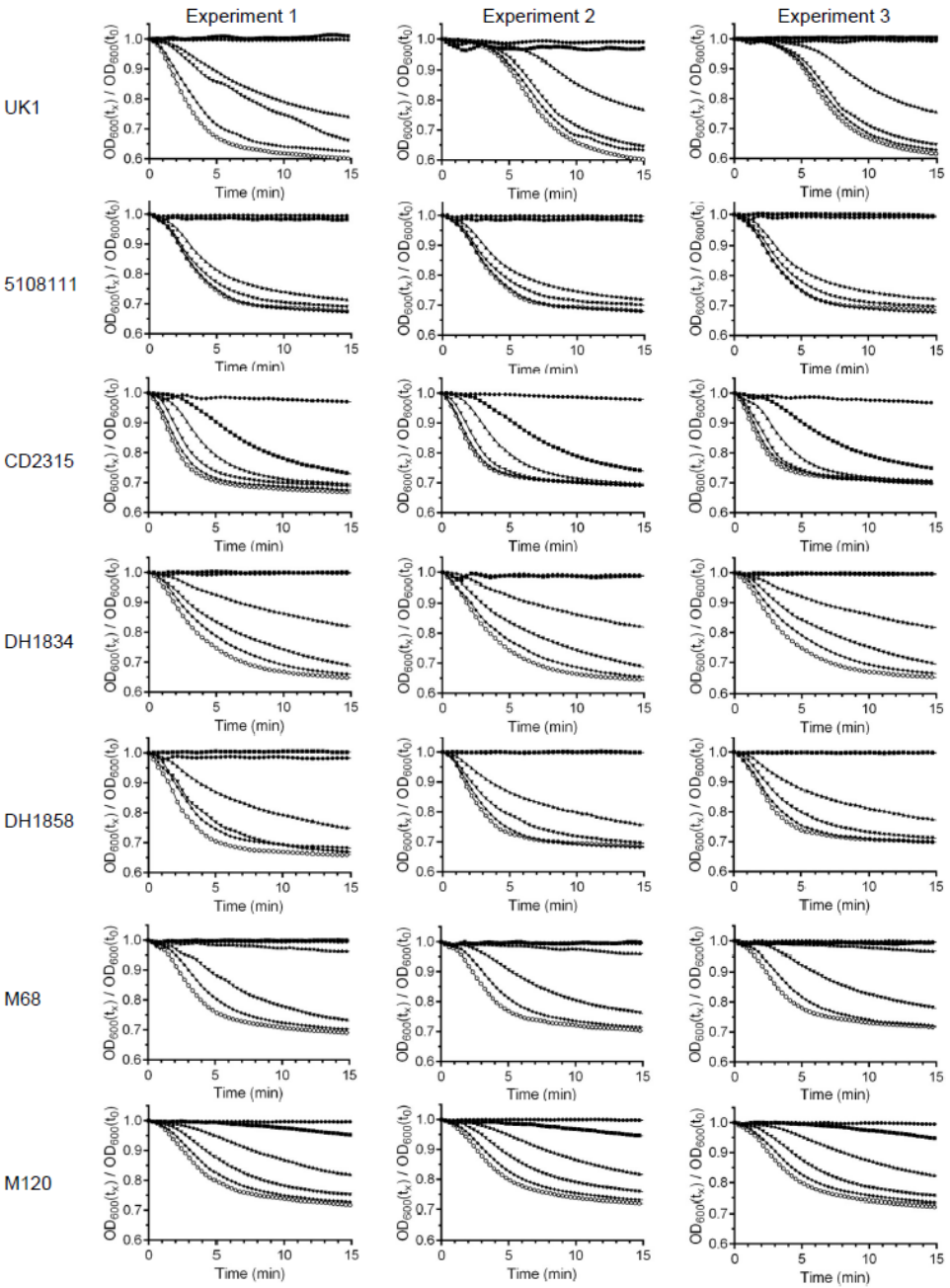


**Figure S 3:** Complete germination data for *C. difficile* spores suspended in medium supplemented with TA and CDCA (reprinted with permission from (121)). Purified *C. difficile* spores of the indicated strains were suspended in BHIS medium supplemented with 1 mM CDCA (●) or medium supplemented with 2 mM (■), 5 mM (▲), 10 mM (▼), 20 mM (♦) or 50 mM (○) TA and 1 mM CDCA. Germination was monitored at OD600 as described previously. Experiments were performed in triplicate and each experiment is shown. Data could not be displayed as the average due to inconsistent time points generated by the spectrophotometer between experiments.

