SIGNIFICANCE OF AMINO ACID(S) DURING *CLOSTRIDIUM DIFFICILE* SPORE GERMINATION

A Dissertation

by

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ABSTRACT

Clostridium difficile spore germination is critical for the transmission of disease. *C. difficile* spores germinate in response to cholic acid derivatives, such as taurocholate (TA), and amino acids, such as glycine or alanine. Many endospore-forming bacteria embed alanine racemases into their spore coats and these enzymes are thought to convert the L-alanine germinant into D-alanine, a spore germination inhibitor. *C. difficile* packages the Alr2 alanine racemase into the spore. Here, I describe my findings that *alr2* mutant spores more readily germinate in response to L-alanine as a co-germinant. Surprisingly, D-alanine also functioned as a co-germinant. Finally, I demonstrated that L- and D-serine are also co-germinants for *C. difficile* spores and *C. difficile* Alr2 can accommodate both alanine and serine as substrates.

During the analysis on Alr2, I found that D-alanine can function as a co-germinant for *C. difficile* spores at 37 ºC but not at 25 ºC. Because most germination assays are conducted at room temperature, I tested the ability of other amino acids to act as co-germinants with TA at 37 ºC and found that many amino acids previously categorized as non-co-germinants are actually co-germinants at 37 ºC.

Even though *C. difficile* spore germination is known to require an amino acid co-germinant, the amino acid spore germinant receptor was unknown. In search of the amino acid germinant receptor, I used EMS mutagenesis as a strategy to generate mutants with altered requirements for the amino acid co-germinant, similar to the strategy used previously to identify the bile acid receptor, CspC. Surprisingly, I identified strains that do not require amino acids as co-germinants, and the mutant spores germinated in response

to TA alone. Upon sequencing these mutants, I identified non-isogenic mutations in *yabG*. For *C. difficile*, the YabG protease is critical for the processing of CspBA to CspB and CspA and preproSleC to proSleC during spore formation. A defined *yabG* mutant exacerbated the EMS mutant phenotype. Moreover, I found that various mutations in *cspA* caused spores to germinate in the presence of TA alone without the requirement of an amino acid. Thus, my study provides evidence that apart from regulating the CspC levels in the spore, CspA is important for recognition of amino acids as co-germinants during *C. difficile* spore germination and that two pseudoproteases (CspC and CspA) function as the *C. difficile* germinant receptors.

DEDICATION

I would like to dedicate this dissertation to a religious scholar Mahendra Adhikari (Guruji) who taught me to have a purpose in life and encouraged me to be who I should be.

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Contributors

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Experiments on ITC in Chapter III were done in collaboration with Dr. Steve Lockless from the Department of Biology. Work in all chapters 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

ADP	adenosine di-phosphate
AGFK	L-asparagine, glucose, fructose and K^+ ions
ANOVA	Analysis of variance
ATP	adenosine tri-phosphate
AU	arbitrary units
BHIS	Brain Heart Infusion, supplemented with 5 g /L Yeast extract
BHIS-TA	BHIS supplemented with taurocholic acid
CaDPA	calcium dipicolinic acid
CDC	Centers for Disease Control and Prevention
CDCA	Chenodeoxycholic acid
CDI	*Clostridium difficile* Infection
CDMM	*C. difficile* minimal media
CDT	*C. difficile* transferase
CPD	cysteine protease domain
CROPs	combined repeated oligopeptides
DNA	Deoxyribonucleic acid
DPA	Dipicolinic acid
EC_{50}	concentration that achieves half-maximum germination rate
EMS	Ethyl methanesulfonate
FDA	Food and Drug administration
FDAA	1-fluoro-2-4-dinitrophenyl-5-L-alanine amide

FMT	fecal microbiota transplantation
FOA	5-fluoroorotic acid
GTD	glucosyltransferase domain
GTP	guanosine triphosphate
HEPES	2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid
HPLC	High Performance Liquid Chromatography
IM	inner membrane
IPTG	Isopropyl β-D-1-thiogalactopyranoside
ITC	isothermal titration calorimetry
K_i	apparent inhibitory constant
K_m	apparent rate of germination
LB	Luria-Bertani medium
MAL	muramic-δ-lactam
NAD^+	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
NAG	N-acetylgycosamine
NAM	N-acetylmuramic acid
OD_{600}	optical density measured at 600 nm
PaLoc	pathogenicity locus
PCR	polymerase chain reaction
PLP	pyridoxal 5’ phosphate
PMSF	phenyl methane sulfonyl fluoride

PMT	photomultiplier tube
PVDF	Polyvinylidene difluoride
RBD	receptor binding domain
RNA	ribonucleic acid
SASPs	small acid soluble proteins
SCLEs	spore cortex lytic enzymes
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNPs	single-nucleotide polymorphism
TA	taurocholic acid
TBS	Tris-buffered saline
TBST	TBS supplemented with 1% (vol / vol) Tween 20
TcdA	*Clostridium difficile* Toxin A
TcdB	*Clostridium difficile* Toxin B
TCEP	Tris(2-carboxyethyl) phosphine
TY	Tryptone yeast medium
UV	Ultraviolet light
WT	wildtype

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CHAPTER I

INTRODUCTION TO *Clostridium difficile* SPORES

1.1 Epidemiology

Clostridium difficile, also known as *Clostridioides difficile* (1), is a Gram-positive, endospore forming bacterium and is a leading cause of antibiotic associated diarrhea and healthcare-associated infections (2). Although *C. difficile* was first isolated from a stool of a healthy infant in 1935, *C. difficile* was only linked with human disease in 1978 (3, 4). Each year, nearly half a million *C. difficile* infections (CDI) are present in the US and lead to approximately 30,000 deaths (2, 5). The financial burden for the treatment of CDI in the healthcare system exceeds more than $4 billion per year (6). Due to its natural and developing antibiotic resistance, the CDC (Centers for Disease Control and Prevention) has listed *C. difficile* as an urgent threat in the US healthcare system (2, 5, 7).

The current treatment options for CDI are broad-spectrum antibiotics, *i.e.*, vancomycin, or fidaxomicin (8). However, the use of broad-spectrum antibiotics has been discouraged due to their continued disruption of the colonic microbiota, which plays a key role in the resistance to colonization by *C. difficile* (9). Due to this continued disruption, a patient will frequently experience recurring disease. Treatments involving the restoration of a healthy microbiome through biotherapeutic strategies such as fecal microbiota transplantation (FMT), have become popular in recent years due to the ~ 90% cure rate (10, 11).

Another biotherapeutic strategy to treat CDI is the use of monoclonal antibodies directed against the *C. difficile* toxins. This strategy protects against recurrence by

mediating early reconstruction of gut microbiota and the cessation of CDI symptoms (12). Interestingly, only Bezlotoxumab (directed against TcdB) protects against CDI (13). In fact, Bezlotoxumab is the first FDA-approved monoclonal antibody shown to have effectiveness in prevention of recurrent CDI. Although, the use of Bezlotoxumab as a treatment is a very compelling therapy against CDI, there are still risks associated with this therapy (13, 14) due to the limited data on the effectiveness against the prevention of CDI.

Another treatment strategy being studied is the use of competitive inhibition with non-toxigenic *C. difficile* strains. Development of vaccines to treat against CDI is another emerging therapeutic treatment (15). Wang *et al*., developed an oral vaccine, mTcd138, which contains only some domains of the toxin and can target both *C. difficile* toxins and colonization factors. They found that in both mice and hamsters that oral immunization with non-toxigenic strains protected fully against the hyper-virulent strain UK6 (ribotype 027) (15). However, a recent Phase III clinical trial that tested the effectiveness of a potential *C. difficile* vaccine failed (14). Since this is very recently published data more study is required for it to be considered as a future CDI treatment.

1.2 Toxin secretions

Upon colonization, *C. difficile* vegetative cells secrete two toxins, TcdA (an enterotoxin) and TcdB (a cytotoxin) (16). The genes encoding these toxins, *tcdA* and *tcdB*, are located within the pathogenicity locus (PaLoc) (17-19). In addition to *tcdA* and *tcdB*, the PaLoc also encodes three other genes, i) *tcdR*, encoding an alternative RNA polymerase sigma factor that is responsible for the expression of *tcdA* and *tcdB* ii) *tcdE*,

encoding a holin-like protein necessary for the extracellular release of both toxins and iii) *tcdC,* encoding an anti-sigma factor that negatively regulates the expression of toxins (16, 20, 21). TcdA and TcdB, share a multi-modular domain structure described as the ABCD model (A: biological activity; B: binding; C: cutting; D: delivery). Region A, located at the N-terminus, contains a glucosyltransferase domain (GTD) that acts on the small GTPases involved in regulation of the cytoskeleton. Region B, located at the *C*-terminus, consists of combined repeated oligopeptides (CROPs) that form the receptor binding domain (RBD) and help in the initial interaction with the host cell. Region C is the cysteine protease domain (CPD) that promotes the auto-catalytic cleavage of the GTD from the larger protein. Region D, located in the middle of the protein, is the delivery domain that is involved in the translocation of the toxin into the cytosol from the acidified endosome (16, 22).

Environmental signals such as nutrient limitation, change in temperature or change in the redox potential regulates the expression of TcdA and TcdB toxins. Once toxins are produced by the vegetative cell, toxins are delivered into the host cell cytosol by binding of RBD to the host cell surface receptor. The two toxins have different receptors in the host. For example, sucrase-isomaltase and the glycoprotein 96 (gp96) produced by the host colonocyte apical membrane recognize TcdA RBD (23) while the chondroitin sulfate proteoglycan 4 (CSPG4), the poliovirus receptor-like 3 (PVRL3) (24), and frizzled proteins (FZD1, 2 and 7) (25) have been identified as TcdB receptors. When the RBD domain bind to their specific receptors, the toxins are internalized through receptor-mediated endocytosis (26). Toxin once internalized, can acidify endosomes leading to

pore formation. The acidification of the endosome alters the structure of the toxin through a pH-induced conformational change that helps in the translocation of the toxin into the host cytosol. As the toxins enter the host cytosol, inositol hexakisphosphate-dependent autocatalytic cleavage releases the GTD of the toxin (27, 28). The GTD then inactivates the Rho-family of small GTPases leading to loss of barrier function and inflammation of the colonic epithelium causing symptoms ranging from mild to severe diarrhea to other possibly fatal conditions such as pseudomembranous colitis and toxic megacolon (20, 29, 30).

Some *C. difficile* strains produce a binary toxin, *C. difficile* transferase (CDT). CDT belongs to the family of binary ADP-ribosylating toxins and consists of two separate toxin components: CDTa - an enzymatic component and CDTb - a binding component (22). CDTa is the biologically active ADP-ribosyltransferase processing component and is required for actin modification. The CDTb component binds to host cells and is required for the translocation of CDTa into the host cytosol (22). Several studies indicate that *C. difficile* strains that produce binary toxin, in addition to TcdA and TcdB, are associated with high mortality rates which make CDI even more challenging for treatment.

1.3 Ultrastructure of *C. difficile* spore

Apart from toxins that cause CDI, *C. difficile* vegetative cells also produce spores, which are critical for the transmission between hosts because of their resistance to environmental factors such as heat, UV, chemicals and, importantly, oxygen (31, 32). The *C. difficile* spore ultrastructure is similar to other endospore-forming bacteria. Endospores are composed of several layers. Located at the center of the spore, the core contains the

DNA, RNA, ribosomal proteins and enzymes that are essential for eventual vegetative growth (15, 33-35). The DNA in the core is protected from UV damage by small acid soluble proteins (SASPs) (36). The core is dehydrated and much of the water in the core is replaced by pyridine-2, 6-dicarboxylic acid (dipicolinic acid; DPA), chelated with calcium (CaDPA), and provides heat resistance to the spores (33, 34). The core is surrounded by an inner membrane (IM) composed of phospholipids with minimal permeability to small molecules, including water. The IM is surrounded by a thin germ-cell-wall layer that becomes the cell wall of the vegetative cell upon outgrowth of the germinated spore. The germ cell wall is composed of layers of peptidoglycan, which is formed by linear chains of two alternating amino sugars, N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) connected by peptide crosslinks (35, 37). The germ-cell wall is surrounded by a thick layer of specialized peptidoglycan layer (the cortex). In *B. subtilis*, the cortex has three different modifications that makes the cortex layer different than the germ-cell wall: i) muramic-δ-lactam (MAL) residues replace 50% of the muramic acid residues in cortex peptidoglycan; (ii) some of the NAM residues are substituted with short peptides lowering the crosslinking in the cortex; and (iii) approximately one fourth of the NAM residues have a single L-alanine residue (35, 37-40). This unique property of the cortex is critical for maintaining the partially dehydrated state of the core as it prevents the core expansion. During germination, which will be discussed below, cortex degrading enzymes recognize the unique modifications so that the germ cell wall is not degraded (41). Surrounding the cortex is the outer spore membrane. Although present as a spore layer, the exact role of the outer spore membrane is not yet understood. The outer spore

membrane is surrounded by several layers of coat proteins. Nearly 30% percent of total spore proteins are present in the coat layer (42). The spore coat provides a barrier against lytic enzymes and toxic chemicals and thus, is important for the protection of cortex layer, which is susceptible to peptidoglycan-degrading enzymes such as lysozyme (32). The coat layers also have an important function in the interaction of the spores with germination-inducing molecules (germinants). Some endospore-forming bacteria produce endospores with an additional layer known as the exosporium, which consists of a paracrystalline basal layer and an external hair-like structure (35, 43). Apart from providing resistance to chemical and enzymatic reactions, the exosporium also provides a hydrophobic surface that enhances the adhesion properties of the spore and may contribute to the pathogenesis of some bacteria (35).

1.4 Sporulation

The signals that trigger the *C. difficile* sporulation pathway are not yet identified. However, many environmental factors, such as nutrient deprivation and quorum-sensing lead to the activation of the sporulation pathway (44). Like other spore-forming organisms (*e.g., B. subtilis*), sporulation in *C. difficile* also contains four morphogenic stages (Figure 1): (i) generation of a smaller compartment (forespore) and larger compartment (mother cell) through an asymmetric division event; (ii) engulfment of the forespore by the mother cell in a phagocytic-like event; (iii) assembly of the cortex and the coat layer; and (iv) release of the mature spore from the mother cell (34).

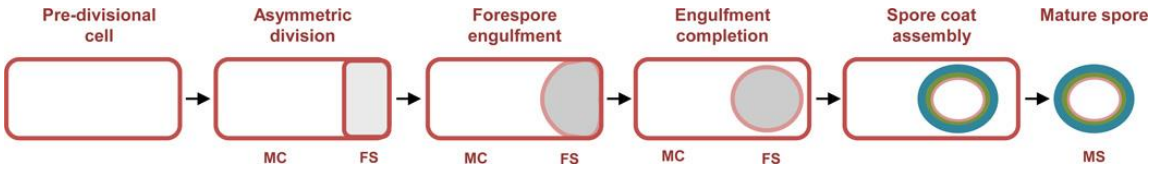

Figure 1. Morphogenic stages during sporulation (reprinted from (34)
During sporulation, a cell divides asymmetrically generating a larger mother compartment and a smaller forespore compartment. After DNA segregation, the mother cell engulfs the forespore followed by formation of spore cortex, inner and outer coat layers. Once spore formation is complete, the mother cell lyses and the mature spore is released.
(MC, mother cell compartment; FS, forespore compartment; MS, mature spore)

In most endospore-forming bacteria, sporulation is activated by phosphorylation of the master transcriptional regulator, Spo0A (45), by several orphan histidine kinases (46). Spo0A expression is under the control of Sigma H (σ^H), an alternative sigma factor that stimulates the production of early sporulation proteins during the post-exponential growth phase. As shown in Figure 2, Spo0A regulates sporulation-specific RNA polymerase sigma factors σ^E and σ^F (34). These σ factors activate compartment-specific transcriptional regulation during sporulation. In *B. subtilis*, activation of the sigma factor occurs in a 'crisscross' pattern between mother cell and forespore. As in *B. subtilis*, *C. difficile*, σ^E and σ^K are mother cell-specific, and σ^F and σ^G are specific to the developing forespore (47).

In both *B. subtilis* and *C. difficile*, activation of σ^F results in the expression of SpoIIR, which interacts with the membrane-bound protease SpoIIGA. SpoIIGA processes pro-σ^E to the active σ^E form in the mother cell. The activation of σ^E leads to the expression of genes whose products are required for the mother cell to engulf the forespore. In the forespore, σ^F regulates the expression of SpoIIQ, and, in the mother cell, σ^E leads to

expression of SpoIIIA-H (34). These proteins are required to form a channel between the inner and outer forespore membranes. σ^E controls expression of SpoIIID, which positively regulates the expression of *sigK* in the mother cell (47). SigK regulates the late stage of sporulation where many genes encoding the spore coat and spore exosporium proteins are synthesized.

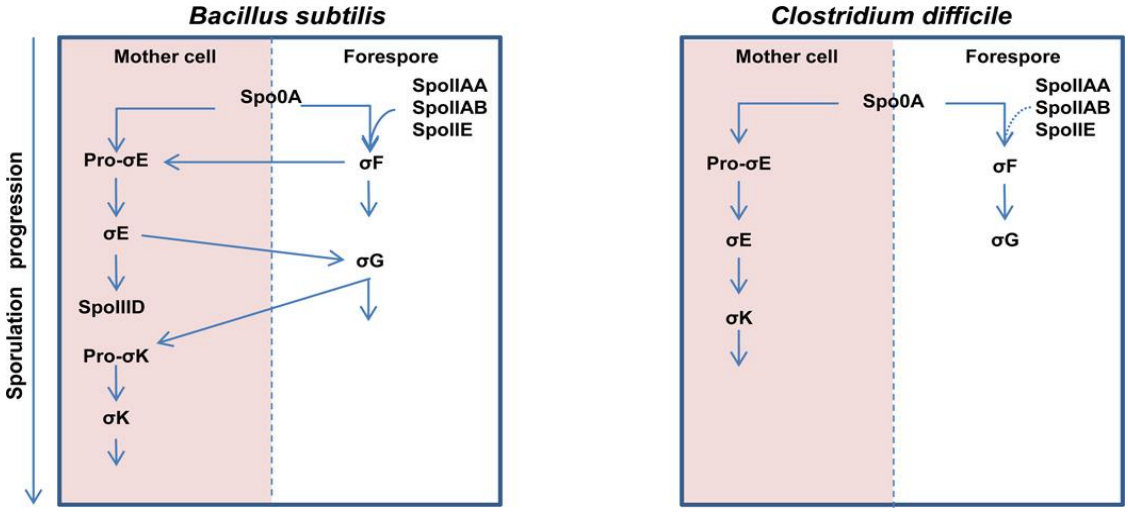

Figure 2. Comparison of activation of sigma factor in *Bacillus subtilis* and *Clostridium difficile.* (reprinted from (34)
Models of global regulatory pathway of the four-major sporulation specific factors in *B. subtilis* and *C. difficile*.

After the completion of engulfment, two types of peptidoglycans are layered between the inner and outer membranes that surround the prespore. A thin layer of germ cell peptidoglycan is layered on the surface of the inner spore membrane. The germ cell wall serves as the wall of newly formed vegetative cell after spore germination. The cortex layer is assembled on the inner surface of the outer prespore membrane and is distinct in chemical composition compared to germ cell wall peptidoglycan. During spore cortex

synthesis, the protein coat is deposited around the outer surface of the outer membrane, subsequently the exosporium is assembled.

The composition of the coat in *C. difficile* differs from *B. subtilis*. Out of 70 *B. subtilis* spore coat proteins, only 18 orthologues are found in *C. difficile* (48). In *B. subtilis*, coat assembly occurs when mother cell encloses the forespore with a series of proteinaceous shells. These shells include the basement layer, the inner coat, the outer coat, and the crust layers. The formation of the first three coat layers involves the SpoVM, SpoIVA, SpoVID, SafA, and CotE proteins. Interestingly, *C. difficile* only encodes orthologues of SpoIVA, SpoVM and CotE (49-51). SpoIVA is essential for coat biogenesis in both *B. subtilis* and *C. difficile*. In *C. difficile*, recently identified SipL, is hypothesized to function like *B. subtilis* SpoIVD. SipL binds to SpoIVA, and the SipL/SpoIVA complex is required as a foundation for the spore coat assembly (51). In addition, *B. subtilis* SpoIVA recruits another morphogenetic protein, SafA, while CotE is recruited by both SpoIVA and SpoVID (34). *C. difficile* encodes only a CotE orthologue. Whether or not other proteins are recruited by SpoIVA and SipL is unknown.

In both *Bacillus anthracis* and *C. difficile* spores, the exosporium surrounds the coat layers. Similar to what is observed in *B. anthracis* spores, the *C. difficile* exosporium also lacks a clear gap/inner coat space. *C. difficile* strain 630 encodes three paralogs of the *B. anthracis* collagen-like glycoprotein BclA (*bclA1* [CD0332], *bclA2* [CD3230], and *bclA3* [CD3349]) (43, 52). These proteins contain three domains: (i) an N-terminal domain of variable size; (ii) a collagen-like domain; (iii) and a C-terminal domain (53). However, the particular role of the other BclA paralogs in *C. difficile* is not clear. Recently, Barra-

Carrasco *et al.* found that the cysteine-rich exosporium protein CdeC (CD1067) is unique to *C. difficile* and is essential for the proper assembly of the exosporium. Mutations in *cdeC* dramatically decrease the abundance of the exosporium in the spores resulting in defects in coat and exosporium assembly (54).

1.5 *C. difficile* spore germination

C. diffiicle can only become pathogenic when it transforms from a spore to a vegetative cell, through a process known as germination. Upon sensing various species-specific germination cues, dormant spores awake and become metabolically active. Spore germination has been studied extensively in *B. subtilis*. In *B. subtilis*, various agents (*e.g.* amino acids, sugars, CaDPA, dodecyl amine or peptidoglycan fragments) trigger *B. subtilis* spore germination (55, 56). However, in nature, germination of *B. subtilis* spores by nutrient is most likely. *B. subtilis* spores germinate in response to L-alanine or to the combination of L-asparagine, D-glucose, D-fructose and potassium ions (AGFK), which bind to spore-specific protein complexes, germinant receptors that are located in the IM. The *B. subtilis* GerA germinant receptor (a combination of the GerAA, GerAB and GerAC proteins) recognizes L-alanine as a germinant (57). *B. subtilis* spores also germinate in response to AGFK via cooperative action between GerB and GerK (56, 58). Only the L-form of amino acids can trigger germination of *B. subtilis* spores. In fact, some D-amino acids inhibit the germination of *B. subtilis* spores (59).

In contrast to *B. subtilis*, *C. difficile* does not encode orthologues of the Ger-type germinant receptors. Germination by *C. difficile* spores is triggered in response to certain bile acids [cholic acid-derivatives, such as taurocholate (TA)] in combination with certain

amino acids (*e.g.* glycine or L-alanine) (60). The bile acid germinant receptor, CspC was identified in a random mutagenesis screen (61). A single point mutation in *cspC* resulted in loss of recognition of TA as a germinant. In addition, *C. difficile* spore germination was inhibited by chenodeoxycholic derivatives (CDCA). Similarly, a single substitution in CspC (G457R) resulted in spores no longer being inhibited by CDCA. Instead, these mutant spores recognized CDCA as a germinant (61). These results suggested that CspC is the bile acid germinant receptor and is important for recognition of both TA (an activator) or CDCA (an inhibitor) during germination of *C. difficile* spore (61-63).

The Csp locus was originally studied in *Clostridium perfringens (64). C. perfringens* encodes three Csp proteases, CspB, CspA, and CspC. Each are predicted to cleave the inhibitory pro-peptide from proSleC, a spore cortex hydrolase that degrades the cortex peptidoglycan, thereby activating the protein. In *C. perfringens* spores, although CspB, CspA and CspC are present, germination occurs similar to that of *B. subtilis* due to presence of orthologues of GerA, GerB and GerK (64-66).

In *C. difficile,* CspB and CspA are encoded by one ORF upstream of *cspC,* and the resulting protein (CspBA) is post-translationally processed into CspB and CspA and then further processed by the sporulation specific protease, YabG. To date, YabG has only been studied in *B. subtilis* where YabG processes several coat related proteins (67, 68). Clearly, the proteins targeted by the YabG protease in *B. subtilis* and *C. difficile* are different (67, 69). In *C. difficile*, apart from the processing of the CspBA interdomain processing, YabG is also responsible for processing preproSleC into the proSleC form found in the spore. The *yabG* mutant spores accumulate mostly preproSleC and CspBA (69).

In our original working model for spore germination (Figure 3B), we hypothesized that proSleC, CspB, CspC and CspA are in a complex where proSleC is held in an inactive state by its propeptide. CspC, the bile acid receptor, is activated by TA, which leads to the activation of the CspB protein. The CspB serine protease cleaves proSleC into its active form. Activated SleC then degrades the cortex layer and results in the core releasing CaDPA, in exchange for water, through the SpoVA mechanosensing channel (70). At the time we generated this model, the role of CspA in spore germination was unknown. However, deletion of CspA greatly affects the incorporation of CspC into the spore, thereby influencing germination due to lack of the bile acid germinant receptor (69). During *C. difficile* spore germination amino acids are also required as a co-germinant along with TA. However, it is not understood where and how amino acids are playing a role during germination.

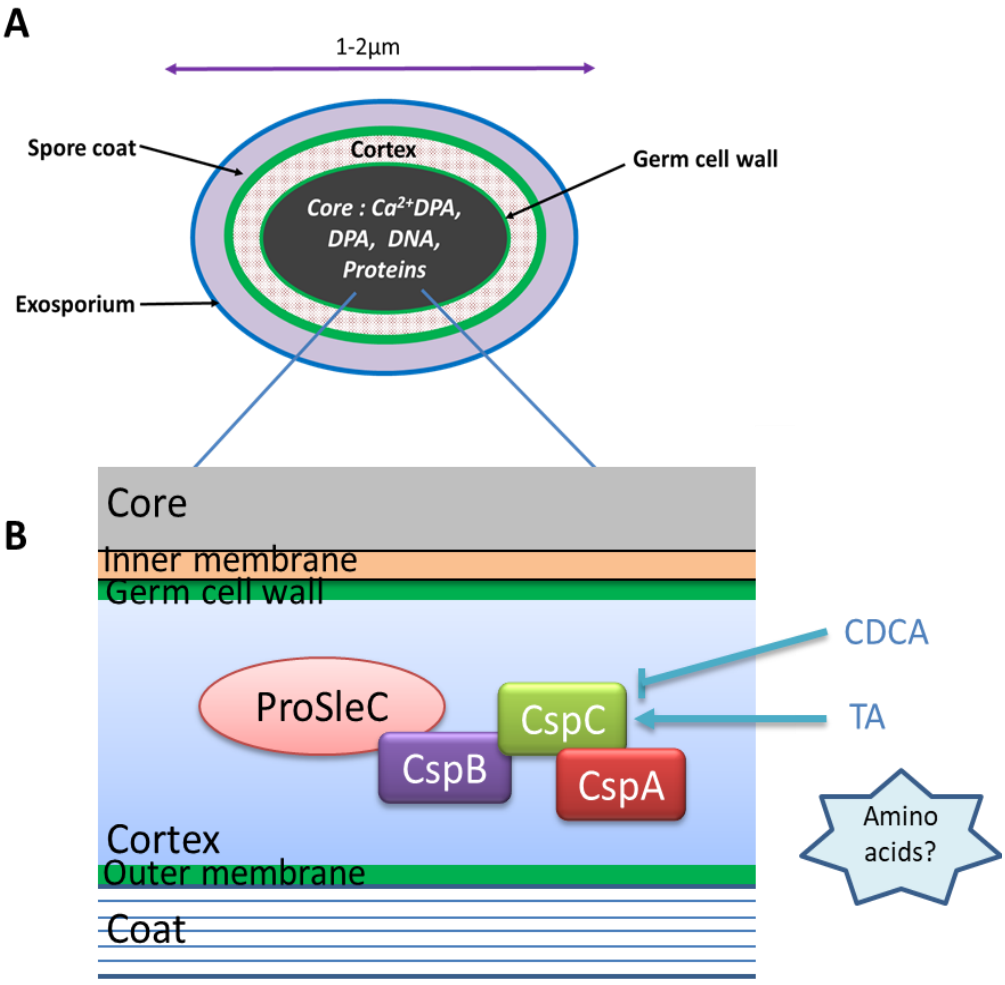

Figure 3. Working model for *C. difficile* spore germination

A) Overall architecture of a dormant spore. The core contains CaDPA, DNA etc. and is surrounded by inner membrane, germ cell wall, cortex, coat layers and an exosporium. B) Prior to publication of this thesis, our working model for *C. difficile* spore germination hypothesized that proSleC is forms a complex with CspB, CspC and CspA in the cortex. CspC, the bile acid germinant receptor, is activated in response to cholic acid-derivatives and inhibited by chenodeoxycholic acid-derivatives. Activation of CspC leads to activation of the CspB protease, which cleaves pro-SleC to its active form. Activated SleC hydrolyzes the cortex which results in the release of CaDPA from the core. An amino acid, such as glycine, is also required in the germination process, though was not clear where and how it binds.

1.6 Proteins indirectly involved during germination by *C. difficile* spores

Recent studies on *C. difficile* spore germination have identified several other proteins that are involved in spore germination. GerS is the second most highly expressed protein during sporulation and also plays a role in germination, encodes a lipoprotein; a *gerS* mutant germinates poorly (71). Even though SleC is activated in *gerS* mutants in response to germinants, these mutants failed to degrade the cortex and as a result, do not release DPA. Originally, GerS was hypothesized to be a part of the germination complex, potentially by anchoring proSleC to the inner leaflet of the outer spore membrane (71). However, in subsequent work, GerS, along with CwlD (a muramoyl-L-Alanine amidase) (*i.e.*, MAL) and PdaA (a polysaccharide deacetylase) were shown to be required for the cortex-specific modifications of peptidoglycan. In mutants where these modifications are hindered, SleC fails to degrade the cortex (72).

A separate study identified GerG as another *C. difficile* germination protein. GerG which encodes a protein with gel forming properties (73). A deletion of *gerG* results in a reduced response to bile acid germinants. GerG is required for the incorporation of important germination proteins such as CspB, CspA and CspC into spores. While CspA also controls the incorporation of CspC, it was shown that GerG acts upstream of CspA and is responsible for incorporation of all three Csp proteins in the spore (73).

1.7 Importance of amino acid co-germinants during *C. difficile* spore germination

Although bile acids are important, germination of *C. difficile* spores cannot occur without an amino acid co-germinant and, prior to publication of this thesis, the amino acid germinant receptor was still unknown. Glycine is the most efficient co-germinant but other

L-forms of amino acids, such as L-alanine or L-cysteine, can also efficiently trigger *C. difficile* spore germination (74). Unlike *B. subtilis*, *C. difficile* spore germination is not inhibited by the D-forms of amino acids. However, in a previous study, D-amino acids did not activate *C. difficile* spore germination (74). In other spore-forming bacteria, amino acid racemases play important roles in spore germination (as well as during a vegetative growth). In *B. subtilis*, the spore specific-alanine racemase converts L-alanine to D-alanine to suppress spore germination in response to L-alanine (75). This is thought to prevent germination of the developing spore within the mother cell where L-alanine is present. In *C. difficile*, the *alr2* alanine racemase is encoded downstream of *gerS* and is very highly expressed during sporulation (71). However the inactivation of *C. difficile alr2* had minimal impact on *C. difficile* spore germination, when analyzed in rich medium (71). Since all amino acids are present in rich medium, under this condition the effect of Alr2 during *C. difficile* spore germination might be masked.

Prior work on amino acid co-germinants in *C. difficile* spore germination has suggested that *C. difficile* spores contain an amino acid germinant receptor that recognizes multiple amino acids as co-germinants(74). Several amino acid analogs were tested to understand is the amino acids interact with a putative receptor by analyzing the kinetics of spore germination. The study showed that the *C. difficile* germination machinery recognizes different amino acid side chains. In addition, the putative glycine receptor requires both a free carboxylate and a free amino group to recognize glycine, however, the binding site is flexible enough to accommodate longer separations between the two functional groups (74). Moreover, D-amino acids did not contribute to germination of *C.*

difficile, suggesting that putative germinant receptor is specific to L-form of amino acids. Among various L amino acids tested, apart from L-alanine, only L-cysteine, L-phenylalanine and L-arginine were able to function as co-germinants. Another study showed that L-histidine could also contribute to germination of *C. difficile* along with TA (76). Also, because none of the amino acid analogs was able to compete with glycine to inhibit *C. difficile* spore germination, the study suggested that the functional groups in the amino acid moieties are needed for both binding and activation of the putative amino acid germination receptor (74).

1.8 Role of calcium during germination

Recently, calcium was identified as an important contributor to spore germination, though its precise role remains unclear (77). Calcium may function as a bona fide spore co-germinant, a co-factor for a process essential for spore germination and/or as an enhancer for amino acids. CaDPA also induce germination in *Bacillus subtilis* spores (77). Spores of several *Bacillus* species contain two cortex lytic enzymes, SleB and CwlJ (78). While SleB is activated when a nutrient germinant bind to its respective receptor, CwlJ is activated in the presence of endogenous or exogeneous CaDPA (56, 79). *C. difficile,* however does not encode orthologues of CwlJ, and only encodes SleC, suggesting that germination by endogenous CaDPA and TA in *C. difficile* does not follow the same pathway was in *B. subtilis*. Prior work has shown that the cortex degrading enzyme SleM of *C. perfringens* require divalent cations (including Ca^{2+}) for activity (64). Thus in *C. difficile*, Ca^{2+} may be required for the activity of the cortex degrading enzyme SleC.

1.9 Environmental factors affecting germination

The most obvious environmental factor that influences spore germination are the concentration of the germinants. Optimum concentrations of both amino acid and bile acids are required for the germination of the *C. difficile* spores. The EC_{50} (the concentration that achieves half-maximum germination rate) of TA is approximately 2 mM. and the EC_{50} of glycine is approximately 1 mM (80). Interestingly, the postprandial bile acid concentration in the human distal ileum averages 2 mM, whereas postprandial bile acid concentration in the proximal ileum averages 10 mM (81, 82). Similarly, with amino acids being a large part of human diet, they are present at high in the gut.

Another important factor influencing germination is pH. The optimum pH required for the germination of *C. difficile* spores is between 6.3 to 7.5. Normally *in vitro* germination is performed at pH 7.5. Though alkaline pH (8.2-8.4) marginally reduced the germination of *C. difficile* spores, an acidic pH significantly reduced germination (63). *In vivo*, the stomach pH ranges between 1.5 – 2.0 (63), suggesting that *C. difficile* spores are unable to germinate under these conditions. The colonic environment can be acidic or neutral depending on the region. For example, in the small intestine, the pH ranges between 6.0 – 7.5, and, similarly in the large intestine the pH ranges between 5.8 – 7.0, which is suitable pH for the germination of the *C. difficile* spores (83).

Temperature also plays a significant role during germination. The rate of germination significantly increases when the temperature is increased from 20 °C to 37 °C, however there was no effect on the extent of germination, suggesting that at both temperatures, spores can germinate completely but at different rates (63).

In other spore-forming organisms, the medium in which spores were prepared influences the rate of germination by increasing in the number of germinant receptors (84, 85). In *C. difficile*, it has only been reported that preparation of spores in different nutrient media increases the number of spores compared to BHIS medium (51). However, it has not been shown whether there is an effect of different media on germination, or if different media cause a change in the germination specific proteins of *C. difficile* spores.

CHAPTER II

A *Clostridium difficile* ALANINE RACEMASE AFFECTS SPORE GERMINATION AND ACCOMMODATES SERINE AS A SUBSTRATE*

In Chapter II, we identified the role of alanine racemase (Alr2) during *C. difficile* spore germination. We also looked for other substrate of Alr2 other than alanine. The ITC work on this chapter was done in collaboration with Dr. Steve W. Lockless.

2.1 Introduction

Clostridium difficile infection (CDI) has become one of the most common hospital acquired infections in the United States (7, 86). Antibiotic use is the major risk factor associated with CDI (86). Treatment of potential hosts with broad spectrum antibiotics leads to major alterations in the normally-protective colonic microbiota and permits colonization of that host by *C. difficile* spores (86). Though antibiotics (*e.g.*, vancomycin) are available to treat CDI, the continued perturbation to the colonic microbiota by these antibiotics leads to multiple rounds of recurring infections (10). It is for this reason that the Centers for Disease Control and Prevention have listed *C. difficile* as "an urgent threat" regarding the antibiotic associated threats to the United States (7).

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Although *C. difficile* is a strict anaerobe, it survives in aerobic environments and transmits between hosts in the spore form (87). Spores are metabolically dormant forms of bacteria and formed from vegetatively growing cells, often, upon nutrient limitation. During spore-formation, a vegetative cell asymmetrically divides into a mother cell compartment and a forespore compartment (88). Through coordinated gene expression, the two compartments mature the forespore into a multilayered spore that can resist harsh conditions (*e.g.*, radiation, heat, antibiotics, etc.) (31). Spores are composed of a DNA-containing core where much of the water has been replaced with dipicolinic acid [often as a calcium chelate (CaDPA)], an inner membrane, a thin peptidoglycan layer, a thick specialized peptidoglycan-containing cortex layer, an outer membrane, a coat layer and, in some organisms, an exosporium layer (31, 88, 89).

In a host, these spores must exit dormancy (germinate) to generate the actively growing, toxin-producing, cells that elicit the primary symptoms of disease. *C. difficile* spore germination is stimulated by the combinatorial action of certain bile acids and certain amino acids (60, 62, 74, 90). *C. difficile* spore germination is activated by cholic acid-class bile acids while chenodeoxycholic acids competitively inhibit cholic acid-mediated germination (60, 62). Though bile acids are important for germination, they are not sufficient to activate germination on their own. Amino acids are also required for germination and the most effective co-germinant is glycine (60). However, other amino acids can function as co-germinants with varying efficiencies (*e.g.*, alanine) (74).

In most organisms, germinants interact with their cognate receptors at the inner membrane of the spore (89). These *ger*-type germinant receptors bind to germinants and

lead to the release of CaDPA from the core in exchange for water (89). Subsequently, the spore cortex is degraded through the action of spore cortex lytic enzymes (SCLEs). *C. difficile* does not encode orthologues of the classical *ger*-type germinant receptor and, instead, uses a novel pathway for initiating spore germination where the CspC pseudoprotease is the bile acid germinant receptor (61, 88, 91). We hypothesized that CspC transmits a bile acid signal to the CspB protease which then cleaves the SCLE, pro-SleC, to an active form (41, 92, 93). Activated SleC degrades the cortex leading to CaDPA release, due to osmotic changes in the core resulting from to cortex degradation (70, 93).

Recently, GerG and GerS were identified as important players in *C. difficile* spore germination. Encoded downstream of *gerS* is *alr2*, coding for an alanine racemase (71, 73). Alanine racemases enzymatically convert L-alanine to the D-alanine stereoisomer and are known to be involved in spore development and germination (94, 95). During spore development, the alanine racemase converts the L-alanine germinant to the D-alanine inhibitor in order to prevent germination of the developing spore within the mother cell (55, 95-97). During *B. subtilis* spore germination, the alanine racemase converts L-alanine to D-alanine to suppress spore germination, a process termed 'autoinhibition' (97, 98); D-alanine does not inhibit germination by other *B. subtilis* germinants and is specific to the GerA germinant receptor (55). Autoinhibition of spore germination occurs, presumably, until the abundance of L-alanine accumulates to sufficient levels to overwhelm the racemase and stimulate spore germination (95). In *C. difficile* only the L-form of alanine can trigger spore germination when added with taurocholic acid (TA) (74). Unlike what

is seen for *B. subtilis* spore germination, D-alanine does not inhibit germination by *C. difficile* spores (74).

Interestingly, in the same study that identified GerS as a novel regulator of spore germination, inactivation of *C. difficile alr2* had minimal impact on *C. difficile* spore germination when analyzed in rich medium (71). However, we hypothesized that *alr2* may play an important role during spore germination in defined medium in the presence of alanine and TA as germinants. Indeed, we find that *C. difficile alr2* mutants play an important role during spore germination – by converting D-alanine to the L-alanine co-germinant. Interestingly, we find that Alr2 also interconverts L- and D-serine and that these amino acids are germinants for *C. difficile* spores. Finally, we determine that the affinity of Alr2 for amino acids may be destabilized by the bound cofactor because an Alr2 mutant that does not bind to its cofactor has an affinity for L / D alanine that is ~80x greater to that of the wildtype protein. Our results shed new light on mechanisms of *C. difficile* spore germination and suggest that *C. difficile* spores are equipped to recognize more amino acids as co-germinants than previously thought.

2.2 Materials and Methods

2.2.1 Strains and growth conditions

C. difficile UK1 and derivatives were routinely grown at 37 °C in an anaerobic environment (10% H_2, 5% CO_2, 85% nitrogen) in Brain Heart Infusion supplemented with 5 g / L yeast extract and 0.1% L-cysteine (BHIS) medium. *E. coli* was routinely grown in LB medium at 37 °C. *B. subtilis* was grown in LB medium. Antibiotics were added when

necessary (20 µg / mL chloramphenicol, 10 µg / mL thiamphenicol, 20 µg / mL lincomycin, 50 µg / mL kanamycin, 100 µg / mL ampicillin).

2.2.2 Molecular Biology

A mutation in *C. difficile alr2* mutant was generated by retargeting the pJS107 TargeTron plasmid, as described previously (61). Primers to generate *C. difficile* RS07 and its complementing plasmid (pRS89) are listed in Table 3. Briefly, potential insertion sites for the group II intron were determined using the Targetronics algorithm (Targetronics, LLC.) and a potential insertion site was identified in the sense orientation at nucleotide 138 of the *alr2* coding sequence based upon the sequenced *C. difficile* R20291 strain (*C. difficile* UK1 is not a sequenced strain but is closely related to the R20291 strain). A gBlock (Integrated DNA technologies) was synthesized and cloned into the TOPO-ZeroBlunt cloning vector and transformed into *E. coli* DH5α, generating pRS85. The retargeting fragment was sub-cloned into pJS107 at the HinDIII and BsrGI restriction sites, transformed into *E. coli* DH5α, yielding pRS86. The *alr2* complementing plasmid (pRS89) was generated by amplifying the P1 promoter and the *alr2* coding sequences using Phusion DNA polymerase (New England Biolabs). The resulting fragments were cloned into the *B. subtilis – C. difficile* shuttle vector, pJS116, at the XbaI and XhoI restriction sites using Gibson Assembly (99). The plasmid used to express recombinant Alr2 protein was created by amplifying the *alr2* gene from *C. difficile* UK1 using primers 5'pET_alr and 3'pET_alr (Table 3) and cloned into expression vector pET222b at the XhoI and NdeI restriction sites. The resulting plasmid was named pJS164. Similarly, the plasmid for the expression of Alr_{K39A} was created by using overlapping PCR

using primers 5'alr L39A and 3'alr L39A (Table 3), to create a change AA into GC such that the resulting amino acid would change from lysine to alanine. The amplified fragment with the mutation was stitched together using 5' alr and 3' alr primers and cloned into pET22b expression vector at XhoI and NdeI site and the resulting plasmid was named as pRS100. The DNA sequences for all plasmids were verified by sequencing (Eurofins).

2.2.3 Conjugation and mutant selection

Both pRS86 and pRS89 were inserted into *C. difficile* UK1 via conjugation with the Tn916-containing *B. subtilis* BS49 as a donor. *B. subtilis* was transformed with these plasmids following standard protocols and transformants were confirmed using PCR. *B. subtilis* BS49 containing pRS86 or pRS89 was conjugated with *C. difficile* UK1, as described previously (61). After screening for tetracycline-sensitive and thiamphenicol-resistant colonies (transposon-negative, plasmid-positive), the colonies were confirmed to have the desired plasmid using PCR. To isolate a TargeTron insertion into *alr2*, the above isolates were spread onto BHIS plates containing 20 µg / mL lincomycin and incubated for 24-36 hrs to obtain colonies. Lincomycin-resistant colonies were isolated and tested for insertion of TargeTron by PCR using the primers 5'alr and 3'alr (Table 3). An isolate with the desired group II intron insertion was identified and this strain was renamed *C. difficile* RS07.

2.2.4 Preparation of spores

All spores were purified from BHIS agar medium as described previously (60, 61, 90). Briefly, both wild type *C. difficile* UK1 and *C. difficile* RS07 were grown on BHIS agar medium and *C. difficile* RS07 pRS89 was grown on BHIS agar media supplemented

with 5 µg / ml thiamphenicol. Strains were grown for 4-5 days before isolating the growth and suspending in 1 ml sterile water per plate. The suspension was stored overnight at 4 °C to promote release of the spores from the mother cells. The suspensions were then washed five times with sterile water to remove cell debris. The resulting spores were purified using a bed of 60 % sucrose by centrifuging at 4,000 x *g* for 30 minutes. After centrifugation, the sucrose was removed and the spores at the bottom of the tube were isolated and washed five times with sterile water to remove the sucrose. The spores were >99 % pure and heat-activated at 65 °C for 30 min before use.

2.2.5 Spore germination

Spore germination was analyzed at 25 °C or 37 °C using both a DPA release assay and an OD assay both. All assays were carried out in 100 µL total volume using buffer containing a final concentration of 50 mM HEPES, 100 mM NaCl (pH 7.5), 10mM TA and varying concentrations of amino acids. The DPA release assay was performed, as described previously, using final spore density of 0.5 OD with 30mM of amino acids and 250 µM of $TbCl_3$ in a 96-well plate reader at low PMT settings (Excitation at 270, emission at 420nm) (70). The OD-based germination assay was also carried out using a plate reader at 595 nm with final 0.5 OD spores and 30mM of amino acids.

For the calculation of EC_{50}, the DPA release assay was used at 37 °C with increasing concentrations of amino acids (0 – 200 mM and a final OD_{600} = 0.3 spores). The rates of germination were determined using the slopes of the linear portions of the germination plots. Data are reported as the averages from three independent experiments with one standard deviation from the mean.

2.2.6 Purification of Alr2 and Alr_{K39A}

pJS164 and pRS100 were transformed into *E. coli* BL21(DE3) *slyD::kan*. To express and purify $Alr2_{6His}$, cells were grown at 37 °C to an OD_{600} = 0.8 in 2xTY medium supplemented with ampicillin before inducing for 4 hours at 37 °C in the presence of 500 µM IPTG. Cells were pelleted at 6,000 x *g* for 15 minutes in a Sorvall centrifuge (Beckman Coulter). Pellets were suspended in lysis buffer (LIB) containing 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 1 mM pyridoxyl-5'-phosphate (PLP), 1 mM (tris (2-carboxyethyl) phosphine (TCEP), 15 mM imidazole and protease inhibitor (1mM PMSF) before freezing at -80 °C.

The cell pellets were thawed and suspended in LIB (25 ml per 1L of cell pellet). Lysozyme and DNase were added and the cell suspension was incubated on ice for 30 min. Cells were lysed using sonication. The sonicated extract was clarified by centrifugation at 15,000 X *g* for 30 minutes at 4 °C. $Alr2_{6his}$ was purified from the extract by nickel-affinity chromatography. Beads were washed once in wash buffer LIB containing 30 mM imidazole. The recombinant protein was eluted from the beads using the wash buffer with 500 mM imidazole. The eluted protein was dialyzed in LIB for 3 hrs and further purified by gel filtration chromatography (Akta Pure, GE Health) on a Sephedex G200 size exclusion column. Protein was injected and separated at 1 mL / min using LIB. Protein was detected at 280 nM and the eluted protein was collected (Figure S3).

2.2.7 Enzymatic assays and HPLC separation of stereoisomers

L- to D-alanine and L- to D-serine conversion (and *vice versa*) was initiated by adding 500 ng of purified Alr2 to 50 mM amino acid in 50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 1 mM PLP and 1 mM TCEP [total reaction volume was 100 µL (155 nM)]. The reactions were incubated at 37 °C for 1 hour.

In order to assay for the presence of L and D forms of the tested amino acids, 200 µL of 1 % FDAA (Marfey's reagent) in acetone and 40 µL of 1 M sodium bicarbonate was added to the reaction conditions above and incubated for 1 hour at 40 °C (100). After the 1 hour incubation, 20 µL of 2 M HCl was added to stop the reaction. The reacted material was separated by reverse-phase HPLC (Prominence system, Shimadzu) using a Thermo Scientific Synchronis C18 column. Three microliters of the reacted material was injected and separated at a 0.65 ml / min flow rate of buffer A (50 mM triethylammonium phosphate pH 3.5 in 25% methanol) and a gradient of 0 % buffer B (50 mM triethylammonium phosphate pH 3.5 in 75 % methanol) to 100 % buffer B in 40 min before returning to 0 % buffer B for another 30 minutes (70 minutes total time). Elution of the FDAA and FDAA-labeled compounds was detected by absorbance at 340 nm and peak area was quantified.

2.2.8 Isothermal Titration Calorimetry

All ITC experiments were done using a MicroCal iTC200 System (Malvern) at a constant temperature of 25 °C. The ITC base buffer was 50 mM Tris-HCl (pH 7.5), 250 mM NaCl and 1 mM PLP. In the ITC chamber, wild-type Alr2 was used at a concentration of 32 – 45 µM in the ITC base buffer, while $Alr2_{K39A}$ was at 100 µM. In the syringe, D-

and L-alanine ligands were dissolved in ITC base buffer at 200 mM for wild-type protein or 20 mM for the $Alr2_{K39A}$ experiments, and D- and L-serine were made to 200 mM. All solutions were filtered or centrifuged at 18,000 x g for 5 minutes prior to use. Thermograms were processed and fit using a single-ligand model (with *n* fixed to unity), using Origin software, to obtain *K* (association constant) and ΔH (enthalpy). The dissociation constant (K_D) was calculated from the relationship $K_D=1/K$.

2.3 Results

2.3.1 C. difficile alr2 is not required for spore germination in rich medium

C. difficile alr2 (encoding an alanine racemase) is the sixth most expressed sporulation protein (101). Prior work has shown that, though highly expressed, Alr2 seems to play little to no role during *C. difficile* spore germination in rich medium supplemented with TA germinant (71). However, given the diverse signals present in rich medium, we hypothesized that *C. difficile alr2* may play a role similar to what is observed in other spore-forming bacteria by affecting germination in response to L-alanine. Towards this goal, we generated a TargeTron mutation in *C. difficile* UK1 *alr2*. When suspended in rich, BHIS, medium supplemented with TA (BHIS-TA), wild type *C. difficile* UK1 spores rapidly germinate as shown as a decrease in the optical density of the suspension (Figure 4A). Similarly, *C. difficile* RS07 (*alr2::ermB*) spores also rapidly germinated in BHIS-TA (Figure 4B) and when the *alr2* disruption was complemented by expressing *alr2* from a plasmid, no difference was observed compared to the other tested strains (Figure 4C).

These results confirm prior observations that Alr2 plays little role during germination of *C. difficile* spores in rich medium.

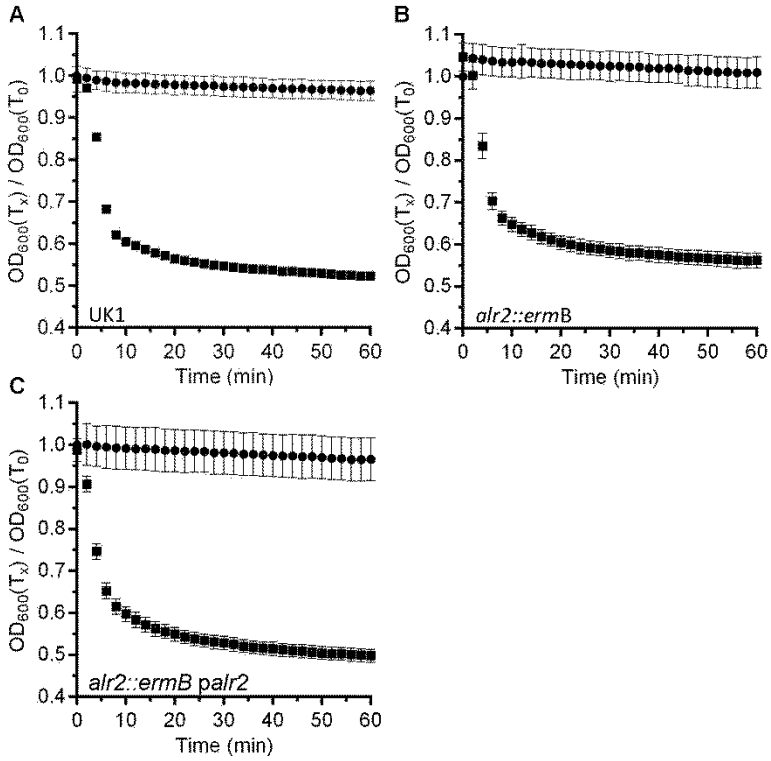

Figure 4. Alr2 does not contribute to germination by *C. difficile* spores in rich medium. Purified spores from (A) wildtype *C. difficile* UK1 or (B) *C. difficile* RS07 (*alr2::ermB*) or (C) *C. difficile* RS07 pRS89 (p*alr2*) were suspended in rich, BHIS medium (●) or BHIS supplemented with 10 mM TA (■) at 25 °C. The OD_{600} of the suspension was monitored over time. Data points represent the average from three independent experiments and error bars represent the standard deviation of the mean.