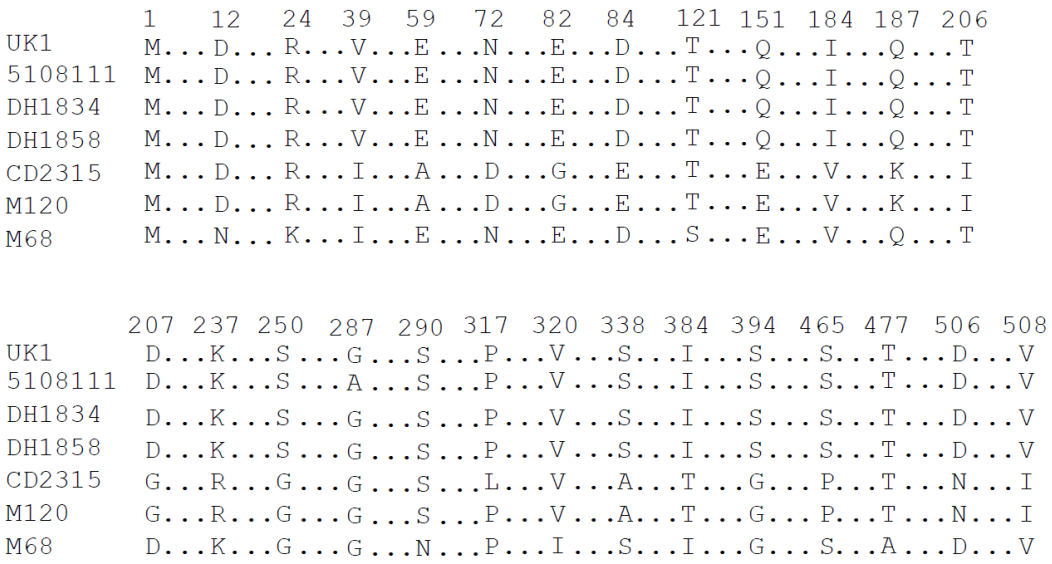


**Figure S 4:** Alignment of CspC protein sequences (reprinted with permission from (121)). The cspC gene was amplified from each of the *C. difficile* strains used in this study and sequenced. The translated sequence was aligned using the Clustal W method and only the sites where substitutions occurred are listed.

```
          1   62  110 211 267 287 311 360 362 367
UK1       M...I...D...R...Q...I...A...K...V...D
5108111   M...I...D...R...Q...I...A...K...V...D
DH1834    M...I...D...R...Q...I...A...K...V...D
DH1858    M...I...D...R...Q...I...A...K...V...D
CD2315    M...V...N...K...R...V...T...R...I...N
M120      M...V...N...K...R...V...T...R...I...N
M68       M...I...D...R...Q...I...A...K...V...D

         408 446 499 523
UK1       V...I...G...I
5108111   V...I...G...I
DH1834    V...I...G...I
DH1858    V...I...G...I
CD2315    I...V...V...V
M120      I...V...V...V
M68       I...V...G...I
```

**Figure S 5:** Alignment of CspB protein sequences (reprinted with permission from (121)). The cspB gene was amplified from each of the *C. difficile* strains used in this study and sequenced. The translated sequence was aligned using the Clustal W method and only the sites where substitutions occurred are listed.