2.3.2 C. difficile alr2 is dispensable for germination in response to L-alanine but essential for germination in response to D-alanine

To test the effects of an *alr2* disruption on *C. difficile* spore germination, we analyzed spore germination in defined medium. *C. difficile* UK1 spores were suspended in buffer supplemented with TA and glycine or TA and L-alanine or TA and D-alanine and

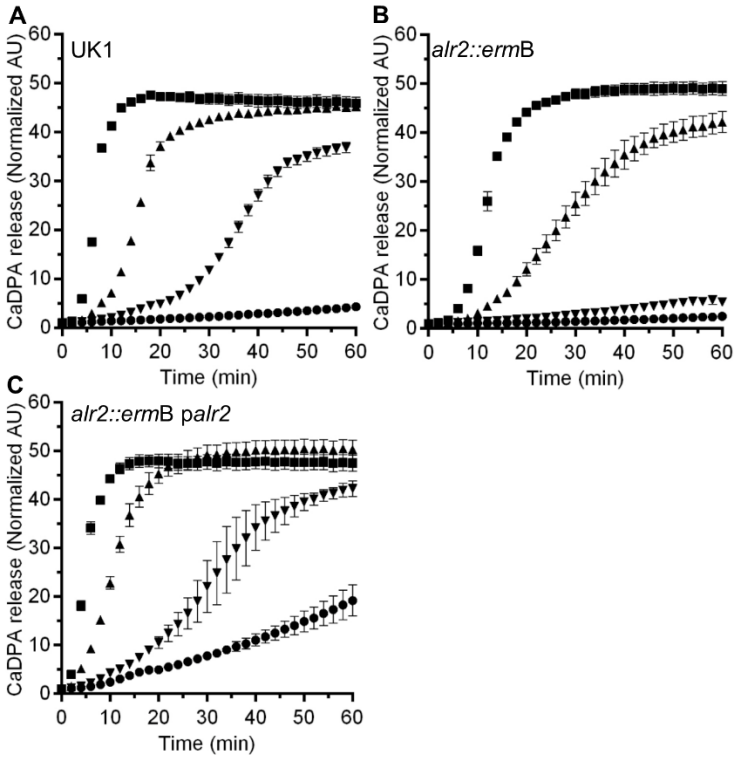

Figure 5. Alr2 affects L/D-alanine-mediated germination by *C. difficile* spores. Purified spores from (A) wildtype *C. difficile* UK1 or (B) *C. difficile* RS07 (*alr2::ermB*) or (C) *C. difficile* RS07 pRS89 (p*alr2*) were suspended germination buffer supplemented with TA alone (●), or supplemented with glycine (■) or L-alanine (▲) or D-alanine (▼). CaDPA release from the germinating spores was monitored at 37 °C as an increase in Tb^{3+} fluorescence over time. Data points represent the average from three independent experiments and error bars represent the standard deviation of the mean.

germination was monitored at 25 °C (Figure S1). As expected, wild type *C. difficile* UK1 germinated in response to glycine and in response to L-alanine (Figure S1A). *C. difficile* UK1 spores did not initiate germination in response to TA and D-alanine. When *C. difficile* RS07 was tested for germination in response to TA and glycine or TA and L-alanine or TA and D-alanine, we observed a decrease in the ability of the strain to germinate in response to L-alanine but not in response to glycine (D-alanine, again, had no effect on *C.*

difficile spore germination) (Figure S1B). This defect in germination in response to L-alanine could be complemented to near wild type levels (Figure S1C).

To date, most germination assays in our lab, and others, were performed at room temperature. Though germination often proceeds quickly at this temperature, we reasoned that some *C. difficile* spore enzymes may best function at 37 °C (a physiologically-relevant temperature). Therefore, we again tested germination of wildtype *C. difficile* spores in response to TA and glycine or TA and L-alanine or TA and D-alanine. As expected, wildtype *C. difficile* UK1 spores germinated in response to TA and glycine or TA and alanine (Figure 5A). Surprisingly, *C. difficile* UK1 spores also germinated using D-alanine as a co-germinant with TA (Figure 5A). *C. difficile* RS07 spores germinated similar to wildtype UK1 spores in TA and glycine or TA and L-alanine (Figure 5B). Interestingly, *C. difficile* RS07 spores were unable to respond to D-alanine as a co-germinant with TA at the concentration tested (Figure 5B). This defect in germination could be restored by expressing *alr2* from a plasmid (Figure 5C). These results suggest that the Alr2 alanine racemase has a role in recognizing D-alanine as a co-germinant or that it converts D-alanine to L-alanine which then functions as a co-germinant with TA.

2.3.3 C. difficile RS07 spores more-readily germinate in response to L-alanine as a co-germinant with TA

Alanine racemases are known to be involved in spore germination, but all studied to date are thought to covert L-alanine to D-alanine which then acts as an inhibitor of L-alanine-mediated germination and it is unclear if D-alanine conversion to L-alanine is important in these organisms. During germination, *C. difficile alr2* may convert D-alanine

to L-alanine which would stimulate spore germination in combination with TA. However, because L-alanine is a germinant (and D-alanine does not inhibit *C. difficile* spore germination) if Alr2 converts L-alanine to D-alanine during germination, *C. difficile* RS07 spores may be more sensitive to the L-alanine co-germinant. To quantify this, we determined the EC_{50} values for L-alanine in *C. difficile* UK1 spores, *C. difficile* RS07 spores and *C. difficile* RS07 p*alr2* spores. Though spore germination is a multi-enzyme process, these kinetic measurements allow for the quantitative interaction of the spores with activators and inhibitors of germination (80, 102-108). When *C. difficile* UK1 spores were suspended in buffer supplemented with TA and increasing concentrations of L-alanine we observed an increase in the germination rate. Using the rates and concentrations tested, we found that wildtype *C. difficile* spores had an EC_{50} of approximately 5.5 mM for L-alanine (Table 1). *C. difficile* RS07 spores were more sensitive to L-alanine as a co-germinant with an EC_{50} value of 2.7 mM suggesting that Alr2 is able to convert L-alanine to D-alanine (Table 1). When the complemented strain was analyzed, the EC_{50} value was higher than wildtype, 10.7 mM, suggesting that overexpression of Alr2 drives L-alanine to D-alanine more than what is observed in wildtype (Table 1).

Next, we determined the EC_{50} values for D-alanine during spore germination. *C. difficile* UK1 demonstrates an EC_{50} of 11.5 mM for D-alanine. If Alr2 is converting D-alanine to L-alanine during germination, the EC_{50} value should increase in *C. difficile* RS07. Indeed, *C. difficile* RS07 spores were less sensitive to D-alanine as a germinant and yielded an EC_{50} value of 24.2 mM, weaker than wildtype. When *alr2* was expressed from a plasmid, the EC_{50} decreased to 6.6 mM due to its conversion to L-alanine. The EC_{50} of

C. difficile RS07 p*alr2* with D-alanine was similar to the EC_{50} of wildtype spores and L-alanine. Importantly, because Alr2 affected germination in response to L-alanine and D-alanine, the EC_{50} values for *C. difficile* UK1 are an approximation for these amino acids (some of the L-alanine is converted to D-alanine and vice versa during germination). Taken together, these results suggest that D-alanine can be converted to L-alanine which functions as a co-germinant. Moreover, these results demonstrate that D-alanine, itself, is a co-germinant for *C. difficile* spores.

Table 1 EC_{50} values (mM) for amino acids and the *C. difficile* spore

	***C. difficile* strain**		
	UK1 (wildtype)	**RS07 (*alr2::ermB*)**	**RS07 pRS89 (*alr2::ermB* p*alr2*)**
L-alanine	5.5 ± 1.1	2.7 ± 1.8	10.7 ± 2.5
D-alanine	11.5 ± 2.3	24.2 ± 6.6	6.6 ± 1.0
L-serine	8.2 ± 0.8	2.5 ± 1.7	4.9 ± 0.5
D-serine	7.8 ± 1.2	19.1 ± 5.0	CND

The average and standard deviation for three biological replicates are shown.
CND: could not determine
EC_{50}: concentration that achieves half-maximum germination rate

2.3.4 Alr2 interconverts L-serine and D-serine

A prior study determined a crystal structure of a *C. difficile* alanine racemase (109). Though not known at the time, this racemase is Alr2. When recombinantly expressed and purified Alr2 (Figure S2) was suspended in reaction buffer, reacted with L or D-alanine before labeling and separating by HPLC. As shown in Figure 6A, when incubated with L-

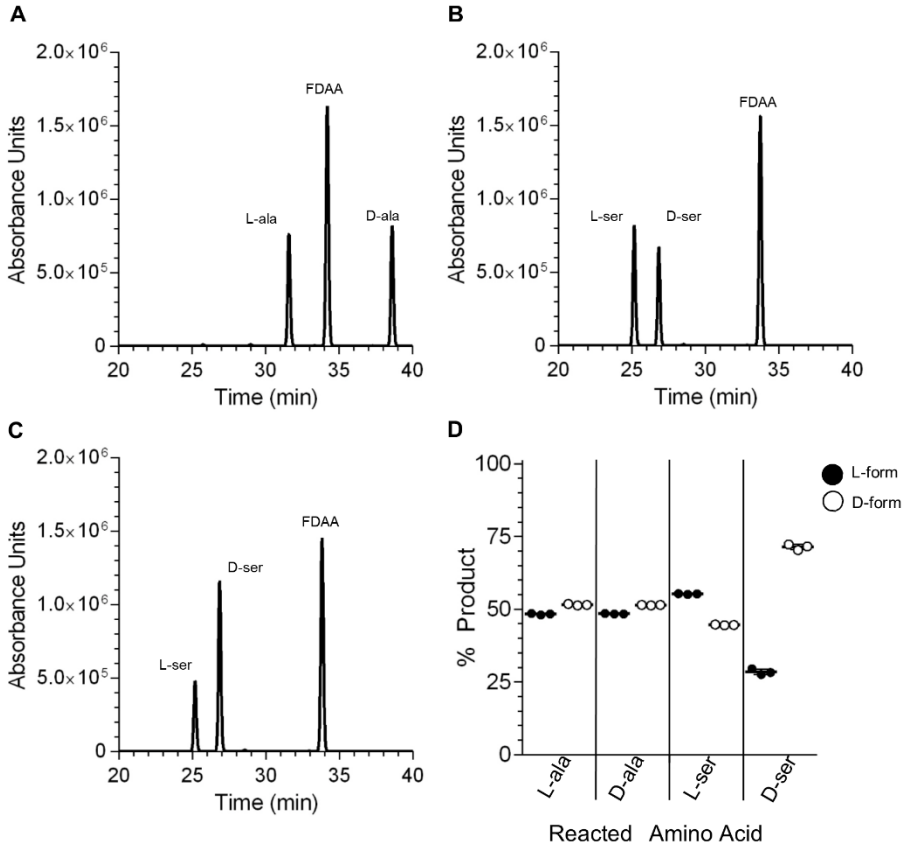

Figure 6. Alr2 interconverts L/D alanine and serine. Recombinantly expressed and purified Alr2 was incubated with (A) L-alanine or (B) L-serine or (C) D-serine for 1 hour. The reaction was labeled with FDAA and separated by HPLC. The HPLC retention times of each amino acid were determined using labeled amino acid standards. (D) Peak areas from each reaction were quantified and presented as % product generated from a reaction involving either L-alanine, or D-alanine, etc. and each data point is shown. Each bar represents the average peak area from three technical replicates. The error bars represent the standard deviation from the mean and, in most cases, are smaller than the bars.

alanine, Alr2 converted L-alanine to an approximately equal amount of D-alanine (Figure 6D); we use >98% pure D or L-amino acid for these studies and the opposite enantiomer is extremely low in these studies (Figure S3). During the course of these experiments, we included L-serine as a would-be negative control for conversion. To our surprise, Alr2

converted L-serine to an approximately equal amount of D-serine (Figure 6B). When tested with D-serine, Alr2 did not convert as much and nearly 75% of the D-serine (Figure 6C, quantified in Figure 6D) remained in the D-form. Because Alr2 converted L- and D-serine, we tested every other, soluble, amino acid (data not shown). No other amino acid was converted by Alr2 indicating that Alr2 has specificity to the L and D forms of alanine and serine.

Table 2 ITC analysis of Alr2 and $Alr2_{K39A}$ binding to L/D alanine & serine

	$Alr2_{WT}$		**$Alr2_{K39A}$**	
	K_D (mM)	**ΔH**	**K_D (μM)**	**ΔH**
L-alanine	9.2 ± 0.7	-7 ± 0.7	64 ± 6	-1.1 ± 0.1
D-alanine	9.2 ± 0.4	-7.8 ± 0.7	46 ± 9	-1.7 ± 0.1
L-serine	14 ± 1	-16 ± 1	CND	CND
D-serine	17 ± 1	-21 ± 0.2	CND	CND

The average and standard error of the mean for at least three technical replicates are shown. The differences in binding between L-alanine and D-alanine or L-serine and D-serine are not statistically significant. The difference in the binding of Alr2 to L-alanine and L-serine or D-alanine and D-serine are significant (p-val < 0.05 and p-val < 0.01, respectively).
CND: could not determine

2.3.5 L- and D-serine are co-germinants for C. difficile spores

Because Alr2 interconverted L and D serine, we reasoned that *C. difficile* spores may respond to L-serine as a germinant. Thus, we determined the EC_{50} values for L- and D-serine during *C. difficile* spore germination, as described above (Table 2). Wildtype *C. difficile* UK1 displayed an EC_{50} for L-serine of 8.2 mM. This EC_{50} decreased to 2.5 mM

in the absence of *alr2*. Complementation of *C. difficile* RS07 lead to a decrease in EC_{50} from the mutant levels to 4.9 mM but did not restore the EC_{50} to wildtype levels. Interestingly, *C. difficile* spores showed an EC_{50} for D-serine similar to that of L-serine. Also, similar to *C. difficile* RS07 and D-alanine, D-serine was a poor germinant in the absence of *alr2* (EC_{50} = 19.1 mM). Unfortunately, we were unable to complement the phenotype in response to D-serine, for unknown reasons. These results suggest that both L- and D-serine are co-germinants *C. difficile* spores.

2.3.6 Determining the affinity of Alr2 and Alr2$_{K39A}$ to L/D alanine and serine

Previously, kinetic analysis of L- to D-alanine conversion (and vice versa) was used to determine the affinities of Alr2 to the alanine isoforms (109). Because we found that Alr2 can convert L- and D-serine, we tested the affinity of Alr2 to alanine and serine by isothermal titration calorimetry (ITC) (Figure 7). As shown in Figure 7A, when L-alanine was injected into the ITC cell, we observed exothermic binding between Alr2 and L-alanine which could be saturated. We found that Alr2 had similar affinities for L-alanine (9.2 mM) and D-alanine (9.2 mM). The affinities for L-serine (14 mM) and D-serine (17 mM) were weaker (Table 2). Because the ITC method injects ligand into the ITC cell, Alr2 converted the injected ligand to its isomer (Figure S4).

Because of the conversion of the L to D-form (and vice versa) in the ITC cell, the affinity of Alr2 for each of the tested amino acids could be an average of the affinities for tested and converted amino acids. For this reason, we engineered a mutation that disrupts the binding of the co-factor that is essential for catalysis (pyridoxal 5' phosphate). The Alr2$_{K39A}$ mutant was confirmed to be enzymatically dead by HPLC analysis of Alr2$_{K39A}$

reactions with L- and D-alanine (data not shown). When the binding of $Alr2_{K39A}$ to L- or D-alanine was tested by ITC, we observed significantly less signal (Figure 7B) compared to that of wildtype Alr2 (Figure 7A). However, we observed a signal that could be saturated (Figure 7B). From this data, we determined that $Alr2_{K39A}$ had an affinity of 65 µM for L-alanine and 50 µM for D-alanine (140 times that of wildtype for L-alanine and 184 times that of wildtype for D-alanine). Unfortunately, we could not determine the affinity of Alr_{K39A} and L-serine or D-serine, the signal to noise ratio was much too great to yield usable data.

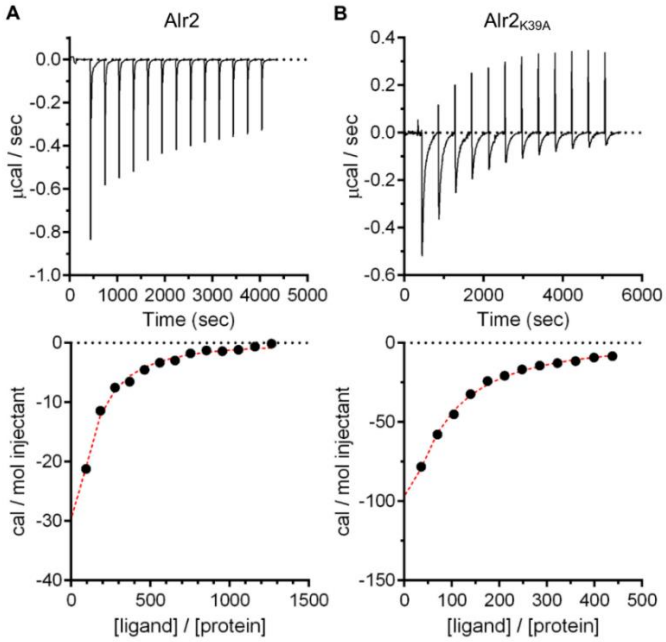

Figure 7. Binding of $Alr2_{WT}$ and $Alr2_{K39A}$ to L-alanine. Recombinantly expressed and purified (A) $Alr2_{WT}$ or (B) $Alr2_{K39A}$ was tested for their ability to bind to L-alanine by ITC. The thermograms are shown in the top panels. A binding isotherm was generated from the total heat following each ligand injection and is plotted in the bottom panel. The data are representative of at least three technical experiments. The summarized data can be found in Table 2.

2.4 Discussion

Spore germination is most-often triggered by L-alanine (88). In the model spore forming organism, *B. subtilis*, L-alanine interacts with the GerAA-AB-AC germinant receptor (89). This interaction leads to the release of CaDPA from the spore core and subsequent cortex degradation. The GerA germinant receptor is conserved across most spore-forming bacteria (110). In *B. anthracis*, and other organisms, L-alanine is a germinant whose action is dependent on the presence of the GerA germinant receptor. Importantly, *C. difficile* does not encode orthologues of the *gerA* germinant receptor but germinates through a novel spore germination pathway (61, 70, 88, 91, 93).

The bile acid germinant receptor, CspC, activates germination through an unknown mechanism (61). Subsequently, the CspB protease activates pro-SleC by cleaving the N-terminus from the protein, generating active cortex hydrolase (92). SleC then degrades the cortex resulting in osmotic changes that are perceived at the inner spore membrane (70, 93). This change in osmotic pressure at the inner spore membrane is relieved by the SpoVAC mechanosensing protein and results in CaDPA release from the core (70). Though bile acids are necessary to trigger germination, they are not sufficient. An amino acid signal is required to function as a co-germinant (60).

The most effective amino acid co-germinant is glycine (60). However, other amino acids can function as co-germinants (88). Previously, Howerton, Ramirez and Abel-Santos (2011) found that L-alanine could function as a co-germinant with TA for *C. difficile* spores (74). In their study, they found that D-alanine did not inhibit L-alanine-mediated spore germination, unlike what is observed in other spore-forming bacteria. Importantly,

the authors found that D-alanine did not function as an activator of spore germination at 30 °C (74). Herein, we found that D-alanine can stimulate spore germination in combination with TA at 37 °C. Moreover, in the same study, L-serine was nearly inactive as a co-germinant (L-serine generated ~5% germination when compared to glycine); D-serine was not tested (74). However, we find that both L-serine and D-serine can function as co-germinants for *C. difficile* spores at 37 °C. Our data suggest that temperature is an important consideration when determining activators and inhibitors of *C. difficile* spore germination.

Surprisingly, *C. difficile* Alr2 converted L-serine to D-serine (and vice versa). The affinities of Alr2 to L-serine and D-serine were similar, but the difference was not statistically significant. When tested for the ability to convert L-serine to D-serine, we observed that Alr2 generated nearly 50 % D-serine, suggesting that Alr2 efficiently converts L-serine to D-serine. When Alr2 was given with D-serine as a substrate, Alr2 only generated approximately 25 % L-serine from 100 % D-serine. Because the binding affinities of Alr2 and L- or D-serine are similar, this could indicate that the rate of conversion of D-serine to L-serine is much slower than the reverse reaction. Due to the conversion of L / D alanine and serine during ITC, we tested the binding of $Alr2_{K39A}$ to these amino acids. Though the K_D for $Alr2_{K39A}$ and L- and D-serine could not be determined due to a low signal to noise ratio, we found that the K_D for L- and D-alanine to be much lower than the K_D for the wildtype protein (Table 2). This suggests that the PLP co-factor may destabilize the interaction of Alr2 with its substrate, potentially as part of the catalytic mechanism.

Alanine racemases play important roles during the growth of Gram-negative and Gram-positive bacteria (94). These racemases are used to convert L-alanine to the D-alanine that is used to synthesize stem peptides during cell wall synthesis (94). As such, these proteins can be targeted by antibiotic treatment to prevent cell wall synthesis. D-cycloserine is a commonly used antibiotic that targets alanine racemase, indicating that the racemization of alanine is important for cellular physiology (109). Alr2 can be inactivated by D-cycloserine but *C. difficile* is naturally resistant to the antibiotic action of D-cycloserine and D-cycloserine is included in medium that is selective for *C. difficile* growth (109, 111). This suggests that the Alr2 that is incorporated into the *C. difficile* spores is unlikely to be used during cell wall synthesis. *C. difficile* encodes other amino acid racemases. For example, CDR20291_2507 is annotated as a putative alanine racemase. However, there are several other putative amino acid racemases, including a putative VanTG orthologue (serine / alanine racemase). It is likely that one of these other racemases is insensitive, or has reduced sensitivity, to D-cycloserine. We tested the effects of pre-exposure of D-cycloserine to *C. difficile* spores as a means to inactivate Alr2 to prevent the conversion of amino acids in the *C. difficile* spore. Unfortunately, D-cycloserine functioned as a weak co-germinant (data not shown). Because of the background spore germination generated by D-cycloserine, we could not inactivate Alr2 chemically.

Autoinhibition of spore germination is important for the development of a spore and ensuring that the spore germinates under appropriate conditions (95, 97). Alanine racemase is important for autoinhibition by preventing premature germination of the

developing spore within the mother cell or under less-than-suitable environmental conditions. We did not observe a difference between spores produced by the wildtype UK1 strain and spores produced by the *alr2* mutant strain. Because *C. difficile* spore germination is activated in response to bile acids and an amino acid co-germinant, it is not surprising that the *alr2* mutation did not affect spore formation. The *alr2* mutation resulted in an increased sensitivity to L-alanine and a decreased sensitivity to D-alanine (Table 1). Because D-alanine does not inhibit *C. difficile* spore germination, autoinhibition is minimal during *C. difficile* spore germination and only applies to the conversion of a very good germinant to a weak germinant.

A prior study analyzed the proteome of *C. difficile* strain 630 spores (112). In this study, Alr2 was found to be extracted by incubating spores in 2-mercaptoethanol (a condition that solubilizes coat proteins) (112). Though the location of Alr2 has not been determined, this would suggest that it resides outside of the spore core where it would be available to convert L / D alanine and serine. In this same study, no serine racemase was found (112). But, the authors found that *C. difficile* 630 CD3237 (a putative proline racemase) was present in the spore extracts. Though proline has not been described as a germinant for *C. difficile* spores, the inclusion of a proline racemase into the spore could signal the importance of proline racemization for *C. difficile* physiology, potentially for use in Stickland metabolism (113).

This study builds upon the hypothesis that the unidentified amino acid germinant receptor has broad specificity. Prior work has shown that the amino acid germinant receptor may require both a free carboxylate and a free amino a group (74). In this study,

some amino acids did not function as co-germinants (*e.g.*, L-histidine or L-serine). Our findings suggest that either there are multiple receptors that each recognize different amino acids as co-germinants, or that the amino acid germinant receptor can accommodate a diverse range of amino acids (both L and D forms). Because studies on *C. difficile* spore germination have been conducted at lower than physiological temperatures, it would be worthwhile to revisit the requirements of the amino acids to function as co-germinants at 37 °C.

Table 3 Oligonucleotides used in Chapter II

Primer Name	**Primer sequence**
R20291_alr 138s (gBlock)	TTCCCCTCTAGAAAAAAGCTTATAATTATCCTTATATGGCCATGGTGTGCGCCCAGATA GGGTGTTAAGTCAAGTAGTTTAAGGTACTACTCTGTAAGATAACACAGAAAACAGCCA ACCTAACCGAAAAGCGAAAGCTGATACGGGAACAGAGCACGGTTGGAAAGCGATGAC TTACCTAAAGACAATCGGGTACGACTGAGTCGCAATGTTAATCAGATATAAGGTATAA GTTGTGTTTACTGAACGCAAGTTTCTAATTTCGATTCCATATCGATAGAGGAAAGTGTC TGAAACCTCTAGTACAAAGAAAGGTAAGTTAGCACCATGGACTTATCTGTTATCACCA CATTTGTACAATCTG
5’ alr	CATGCAAAAAATAACAGTG
3’ alr	TTATTTTAGCAAATAACTGTTT
5' pJS116_XbaI_P1-prom	TACGAATTCGAGCTCGGTACCCGGGGATCCTCTAGACCAGTTGTAGATTCAGAGAAT A
3' alr_P1-Prom	TGCCCATGTAGGCACTGTTATTTTTTGCATCACACCTCCTACTTCAGTTT
5' P1-Prom_ alr	TACAATGAATAAACTGAAGTAGGAGGTGTGATGCAAAAAATAACAGTGC
3' pJS116_XhoI_alr	CCAGTGCCAAGCTTGCATGTCTGCAGGCCTCGAGTTATTTTAGCAAATAACTGTTTA TTT
5'pET_alr	GTTTAACTTTAAGAAGGAGATATACATATGCAAAAAATAACAGTGCCTACATG
3'pET_alr	ATCTCAGTGGTGGTGGTGGTGGTGCTCGAGTTTTAGCAAATAACTGTTTATTTGTAC
5' alr L39A	AAGATTTGTGGAGTAATAGCAGCTGATGCATATGG
3' alr L39A	GTCCATATGCATCAGCTGCTATTACTCCACAAAT

CHAPTER III

HIERARCHICAL RECOGNITION OF AMINO ACID CO-GERMINANTS DURING *Clostridioides difficile* SPORE GERMINATION[†]

In Chapter III, we analyzed the efficiency of amino acid to which putative amino acid germinant receptor is bound by measuring DPA release.

3.1 Introduction

Clostridioides difficile (recently renamed from *Clostridium difficile* (114, 115)) is a Gram-positive, spore-forming, obligately anaerobic human pathogen that causes symptoms ranging from mild diarrhea to life threatening colonic inflammation (116, 117). Antibiotic use is common in the healthcare environment, and prior antibiotic treatment, either related or unrelated to *C. difficile* infection (CDI), is the greatest risk factor for CDI. A healthy gut microbiota protects against CDI and the use of broad spectrum antibiotics disrupts the gut microbiota so that the gut becomes a suitable environment for *C. difficile* colonization (116-118). During colonization and disease, *C. difficile* forms metabolically dormant spores that are resistant to adverse environmental factors including most disinfectants (34, 88, 118).

Bacterial endospores are metabolically dormant structures that some bacteria produce when the cells are under nutrient deprivation or other environmental stresses.

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Similar to what is observed in *Bacillus subtilis*, a model organism for studying spore germination, the *C. difficile* spore core contains DNA, RNA, and the proteins necessary for the spores to outgrow after germination initiates (34, 88). The core is rich in calcium dipicolinic acid (DPA) which contributes to the spores' significant resistance to heat (31, 32, 89, 119). The core is surrounded by an inner membrane, a germ cell wall layer, a cortex layer, an outer membrane, a spore coat layer and an exosporium layer (88). To initiate germination, small molecule germinants must penetrate these layers to interact with their receptors. Because *C. difficile* is found in the gut, the key germination signals that its spores respond to are bile salts, such as taurocholate (TA), and amino acids, such as glycine (60). Spore germination is a crucial step in causing CDI, therefore it is an attractive therapeutic target for treatment (61, 120).

Though bile acids are essential signals for *C. difficile* spore germination, they are not sufficient (60, 61). *C. difficile* spores also require an amino acid signal (*e.g.*, glycine) to trigger the germination process (60, 74, 104). Even though some of the amino acids that trigger germination in other organisms can activate germination in *C. difficile, C. difficile* does not encode orthologues of the well-studied *ger* receptors (61, 91). In fact, *C. difficile* spores germinate through novel spore germination pathway involving activation of a pseudoprotease, CspC, present within the cortex layers (88, 93). In our working model, when taurocholate binds to CspC, the protein becomes activated such that it transmits its signal to the CspB protease, by an unknown mechanism (70, 88, 93). Activated CspB cleaves pro-SleC into its active, cortex-degrading, form (41, 92). DPA is then released from the core, the core becomes hydrated, and the germinated spore prepares for from the

core, the core becomes hydrated, and the germinated spore prepares for outgrowth (70, 93).