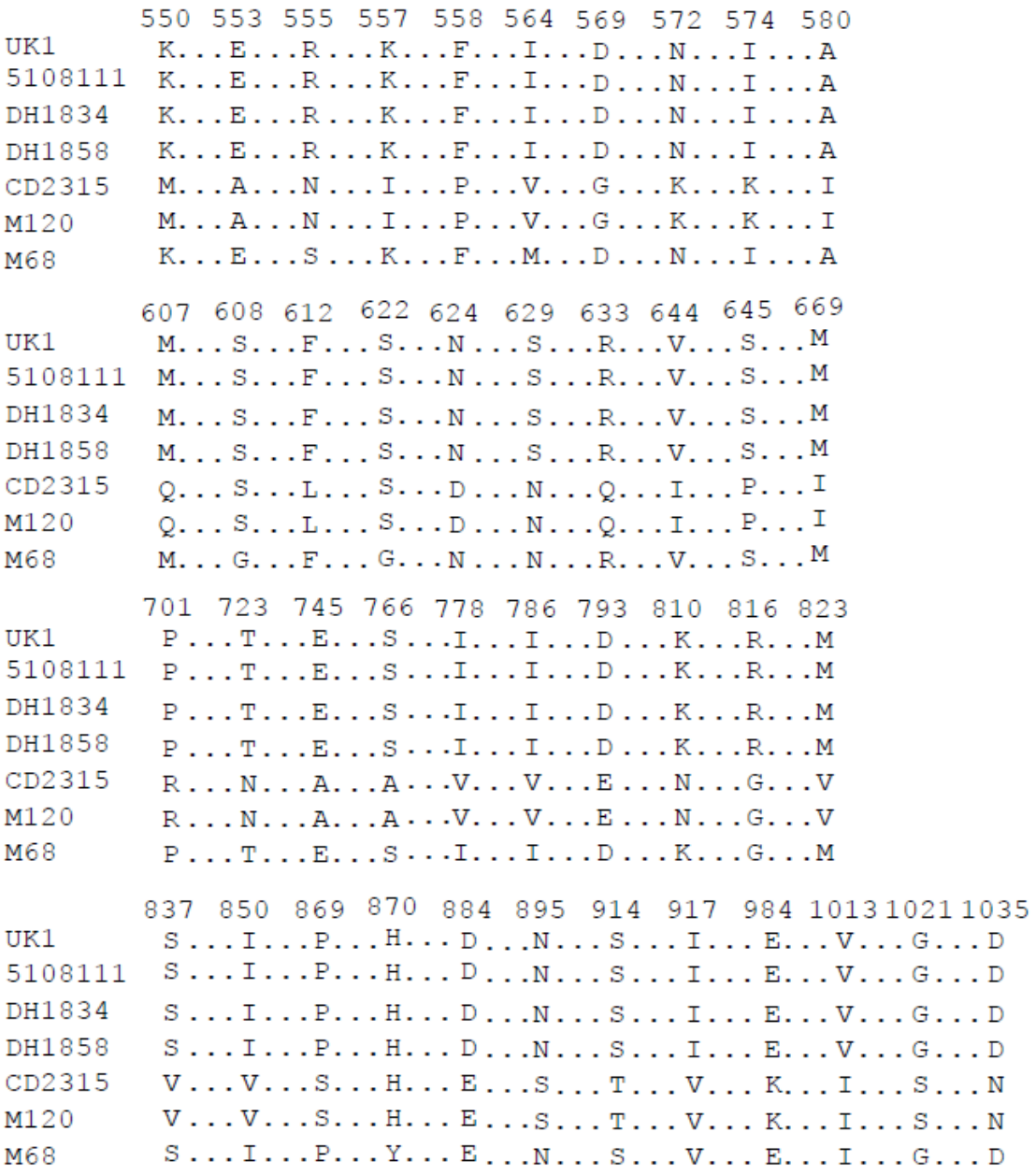


**Figure S 6:** Alignment of CspA protein sequences (reprinted with permission from (121)). The cspA gene was amplified from each of the *C. difficile* strains used in this study and sequenced. The translated sequence was aligned using the Clustal W method and only the sites where substitutions occurred are listed.

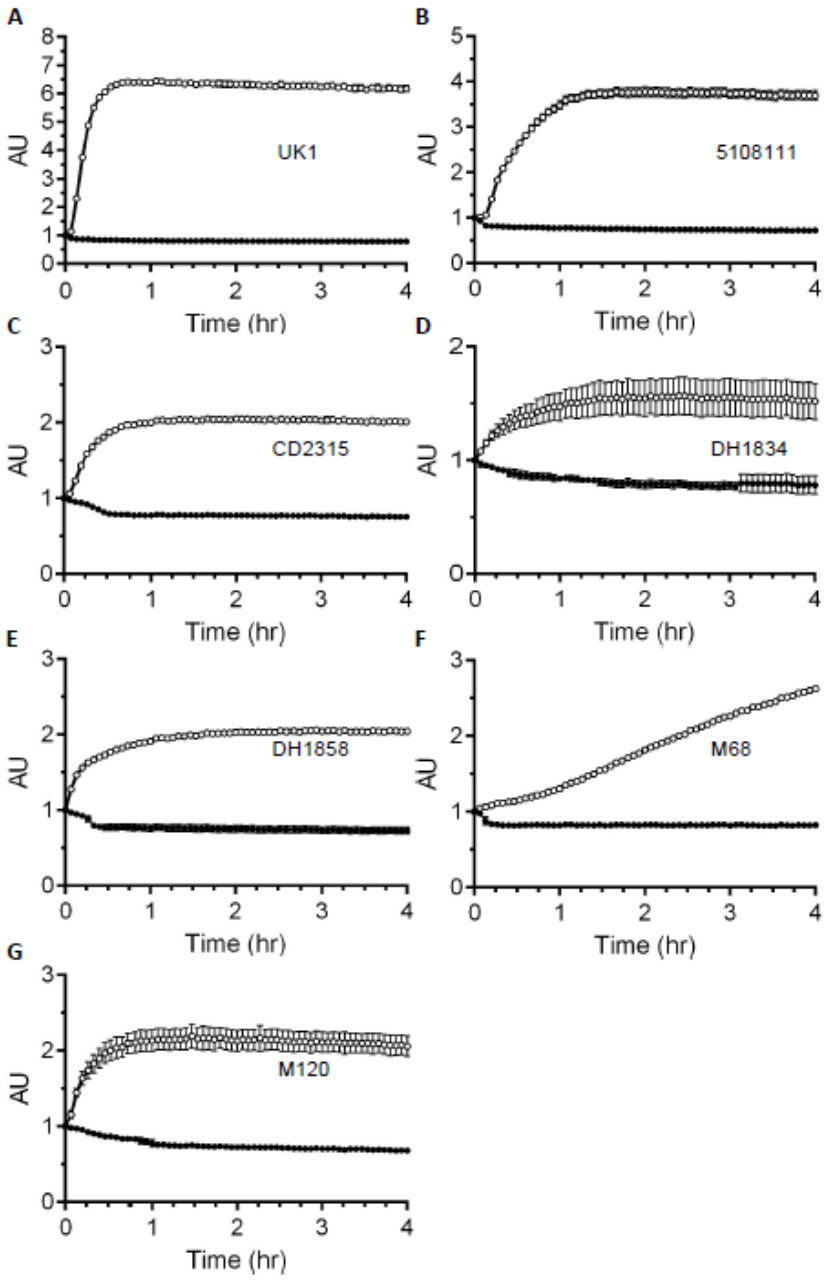


**Figure S 7:** *C. difficile* spores require TA to initiate germination (reprinted with permission from (121)). Purified *C. difficile* spores were suspended in buffer supplemented with 100 mM glycine (●) or glycine and 10 mM taurocholic acid (○) and DPA release during spore germination was monitored using Tb3+ fluorescence, described previously, for 4 hours. (A) *C. difficile* UK1, (B) *C. difficile* 5108111, (C) *C. difficile* CD2315, (D) *C. difficile* DH1834, (E) *C. difficile* DH1858, (F) *C. difficile* M68, (G) *C. difficile* M120. Data points represent the average of 3 independent experiments and error bars represent the standard error of the mean.