Though bile acids are important, germination of *C. difficile* spores cannot occur without a co-germinant (historically an amino acid, but calcium may play an unknown role (77)) and the amino acid germinant receptor is still unknown. Amino acids are frequently used as germinants. For example, in *B. subtilis*, spores germinate in response to L-alanine or to the combination of L-asparagine, D-glucose, D-fructose and potassium ions (AGFK) (121). The GerA germinant receptor (a combination of GerAA, GerAB and GerAC proteins) of *B. subtilis* recognizes L-alanine as a germinant. Prior work has suggested two possibilities regarding the identity of the amino acid germinant receptor in *C. difficile*: (i) there are multiple amino acid-recognizing germinant receptors; or (ii) the receptor recognizes multiple amino acids as germinants (74).

In *C. difficile*, glycine is the most effective co-germinant and can be found in most rich media and in an antibiotic-treated host environment; indeed glycocholate, a host derived bile acid, can be deconjugated to glycine and cholate (122, 123). Other L-forms of amino acids (*e.g*, L-alanine or L-cysteine) also trigger *C. difficile* spore germination when added with TA (74, 124). Yet, not all amino acids (*e.g.*, L-lysine or L-methionine) were identified as co-germinants (74).

Previously we showed that at a physiologically-relevant temperature, amino acids that were reported not to be co-germinants (124) did trigger germination when added with TA (*e.g.*, D-alanine or D-serine). These results led us to revisit germination of other amino acids which have not been previously shown to stimulate germination at room

temperature. Since many enzymes have an optimal temperature of 37 ºC, we tested the germination with TA and other amino acids at room temperature as well as at 37 ºC. To quantify the interaction of the amino acids with the *C. difficile* spore, we calculated the EC_{50} values for many amino acids from the data generated from the DPA release assay, which serves as a measure for spore germination. Based on the EC_{50} values, we were able to rank the amino acids from most effective to least effective as co-germinants. However, a few other amino acids such as L-isoleucine, L-leucine, and L-valine were still not able to stimulate germination at 37 ºC. This leads us to two more hypotheses regarding the role of amino acids, (i) the amino acid receptor has specificity to certain amino acids and this specificity decreases as the temperature is increased to 37 ºC (*e.g.*, in a host), (ii) any amino acid can act as the second signal, but the affinity of the amino acid for its receptor varies.

3.2 Materials and Methods

3.2.1 Strains and their growth conditions

C. difficile strains UK1 (027 ribotype, (61, 103, 125)) and M68 (017 ribotype, (126, 127)) were grown on BHIS agar medium (37 g of brain heart infusion, 5 g of yeast extract and 1g of cysteine per liter) at 37 ºC under anaerobic conditions (10% H_2, 5% CO_2, 85% N_2) for 3-4 days before harvesting for spore purification.

3.2.2 Spore purification

Spores derived from the UK1 and M68 strains were purified as described previously (70, 93). Briefly, the strains were grown on BHIS agar medium (20-30 plates) before scraping into microcentrifuge tubes containing 1 mL sterile water and kept at 4 ºC

overnight. The suspension containing spores and vegetative cell debris was washed with water five times by centrifuging for 1 min at 14,000 x g per wash. Spores were combined into 2-4 mL of water and further purified with a sucrose gradient (added 2 mL spores on top of 8mL of 60% sucrose and centrifuged at 4,000 x g for 20 min). The supernatant containing cell debris was discarded and the remaining pellet was washed again five times with sterile water. The spores were stored at 4 ºC until use and heat activated at 65 ºC for 30 minutes before use.

3.2.3 Germination assay

To compare the germination of spores at two different temperatures, spores was analyzed at 25 ºC and 37 ºC by measuring both OD_{600} and DPA release. For the OD assay, the germination was carried out in clear Falcon 96-well pates in a final volume of 100 µL and final concentration of 10 mM TA, 30 mM amino acid, 50 mM HEPES, 100 mM NaCl pH 7.5. All amino acids stocks were dissolved in water and pH was adjusted to 7.5 and filtered sterilized before use.

Similarly, 96-well black opaque plates were used to measure DPA released from the spores when exposed to a final concentration of 10 mM TA, 30 mM amino acid, 250 µM $TbCl_3$, 50 mM HEPES, 100 mM NaCl at pH 7.5. Spores were added to final OD_{600} of 0.5 and germination was analyzed for 1 hr using a plate reader (spectramax M3). Tb fluorescence was monitored at 275 nm excitation and 545 nm emission.

3.2.4 Calculation of EC_{50} values

DPA release was measured using concentration of solutions described earlier with some changes (80, 124). Various amounts of amino acids ranging from 0 mM to 500 mM

were used for the DPA release and this assay was run at 37 ºC for 2 hours. The values acquired from the DPA release were normalized and any DPA release that occurred in the absence of amino acid was subtracted before calculating the EC_{50} values for each amino acid. EC_{50} values were calculated by deriving a rate curve from slopes of the germination plot against time. All germination experiments were done in triplicate and the average mean and standard deviation values were reported.

3.3 Results

3.3.1 Effect of temperature on germination by C. difficile spores

Bile acids are essential, but not sufficient, to stimulate *C. difficile* spore germination. Amino acids are a required second signal. The best amino acid co-germinant is glycine, but others can substitute (*e.g.,* L-alanine) (60, 74, 88). Previously, we demonstrated that temperature plays an important role in germination of *C. difficile* spores; other amino acids were revealed to be co-germinants only when *C. difficile* spore germination was tested at 37 ºC (124). To further investigate the effect of this physiologically-relevant temperature on *C. difficile* spore germination, we compared the germination of glycine, L-alanine and D-alanine to induce germination at 25 ºC and at 37 ºC (Figure 8). When *C. difficile* UK1 spores were germinated with TA and glycine at 25 ºC, the OD of the spore suspension rapidly dropped and DPA was rapidly released from the spore core (Figure 8A). When germinated at 37 ºC, the rate of OD drop and DPA release increased (Figure 8B). We also observed the same trend for L-alanine (Figure 8C and 8D). When we tested D-alanine at 25 ºC, we observed little change in the OD of the

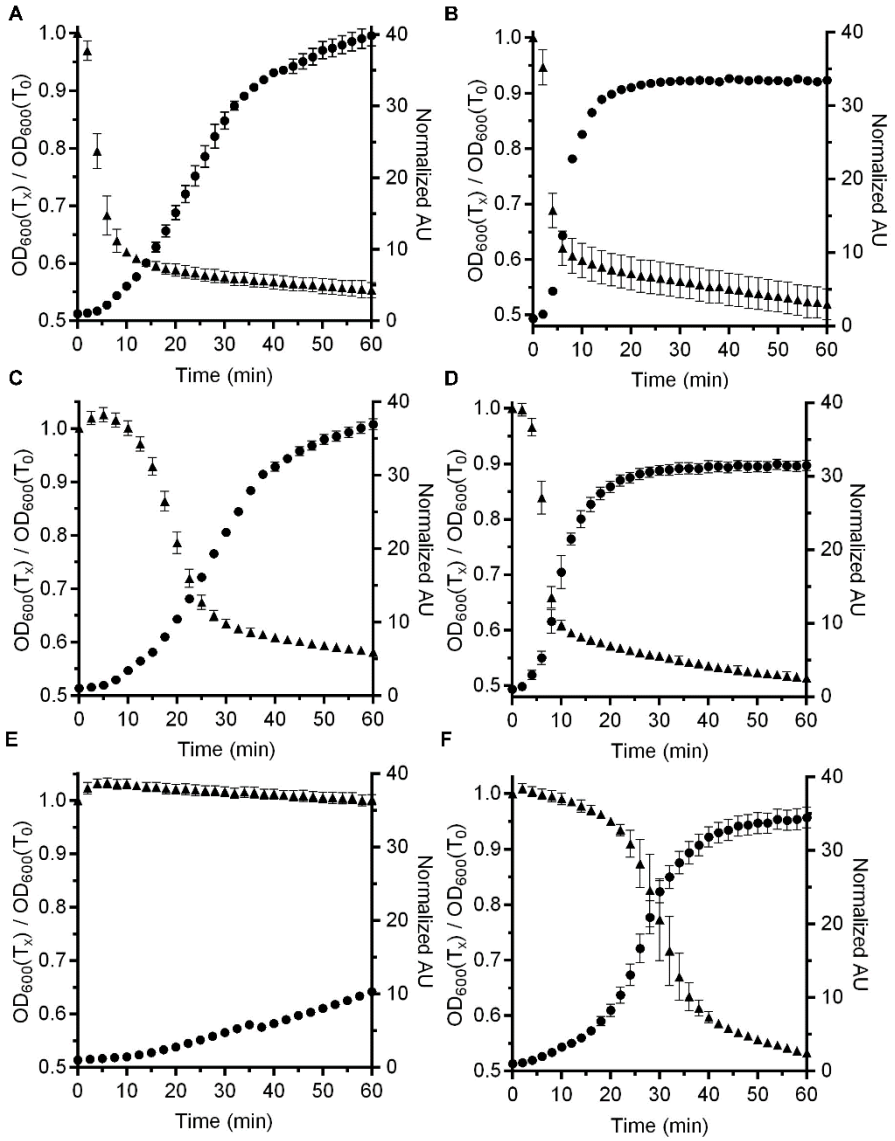

Figure 8. Comparison of *C. difficile* spore germination at 25 °C and 37 °C. Purified *C. difficile* UK1 spores were suspended in buffer supplemented with 10 mM taurocholate and 30 mM of the indicated amino acid. The change in OD_{600} during germination (▲) and the release of DPA (●) was measured over time at 25 °C (A, C, E) or 37 °C (B, D, F). Glycine (A & B), L-alanine (C & D) or D-alanine (E & F) were tested as co-germinants. The data represent the average of three independent measurements and error bars represent the standard deviation from the mean.

spore suspension and a minor amount of DPA release (Figure 8E). However, when analyzed at 37 ºC, D-alanine functioned as a strong co-germinant for *C. difficile* spores (Figure 8F). Although, the efficiency with which D-alanine functioned as a co-germinant was less than that of L-alanine and glycine. These results suggest that there may be a hierarchy for amino acids functioning as co-germinants at 37 ºC.

3.3.2 Identifying other co-germinants for C. difficile spore germination

Because D-alanine functioned as a co-germinant at 37 ºC but not at 25 ºC, we reasoned that there may be other amino acids that can act with TA to stimulate *C. difficile* spore germination at 37 ºC. Thus, we tested every other soluble amino acid for its ability to function as a co-germinant; L-tyrosine and L-tryptophan were insoluble. As we hypothesized, other amino acids were co-germinants with TA when incubated with spores at 37 ºC. For example, L-lysine was a very poor germinant at 25 ºC (Figure 9A) but led to a substantial decrease in the OD of the spores and an increase the release of DPA when tested at 37 ºC (Figure 9C). Importantly, not every tested amino acid functioned as a co-germinant with TA. Incubation of *C. difficile* spores with TA and L-isoleucine at 25 ºC (Figure 9B) or at 37 ºC (Figure 9D) did not lead to an appreciable decrease in OD or DPA release. These results suggest that other amino acids function as co-germinants with TA but not all amino acids are recognized as co-germinants during *C. difficile* spore germination.

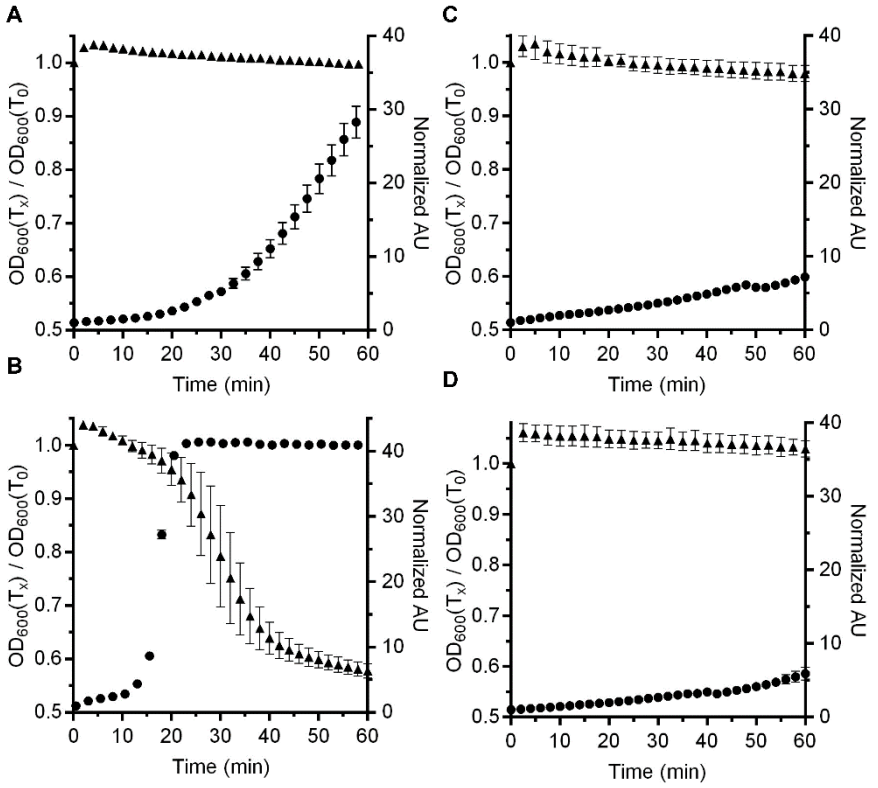

Figure 9. Analysis of the effects of different co-germinants on *C. difficile* spore germination. Purified *C. difficile* UK1 spores were suspended in buffer supplemented with 10 mM taurocholate and 30 mM of the indicated amino acid. The change in OD_{600} during germination (▲) and the release of DPA (●) was measured over time at 25 °C (A & C) or 37 °C (B & D). L-lysine (A & B) or L-isoleucine (C & D) were tested as co-germinants. The data represent the average of three independent measurements and error bars represent the standard deviation from the mean.

Table 4 EC_{50} values for amino acids and *C. difficile* UK1 spores

Amino Acids	*C. difficile* UK1
	EC_{50} (mM)
glycine	0.19 ± 0.01
L-alanine	1.1 ± 0.2
taurine	2.5 ± 1.6
L-glutamine	3.1 ± 2.1
L-histidine	4.1 ± 1.6
L-serine	5.6 ± 0.5
L-arginine	6.1 ± 0.6
L-lysine	13.8 ± 1.3
L-threonine	14.4 ± 3.2
D-alanine	15.1 ± 9.1
D-serine	24.3 ± 1.6
L-proline	28.5 ± 5.2
L-methionine	35.2 ± 10.5
D-lysine	72.8 ± 36.1
L-glutamate	98.7 ± 45.6
L-aspartic acid	115 ± 17
L-leucine	>500 mM
L-isoleucine	>500 mM
L-valine	>500 mM
L-phenylalanine	>500 mM
L-tyrosine	Not soluble
L-tryptophan	Not soluble
L-cysteine	Interfered with DPA measurements

Table 5 EC_{50} values for amino acids and *C. difficile* M68 spores

Amino Acids	*C. difficile* M68
	EC_{50} (mM)
glycine	0.48 ± 0.2
L-arginine	3.6 ± 0.7
L-alanine	5.1 ± 0.7
taurine	7.7 ± 1.9
L-glutamine	10.1 ± 0.6
L-histidine	12.4 ± 5.8
L-lysine	18.5 ± 4.6
L-serine	19.4 ± 4.2
D-alanine	25.0 ± 6.6
D-serine	54.4 ± 2.6
D-lysine	56.9 ± 19.4
L-methionine	58.7 ± 4.6
L-threonine	59.8 ± 25.3
L-proline	73.2 ± 18.0
L-aspartic acid	425 ± 170
L-glutamate	CND
L-leucine	>500 mM
L-isoleucine	>500 mM
L-valine	>500 mM
L-phenylalanine	>500 mM
L-tyrosine	Not soluble
L-tryptophan	Not soluble
L-cysteine	Interfered with DPA measurements

EC_{50} values were calcuated from the germination rate curves as described in the materials and methods. The values reported are the averages from three independent experiments with the standard deviation from the mean.

3.3.3 Determining the effectiveness of various amino acids as a co-germinant

To test if there indeed is a hierarchical rank of amino acids that function as co-germinants, we determined the EC_{50} values for each amino acid. These values have been used to provide information about how germinants interact with the *C. difficile* spore (74, 80, 90, 102-105, 107, 108, 124, 128). To calculate the EC_{50} values we incubated *C. difficile* UK1 spores in buffer supplemented with 10 mM TA and increasing concentrations of tested amino acid; the pH of the germination solution was adjusted to pH 7.5 before spores were added. The release of DPA during *C. difficile* spore germination was monitored for up to 2 hours at 37 ºC. The values were normalized and plotted against time. From this data, the maximum rates of DPA release were determined and used to calculate the EC_{50} using the Michaelis-Menten equation, as described previously (74, 80, 90, 102-105, 107, 108, 124, 128). Indeed, we observed a hierarchical order of amino acids that can stimulate germination in combination with TA (Table 4). As expected, glycine functioned as the best co-germinant and yielded an EC_{50} of ~190 µM. This was followed by L-alanine (1.1 mM), taurine (2.5 mM), L-glutamine (3.1 mM) and L-histidine (4.1 mM). For the *C. difficile* UK1 strain the order was glycine > L-alanine > taurine > L-glutamine > L-histidine > L-serine > L-arginine > L-lysine > L-threonine > D-alanine > D-serine > L-proline > L-methionine > D-lysine > L-glutamate > L-aspartic acid.

In order to determine if spores from a different *C. difficile* isolate germinate with the same rank order of amino acids, we determined EC_{50} values for each amino acid and

C. difficile M68 spores. There was a slight difference in the rank order for the two strains of *C. difficile* that we tested (Table 5). *C. difficile* M68 yielded a rank order of: glycine > L-arginine > L-alanine > taurine > L-glutamine > L-histidine > L-lysine > L-serine > D-alanine > D-serine > D-lysine > L-methionine > L-threonine > L-proline > L-aspartic acid.

The most effective amino acid co-germinant was glycine and the least effective co-germinant was L-aspartic acid for both the strains. Unfortunately, we could not determine the EC_{50} values for the remaining amino acids. Even though L-cysteine was shown to be a germinant in a prior report (74), L-cysteine interfered with the DPA release assay. L-tyrosine and L-tryptophan were not soluble. L-phenylalanine, L-valine, L-leucine, L-isoleucine, L-asparagine were very poor germinants at the concentrations that were soluble.

3.4 Discussion

Apart from the bile acid signal, amino acids are also an important signal for *C. difficile* spore germination (88). Glycine was first identified as the co-germinant required to initiate *C. difficile* spore gerimination, though other amino acids could substitute for glycine (60, 74). Later, the requirements for the amino acid structure in functioning as a co-germinant were tested by Howerton & Abel-Santos (74). Here, the authors found that the EC_{50} for glycine was approximately 8.7 mM^2 for the *C. difficile* CD630 strain, using the OD assay as a readout . Importantly, these two studies analyzed germination at either room temperature or 30 ºC (60, 74). Recently, we found that when *C. difficile* spore germination occurs at 37 ºC, the spores recognize amino acids previously reported to not activate germination (*e.g.*, D-alanine) (124). Therefore, we hypothesized that other amino

acids may be recognized by the spore when tested at physiologically relevant concentrations.

When tested at 37 ºC, we observed that *C. difficile* spores recognized a diverse set of amino acids as co-germinants. Using two different *C. difficile* strains (UK1 and M68, ribotypes 027 and 017, respectively), we found that glycine is the most preferred amino acid co-germinant (Table 4). By using kinetic analysis of spore germination, we calculated the EC_{50} of glycine to be 190 µM and 420 µM for *C. difficile* UK1 and *C. difficile* M68, respectively (Table 4, Table 5). These values are below the previously-reported value for glycine (74). There are several factors that might have affected germination in response to these amino acid co-germinants. For example, the temperature previously used to test germination, strain considerations and the assay conditions. All prior studies that determined the EC_{50} values for germinants / co-germinants and the *C. difficile* spore used the OD assay for the measurements (74, 80, 90, 102-104, 128). Here, we used a DPA release assay to calculate the EC_{50} in order to test germination at 37 ºC. The SpectroMax plate reader we routinely use for these assays will also accommodate OD measurements at 37 ºC. Importantly, though, due to the small light path of the 96-well plate, much higher spore amounts are needed to achieve an OD_{600} ~0.5 so that we can monitor sufficient OD change during germination. These higher spore concentrations lead to a concominant increase in the unidentified amino acid co-germinant receptor concentration(s) which would influence EC_{50} calculations (an increased receptor concentration would lead to an increase in the EC_{50} value).

One of the reasons why glycine might be more effective as a co-germinant is because of its size. Glycine is the simplest amino acid, which may help with diffusion through the spore layers. Importantly, though, size alone cannot explain why glycine functions as the best amino acid co-germinant. Since, the next best amino acid co-germinants were L-alanine, taurine and L-glutamine (for *C. difficile* UK1) and some of these amino acids are larger than L-serine, which functioned as a poorer co-germinant (Table 4). These observations also held true for *C. difficile* M68 spore germination (Table 5). Though the hierarchical order of co-germinant efficacy was slightly different, L-serine was a poorer spore germinant than L-arginine and L-alanine (Table 4).

Glycine is also important for *C. difficile* growth because it can be used in Stickland reactions to produce energy (113, 129). During Stickland-based metabolism, amino acids are oxidatively decarboxlyated or deaminated to produce ATP and NADH (129). In the reductive branch, D-proline or glycine are reduced by proline reductase or glycine reductase, respectively, to regenerate NAD^+ (129). *C. difficile* spores can use both glycine and D-proline as amino acid co-germinants (Table 4). However, in the two *C. difficile* strains tested, glycine was the best co-germinant while D-proline was a poor co-germinant (Table 4). An interesting hypothesis is that germinating *C. difficile* spores may preferentially utilize glycine as an energy source during outgrowth of a vegetative cell. In a prior publication, D-proline was found to be the preferred amino acid for vegetative growth while glycine was less important (113). It would be interesting to test the effects of glycine and proline during outgrowth of *C. difficile* spores.

C. difficile UK1 and M68 strains are different ribotypes (UK1 is 027 while M68 is 017). In these two ribotypes, it was previously reported that the EC_{50} for TA was similar (~3 mM) (80, 90). However, we found that the EC_{50} values of amino acid co-germinants varied between the two strains. We found that the hierarchy of amino acids that functioned as co-germinants differed except for glycine (the most efficient), and aspartic acid (the least efficient). Interestingly, germination of *C. difficile* UK1 was more efficient with most amino acids than was observed for *C. difficile* M68 strain with some exceptions (*e.g.*, L-arginine and D-lysine had EC_{50} values lower for M68).

In a previous report, L-methionine, L-serine, L-lysine, L-histidine, and L-aspartic acid were not co-germinants for *C. difficile* spores (74). However, we found that at 37 ºC, these amino acids functioned as co-germinants to trigger germination. Previously, *C. difficile* CD630 (ribotype 012) was used to characterize germination in response to different amino acid co-germinants. In that study, 12 mM of amino acids was used to test amino acids as co-germinants. We found that the EC_{50} values for some of these amino acids to be below the 12 mM concentration used in the prior work. Thus, it is likely that 12 mM was not sufficient to trigger germination in this study at the lower temperature (74). These results support the hypothesis that temperature at which germination is tested / occurs is an important consideration for *C. difficile* spore germination.

In our study, there were several amino acids whose EC_{50} values could not be calculated. L-phenylalanine and L-cysteine were previously shown to function as co-germinants . However, L-cysteine interfered with DPA release assay and L-phenylalanine did not dissolve at a concentration necessary for calculating the EC_{50} value, and, thus, we

could not determine their EC_{50} values. Also, we found that L-glutamate is a slow co-germinant, but we could not determine the EC_{50} value for the M68 strain (for unknown reasons, the kinetic analysis of spore germination with L-glutamate as a co-germinant yielded negative EC_{50} values).

Consistent with a prior study that analyzed germination at a 12 mM concentration, we found L-valine, L-isoleucine and L-leucine were poor germinants, even at the highest tested concentration (500 mM) [Table 4]. Although nonpolar, like glycine or alanine, these amino acids have branched methyl groups attached to their side chains closer to the alpha carbon. We also noticed that negatively charged amino acids, such as glutamic acid and aspartic acid, have higher EC_{50} values, making them poor co-germinants. However, positively charged amino acids were better co-germinants even though they have longer side chains.

Even though the D-form of amino acids are not normally recognized as activators of germination, we observed that the D-forms of amino acids could function as co-germinants with TA. However, these forms were weaker germinants compared to the L-form. We know from a previous report that the *C. difficile* alanine racemase, Alr2, has affinity for and can interconvert both L/D-alanine and L/D-serine (74, 124). Thus, it is possible that other racemases could be part of the spore coat and influence the ability of D-form amino acids to function as co-germinants. In fact, a prior study by Lawley and colleagues that characterized the spore proteome found that a putative proline racemase (CD3237) was present (112).

An important question is if our findings are physiologically (*i.e.*, *in vivo*) relevant. The concentration of most of the amino acids found in the distal colon are less then 3.5 mmol / kg. Physiologically, the concentration of alanine is ~0.89 mmol / kg and glycine is ~1.79 mmol / kg. The EC_{50} for glycine is 190 μM for UK1, which is below the glycine concentration in the distal colon (83, 130). Interestingly, we found that taurine was a good co-germinant for *C. difficile* spores in both the UK1 (2.5 mM) and M68 isolates (7.7 mM). In the gut, taurine is normally found to be conjugated to cholic acid (taurocholic acid, a very effective bile acid germinant) (122, 123). In healthy individuals, taurine was found to be at 3.53 mmol / kg in the descending colon and 2.89 mmol / kg in the rectum (83). This concentration is well within the EC_{50} range and suggests that glycine and taurine could effectively be used as co-germinants in a healthy host (the concentration in an antibiotic treated host was not analyzed in the prior study).

Here, our findings suggest that *C. difficile* spores can respond to a variety of amino acids as co-germinants with taurocholic acid. Interestingly, there appears to be a hierarchy in recognition of these amino acids with glycine, alanine and taurine functioning as the most efficient co-germinants. To date, the germinant receptor that recognizes the amino acid co-germinants has not been identified. Because *C. difficile* is well-equipped to respond to a variety of amino acids as co-germinants, it either uses several different / redundant receptors to repond to this variety of germinants, or, as hypothesized previously, the yet-to-be-identied receptor is significantly promiscuous (74).

CHAPTER IV

THE REQUIREMENT FOR THE AMINO ACID CO-GERMINANT DURING *Clostridium difficile* SPORE GERMINATION IS INFLUENCED BY MUTATIONS IN *yabG* AND *cspA*

In Chapter IV, I have used various techniques to identify the amino acid germinant receptor. I successfully demonstrated that CspA is the amino acid germinant receptor recognized by various amino acids during germination. Based on my results, I proposed a new model for *C. difficile* spore germination.