*cspBA*

```
P. bifermentans   VFFINYEVIVKYNGDISEIEKDLGVSVEKLGYNYAIIITDSAEKIDLLLNYPQIEYLEKP
C. difficile      VIIINYELIVKYNGDILRLEEELGVSVEILNSSYAIITSSNEEDVNTLLTYPEIEFIEKP
                  *::****:******** .:*::****** *. .**** :.. *.:: **.**:**::***

P. bifermentans   FILTTQDIQSFSRTGITRFKNTNRLTGKGTIIGIIDSGIDYNIDLFKDSNGNSKILYYWD
C. difficile      FILQTQDVQSFSSTGITGFKNRTGLTGKGTIIGIIDSGIDYTLPVFRDSDGRSKILYYWD
                  *** ***:**** **** *** . **********************.: :*:**:*.********

P. bifermentans   QSIKGKPPEGFQYGSLYTNEDINKAIKRELNIPISPTSTHGTHVAGIACQIAEEANLIVV
C. difficile      QSIQGNPPEGFREGTLYTNEDINNAIDGSMYIPISTTSLHGTHVAGICATIASDARIIVV
                  ***:*:*****: *:********:**. .: ****.** ********.. **.:*.:***

P. bifermentans   RVGSVVTDVFSKSTEFMRAIKFILDKALELKMPAAINISYGSNEGSHRGLSLFEQYIDDM
C. difficile      RVGNIQTDIFSRSTEFMRAIKFILDRALELRMPVTLNISYGSNEGSHRGTSLFEQYIDDM
                  ***.: **:**:**************:****:**.::************** **********

P. bifermentans   SAFWKNNIVVAAGNNGDKDGHKSIKLDK-ETTEVEFVVGENEKILNINIWPEFIDDFSVY
C. difficile      CLFWKNNIVVAAGNNADKGGHKRIRLQNNITEEVEFIVGEGERILNINIWPDFVDDFSVH
                  . *************.**.*** *:*::  * ****:***.*:********:*:*****:

P. bifermentans   IVNPSNIKSQEISLTSGEIKNVLGGTRVKGYFYPITPYSLSRRISVQLTSPTFINPGIWK
C. difficile      LVNPSNNQTQAISLTSGEIRNTLGETRITGYFYPIAPYSLTRRVTLQLSSNTQITPGLWK
                  :***** ::* ********:*.** **:.******:****:**:::**:* * *.**:**

P. bifermentans   LVFTPINIVMGDISIYLPTSEGISKDTRFLEANKNLTVTVPGTANKVITVGSFNSTTDTV
C. difficile      IVFEPIDIVTGNVNIYLPTSEGLNRNTRFLIPTQELTVTVPGTASRVVTVGSFNSRTDIV
                  :** **:** *:::********:.::**** ..::**********.:*:******* ** *

P. bifermentans   SIFSGEGDIDQNVYKPDLLAPGENILSVLPGGSIGALTGTSMATPHVTGVVSLLMQWGIV
C. difficile      SIFSGEGDTQLGVFKPDLLAPGEDIISFLPGGTSGALTGTSMATPHVTGVCSLFMEWGIV
                  ******** : .*:*********:*:*.****: ********************* **:*:****

P. bifermentans   DKNDLFLYSQKIKAFLLKEAKRNSTYTYPNNPMGFGFLDLTEVRLDNISNLNKEYDLLYR
C. difficile      NGNDLFLYSQKLRALLLKGARRLSNQSYPNNSSGFGFLNLSDIDLYTLSSINQDLETEDI
                  : *********::*:*** *:* *. :****. *****:*::: * .:*.:*:: :

P. bifermentans   KRKKKVKELSRLDLPLDLAFRYHVEHG----------------------------PNFEE
C. difficile      GYRSINKSFKDEENRYKFIDGYNIQIHNDLENEIYISKNASRQSGILSGIDVVHTPEFEE
                    :.  *.:.  :   .:   *:::                                *:***

P. bifermentans   ELKAIGLDYTYYEISDTEGVLTLPIKDPKRYNKLLKIEGFKFIASSLVMNQLGTISRDTS
C. difficile      ELAGLGMSQNFFKISDSLGVLSINNTDYSSIQRVLQLPSIIRTVSTTKMTLLGEINRGTF
                  ** .:*:. .:::***: ***::  .* .  :::*:: .:   .*:  *. ** *.*.*

P. bifermentans   NGVVAKEEIGANFLQNNLNIPITGSGVLVAVLDSGIDYLHEDFIYPDKTSKIAYIWDQTE
C. difficile      GGVVATEEMGVNFFKNNPNININITGRGTLISIADTGIDYLHPDFIYPDGTSKIVYLWDQTK
                  .****.**:*.**::** ** *** *.*::: *:****** ****** ****.*:****:

P. bifermentans   DGNPPKGYKIGTEYSREDINKAIQANDNNLTKDETGHGTMISGICSGLGNLNKQYSGVAP
C. difficile      EGTPPDGFYIGTEYTREDINRAIAENDPSLSQDEVGQGTMLSGICSGLGNVNSEYAGIAE
                  :*.**.*: *****:*****:**  ** .*::**.*:***:*********:*.:*:*:*

P. bifermentans   DSELIIVKLKKIEGNYDSTFLKAGVMYAYEKSIELNKPIVINLSLGSNNLIGAKENTLRK
C. difficile      DSELIIIKLGKIDGFYNSAMLFAASQYAYKKAFELRRPLVINMSLGTSSLAGLTNRSNSE
                  ******:** **:* *:*::* *.  ***:*::**.:*:***:***:..* * .:.:  :

P. bifermentans   EPHFARGVCIVSAVGNEGNTQTHSMGKVNFVGQQKDIEIEIFEDESLLEINIWVNKPDKI
C. difficile      KAFFTRGLCITAGAGNEGNTQTHTSGIIPHVGGSVEVELELNEDEEELSLELWLNRPDKA
```

```
                  :..*:**:**.:..*********: * : .** . ::*:*: ***. *.:::*:*:***

P. bifermentans   SAAVIAPSGEQSKFITVSSYNEVSGLFDLESTWYVITYVYPTEYSGQEQITVLLKNATKG
C. difficile      DVIIVSPTGEESKSVGISNYNKVTGLFDLEGTEYSITYIYPTTFSGQQFTNVTLKNAKRG
                  .. :::*:**:** : :*.**:*:******.* * ***:*** :***:  .* ****.:*

P. bifermentans   IWKIRLRGEYITNGIYNAYLPNRAIINPGTKFRDSTSAYTINYPATYRDVISAGAYNLVE
C. difficile      VWKIRLVGVYIITGRYNLYLPNRELLKSGTRFREVDPFYTINYPAIQDDLITVGAYNTIN
                  :***** * ** .* ** ***** :::.**:**:  . *******   *:*:.**** ::

P. bifermentans   NSIWPPSSRGPTINGLLRPDIVAPGVNIISTYPGNTYATLTGTSASGAYLAGSVALYLQY
C. difficile      GSLWQSSSRGPTIEDRLKPDIVAPGVNIIAAYPGNTYATITGTAAASAHAAGAAAMYFQY
                  .*:* .*******:. *:***********::********:***:*:.*: **:.*:*:**

P. bifermentans   TLVDNYYPLKGFTNIIRTYIRAGAKRNSEIVYPNDIYGYGILDIRGAFDQLK
C. difficile      TFVDGRYPNQAYVQKIKTFMQAGARKDSNTVYPNTNSGYGLLDVRGMFDVLR
                  *:**. ** :.:.: *:*:::***:::*: ****   ***:**:** ** *:
```