4.1 Introduction

Clostridioides difficile (formerly *Clostridium difficile*) (1, 114, 115) is a Gram-positive, spore-forming pathogenic bacterium, and has become a leading cause of nosocomial diarrhea in the United States (7, 86). *C. difficile* infection (CDI) is commonly the result of disruption to the gut microflora caused by antibiotic use (86, 117, 131). Due to the broad-spectrum nature of many antibiotics, alterations to the ecology of the colonic microbiome results in the loss of the colonization resistance that is provided by the

microbiota. Subsequently, patients are treated with other, broad-spectrum, antibiotics (*e.g.*, vancomycin or fidaxomicin) which treat the actively growing, toxin-producing, vegetative cells (132). Although these antibiotics alleviate the primary symptoms of disease, the continued disruption to the colonic microbiome results in frequent CDI recurrence. The symptoms of CDI are caused by the actions of two secreted toxins. TcdA (an enterotoxin) and TcdB (a cytotoxin) are endocytosed by the colonic epithelium and inactivate the Rho-family of small GTPases leading to loss of barrier function and inflammation of the colonic epithelium (117).

Though *C. difficile* vegetative cells produce the toxins that cause CDI, they are strictly anaerobic and only survive short periods of time outside the anaerobic colonic environment (133). However, the spores that are produced by the vegetative form are critical for transmission between hosts because of their resistance to environmental factors such as heat, UV, chemicals and, importantly, oxygen (34, 87, 88, 134, 135). The overall architecture of *C. difficile* spores is conserved among all endospore-forming bacteria. The centrally-located core is composed of DNA, RNA, ribosomes and proteins necessary for the outgrowth of a vegetative cell, post germination (34, 135). The DNA in the core is protected from UV damage by small acid soluble proteins (SASPs) and much of the water in the core is replaced by pyridine-2, 6-dicarboxylic acid (dipicolinic acid; DPA), chelated with calcium (CaDPA), which provides heat resistance to the spores (31, 34, 135). The core is surrounded by an inner membrane composed of phospholipids with minimal permeability to small molecules, including water (31). A thin germ-cell-wall layer surrounds the inner membrane and becomes the cell wall of the vegetative cell upon

outgrowth. A thick layer of specialized peptidoglycan (cortex) surrounds the germ cell wall and helps constrain the core against osmolysis (31). Finally, surrounding the cortex is the outer membrane which serves as an organization structure / point for the coat layers (34, 49, 51, 136). All these features of endospores contribute to ensuring that the spores remain metabolically dormant.

Though dormant, spores still sense their environment for species-specific germination-inducing small molecules and, when appropriate germinants are present, initiate the process of spore germination. Much of our knowledge of spore germination comes from studies in *Bacillus subtilis* (a model organism for studying spore formation and germination). In *B. subtilis*, and most other endospore-forming bacteria, germination is activated upon binding of the germinants to the Ger-type germinant receptors that are deposited in or on the inner spore membrane (89, 121). In *B. subtilis*, this event triggers an irreversible process whereby CaDPA is released through a channel composed of the SpoVA proteins (137-143). The release of CaDPA is an essential step for the germination process because it results in the rehydration of the spore core and permits the eventual resumption of metabolic activity. In *B. subtilis*, the cortex peptidoglycan layer is then degraded, and this event can be activated by the CaDPA that is released from the core (78).

In contrast to *B. subtilis*, *C. difficile* does not encode orthologues of the Ger-type germinant receptors suggesting that *C. difficile* spore germination is fundamentally different than what occurs in most other studied organisms (88). Germination by *C. difficile* spores is triggered in response to certain bile acids in combination with certain

amino acids (60, 88, 144). In all identified *C. difficile* isolates, the cholic acid-derivative, taurocholate (TA), is the most efficient bile acid at promoting spore germination, and glycine is the most efficient amino acid co-germinant (80, 145, 146). Recently, calcium was identified as an important contributor to spore germination, though its role remains unclear (77). Calcium may function as a bona fide spore co-germinant, a co-factor for a process essential for spore germination and / or as an enhancer for amino acids (77, 134).

In a screen to identify ethyl methane sulfonate (EMS) generated mutants that could not respond to TA as a spore germinant, we identified the germination-specific, subtilisin-like, pseudoprotease, CspC, as the *C. difficile* bile acid germinant receptor (61). Prior to work performed in *C. difficile*, the Csp locus was best studied in *Clostridium perfringens* (64, 147-149). *C. perfringens* encodes three Csp proteases, CspB, CspA, and CspC, that are predicted to cleave the inhibitory pro-peptide from proSleC, a spore cortex hydrolase that degrades the cortex peptidoglycan, thereby activating the protein (64, 147-149). In *C. difficile*, CspB and CspA are encoded by one ORF and the resulting protein is post-translationally processed into CspB and CspA and then further processed by the sporulation specific protease, YabG (69, 92, 135, 150). CspC is encoded downstream of *cspBA* and is part of the same transcriptional unit. Interestingly, the catalytic residues in CspA and CspC are lost, suggesting that only the CspB protein can process proSleC to its active, cortex degrading form (61, 92). Although present in the spore, and essential for *C. difficile* spore germination, the CspA pseudoprotease has only been shown to regulate the incorporation of CspC into the spore (69, 150).

In our working model for spore germination, activation of CspC by TA leads to the activation of the CspB protein which cleaves proSleC into its active form. Activated SleC degrades cortex and the core releases CaDPA in exchange for water by a mechanosensing mechanism (70). Because the receptor with which amino acids interact is unknown, we sought to screen for chemically-generated mutants that have altered amino acid requirements during spore germination [similar to the strategy used to identify the bile acid germinant receptor (61)]. Here, we report that a mutation in *yabG* gene results in strains whose spores no longer respond to or require amino acid co-germinants but respond to TA alone as a spore germinant. We hypothesize that the misprocessing of CspBA in the *yabG* mutant leads to this phenotype and provide evidence that short deletions in CspA alter the requirements for a co-germinant during spore germination. Our results suggest that CspA may be the amino acid co-germinant receptor.

4.2 Materials and methods

4.2.1 Growth conditions

C. difficile strains were grown on BHIS agar medium (Brain heart infusion supplemented with 5 g / L yeast extract and 1 g / L L-cysteine) in an anaerobic chamber (Model B, Coy Laboratories Grass Lake, MI) at 37 °C (85% N_2, 10% H_2, and 5% CO_2). Antibiotics were added as needed (15 µg / mL of thiamphenicol, 10 µg / mL lincomycin, and 20 µg / mL uracil). Deletion mutants were selected on *C. difficile* minimal medium (CDMM) supplemented with 5 µg / mL 5-fluoroorotic acid (FOA) and 20 µg / mL uracil. *Bacillus subtilis* was used as a conjugal donor strain to transfer plasmids into *C. difficile*

and was grown on LB medium with 5 µg / mL of tetracycline and 2.5 µg / mL of chloramphenicol. Conjugation was performed on TY medium (3% Bacto Trypone, 2% yeast extract, and 0.1% thioglycolate) with or without uracil. *E. coli* DH5α and *E. coli* MB3436 were grown on LB medium supplemented with 20 µg / mL chloramphenicol at 37 °C.

4.2.2 Spore purification

C. difficile spores were purified as described previously (70, 80, 93, 144). Briefly, the strains without plasmids were grown on BHIS agar medium while strains with plasmid were grown on BHIS agar medium supplemented with 5 µg / mL thiamphenicol and allowed to grow for 4 days. Cells from each plate were scraped into 1 mL sterile water and incubated at 4 °C overnight. Next, the cells were washed five times with water and combined into 2 mL total volume. The washed spores were layered on top of 8 mL of 60% sucrose and centrifuged at 4,000 X g for 20 minutes. The supernatant was discarded, and the spores were washed five more times with water and incubated at 4 °C until use.

4.2.3 EMS mutagenesis and phenotype enrichment

An overnight culture of wild type *C. difficile* UK1 vegetative cells were diluted to an OD_{600} of 0.05 into two, separate 15 mL falcon tubes containing BHIS liquid and grown for 3 - 4 hrs. To one of the cultures, ethyl methane sulfonate (EMS) was added to a final concentration of 50 µg / mL; the other culture was untreated for use as a negative control. The cultures were grown for 3 hours and then centrifuged at 3,000 X g for 10 min. The supernatants were discarded, and pellets were washed two more times with BHIS. After the final wash, the pellets were suspended in 40 mL BHIS medium and allowed to recover

overnight. The recovered EMS-treated cells were then plated onto 10 - 12 BHIS plates (25 µL on each plate) to produce spores. Spores were purified from the EMS-treated strain as described above. Purified spores were heat activated at 65 °C for 30 minutes before enrichment as shown in Figure 10A. The EMS-treated spores were treated for 15 minutes with HEPES buffer (50 mM HEPES, 100 mM NaCl, pH 7.5) supplemented with 10 mM TA and 10 mM betaine and then washed twice with buffer. Germinated, washed spores were plated onto BHIS agar medium for spore formation. This procedure was repeated iteratively for 4 – 5 times before isolating candidate strains for phenotypic screening.

4.2.4 Characterizing mutant phenotypes

The mutant spores were initially characterized by measuring the CaDPA release from germinating spores. Spores were heat activated at 65 °C for 30 minutes and suspended in water at an OD_{600} = 50. The spores were then added to final OD of 0.5 in 100 µL final volume of HEPES buffer containing 250 µM Tb^{3+}, 10 mM TA and / or 10 mM betaine in a 96 well plate. The CaDPA release was measured using a SpectraMax M3 plate reader for 30 minutes at 37 °C with excitation at 270 nm and emission at 545 nm with a 420 nm cutoff.

The phenotype of the *yabG* mutant (RS08) and *cspBA* deletion mutants were determined by measuring changes to the OD_{600} and release of DPA. OD_{600} was monitored at 37 °C for 1 – 2 hrs using the plate reader. The spores were added to a final OD of 0.5 in HEPES buffer supplemented with 30 mM glycine alone or 10 mM TA alone or 10 mM TA and 30 mM glycine in 100 µL final volume. CaDPA release was measured as described above with spores containing final OD_{600} of 0.25.

4.2.5 DNA sequencing

High-quality genomic DNA was purified from logarithmically growing *C. difficile* cells (151). Genomic DNA from the EMS-mutant strains and the wild type parent was sent for paired end sequencing at Tufts University Genomics Core. Reads were aligned to the R20291 genome using DNASTAR software and SNPs determined (DNASTAR, Madison, WI). RAW sequence (fastq) reads were uploaded to the NCBI Sequence Read Archive as follows (Table 6): *C. difficile* UK1 parent (SRS3677310), Mutant 20C (SRS3677309), Mutant 27E (SRS3677307), Mutant 30A (SRS3677308), Mutant 30C (SRS3677311), Mutant 31D (SRS3677312).

4.2.6 Molecular Biology

The oligonucleotides used for making the strains and plasmids used in this study are listed in Table 9. *yabG* and *sleC* mutants were created in the *C. difficile* R20291 background by using the TargeTron mutagenesis system. The potential insertion sites for targeting the group II intron were found using the Targetronics algorithm (Targetronics, LLC.) and a gBlock (Integrated DNA Technologies, San Jose, CA) of the group II intron targeted to *yabG* at the 279th position was ordered (Table 9) and cloned into pJS107 at the HinDIII and BsrGI restriction sites using Gibson assembly. The ligation was then transformed into *E. coli* DH5α. The site to engineer a TargeTron insertion in *sleC* (pCA6) was previously described for *C. difficile* UK1 (93) and the same protocol was used to engineer the insertion into R20291. The TargeTron insertion plasmids were isolated from *E. coli* DH5α and transformed into *E. coli* MB3436 (a *recA*$^{+}$ strain) and then into *B. subtilis* (BS49). Conjugation between *B. subtilis* and *C. difficile* R20291 was performed on TY-

agar medium for 24 hrs before plating onto selection plates. Once the plasmid was inserted into *C. difficile*, the colonies were screened for tetracycline-sensitive and thiamphenicol-resistant colonies and confirmed with PCR. *yabG* or *sleC* TargeTron mutants were selected by plating the colonies onto BHIS supplemented with lincomycin. Lincomycin-resistant colonies were tested by PCR and confirmed by sequencing the mutation. *yabG* and *sleC* TargeTron mutants were renamed as RS08 and RS10, respectively (Table 8).

A *yabG* complementing plasmid (pRS97) was created using primers 5' XbaI_Prom_YabG and 3' YabG_XhoI to amplify the *yabG* promoter and *yabG* coding regions and cloned into pJS116. The complementing plasmid was then inserted into *C. difficile* RS08 by conjugation with *B. subtilis*, as described above.

To engineer the required site-specific deletions, the *pyrE*-mediated allelic exchange strategy was used with the *C. difficile CRG2359* strain (R20291 Δ*pyrE*). Briefly, 1 kb upstream and 1 kb downstream fragments that surround the desired mutation in *cspBA* or *preprosleC* were cloned into pJS165 using primers listed in Table 9. The plasmids were inserted into *C. difficile* CRG2359 strain using *B. subtilis* conjugation as described above. The strains containing the plasmids were then passaged several times to encourage the formation of single recombinants before passing onto CDMM-FOA-uracil medium. Thiamphenicol-sensitive candidate strains were tested by PCR for the desired mutations and confirmed by sequencing for the mutagenized regions. Where indicated, *pyrE* was restored to wild type using pRS107 (Table 7).

4.2.7 Western blot

Samples were prepared for CspB, CspA, and CspC western blot by extracting

soluble proteins from 2x109 / mL spores (R20291, yabG::ermB, yabG::ermB pyabG). For the protein standard, recombinant CspB, SleC and CspC protein were purified using a previously described protocol (80). Standard amount of protein or number of spores were solubilized in NuPAGE sample buffer (Life Technologies) and heated at 95 °C for 20 minutes. Equal volume of spore extracts and recombinant CspB, CspC or SleC standard proteins were separated by SDS-PAGE. Proteins were then transferred onto low-fluorescence polyvinylidene difluoride membrane (PVDF) at 30V for 16 hours. The membranes were then blocked in 10% skimmed milk in TBS (Tris-buffered saline) and washed thrice with TBS containing 0.1% (vol / vol) Tween-20 (TSBT) for 20 minutes each at room temperature. The membranes were then incubated with anti-CspB, anti-CspC or anti-SleC antibodies for 2 hours and washed with TSBT thrice. For the secondary antibody, AlexaFlour 555-labeled donkey anti-rabbit antibody was used to label the membranes for 2 hours, in the dark. The membranes were washed again, thrice, with TBST, in the dark, and scanned with GE Typhoon Scanner using Cy3 setting, an appropriate wavelength for the Alexa Flour 555 fluorophore. The fluorescent bands were quantified using ImageQant TL 7.0 image analysis software. Intensity of the extracted protein in each blot was compared to the standard curve that was generated from the recombinant protein included on each blot.

To analyze SleC activation, equal number of spores were suspended in HEPES buffer supplemented with 30 mM glycine or 10 mM TA or 10 TA and 30 mM glycine and incubated at 37 °C for 1 hr to 2 hrs. The samples were then centrifuged at 15,000 X g for

1 minute and pellets were suspended in NuPAGE sample buffer and heated for 20 min at 95 °C. The suspension was centrifuged at max rpm for 10 min. The supernatant was separated and transferred into new tubes. The samples were stored at -20 °C until use. For CspC, CspA and CspB western blots, an equal number of spores were suspended in HEPES buffer and boiled to extract the protein and loaded in 10% SDS- PAGE gel. The spore extracts were then transferred into nitrocellulose membrane for western blot analysis.

4.2.8 Statistical analysis

All germination assays were performed in triplicate and data points represent the average of three independent experiments. Error bars represent the standard error of the mean. A 1-way ANOVA with Tukey's multiple comparisons test was used to compare the quantified protein amounts. For quantification of proteins, each blot was loaded with 5 standard proteins and three spore samples.

4.3 Results

4.3.1 Identifying C. difficile mutants with altered co-germinant requirements

In order to identify the receptor with which amino acid co-germinants interact, we used a strategy that was previously used to identify the bile acid germinant receptor (CspC) (61). Although other strategies, such as Tn-seq, could be used to generate random mutations, most of these will result in germination null phenotypes and do not permit the screening of subtler phenotypes. As shown in Figure 10A, wild type *C. difficile* UK1 vegetative cells were exposed to EMS and recovered. Purified spores derived from the

mutagenized bacteria were then germinated in buffer supplemented with 10 mM TA and 10 mM betaine. The structural difference between glycine and betaine is the presence of three methyl groups attached to the N-terminus rather than two hydrogen atoms. Because betaine is a glycine analog and does not stimulate spore germination when added with TA (74), we hypothesized that we could isolate change-of-function mutants that recognize betaine as a germinant or those that no longer require glycine as a co-germinant. Spores incubated in the presence of buffered TA and betaine were then plated and allowed to form spores. Potential mutants were enriched with this strategy 4 - 5 times before isolating colonies and screening for phenotypes. Across several mutagenesis experiments, the most commonly-observed phenotypes were strains that did not require the co-germinant glycine to germinate and germinated in response to taurocholate only (TA-only). As shown in Figure 10B, wild type *C. difficile* UK1 spores required both TA and glycine to stimulate the release of CaDPA from the core. However, spores purified from isolates derived from separate EMS mutageneses released CaDPA in the presence of TA only (Figure 10C, 10D, and 10E). Importantly, though, these mutants still responded to glycine, the germination efficiency/rate increased upon glycine addition. To identify the mutation(s) that caused this phenotype, five different mutant strains (isolated from 4 independent EMS mutageneses) and a wild-type control were sent for genome re-sequencing. Surprisingly, when the sequences of the mutant strains were compared to the sequence of the wild-type parent, we identified SNPs common to all 5 mutants in the coding region or the promoter region of *yabG*, a sporulation-specific protease (Table 6).

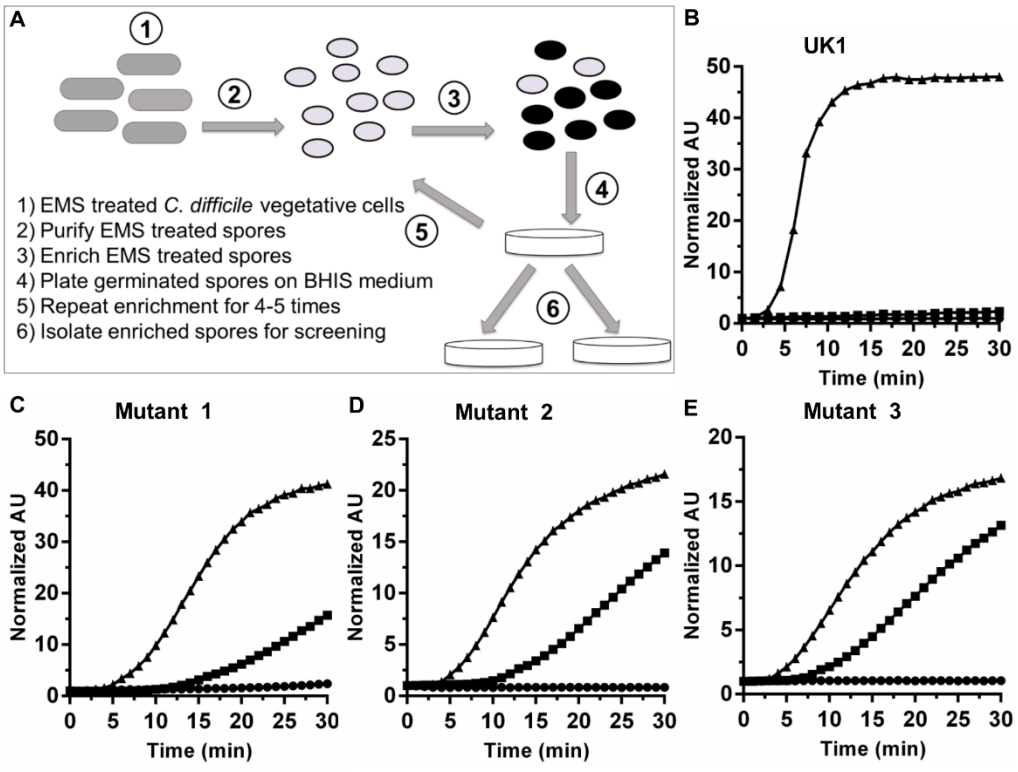

Figure 10. EMS mutagenesis generates *C. difficile* strains with altered requirements for the amino acid co-germinant. (A) Schematic of the EMS mutagenesis strategy where (1) *C. difficile* UK1 vegetative cells were treated with EMS, (2) EMS mutagenized cells were recovered and allowed to form spores, (3) purified spores were suspended for 15 minutes in germination buffer supplemented with 10 mM TA and 10 mM betaine and then washed twice with buffer alone, (4) germinated spores were plated onto BHIS agar medium to permit colony formation, (5) strains were enriched by exposure TA and betaine, iteratively, 4 – 5 times, and (6) spores from the mutant strains were isolated for screening. The germination phenotype of wild type *C. difficile* UK1 (B) and mutant spores (C – E) were screened by measuring CaDPA release in presence of (●) 30 mM glycine, (■) 10 mM TA or (▲) 10 mM TA and 30 mM glycine. The data points represent the data from a single germination experiment. Germination plots performed in triplicate yielded error bars that obscure the data. For transparency, all germination plots can be found in Figure S5.

Table 6 Location of the mutations in *yabG* found in the EMS mutant strains

Mutant	Reference	Coding region	Promoter region
20C (Mutant1)	4048886	G-T(Ala46Asp)	
27E (Mutant 2)	4049063		G-42A
30A (Mutant 3)	4048565	G-A (Pro153Leu)	
30C	4048913	C –T (Gly37Glu)	
31D	4049030		C-8T

Nucleotide positions in the promoter region are mapped from the ATG of the open reading frame (the *yabG* transcriptional start site is unknown)

4.3.2 Characterizing the function of yabG

To confirm that the TA-only phenotype is caused by a mutation in *yabG*, we inserted a group II intron into the *yabG* gene of *C. difficile* R20291 using TargeTron technology. Germination of the *C. difficile yabG::ermB* mutant (RS08) spores was compared to wild type using both OD_{600} and CaDPA release assays (Figure 11). As shown in Figure 11A, wild-type *C. difficile* R20291 spores required both TA and glycine in order to germinate. Interestingly, the RS08 mutant spores germinated in response to TA-only and this germination phenotype was not enhanced by the addition of glycine (in contrast to the phenotype of the EMS-mutant spores) (Figure 11B). We also tested L-alanine as a co-germinant instead of glycine (Figure S6), however, the spores did not show any enhancement in germination with the addition of L-alanine and germinated only in response to TA alone. When the mutation was complemented *in trans* by expression of *yabG* from a plasmid (pRS97), the spores again recognized glycine as a germinant (Figure 11C). The TA-only phenotype in the mutant spores was also

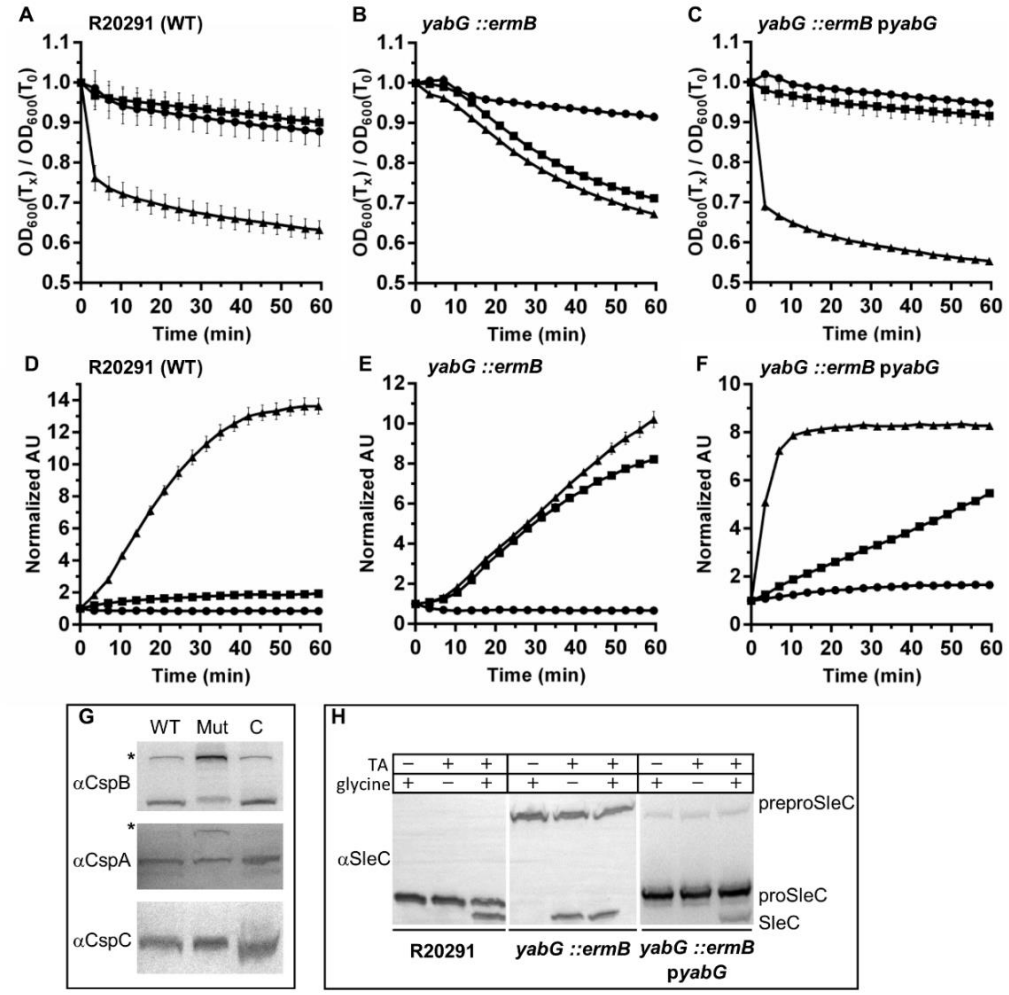

Figure 11. A mutation in *C. difficile yabG* results in spores that do not respond to amino acids as co-germinants. Spores derived from *C. difficile* R20291, *yabG::ermB* and *yabG::ermB* p*yabG* strains were suspended in buffer supplemented with (●) 30 mM glycine or (■) 10 mM TA or (▲) 10 mM TA and 30 mM glycine. Germination was monitored at OD_{600} (A, B, C) or by the release of CaDPA in presence of 250 µM Tb^{3+} (D, E, F), respectively. Data points represent the averages from three, independent experiments and error bars represent the standard error of the mean. (G) Equal numbers of spores were extracted with NuPAGE buffer and separated by SDS-PAGE followed by immunoblotting with antisera specific for each listed protein. (H) Equal numbers of spores were incubated in 30 mM glycine or 10 mM TA or 10 mM TA and 30 mM glycine. Spores were then extracted and separated by SDS-PAGE. SleC activation was analyzed using antisera specific for the SleC protein (the SleC antibody detects the preproSleC, proSleC and activated SleC forms).

confirmed by CaDPA release and compared to spores from both wild type and the complemented strain (Figure 11D, 11E, 11F).

In prior work, a *yabG* mutant strain accumulated unprocessed CspBA into spores (69). To confirm that the generated *yabG* mutant results in the accumulation of CspBA, we extracted spores derived from R20291, RS08 and the complemented strain and separated the extracted protein by SDS-PAGE. The separated protein was then detected using immunoblotting with CspB-specific antisera (Figure 11G). Although the blot showed that some CspBA is remaining in both the wild type and complemented strains, the *yabG* mutant had mostly the unprocessed, CspBA form. Similarly, the CspA western blot showed that the *yabG* mutant had both the CspBA and CspA forms. The mutation in *yabG* did not appear to affect CspC incorporation into the spores although these results are not quantitative.