*cspC*

```
P. bifermentans LEKSYLIIYKGDIASDLKKAGIEKYMILNPSLTVIYVPQNFREETLNRIPSITWWQSSIP
C. difficile    MEKSYCIIYQGDIESALQENGINRYMVLNSQLAVIYVPVDFDETILNNIIQVAWWEESEP
                :**** ***:*** * *:: **::**:**..*:***** :* *  **.* .::**:.* *

P. bifermentans MSSLIEITNNLDEGVSVSDAASTDYIYKNPYIQSTGRNVLIAIIDSGIDYLHPDFMENN-
C. difficile    MSSLIEITNNVNNGETITTAAETDYIYENPYNDITGRGILLAVIDSGIDYLHPDFINDDG
                **********:::* ::: **.*****:*** : ***.:*:*:************:::::

P. bifermentans KTKIISIWDQESEKKNPPDGLIFGSEFTSEDINKAIEENDKTLSEDSIGTGTTAAGIAAG
C. difficile    TSKVLYLWDQEANTNPPPEGFIFGSEFTRSQLNIAINRNDGSLSQDNIGTGTLVSGILAG
                .:*:: :****::.: **:*:******* .::* **:.** :**:*.***** .:** **

P. bifermentans RGNLNSQYKGVAIDSKLVVVKLREYKDTYKKGKINYQGSDFLASIRYVLDVAKKENKNMI
C. difficile    NGRINSQYRGITTESDLIVVKLKSYTDTYYAGRINYSVSDFLAAITYVTNIARTENKPLI
                .*.:****:*:: :*.*:****:.*.***  *:***. *****:* ** ::*:.*** :*

P. bifermentans INLTVGLISKSIVESTMLSTFNELSQPGNIVVSGAGNEGNTDIHYRGNIKNKETVDDIII
C. difficile    INLTIGVKSSAVATTSILDTFNILSSAGVVVVSGAGNQGNTDIHYSGRFSSVGEVQDVII
                ****:*: *.::. :::*.*** **..* :*******:******* *.:..   *:*:**

P. bifermentans QVGEQTNLDIKLVVNGPDKIGAMIISPAGEMSYKIMYSPDYYVYKGKFNLESTPYEMRLS
C. difficile    QDGDDYALDITLNTNGPDKVGAQIISPSGEVSHDIRYSPDFYIYRGKFNLENTTYAMRFI
                * *::  ***.* .*****:** ****:**:*:.* ****:*:*:******.*.* **:

P. bifermentans YPWLESGNEELTISLYDIKPGIWTLRLIPEFIIEGNYDVYLPNKNIISEEARFSDPASEA
C. difficile    YPYITSGKENLEIRLRDIKPGVWILRLTSELIISGEYDIYLPNKNLIAPDTRFLDPDSVA
                **:: **:*:* * * *****:* *** .*:**.*:**:******:*: ::** ** * *

P. bifermentans TISMYAASENVITIGAYNDKTDSIWIGSSKGPVNLDLIKPDIVAPGVDIISTYINSSYNT
C. difficile    TITMYAASDDVITVGTFNNKTDSMWIGSSKGPIRGRGIKPDIVASGVDIISTYKNGTYNT
                **:*****::***:*::*:****:********:.   *******.******** *.:***

P. bifermentans SIGTGVSSSIVSGVLAIILEYITSEYEFAEELLSVQPLKTYLMLGATKKDIYIYPNITQG
C. difficile    GTGTGVSSSIVTGVLALLMEYLEKQDNVPRLSLFTQVLKTYLILGATKLEIYTYPNVSQG
                . *********:****:::**: .: :...  * .* *****:***** :** ***::**
```

```
P. bifermentans YGILNLKNTIVEIAKNFE
C. difficile    YGILNLKNTIQQIANTL-
                ********** :**:.:
```