YabG also was shown to process preproSleC into proSleC (69). Indeed, whereas the wild-type and complemented strains incorporated into spores the processed, proSleC, form, only preproSleC was incorporated into the RS08 strain (Figure 11H). When tested for the processing of SleC during germination, the wild-type and the complemented strains required both TA and glycine to activate SleC. However, *C. difficile* RS08 activated SleC in response to TA alone. These results confirm the TA-only phenotype observed in Figures 11B and 11E and suggest that a protein that is not processed in the *yabG* mutant strain is involved in germinant recognition or regulating the germinant specificity.

4.3.3 Quantifying levels of CspB, CspC and SleC in spores from various strains

Because YabG is a sporulation specific protease, it is possible that the deletion of this protease might alter the amount of germination related proteins (*e.g.*, CspB, CspC, CspA or SleC) that are incorporated in the spore thereby providing the observed phenotype (*i.e.*, increasing the abundance of the germinant receptors could lead to an increase in germinant sensitivity and loss of regulation). Using the previously described method to quantify the protein levels in *C. difficile* spores (80), we quantified the abundance of CspB (and CspBA), CspC and SleC in *C. difficile* RS08 (*yabG::ermB*) and compared them with the abundances in the wild-type and complemented strains (Figure 12).

Introducing the *yabG* complementing plasmid resulted in significantly increased incorporation of proSleC and CspC into spores compared to the RS08 strain (Figure 12A; only the preproSleC form is incorporated into the RS08 strain) or the wild-type strain (Figure 12B), respectively. There were no statistical differences in the abundance of SleC or CspC between the wild-type and mutant strains. Importantly, there was a statistical difference in the abundance of CspB in all pair-wise comparisons of the wild type and complemented strain (there was no quantifiable CspB protein in spores derived from the RS08 strain; Figure 12C). Finally, spores derived from the RS08 strain had significantly more CspBA incorporated than did the wild-type or the complemented strains (Figure 12D). Because spores derived from the complemented strain had increased abundances of proSleC (Figure 12A), CspC (Figure 12B), and CspB (Figure 12C), but did not produce a TA-only phenotype, this suggests that increased abundance of these proteins is not reason for the observed TA-only phenotype in the *yabG* mutant strain. Importantly, though,

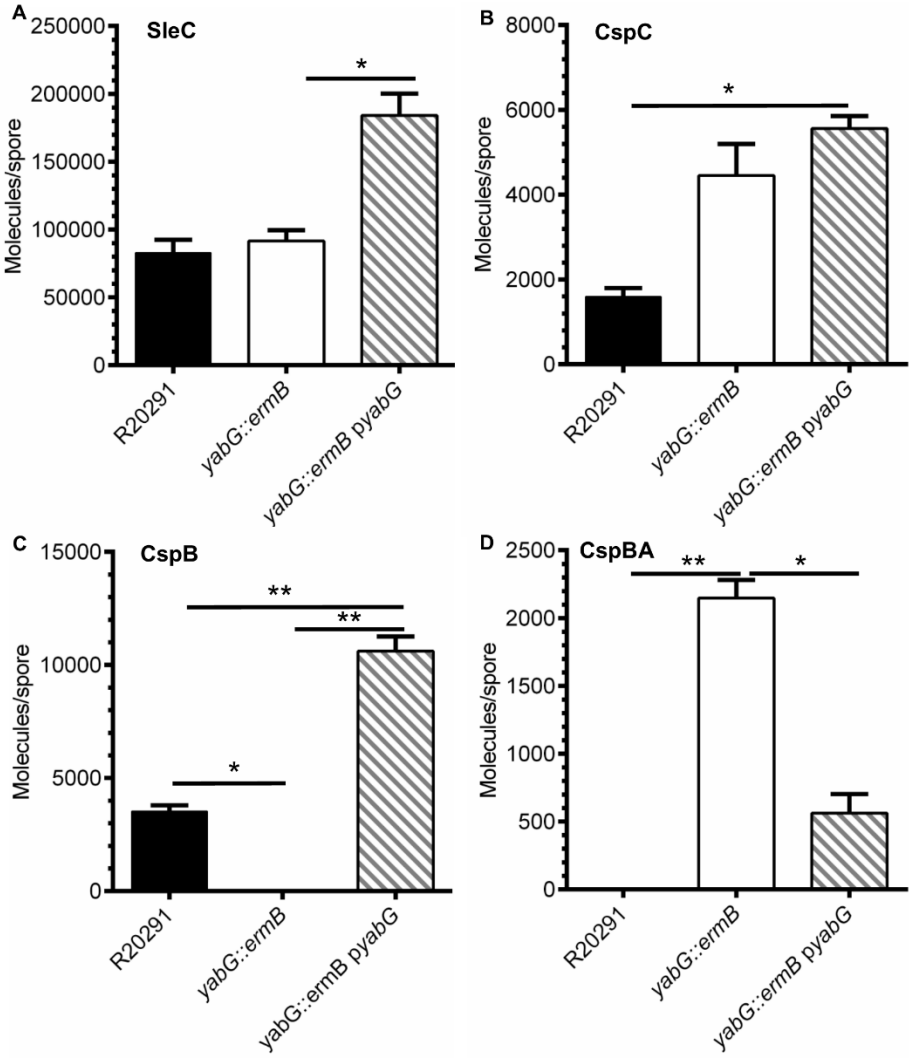

Figure 12. Comparing the abundance of CspB, CspC and SleC in *C. difficile* spores. 2 x 10^9 spores purified from *C. difficile* R20291, the *yabG* mutant and complemented strains and were extracted with NuPAGE buffer. Samples were separated by SDS-PAGE, transferred to low fluorescence PVDF membranes and blotted with antisera specific to the indicated proteins. The primary antibody was detected with a fluorescently conjugated secondary antibody and quantified as described in materials and methods. Quantified proteins are expressed as molecules / spore and are represented in a bar graph form. (A) SleC, (B) CspC, (C) CspB and (D) CspBA. The data presented represent the averages from three independent experiments and error bars represent the standard error of the mean. Statistical significance was determined using a 1-way ANOVA with Tukey's multiple comparisons test (* $p < 0.05$; ** $p < 0.01$). *yabG::ermB* only incorporates the preproSleC form.

the *yabG* mutant accumulated much more CspBA into spores than did the wild-type or the complemented mutant strains (Figure 12D) and only accumulated the preproSleC form (Figure 12H). Therefore, we hypothesized that the presence of full-length CspBA and / or preproSleC could contribute to the observed TA-only phenotype.

4.3.4 Deletions in the cspBA coding sequence lead to the observed TA-only phenotype

Because CspB and CspC are already known to be involved in regulating *C. difficile* spore germination (61, 69, 92, 93, 150), we chose to first focus on the processing of CspBA by YabG. In prior work by Kevorkian *et. al.* (69), the CspBA processing site was hypothesized to occur at or near amino acid 548. To test this hypothesis, we deleted from the *C. difficile* CRG2359 (*C. difficile* R20291 Δ*pyrE*) chromosome 12 aa between CspB and CspA (*cspBA*$_{\Delta 548\text{-}560}$) using *pyrE*-mediated allelic exchange. After confirmation of the engineered mutation in the CRG2359 genome, the *pyrE* gene was restored, and germination of the resulting strain was compared to *pyrE*-restored CRG2359 strain (*C. difficile* RS19; Figure S7A). We found that the CspBA$_{\Delta 548\text{-}560}$ allele did not have any effect on germination (Figure S7B) and it was the same as the germination found in the *pyrE* restored, RS19 strain.

Next, to determine if deletions in the coding region in or between *cspB* and *cspA* affect spore germination, we deleted various regions within the *cspBA* gene in the CRG2359 strain (Figure 13A). The results of the germination phenotype in various deletions are shown in Figure 13. Deletion of 26 codons from the C-terminus of *cspB* (RS20; CspBA$_{\Delta 522\text{-}548}$) did not affect spore germination (Figure 13C) when compared to

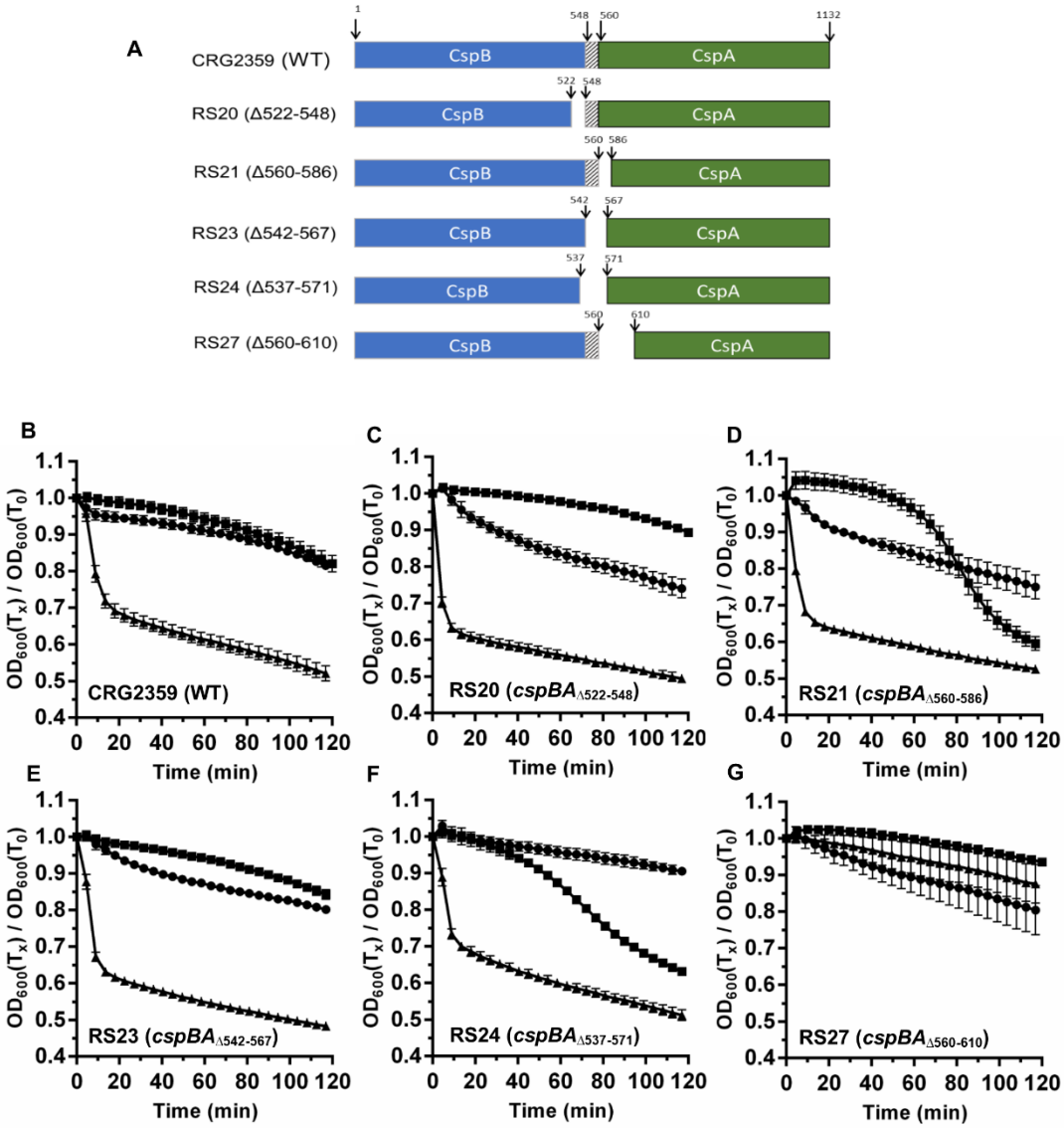

Figure 13. The N-terminus of *C. difficile* CspA is important for regulating germination in response to glycine. (A) Graphical representation of the various deletions introduced into *cspBA* compared to the parental, CRG2359; R20291Δ*pyrE* strain. Spore germination of the indicated strain was monitored at OD_{600} in buffer supplemented with (●) 30 mM glycine or (■) 10 mM TA or (▲) 10 mM TA and 30 mM glycine. (B) CRG2359, (C) RS20 ($cspBA_{\Delta 522\text{-}548}$), (D) RS21 ($cspBA_{\Delta 560\text{-}586}$), (E) RS23 ($cspBA_{\Delta 542\text{-}567}$), (F) RS24 ($cspBA_{\Delta 537\text{-}571}$) and (G) RS27 ($cspBA_{\Delta 560\text{-}610}$).

the wild-type CRG2359 strain (Figure 13B). These results suggest that the C-terminus of CspB is not involved in generating the TA-only phenotype. Interestingly, deletion of 26 codons at the N-terminus of *cspA* (RS21; $CspBA_{\Delta 560\text{-}586}$) resulted in spores that germinated in response to TA-only, after 60 minutes of incubation in the germination solution (Figure 13D). Though deletion of 25 codons in between the *cspB* and *cspA* coding sequences did not result in a TA-only phenotype (RS23; $CspBA_{\Delta 542\text{-}567}$) (Figure 13E), extending the deleted region by another 9 codons into the surrounding region (RS24; $CspBA_{\Delta 537\text{-}571}$) resulted in spores that germinated in response to TA-only, again after 60 minutes of incubation (Figure 13E). Importantly, the spores derived from the RS21 and RS24 strains still respond to glycine as co-germinant, despite also germinating in response to TA-only. Because the TA-only phenotype appeared to be enhanced as more *cspA* was deleted (RS21 to the RS24 strain), we predicted that a larger deletion might result in a phenotype similar to that observed in the *yabG* mutant (which does not recognize glycine as a co-germinant). We found that when 50 codons were deleted from the N-terminus of CspA (RS27) the spores no longer germinated (Figure 13F). These results were confirmed by analyzing the release of CaDPA from the germinating spores (Figure S8).

During construction of the deletion strains, we encountered significant difficulties in restoring the *pyrE* allele to wild type. To circumvent this obstacle, and to understand if *C. difficile* spore germination is affected by the *pyrE* deletion, we restored *pyrE* in the RS21 strain ($CspBA_{\Delta 560\text{-}586}$, $pyrE^{+}$; RS26) and compared with the RS19 strain (CRG2359 with restored *pyrE*). As shown in Figure S9A, *C. difficile* RS19 required both TA and glycine to germinate but the RS26 strain germinated in response to TA-only (Figure S9B).

These results were confirmed by analyzing the release of CaDPA from the spore (Figure S9C and S9D). Finally, we analyzed the activation of proSleC to SleC in response to TA alone. Only the RS26 strain cleaved proSleC in response to TA alone (Figure S9E). These observations are identical to the observations made for the RS21 strain (Figure 13 and Figure S8) and indicate that the *pyrE* allele does not influence *C. difficile* spore germination in the context of these studies.

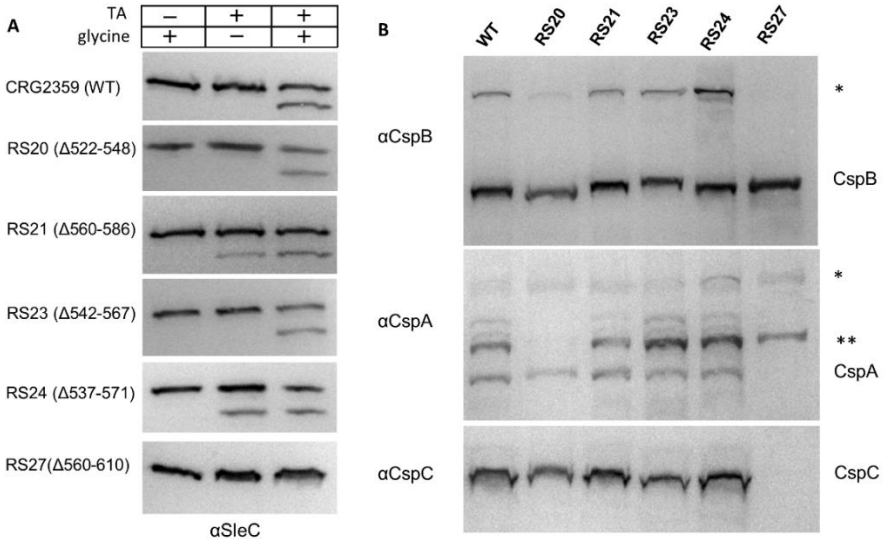

Figure 14. Comparing the effects of mutations in *C. difficile cspBA* on the incorporation and processing of CspB, CspA, CspC and proSleC. Spores derived from the indicated strains were extracted and separated as described in Figure 11. (A) SleC activation was measured in buffer supplemented with 30 mM glycine or 10 mM TA or both 10 mM TA and 30 mM glycine for 2 hours at 37 °C and (B) CspB, CspA, and CspC were detected as described in Figure 11 (*full length CspBA, ** alternative processing of CspA).

To confirm our observations that the strains germinated in response to TA alone, we analyzed by western blot the activation of SleC (Figure 14A). SleC activation in

response to 10 mM TA only occurred in RS21 and RS24 while RS27 did not germinate in response to TA and glycine. The CspB western blot showed that CspBA was processed to CspB and CspA in all of the mutants, compared to wild type (Figure 14B). The CspC western blot did not reveal any differences between the wild type and mutant strains, except for the RS27 strain where no CspC was detected.

4.3.5 Deletion of a hypothesized YabG cleavage site in CspA and preproSleC results in the TA-only phenotype

The data presented above suggests that the N-terminus of CspA is important for regulating spore germination. We had hypothesized that this region is processed by YabG but all the generated *cspA* alleles generated a protein that was processed into the CspB and CspA forms. One way to identify the YabG processing site in CspA is by pulldown of CspA from the spore extract and sequencing the CspA protein using mass spectrometry. Unfortunately, the CspA antibody was unable to immunoprecipitate CspA from the spores due to the quality of the antibody. However, we predicted that the YabG processing site in CspBA might be conserved in preproSleC. Instead of immunoprecipitating CspA, we immunoprecipitated proSleC from spore extracts derived from wild-type spores and *sleC* mutant spores (as a negative control) (Figure 15A). Using the sample from the proSleC pull down, we identified fragments by mass spectrometry of trypsin-digested proSleC (Figure 15B). In this experiment, the most N-terminal fragment identified began with glutamine followed by serine (an SR**Q**S sequence). When we compared this sequence to the protein sequence in CspA, we found that within the N-terminus of CspA there was a

SRQS amino acid sequence that was encompassed within the deletions found in the RS21 strain (a strain that generated a TA-only phenotype).

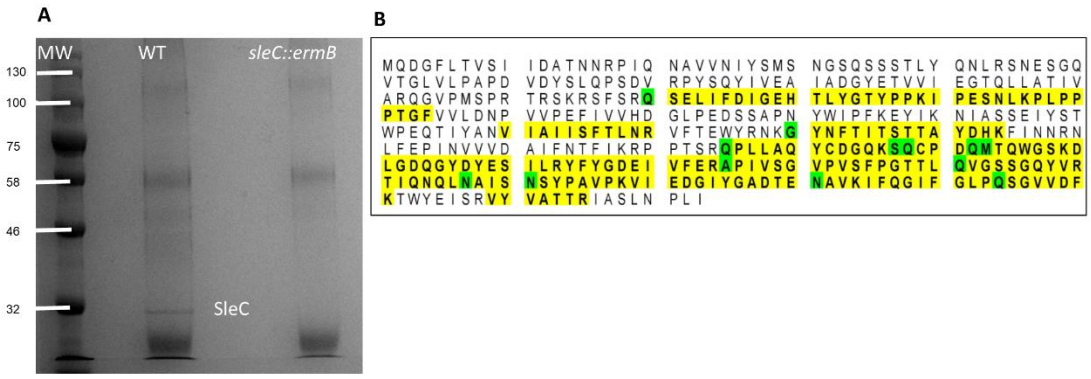

Figure 15. Immunoprecipitation of proSleC from *C. difficile* spores reveals a potential YabG cleavage sequence. (A) Spores derived from *C. difficile* R20291 (WT) and *C. difficile* RS10 (*sleC::ermB*) were disrupted by bead-beating and then proSleC was immunoprecipitated using SleC-specific antisera. Samples were separated by SDS-PAGE gel and stained with Commassie blue. The band corresponding to proSleC was excised from the gel and analyzed by peptide mass finger printing. (B) The sequence of preproSleC is listed. Yellow highlighted regions correspond to fragments that were detected by mass spectrometry and amino acids highlighted in green indicate amino acids that are modified in the MS analysis (*i.e.*, oxidated, deaminated, acetylated or ammonia loss).

We deleted the nucleic acid sequence that encodes this SRQS motif in *sleC*, *cspBA*, or in both genes (Figure 16A). Surprisingly, the deletion of the SRQS site in CspA resulted in spores that germinated in response to TA-only within 40 minutes after germinant addition (Figure 16B). However, deletion of the SRQS motif within preproSleC did not affect spore germination (Figure 16C). When these two deletions were combined in the same strain, the spores had a TA-only phenotype similar to that of spores with a deletion

in CspA alone (Figure 16D and 16A). Next, we confirmed these phenotypes by analyzing the release of CaDPA from the spore (Figure S10). Again, only when the *cspA*$_{\Delta SRQS}$ allele was incorporated into spores did the resulting strains release CaDPA in response to TA-only (Figure S10A and S10C); *sleC*$_{\Delta SRQS}$ spores required both TA and glycine to release CaDPA (Figure S10B).

We next analyzed the activation of SleC for these deletions. proSleC was activated in response to TA-only phenotype in the RS29 (*cspA*$_{\Delta SRQS}$) and the RS31 (*cspA*$_{\Delta SRQS}$ + *sleC*$_{\Delta SRQS}$) strains but not in the RS30 (*sleC*$_{\Delta SRQS}$) strain (Figure 16E). Interestingly, we also noticed that deletion of the SRQS sequence in the RS30 and RS31 strains did not result in the incorporation of full length preproSleC into the spores. Rather, preproSleC was still processed to a size consistent with a proSleC form, potentially suggesting that the SRQS sequence is not the YabG cleavage site or the presence of an alternative YabG processing site in preproSleC. There was no difference in the CspB, CspC and CspA incorporation in these SRQS deletion mutants compared to wild type (CRS2359; Figure 16F). Regardless, our results indicate that due to mis-processing of CspBA, or alterations within the *cspA* sequence, spores lose the requirement for an amino acid co-germinant during spore germination.

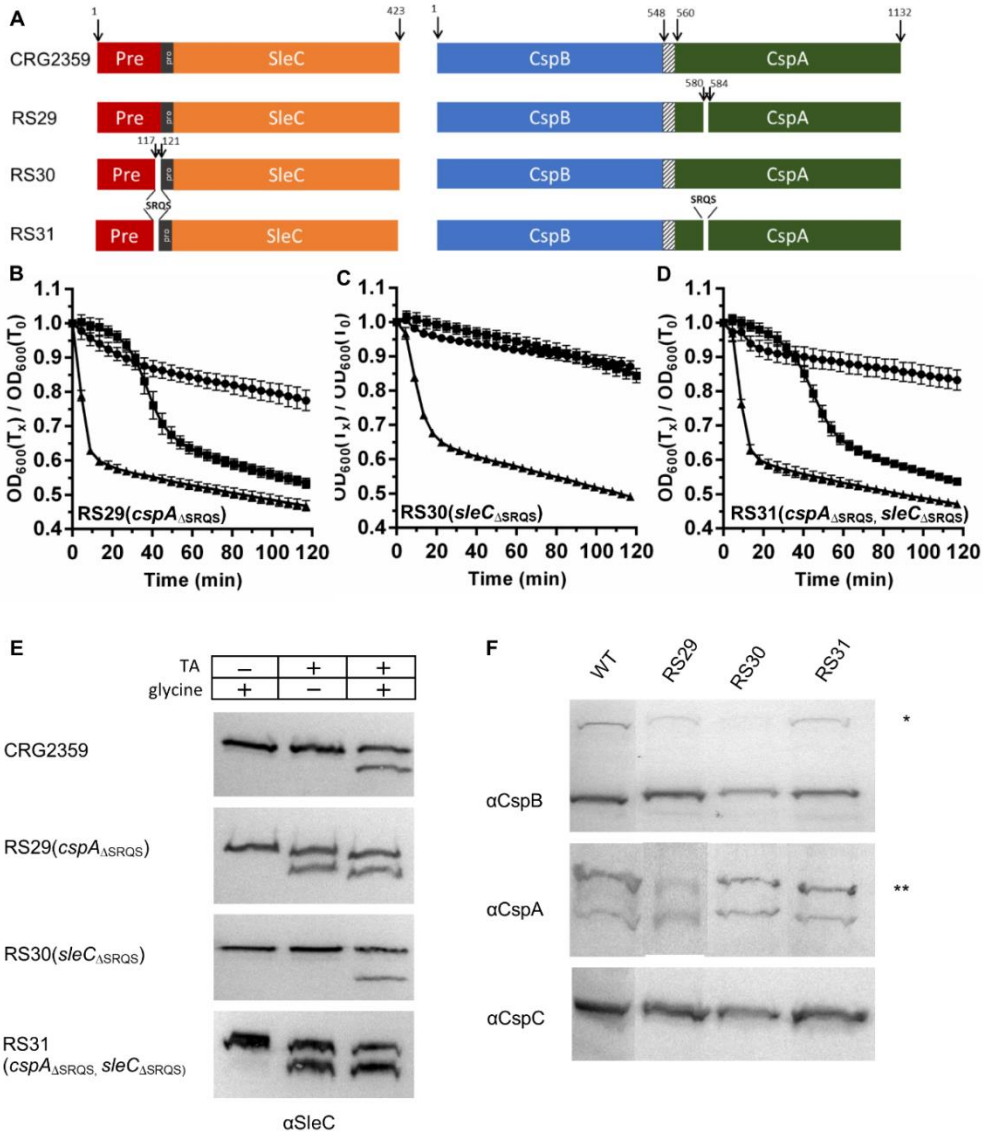

Figure 16. Deletion of the hypothesized YabG cleavage site in CspA results in a TA-only phenotype. (A) Graphical representation of the various deletions introduced into *cspBA* compared to the parental, CRG2359; R20291Δ*pyrE* strain. Spore germination of the indicated strain was monitored at OD_{600} in buffer supplemented with (●) 30 mM glycine or (■) 10 mM TA or (▲) 10 mM TA and 30 mM glycine. (B) RS29 ($cspA_{\Delta SRQS}$), (C) RS30 ($sleC_{\Delta SRQS}$), (D) RS31 ($cspA_{\Delta SRQS}$, $sleC_{\Delta SRQS}$). (E) Spores derived from the indicated strains were extracted and separated as described in Figure 11 and SleC activation was measured in buffer supplemented with 30 mM glycine or 10 mM TA or both 10 mM TA and 30 mM glycine for 2 hours at 37 °C. (F) CspB, CspA, and CspC were detected as described in Figure 11 (*full length CspBA, ** alternative processing of CspA).

4.4 Discussion

Previous studies on *C. difficile* spore germination have hypothesized the presence of an amino acid co-germinant receptor. We used EMS mutagenesis to screen for *C. difficile* mutants whose spores recognize betaine as a germinant. Excitingly, we isolated mutants that did not require an amino acid as a co-germinant, and these EMS-mutants germinated in response to TA-only. However, these mutants still recognized glycine as a co-germinant; the rate of germination increased upon the addition of glycine to the germination solution. Sequencing of these strains revealed common mutations in the *yabG* coding region or promoter region (Table 6). Thus, to confirm the TA-only phenotype observed in the EMS-generated mutants is due to the *yabG* allele, we created a TargeTron mutation in *yabG* of *C. difficile* R20291. In contrast to the EMS-generated mutants, the *yabG* mutant spores did not recognize glycine as a co-germinant (Figure 10C, 10D, 10E), and germinated in response to TA-only (Figure 11B, 11C). These phenotypes could be complemented by expressing *yabG in trans* from a plasmid. Oddly, we noticed that spores derived from the complemented strain released CaDPA in response to TA alone, but this was not observed when germination was measured at OD_{600} (Figure 11F vs. 11C). We further analyzed this observation and found that the supposed CaDPA release observed in the complemented strain in the presence of TA alone was due the presence of Tb^{3+} in the assay (Tb^{3+} is absent from the germination solution in the OD_{600} assay) (Chapter V).