*sleC*

```
P. bifermentans KYIFSFPAFFSLIKFSLPLLKCTSNIIILHRVPFYLLFFKIFTFFNLFYFHHKINLILQM
C. difficile    ------------------------------------------------------------


P. bifermentans VHFKYFIHIYKLIDYLGGIFLYKGLLTIKVTDEITNFPIEGVSINICAMPKEGSTKSKYI
C. difficile    --------------------MQDGFLTVSIIDATNNRPIQNAVVNIYSMSN-GSQSSSTL
                                    : .*:**:.: *  .* **:.. :** :*.: ** .*. :

P. bifermentans YKNLITNSSGMVKKVSLDAPNFIYSQVPNSPRAYSTYILTISKDGYQSVVIQGVQILPLV
C. difficile    YQNLRSNESGQVTGLVLPAPDVDYSLQPSDVRPYSQYIVEAIADGYETVVIEGTQLLATI
                *:** :*.** *. : * **:. **  *.. *.** **:    ***::***:*.*:*. :

P. bifermentans EAIQNISLSKIS----AFTTTNKKIYKIGDNVLYGNYQPKILEDDLKKVP-----YVLPN
C. difficile    VARQGVPMSPRTRSKRSFSRQSELIFDIGEHTLYGTYPPKIPESNLKPLPPPTGFVVLDN
                * *.:.:*  :    :*:  .: *:.**::.***.* *** *.:** :*      ** *

P. bifermentans VVVPEYIIVHDGMPSDKNAPNYWIPFRDYIKNVASSEIYATWPTETIYANVVAIVSFTLN
C. difficile    PVVPEFIVVHDGLPEDSSAPNYWIPFKEYIKNIASSEIYSTWPEQTIYANVIAIISFTLN
                ****:*:****:*.*..********::****:******:*** :******:**:*****

P. bifermentans RVYTEWYRNMGYDFTITSTTAYDHKFIYNRNIFDTISVVVDNIFNVYIQRPKGNPQPLLA
C. difficile    RVFTEWYRNKGYNFTITSTTAYDHKFINNRNLFEPINVVVDAIFNTFIKRPPTSRQPLLA
                **:****** **:************** ***:*:.*.**** ***.:*:**  . *****

P. bifermentans QYCDGIQTQCPGKMTQWGSKYLGDQGYKFDEILRYYYGQDIGLQGTDMIKGVPSSFPGYT
C. difficile    QYCDGQKSQCPDQMTQWGSKDLGDQGYDYESILRYFYGDEIVFERAPIVSGVPVSFPGTT
                ***** ::***.:******* ******.::.****:**::* :: : ::.*** **** *

P. bifermentans LTLWSTGEPVRTIQNQLNAIANAYPALPKVDVDGIYGPKTQESVRKFQEIFRMTQSGNVD
C. difficile    LQVGSSGQYVRTIQNQLNAISNSYPAVPKVIEDGIYGADTENAVKIFQGIFGLPQSGVVD
                * : *:*: ***********:*:***:***  *****..*:::*: ** ** :.*** **

P. bifermentans FATWYAISKIYVAVTKIAEFEI--
C. difficile    FKTWYEISRVYVATTRIASLNPLI
                * *** **::***.*:**.::
```

**Figure S 8:** Alignment of the *C. difficile* and *P. bifermentans* CspBA, CspC and SleC proteins (reprinted with permission from (142)). The *C. difficile* and *P. bifermentans* CspBA, CspC and SleC sequences were aligned in DNASTAR MegAlign using the Clustal W method. The residues at catalytic sites are highlighted in red, the conserved sequences surrounding the catalytic residues are highlighted in black, and deviation from the consensus sequence for S8 proteases are highlighted in green.