To understand if spores derived from the *yabG* mutant recognized other amino acids as co-germinants, we tested L-alanine. L-alanine is the second-best co-germinant during *C. difficile* spore germination with an EC_{50} value of 5 mM (144). Because *C.*

difficile RS08 (*yabG::ermB*) spores did not respond to glycine or L-alanine (Figure S6), these results suggest that the protein(s) YabG processes is / are responsible for recognition of the various amino acids used as co-germinant(s). It is unclear if all of the amino acid co-germinants are recognized by a single protein or if each co-germinant / groups of co-germinants are recognized by unique proteins. However, our results suggest that glycine and other amino acids that trigger germination are recognized by a protein, or a set of proteins, that is processed by YabG.

YabG has been mostly studied in *B. subtilis*. In *B. subtilis* YabG is a sporulation-specific protease, and a *yabG* mutation causes alterations in the coat proteins of *B. subtilis* spores. The orthologues of most *B. subtilis* YabG target proteins are absent in *C. difficile* (*e.g.,* CotT, YeeK, YxeE, CotF, YrbA) (67, 68, 91). In a previous report, YabG was shown to process CspBA and preproSleC during *C. difficile* sporulation (69). However, germination was not significantly altered in the mutant spores compared to wild type spores (germination efficiency decreased from 1 to 0.8 in mutants) (69). Importantly, though, germination efficiency was only tested on BHIS-TA agar medium and not in the presence of TA alone (69).

In prior work by Kevorkian *et. al*. (69), the authors suggested that the CspBA processing site is near-amino acid 548 and is encoded by a linker DNA sequence between *cspB* and *cspA*. To test this, we used allelic exchange to delete the codons encoding amino acids 542-567 regions in *cspBA* (RS23). When the germination phenotype of the RS23 strain was compared with CRG2359, we did not observe a TA-only phenotype (Figure 13A and 13E) and, importantly, CspBA was still efficiently processed (Figure 14). These

results suggest that the predicted 548-560 region as a processing site by YabG is not accurate, or that when the YabG processing site is removed, YabG cleaves the CspBA protein at an alternate site.

Unlike spores derived from the *yabG* mutant, which did not recognize amino acids as co-germinants, the deletions that were engineered in the N-terminus in CspA [*e.g.*, $CspBA_{\Delta 560\text{-}586}$ (RS21) or $CspBA_{\Delta SRQS}$ (RS29)] still responded to glycine as a co-germinant. In order to recapitulate the *yabG* mutant phenotype, we hypothesized that deletion of a larger portion in the N-terminus of CspA might result in a CspA allele that loses the recognition of amino acids as co-germinants. When 50 codons were deleted from the region of CspA encoding the N-terminus ($CspBA_{\Delta 560\text{-}610}$; RS27), the spores no longer germinated (Figure 13G) and no SleC was activated in any of the tested conditions (Figure 14). Moreover, we did not observe functional CspA in the spores derived from the RS27 (although there was a band corresponding to unprocessed CspA similar to what was observed in the other deletion and wild-type strains). Prior work on CspA demonstrated that deletion of CspA results in spores that do not incorporate CspC (69, 150). Here, we found that the 50 amino acids at N-terminus of CspA are important for the incorporation of CspC into spores, probably because CspA is non-functional without the N-terminal domain or the mutant lacks a secretion signal that directs the processed CspA into the spore.

Using immunoprecipitation and mass spectrometry of the immunoprecipitated protein, we identified four amino acids in SleC with identity in CspBA. When this sequence was deleted from CspA (*cspBA*$_{\Delta 580\text{-}584}$), we observed a TA-only phenotype in

the spores suggesting that the SRQS region might be important for CspA activity. Both RS21 (*cspBA*$_{\Delta 560\text{-}586}$) and RS24 (*cspBA*$_{\Delta 537\text{-}571}$) strains generated spores with TA-only phenotypes similar to the SRQS deletion in CspA (RS29; *cspBA*$_{\Delta 580\text{-}584}$). However, only the RS21 strain has the deletion of SRQS region while the SRQS motif is still present in the RS24 strain. Importantly, the SRQS deletion in preproSleC resulted in spores with no TA-only phenotype and the western blot analysis (Figure 16) showed that deletion of the SRQS site in preproSleC resulted in incorporation of the processed, proSleC, into the spore instead of preproSleC form. This indicates that the SRQS sequence is not the YabG processing site in preproSleC (and, potentially CspBA), or that the deletion of the SRQS sequence results in YabG shifting its processing site in preproSleC to a region near the SRQS sequence. Further experiments are required to identify the YabG cleavage sites in spore proteins and how this processing is affected when its recognition site is deleted.

In a recent review on *C. difficile* spore germination (134), the authors build upon a hypothesized model for spore germination and propose a new, "lock and key" model for *C. difficile* spore germination. In the first model, the germinosome complex composed of CspC, CspA, CspB, and proSleC are anchored to the outer spore membrane by GerS (a lipoprotein that is required for spore germination (71) - see below) (88, 134). Upon binding of a germinant (TA with glycine or Ca^{2+}), CspC and CspA are released from this germinosome complex and CspB becomes free to activate proSleC to degrade the cortex. Subsequently, CaDPA is released from the spore core by a mechanosensing mechanism (70, 140). In the second model, CspA and CspC are localized in the coat layer, where TA can bind to CspC. Activation of CspC leads to the transport of glycine and Ca^{2+} through

the outer membrane, by an unknown protein, to the cortex where CspB is held inactive in a complex with GerS and proSleC. In the absence of calcium, glycine is transported to the inner membrane where it activates the release of Ca^{2+} from the core through another unknown process. The released Ca^{2+} traffics to CspB to activate its protease activity. In the presence of calcium and glycine, both are transported in and Ca^{2+} activates CspB. When glycine and calcium bind to CspB, CspB can activate proSleC to degrade the cortex (134).

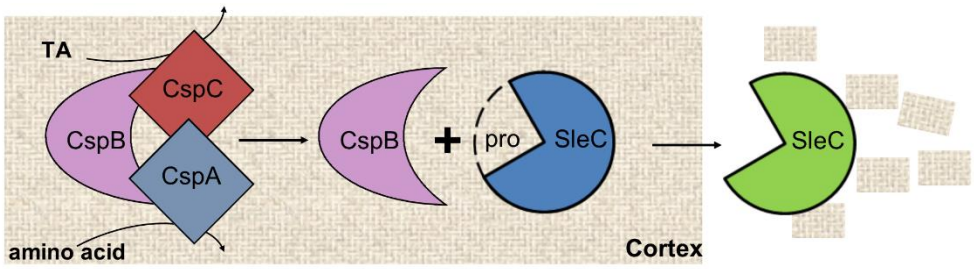

Figure 17. Model for *C. difficile* spore germination. In our working model for *C. difficile* spore germination, CspB protease activity is inhibited by two pseudoproteases, CspC (the bile acid germinant receptor) and CspA (the amino acid germinant receptor; this study). Interaction of CspC with cholic acid derivatives (60) and the interaction of CspA with amino acids (124, 144) results in these two proteins disassociating from CspB. Subsequently, CspB cleaves the inhibitory pro-domain from proSleC thereby activating SleC's cortex degrading activity.

Our results support the first model whereby CspB, CspA, and CspC are in a germinosome complex – similar to the germinosome complex found in *B. subtilis* (152). Recent studies on GerS have shown that GerS likely does not form a complex with other germination proteins (71, 72). Rather, it is required to generate cortex-specific modifications in the spore and thus, important for germination of the spore because the

SleC cortex lytic enzyme depends on cortex-specific modifications to degrade the cortex layer efficiently (72). Based upon prior work from our lab, proSleC is unlikely to be part of this complex because SleC is three to four times more abundant than CspB or CspC, depending upon the strain analyzed (80).

The processing of CspBA depends on the YabG protease and *yabG*-mutant spores incorporate mostly the full length CspBA protein. These spores do not recognize amino acids as co-germinants and germinate in response to TA-only (Figure 11B). We hypothesize CspC (functioning as the bile acid germinant receptor) and CspA (functioning as the amino acid co-germinant receptor) inhibit CspB activity within dormant spores (Figure 17). These two pseudoproteases would regulate the activity of CspB so that it does not prematurely activate proSleC, potentially similar to how other pseudoproteases / pseudokinases regulate activity of their cognate proteins (153-157). Because *yabG* mutant spores package full length CspBA, where CspA is tethered to CspB, CspC alone prevents CspB from cleaving proSleC into its active form, and TA might dislodge CspC from CspB (this is consistent with our prior publication that indicates that CspC may have an inhibitory activity during spore germination (80)). Moreover, our data suggest that the N-terminus of CspA might be important for formation of this hypothesized complex with CspC and / or CspB. When portions of the CspA N-terminus are deleted, the binding of CspA to the complex might become unstable causing CspA to randomly disassociate from the complex. This would result in TA alone stimulating germination by disassociating CspC from CspB. Once CspB is free from the complex, it could then activate many

proSleC proteins to maximize the germination process. Further work is needed to test the biochemical implications of this hypothesis.

Table 7 Plasmid list with primer pairs to make the plasmids

Plasmid	Description	Oligonucleotides used (Table 9)	Reference
pRS92	*yabG* G-block cloned into Zero blunt	813	This study
pRS93	Plasmid to make TT insertion in *yabG*		”
pRS97	*yabG* Complementing plasmid	906/907, 924/925	”
pCA6	Plasmid to make TT insertion in *sleC*		(93)
pMTLYN4	Plasmid to make deletion in *C. difficile* using allelic exchange		(158)
pJS107	TargeTron vector		(61)
pJS116	Empty vector		(61)
pJS165	Tn916Ori cloned into pMTLYN4 to use *B. subtilis* conjugation for gene insertion	207, 208	This study
pRS110	pJS116 with Δ542-567 in CspBA	578/1232, 1233/1056	”
pRS111	pJS116 with Δ537-571 in CspBA	578/1234, 1235/1056	”
pRS112	pJS116 with Δ560-586 in CspBA	578/1236, 1237/1056	”
pRS113	pJS116 with Δ522-548 in CspBA	578/1238, 1239/1056	”
pRS114	pJS165 with 1Kb upstream and 1Kb downstream od Δ542-567 in CspBA	1160, 1163	”
pRS115	Plasmid to make Δ537-571 in CspBA cloned into pJS165	1160, 1163	”
pRS116	Plasmid to make Δ560-586 in CspBA cloned into pJS165	1160, 1163	”
pRS117	Plasmid to make Δ522-548 in CspBA cloned into pJS165	1160, 1163	”
pMTLYN2C	Plasmid to restore *pyrE*		(158)
pRS107	Tn916Ori cloned into pMTLYN2C to use *Bacillus subtilis* for restoring pyrE	207, 208	This study
pRS118	Plasmid to make Δ560-610 deletion in CspA	1160/1287, 1163/1288	”
pRS120	Plasmid to make SRQS deletion in CspA	1160/1329, 1163/1330	”
pRS121	Plasmid to make SRQS deletion in preproSleC	1361/1362, 1363/1364	”

Table 8 Strain list

***C. difficile* Strain**	**Description**	**Plasmids used Table 7**	**Reference**
UK1	Wild type, ribotype 027		(103)
R20291	Wild type, ribotype 027		(159)
CRG2359	R20291 Δ*pyrE*		(158)
RS08	*yabG::ermB* in R20291 background	pRS93	This study
RS10	*sleC::ermB* in R20291 background	pCA6	”
RS19	CRG2359 with restored *pyrE*	pRS107	”
RS20	CRG2359 with Δ522-548 deletion in CspB	pRS117	”
RS21	CRG2359 with Δ560-586 deletion in CspA	pRS116	”
RS23	CRG2359 with Δ542-567 in CspBA	pRS114	”
RS24	CRG2359 with Δ537-571 in CspBA	pRS115	”
RS26	RS21 with restored *pyrE*	pRS107	”
RS27	CRG2359 with Δ560-610 in CspA	pRS118	”
RS29	ΔSRQS (Δ580-584) in CspA	pRS120	”
RS30	ΔSRQS (Δ117-121) in preproSleC	pRS121	”
RS31	ΔSRQS in CspA and preproSleC	pRS120, pRS121	”
Other Strains			
E. coli DH5α	Cloning vector		(160)
E. coli BL21(DE3)	Expression vector		(161)
E. coli MB3436	*recA*$^{+}$ *E. coli* strain		Gift from Dr. Michael Benedik
B. subtilis Bs49	Tn916 donor strain, Tet^{R}		(162)

Table 9 Oligonucleotides used in Chapter IV

Primer #	Name of primer	Primer Sequence (5' to 3')
156	5' tcdB	TTACATTTTGTTTGGATTGGAGGTC
157	3' tcdB	AGCAGCTAAATTCCACCTTTCTACC
160	5' sleC	ATGCAAGATGGTTTCTTAACAGTAAGC
161	3' sleC	TTAAATTAAAGGATTTAAAGAAGCTATTC
173	5' catP 3	ATGGTATTTGAAAAAATTGATAAAAATAG
174	3' catP 2	TTAACTATTTATCAATTCCTGCAATTCG
207	5'Tn916ApaI	AA GGGCCC TAA CAT CTT CTA TTT TTC CCA AAT CC
208	3'Tn916ApaI	AA GGGCCC C960
578	5'pJS116_cspBA 2	CGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAAAAACTATAAAGTTAT AATTGTTGGAGATGCT
813	yabG (279s) gBlock	TTCCCCTCTAGAAAAAAGCTTATAATTATCCTTAGTTCCCAAACTTGTGCG CCCAGATAGGGTGTTAAGTCAAGTAGTTTAAGGTACTACTCTGTAAGATA ACACAGAAAACAGCCAACCTAACCGAAAAGCGAAAGCTGATACGGGAAC AGAGCACGGTTGGAAAGCGATGAGTTACCTAAAGACAATCGGGTACGAC TGAGTCGCAATGTTAATCAGATATAAGGTATAAGTTGTGTTTACTGAACG CAAGTTTCTAATTTCGATTGGAACTCGATAGAGGAAAGTGTCTGAAACCT CTAGTACAAAGAAAGGTAAGTTATGAAGTTTGACTTATCTGTTATCACCA CATTTGTACAATCTG
906	5' XbaI_Prom_YabG	GATCCTCTAGAGAAATGTTTTTTTGACATTAG
907	3' YabG_XhoI	GCCTCGAGCTAATGTAATATTGTTTTTGGC
924	5' pJS116_XbaI_YAbG	TTCGAGCTCGGTACCCGGGGATCCTCTAGAGAAATGTTTTTTTGACATTAG G
925	3' YabG_XhoI_pJS116	GTGCCAAGCTTGCATGTCTGCAGGCCTCGAGCTAATGTAATATTGTTTTTG GC
1056	3' CspC_pJS116	CAAGCTTGCATGTCTGCAGGCCTCGAGTTATCTATAGAGTATTTGCTATCT GTTGAAT
1084	5' pyrE 2	GTCCAGTGTTCTGGGGAG
1085	3 'pyrE 2	AAAATTTACATTTTTTAAGTAACACTATAAATAATTAAGTTTTTA
1160	5' YN4_1Kb UP_CspBdelA	TTCGAGCTCGGTACCCGGGGATCCTCTAGAAAATATAAGTTATGGAAGTA ATGAA
1163	3' 1Kb DN_cspBdelA_YN4	TGCCAAGCTTGCATGTCTGCAGGCCTCGAGCCAAAGTTCTAATGATAATT CTT
1232	3' 1kb UP cspBA_75nt	CTTATATAAATTTCATTCTCTAAGTCATTATATCCAATATCCTCCGTT
1233	5' 1Kb DN cspBA_75nt	AAGATTTAGAAACGGAGGATATTGGATATAATGACTTAGAGAATGAAATT TAT
1234	3' 1Kb UP_cspBA_102nt	ACTTGCATTTTTACTTATATAAATTTCATTCGTTTCTAAATCTTGATTTATA CTA
1235	5' 1Kb DN_cspBA_102nt	CTATCTAGTATAAATCAAGATTTAGAAACGAATGAAATTTATATAAGTAA AAATGC
1236	3' 1Kb UP_cspA_78nt	GGGAGTATGGACTACATCTATTCCTGATAGATCTATAAACTTATACCTATT TTCCTC
1237	5' 1Kb DN_cspA_78nt	GATGAGGAAAATAGGTATAAGTTTATAGATCTATCAGGAATAGATGTAGT CCA
1238	3' 1Kb UP_cspB_78nt	AAACTTATACCTATTTTCCTCATCTTTAAAATCTGATAAATTTAAAAAACC AA
1239	5' 1Kb DN_cspB_78nt	TCAGGATTTGGTTTTTTAAATTTATCAGATTTTAAAGATGAGGAAAATAG GT
1258	3' cspB_A _75aa_pRS114	TTTCATTCTCTAAGTCATTATA
1259	3' cspB_A _102aa_pRS115	ACTTATATAAATTTCATTCGT
1260	3' cspA _76aa_pRS116	TATTCCTGATAGATCTA
1261	3' cspB_76aa_pRS117	CCTCATCTTTAAAATCTGAT
1287	3' 1Kb UP CspA_150nt	TACACCTAATGAATCACTTATCTTAAAAAAATCTATAAACTTATACCTATT TTCCT
1288	5' 1Kb DN CspA_150nt	GATGAGGAAAATAGGTATAAGTTTATAGATTTTTTTAAGATAAGTGATTC ATTAG
1361	5' YN4_SleC_1kB UP	TTCGAGCTCGGTACCCGGGGATCCTCTAGAAGCTAAAAATACAGAGTAAA TAATAC
1362	3' SleC_SQRS Del_1Kb UP	AGTATGTTCTCCTATGTCAAAAATCAACTCGAAAGAACGCTTACTTCTTG
1363	5' SleC_SRQS del_1kB DN	CTCCTAGAACAAGAAGTAAGCGTTCTTTCGAGTTGATTTTTGACATAG
1364	3' YN4_SleC_1Kb DN	TGCCAAGCTTGCATGTCTGCAGGCCTCGAGATAATAAATAATAATTTAGA TAA
1365	3' SRQS del cspA	ATTCCTGATTGTCTACT
1376	5' SleC SRQS del	GCGTTCTTTCTCAAGACAAAGT

CHAPTER V

TERBIUM CHLORIDE INFLUENCES *Clostridium difficile* SPORE GERMINATION, LIKELY AS A CALCIUM SUBSTITUTE

In Chapter V, I found that terbium which is used while measuring germination by DPA release also influences *C. difficile* spore germination similar to calcium. I also found the TA-terbium phenotype can be stronger depending upon the media composition on which the *C. difficile* spores are generated.

5.1 Introduction

Patients treated with broad-spectrum antibiotics have a disrupted gut microbiome and, as a result, are vulnerable to *C. difficile* infection (CDI) due to the loss of the colonization resistance that is provided by the microbiota (131). Upon colonization of the intestinal tract, *C. difficile* vegetative cells elicit the symptoms of disease through the secretion of two toxins, TcdA (an enterotoxin) and TcdB (a cytotoxin) and their eventual endocytosis by the colonic epithelial cells (163). *C. difficile*-infected patients are commonly treated with other antibiotics, such as vancomycin or fidaxomicin, which relieve the primary symptoms of CDI by targeting the actively-growing, toxin-producing, vegetative forms (8). However, and importantly, these antibiotics also disrupt the gut microbiome and may result in recurring episodes of disease(164).

Though the *C. difficile* vegetative cells are strictly anaerobic, they form metabolically dormant spores that are resistant to toxic chemicals, high temperature,

radiation and oxygen (34, 135, 165). As a result of this dormancy, the spores can survive outside the host in the aerobic environmental setting where the strictly anaerobic, vegetative form cannot. Structurally, endospores are composed of several, well-defined layers (166). The endospore core contains DNA, RNA, ribosomes and a large amount of dipicolinic acid (pyridine-2-, 6-dicarboxylic acid; DPA), chelated with calcium (CaDPA) that provides heat resistance (34, 167). The core is surrounded by an inner spore membrane composed of phospholipids, followed by a layer of germ-cell wall (which later becomes the cell wall of the vegetative cell during outgrowth). A thick layer of specialized peptidoglycan, cortex, surrounds the germ-cell wall and protects the spore core against osmolysis. The cortex is surrounded by the outer membrane derived from the mother cell, coat proteins and an exosporium layer (34, 135, 166, 168). When the environmental conditions become favorable, spores germinate and outgrow to the vegetative form (135, 169, 170).

C. difficile spore germination is initiated in response to the combinatorial actions of certain bile acids [*e.g.*, taurocholic acid (TA)] and amino acids (*e.g.*, glycine) (144, 171-174). In prior work, the bile acid germinant receptor was identified as the germination-specific, subtilisin-like, pseudoprotease, CspC (61). More recently, we found that a small mutation in the *cspA* coding region of *cspBA* resulted in spores that germinate in response to TA alone (without the requirement for an amino acid), suggesting that the CspA protein is the amino acid germinant receptor (Shrestha and Sorg, submitted (175)).

In several endospore-forming bacteria, the abundance of the spore germinant receptors is significantly influenced by the medium used to generate the spores (176). *C.*

difficile spores are generally prepared in rich media at an optimal temperature and cell density for higher yields (51). A study by Hornstra *et al.*, demonstrated that in *Bacillus cereus* ATCC 14579, the composition of the sporulation medium increased the transcription of the seven germinant receptors, and the nutrient-induced spore germination was significantly affected (177). Similar results were also observed in other spore-forming bacteria (*e.g., B. subtilis*) (176), however, no studies have been performed in *C. difficile* on how the medium used to generate spores influences the properties of spore germination; although there are studies that show that high spore yield can be obtained when *C. difficile* spores are produced in peptone rich medium, such as 70:30 or SMC (51, 72, 178-181). Here, we report that media composition influences the abundance of the *C. difficile* spore germinant receptors and find that spores prepared on peptone-rich medium can germinate in response to TA and terbium ions (which likely function as a calcium substitute). Because Tb^{3+} ions are often used to detect the presence of the dipicolinic acid that is released during the early events of spore germination (144, 174, 182-187), these findings could be useful for future analysis of *C. difficile* spore germination or for the germination of other bacteria that are influenced by calcium ions.

5.2 Materials and methods

5.2.1 Growth conditions

C. difficile UK1 (ribotype 027) (61, 188, 189) and M68 (ribotype 078) (90, 144, 189) strains were grown on either BHIS agar medium [Brain heart infusion (BHI) supplemented with 5 g / L yeast extract] or 70:30 agar medium [63 g / L Bacto peptone,

3.5 g / L protease peptone, 11.1 g / L BHI, 1.5 g/ L yeast extract, 1.06 g / L tris base and 0.7 g / L ammonium sulfate (NH_4SO_4)] or SMC agar medium (90 g / L Bacto peptone, 5 g / L protease peptone, 1 g / L NH_4SO_4 and 1.5 g / L tris base) or TYG agar medium [(30 g / L Bacto typtone, 20 g / L yeast extract and 10 g / L glucose)] in an anaerobic environment (85% N_2, 10% H_2, and 5% CO_2) (Model B, Coy Laboratories, Grass Lake, MI) at 37 °C. The BHIS, 70:30 and SMC agar media were supplemented with 1 g / L of L-cysteine while TYG agar medium was supplemented with 1 g / L thioglycolate.

5.2.2 Spore purification

C. difficile spores were purified as described previously (144, 174). Briefly, the *C. difficile* UK1 and *C. difficile* M68 strains were grown on either BHIS, 70:30, SMC or TYG agar medium as described above and allowed to grow for 4 days. Cells from each plate were scraped into 1 mL sterile water and incubated at 4 °C overnight. Next, the cells were washed five times with water to remove cell debris and combined into 2 mL total volume. The washed spores were layered on top of 8 mL of 50% sucrose and centrifuged at 4,000 X g for 20 minutes. The spore pellets were separated from the supernatant, and the spores were washed five times with water to remove any sucrose and incubated at 4 °C until use.

5.2.3 Germination of spores isolated from different media

The spores were characterized by measuring DPA release as well as changes to the optical density (OD_{600}) during germination, as described previously (144, 184). Briefly, spores purified from various media were heat activated at 65 °C for 30 minutes and suspended in water at an OD_{600} = 50. In order to measure DPA release, the spores were added to final OD_{600} of 0.25 in 100 µL final volume of buffer (50 mM HEPES, 250 mM

NaCl, pH 7.5) supplemented with 250 µM Tb^{3+} and containing either 10 mM TA or 30 mM glycine or both 10 mM TA and 30 mM glycine in a 96 well black plate. The DPA release was then measured for 2 hours at 37 °C using a SpectraMax M3 (Molecular Devices) plate reader with excitation at 270 nM and emission at 545 nm with a 420 nm cutoff.

In order to measure the germination by optical density, the spores were added to a final OD_{600} of 0.5 in HEPES buffer supplemented with 30 mM glycine alone or 10 mM TA alone or 10 mM TA and 30 mM glycine in 100 µL final volume with or without 250 µM Tb^{3+} in 96 well clear plates. OD_{600} was monitored at 37 °C for 2 hrs. using the plate reader.

5.2.4 Western blot

Samples for SleC activation were prepared by treating UK1 and M68 spores with either 10 mM TA or 30 mM glycine or both 10 mM TA and 30 mM glycine with or without 250 µM Tb^{3+} and incubated at 37 °C for 2 hours. Soluble protein samples were extracted by boiling the spores in NuPage buffer (Life Technologies) at 95 °C for 20 minutes and centrifuged at 20,000 x g for 10 minutes to separate supernatant from the spore pellet. Solubilized proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane for SleC detection by western blot.

In order to quantify amounts of SleC, CspB, and CspC proteins in spores prepared from different media, solubilized proteins were extracted by boiling 2 x 10^9 spores per mL in NuPAGE (Life Technologies) sample buffer at 95 °C for 20 minutes. Equal volume of spore extracts and recombinant CspB, CspC or SleC standard proteins were separated by

SDS-PAGE. Proteins were then transferred onto low-fluorescence polyvinylidene difluoride membrane (PVDF) at 30V for 16 hours. The membrane was then blocked in 10% skimmed milk in TBS (Tris-buffered saline) and washed thrice with TBS containing 0.1% (vol / vol) Tween-20 (TSBT) for 20 minutes each at room temperature. The membranes were then incubated with anti-CspB, anti-CspC or anti-SleC antibodies for 2 hours and washed thrice with TSBT. For the secondary antibody, AlexaFlour 555-labeled donkey anti-rabbit antibody was used to label the membranes for 2 hours in the dark. The membranes were washed again thrice with TBST in the dark, and scanned with a GE Typhoon Scanner using Cy3 setting, an appropriate wavelength for the Alexa Flour 555 fluorophore. The fluorescent bands were analyzed using ImageQuant TL 7.0 image analysis software. Intensity of the extracted protein in each blot was compared to the standard curve that was included on each blot.