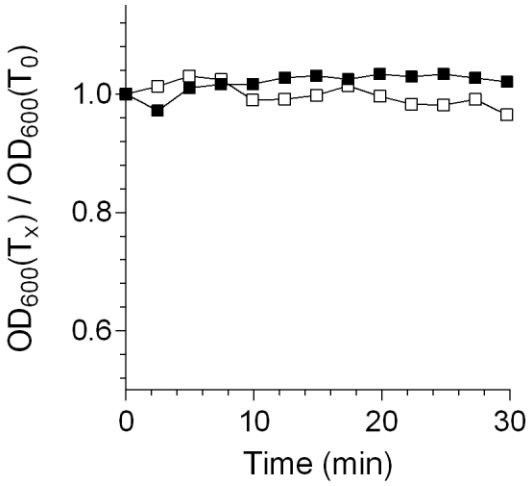


**Figure S9:** Germination of non-heat-treated *P. bifermentans* spores (reprinted with permission from (142)). *P. bifermentans* spores were purified as described in the materials and methods. The un-heat-treated spores were suspended in buffer alone (□) or in buffer supplemented with 50mM Ala, 5mM Phe and 5 mM Arg (■).

APPENDIX B

SUPPLEMENTARY TABLES

**Table S 1:** Strains and plasmids used in Section 2 study (reprinted with permission from (121))

| ***C. difficile* strains** | **Phenotype** | **Reference** |
|---|---|---|
| UK1 | Ribotype 027 | (13, 25, 27) |
| 5108111 | Ribotype 027 | (31) |
| DH1834 | Ribotype 027 | (31) |
| DH1858 | Ribotype 027 | (31) |
| CD2315 | Ribotype 078 | (31) |
| M120 | Ribotype 078 | (60, 61) |
| M68 | Ribotype 017 | (62, 63) |
| JSC11 | *cspC::ermB* | (13, 27) |
| CAA5 | *sleC::ermB* | (27) |
| ***E. coli strains*** | | |
| DH5α | F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(rK- mK+), λ– | (64) |

**Table S1 continued**

| ***C. difficile* strains** | **Phenotype** | **Reference** |
|---|---|---|
| BL21(DE3) | F– ompT gal dcm lon hsdSB(rB- mB-) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5]) | Novagen |
| **Plasmids** | | This study |
| pET22b | Expression vector | (Novagen) |
| pKS02 | *cspB*-expressing plasmid | This study |
| pKS08 | *sleC-expressing plasmid* | This study |
| pEK-K-CspC | Codon optimized *cspC* | This study |
| p*cspC-CO-Δ50CPD* | Codon optimized *cspC*-expressing plasmid | A. Shen (gift) |

**Table S 2:** Primers used for electroporation study (Section 4) to make plasmids

| Oligonucleotide | Sequence | Reference |
|---|---|---|
| 916 | GAAACAGCTATGACCGCGGCCGCTGTATCCA TATGCTACTTTTACAGTCTTCCCTATG | This study |
| 1377 | CTAATCAATTATAATTTTACATAGGTTCTTAT CTATAGAGTATTTGCTATCTGTTG | This study |
| 1378 | GATTCAACAGATAGCAAATACTCTATAGATA AGAACCTATGTAAAATTATAATTG | This study |
| 1379 | CAGTGCCAAGCTTGCATGTCTGCAGGCCTCG AGATGGAAAAATCTTATTGTATAATT | This study |
| 5'Tn916ori | AAGCGGAAGAGCGCCCAATACGCAGGGCCC TAACATCTTCTATTTTTCCCA | McAllister 2017 |
| 3'Tn916ori | TATCTACAATTTTTTTATCCTGCAGGGGGCCC CTAAAGGGAATGTAGATAAATTATTAG | McAllister 2017 |
| 5'pyrE_HR_xyl R 2 | CATTCAAAAGAAGGAAGAACATCAATGCTTC TCGAGCTAGCATAAAAATAAGAAGCCT | This study |
| 3'cas9_Pxyl 2 | TAATCCTATACTATATTTTTTATCCATTTAATT AACTCTCCTCTTTACCCTCCTT | This study |