5.2.5 Calcium measurement

Calcium concentrations in different media were measured using a Ca^{2+} detection assay kit (Abcam, ab102505). Standards for Ca^{2+} were prepared as advised in the kit. The Ca^{2+} concentration was then measured from BHI, BHIS, 70:30, SMC and TYG medium at OD_{575} in the plate reader. The OD_{575} values for the Ca^{2+} measurement were then converted into µg / L. For the control, 23 mg / L $CaCl_2$ suspended in water was used.

5.2.6 Statistical analysis

All germination assays were performed in triplicate and data points represent the average of three independent experiments. Calcium concentrations were measured in triplicate. Error bars represent the standard error of the mean. A one-way ANOVA with

Tukey's multiple comparisons test was used to compare the quantified protein amounts. Each blot was loaded with five standard proteins and three spore samples for quantification of proteins.

5.3 Results

5.3.1 Tb^{3+} enhances the germination of C. difficile spores with TA

Recent work by Kochan *et al.*, on germination by *C. difficile* spores has shown that calcium plays a role as an enhancer of germination when added with TA (179). In several studies, terbium has been used to replace calcium, mostly to observe the calcium binding because terbium fluoresces at certain wavelengths and calcium does not (190, 191). One of the ways germination is observed is through the measurement of the DPA that is released from the spore core. Upon recognition of germinants by *C. difficile* spores, the cortex layer is degraded by the cortex lytic enzyme (SleC) and, subsequently, DPA is released from the core (192). In the presence of terbium ions, the released DPA can associate with terbium and the terbium-DPA complex can be detected in a FRET-like assay.

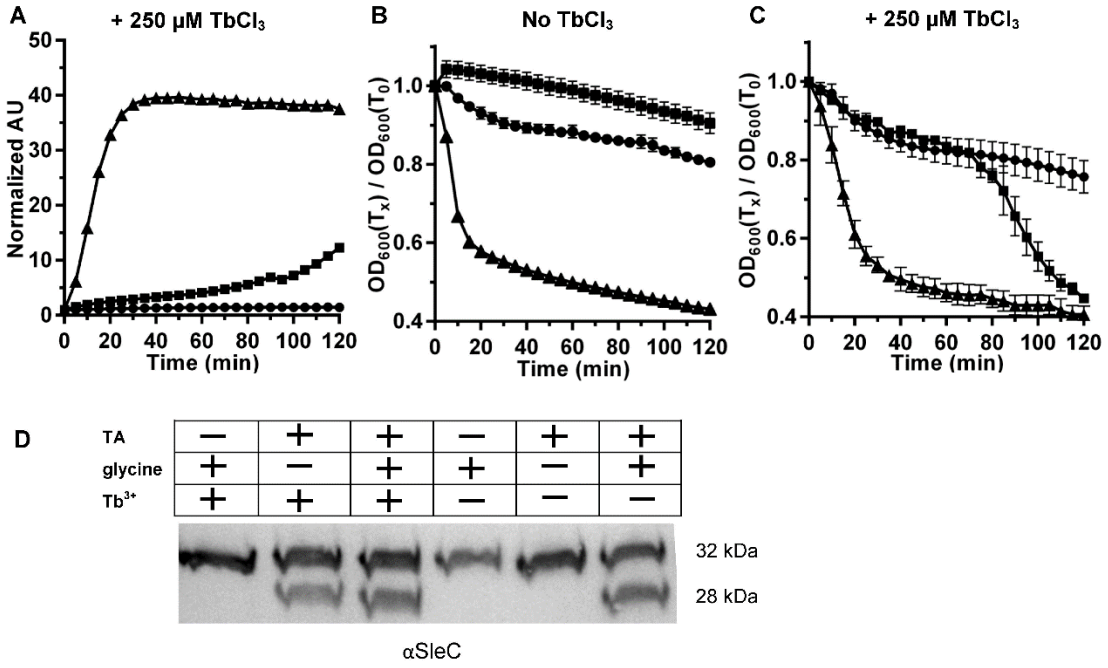

Figure 18. Comparison of germination by *C. difficile* UK1 spores in presence/absence of terbium. *C. difficile* UK1 spores were prepared from BHIS medium as described in the materials and methods. (A) CaDPA release from the purified spores was analyzed by suspending the spores in buffer supplemented with 250 µM $TbCl_3$ and (●) 30 mM glycine, (■) 10 mM TA or (▲) 10 mM TA and 30 mM glycine. The extent of germination was also analyzed by determining OD_{600} over time. Germination by *C. difficile* spores was analyzed in the absence of $TbCl_3$ (B) or in the presence of 250 µM $TbCl_3$ (C). Data points represent the averages from three independent experiments and error bars represent the standard error of the mean. (D) Equal numbers of spores were incubated with or without 250 µM $TbCl_3$ in 30 mM glycine or 10 mM TA or 10 mM TA and 30 mM glycine for 2 hours and soluble proteins were extracted with NuPAGE buffer and separated by SDS-PAGE followed by immunoblotting with SleC- specific antisera.

During some of my experiments analyzing the requirements of *C. difficile* spore germination, we found that, at times, wild type *C. difficile* spores would seemingly release DPA in response to TA alone. To investigate this phenomenon further, we measured DPA released from spores that were prepared on BHIS medium. We found that these spores

started to release DPA in response to TA alone after 1 hr 30 min incubation in these conditions (Figure 18A). However, when germination was measured by OD at 600 nm, in absence of terbium, the OD did not change with TA alone (Figure 18B). Surprisingly, when the germination was measured by OD in presence of terbium, the OD changed in response to TA alone (Figure 18C) – the germination rate was much faster when glycine was added as a co-germinant. To confirm that our observations were due to the initiation of spore germination and not just an artifact of the assay conditions, we tested the activation of SleC after two hours of incubation in the indicated germination condition. We found that in presence of terbium, SleC is activated when the spores were treated with TA alone, similar to the activation of SleC in response to both TA and glycine (Figure 18D). These results suggest that terbium might be involved in germination of the spores, potentially similar to the response of *C. difficile* spores to calcium.

5.3.2 The TA-terbium response increased when spores were prepared in peptone rich medium

Many *C. difficile* strains do not efficiently form spores in liquid BHIS medium. Therefore, most studies produce spores on agar medium (*e.g.*, BHIS, 70:30, or SMC) (51).To investigate if our observation that spores germinate in response to TA and Tb^{3+} was due to how spores were prepared (*i.e.*, BHIS agar medium, on which our laboratory normally produces spores), we prepared spores on BHIS, 70:30, SMC or TYG agar medium and tested how Tb^{3+} influences germination of these spores. Interestingly, we found that the TA-terbium phenotype increased when spores were prepared in the peptone rich media, when compared to BHIS agar medium. In figure 19A, 19D, and 19G we

compared DPA release of wildtype *C. difficile* UK1 spores prepared from 70:30, TYG or SMC agar medium, respectively. We found that the influence of Tb^{3+} on *C. difficile* spore germination was greater in these media compared to BHIS-prepared spores (Figure 18A). Similar to what we observed for BHIS-prepared spores, none of these spores germinated, as measured by changes in OD_{600}, in the absence of Tb^{3+} (Figure, 19B, 19E, and 19H). However, when terbium was added with TA, the OD of the spore suspension dropped in response to TA and Tb^{3+} (Figure 19C, 19F and 19I). We also confirmed that the activation of SleC occurred in presence of TA and Tb^{3+} (Figure S11). These results strongly suggest that the medium used to prepare spores plays a role in the TA-terbium phenotype; the spores become more responsive to terbium when produced on a more peptone rich media (*e.g.*, 70:30, SMC and TYG).

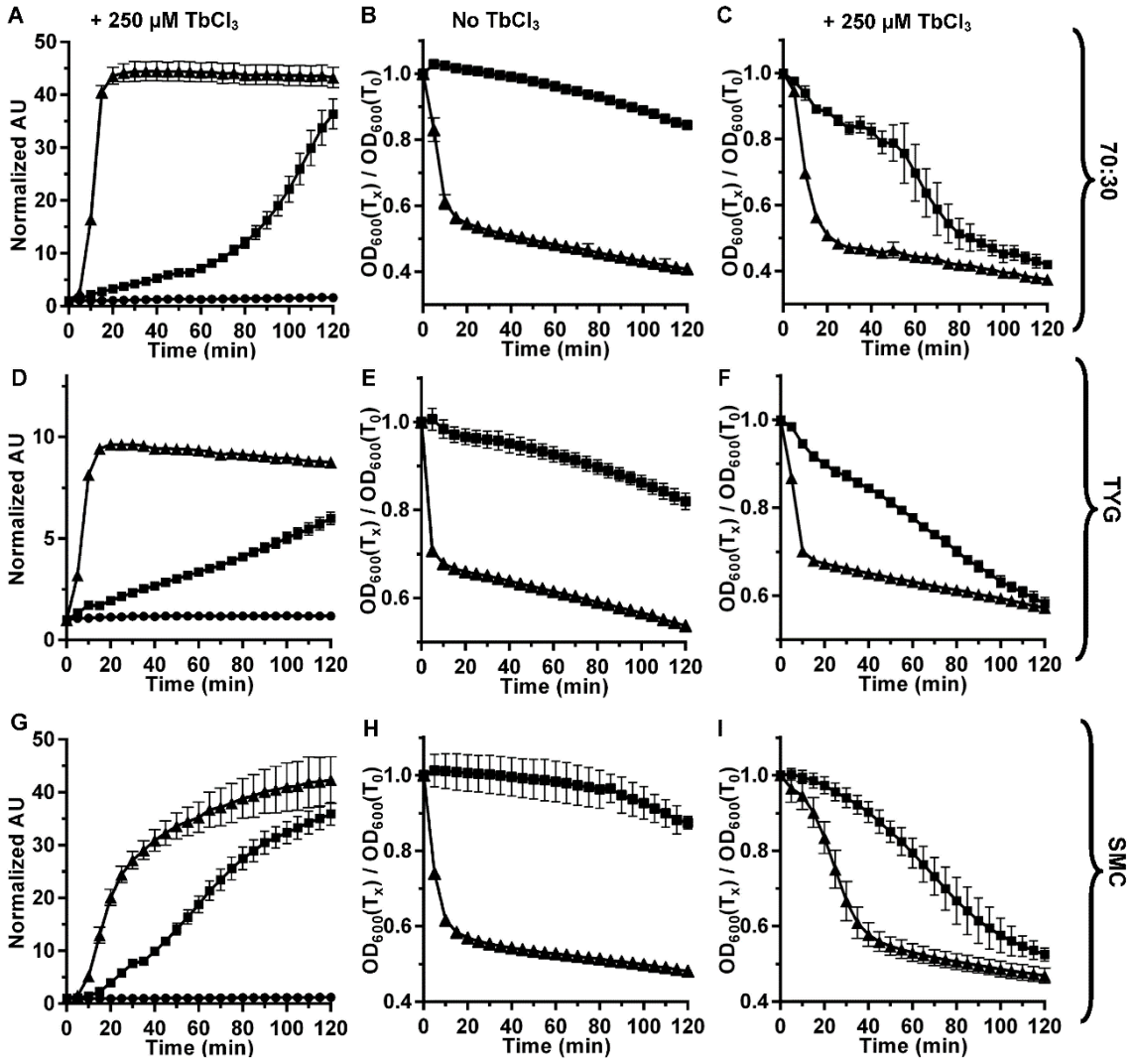

Figure 19. Comparison of germination by *C. difficile* UK1 spores prepared in different media. The germination phenotype of wild type *C. difficile* UK1 spores prepared from (A, B, C) 70:30 medium, (D, E, F) TYG medium or (G, H, I) SMC medium were analyzed as described in Figure 18 (●) 30 mM glycine, (■) 10 mM TA or (▲) 10 mM TA and 30 mM glycine. Data points represent the averages from three, independent experiments and error bars represent the standard error of the mean.

5.3.3 TA-terbium phenotype is found in a different C. difficile ribotype

Next, we wanted to test if the TA-terbium phenotype is only true for the *C. difficile* UK1 strain or if it is observed in another ribotype. In our prior work on *C. difficile* spore germination, we used the *C. difficile* M68 strain (ribotype 078) to test the impact of muricholic acids and the effect of different amino acids on *C. difficile* spore germination (90, 144). Thus, we used this strain to investigate the impact of Tb^{3+} on germination by *C. difficile* spores. As for *C. difficile* UK1, we prepared *C. difficile* M68 spores in BHIS, 70:30 or SMC agar medium (M68 strains did not form spores on TYG medium for unknown reasons) and measured DPA release (Figure 20A, 20D and 20G). The M68 strain also germinated in response to TA and Tb^{3+} and, similar to *C. difficile* UK1 spores, this phenotype was stronger for spores prepared from 70:30 and SMC compared to spores prepared from BHIS agar medium. Similarly, when we tested germination by OD, in the absence of terbium, spores did not germinate in response to TA alone (Figure 20B, 20E and 20H). However, the addition of Tb^{3+} to the germination solution resulted in germination (Figure 20F and 20I); BHIS prepared spores (Figure 20C) had a much milder response to $TbCl_3$ addition than did 70:30- or SMC-prepared spores (Figure 20F and 20I). These observations were also confirmed, as for *C. difficile* UK1, by analyzing SleC activation (Figure S12).

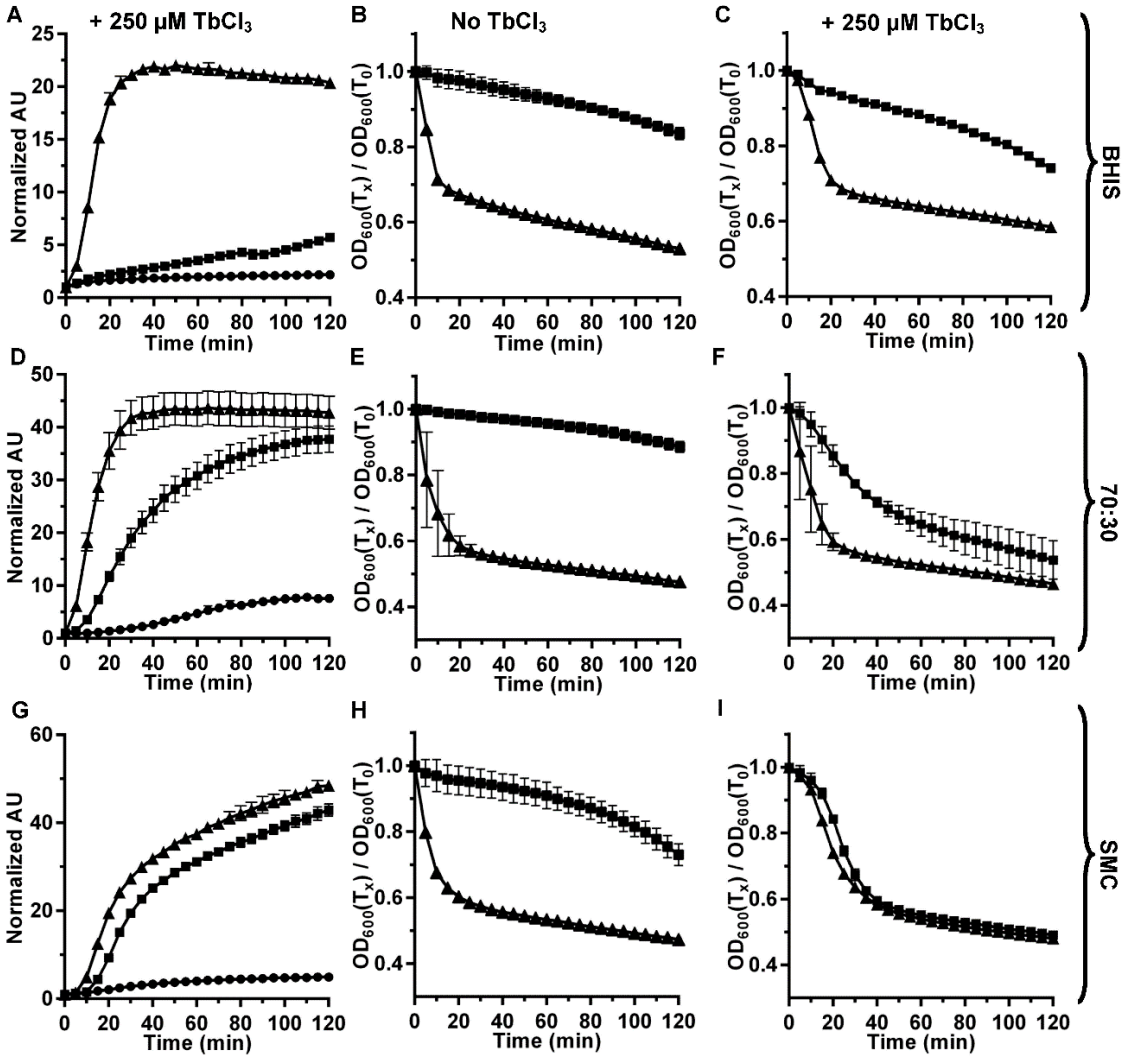

Figure 20. Comparison of germination by *C. difficile* M68 spores prepared in different media. The germination phenotype of wild type *C. difficile* M68 spores prepared from (A, B, C) BHIS medium, (D, E, F) 70:30 medium and (G, H, I) SMC medium were analyzed as described in Figure 18 (●) 30 mM glycine, (■) 10 mM TA or (▲) 10 mM TA and 30 mM glycine. Data points represent the averages from three, independent experiments and error bars represent the standard error of the mean.

5.3.4 Medium composition influences the abundance of C. difficile spore germinant receptors but does not explain TA-Tb^{3+} phenotype

In *B. subtilis*, it was shown that spores prepared on different media resulted in changes to the amount of Ger proteins found within the spores (176). We hypothesized that spores prepared in peptone rich media may have increased amounts of the germinosome proteins (*i.e.*, CspB, CspC or SleC) which could influence the sensitivity of spores to terbium. To test this hypothesis, we quantified the amount of CspB, CspC and SleC proteins purified from the different media mentioned above using a previously described technique (due to the quality of our CspA antibody, we could not quantify the abundance of CspA in the spores). We found that there was not a significant increase in SleC levels for *C. difficile* UK1 or *C. difficile* M68 spores that were purified from different media (Figure 21A and 21B, respectively). We observed an increase in CspB protein purified from 70:30, TYG or SMC media compared to BHIS-prepared medium for spores derived from the *C. difficile* UK1 strain (Figure 21C). However, for *C. difficile* M68, spores prepared from BHIS contained a larger amount of CspB than did spores prepared on 70:30 or SMC media (Figure 21D). We also observed small differences in abundance of CspC in both the strains purified from different media (Figure 21E, 21F). But, because CspC abundance in *C. difficile* UK1 SMC-prepared spores was lower than in TYG and *C. difficile* M68 SMC-prepared spores was more abundant the abundance of CspC is not likely to contribute to the observed phenotypes. These results suggest that producing *C. difficile* spores on different media can influence the abundance of some spore proteins. However, we did not

observe a correlation between medium and the Tb^{3+} phenotype observed in Figures 18, 19 and 20.

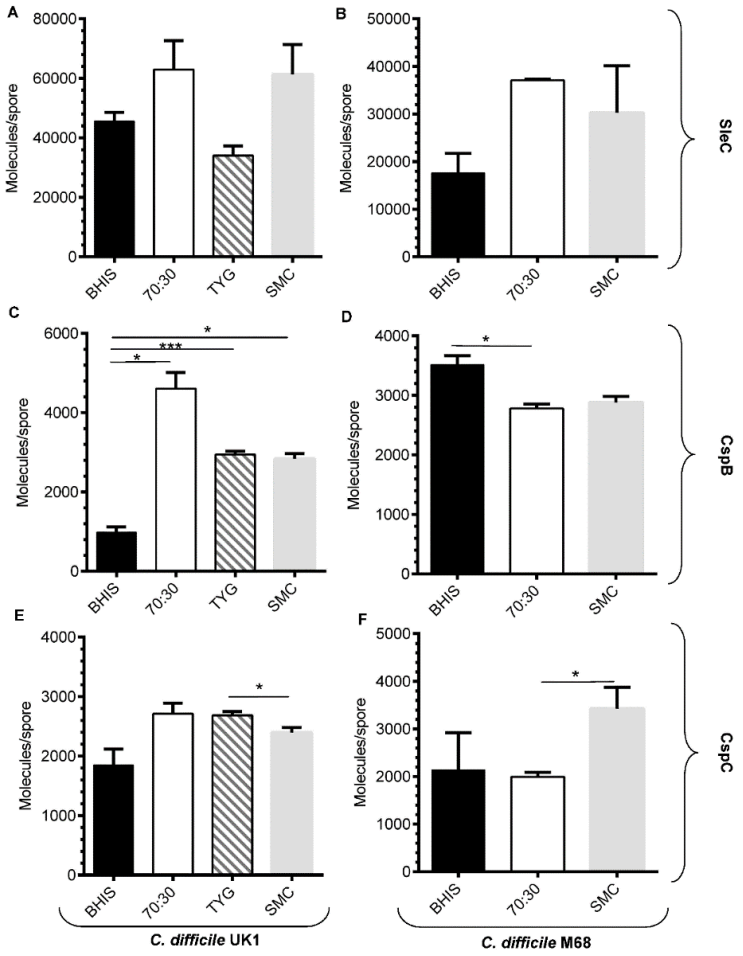

Figure 21. Quantifying the abundance of CspB, CspC and SleC in *C. difficile* spores. 2 x 10^9 spores purified from *C. difficile* UK1 strain (A, C, E) or the *C. difficile* M68 strain (B, D, F) that were generated in BHIS medium, 70:30 medium, TYG medium or SMC medium. The proteins were extracted with NuPAGE buffer and samples were separated by SDS-PAGE, transferred to low fluorescence PVDF membranes and blotted with antisera specific to the indicated proteins. Quantified proteins are expressed as molecules / spore and are represented in a bar graph form (A, B) SleC, (C, D) CspB and (E, F) CspC. The data presented represent the averages from three independent experiments and error bars represent the standard error of the mean. Statistical significance was determined using a one-way ANOVA with Tukey's multiple comparisons test (* $p < 0.05$; *** $p < 0.001$).

5.3.5 The calcium concentrations are similar in different media

In several studies, terbium has been used to substitute calcium. Previous studies by Kochan *et al.* have also shown that calcium is involved in enhancing germination by *C. difficile* spores in the presence of germinants (TA and glycine) (178, 179). We hypothesized that TA-terbium is functioning similar to TA-calcium and that a difference in the calcium concentration in the media used to prepare spores might contribute to spores being sensitive to calcium or terbium in these germination assays. In order to test this hypothesis, we used a colorimetric assay to detect calcium concentration in various media. However, we observed no significant difference in the calcium concentration in BHIS, 70:30, SMC or TYG media (Figure 22). These results suggest that an unidentified factor contributes to the sensitivity to terbium during *C. difficile* spore germination and that to detect the release of DPA from germination of *C. difficile* spores, the concentration of terbium should be optimized so as not to influence germination during the assay conditions.

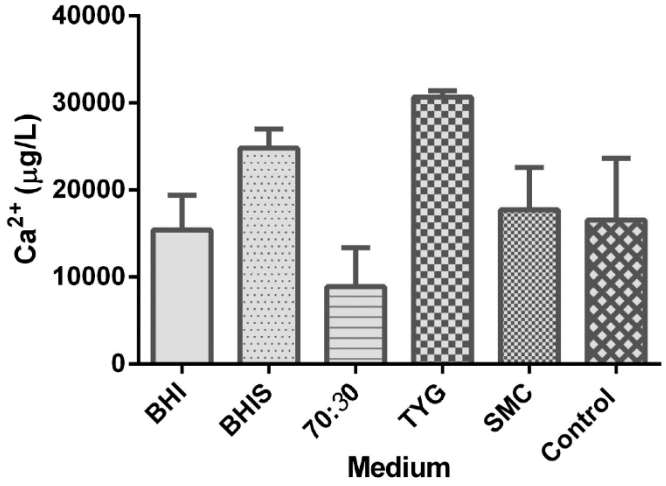

Figure 22. Detection of calcium concentration in different media types. Bar graph representing the calcium concentration in µg / L from BHI, BHIS, 70:30, TYG and SMC media. 23 mg/mL of calcium chloride dissolved in water is used as a control.

5.4 Discussion

Growth media plays a significant role in formation of spores (51, 176, 177). A common medium used for the growth of *C. difficile* vegetative cells is BHIS agar medium; however, *C. difficile* spores are frequently produced in / on 70:30 or SMC medium because of the improved spore yield in these media (51, 72, 178-181). Importantly, the different composition of sporulation media can impact the germination phenotype of the spores. For example, Ramirez-Peralta *et al.*, showed for *B. subtilis* spores the medium composition had a significant effect on the rate of spore germination (176). The rate of germination of *B. subtilis* spores prepared from nutrient-rich, liquid medium was significantly higher compared to nutrient-poor, liquid medium. Similarly, we found that germination of spores prepared under different sporulation conditions (media) resulted in significant differences in germination. Specifically, when we prepared spores from the

BHIS medium, at the end of 2 hours germination we observed slight increase in DPA release in presence of TA alone (Figure 18A); the release of DPA corresponds to germination. Oddly, when the OD assay was used to measure the germination, there was no germination in TA alone (Figure 1B). The difference between the assays is the addition of terbium to monitor DPA release. When terbium was added in the OD assay, we observed that, similar to DPA increase in TA alone, germination was observed with OD within 2 hours suggesting that it was terbium, along with TA, that induced the germination of the spores. Although *C. difficile* is known to germinate in the presence of bile acids (*e.g.*, TA) and amino acids (*e.g.,* glycine), calcium has been identified as an important contributor to spore germination, though its role remains undefined (134, 178, 179). It is not clear whether calcium functions as a bona fide spore co-germinant, a co-factor for a process essential for spore germination and / or as an enhancer for amino acids. But, removal of calcium by chelation prevents spore germination, indicating that calcium is essential for *C. difficile* spore germination, and addition of calcium increases the sensitivity of spores to germinants (179).

We found that the TA-terbium germination phenotype increased when spores were prepared from peptone rich medium, such as 70:30, SMC or TYG, and compared to BHIS-prepared spores. As shown in Figure 19, when spores were produced from 70:30, TYG or SMC medium, the spores germinated in response to TA-terbium within 1 hour. The TA-terbium phenotype was stronger in spores prepared from SMC medium followed by TYG, 70:30 then BHIS medium. These results were also true for the *C. difficile* M68 strain (Figure 20). We found that in wildtype *C. difficile* UK1 spores, CspB abundance was

significantly increased in SMC, 70:30 or TYG medium compared to BHIS medium, but this was not true for *C. difficile* M68 spores. Unfortunately, we could not analyze the abundance of CspA in this study. It is possible that CspA abundance correlates with the observed phenotype, but we cannot test this directly.

We hypothesized that the calcium concentration in BHIS medium was greater, thus reducing the effect of Tb^{3+} on germination (*i.e.*, the spore would already be saturated with Ca^{2+} and be less influenced by Tb^{3+}). However, there were no significant differences in the calcium concentration in different media (Figure 22). Although the mechanism for the increase in terbium sensitivity in spores prepared in different media is not known, there could be various other media components that might relate to the terbium sensitivity. However, we feel that it is important to report the impact of Tb^{3+} on *C. difficile* spore germination because analysis of DPA release is a common screening technique in spore germination studies. An increase in sensitivity to terbium may be confused with a *bona fide* germination phenotype and it is important to confirm the germination phenotype with different assays, such as the OD assay or SleC activation during germination. Finally, we recommend that the Tb^{3+} concentration used for each *C. difficile* strain be titered accordingly so as not to influence the germination process.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Although, germination of *Clostridium difficile* spores is a critical step in *C. difficile* pathogenesis, the mechanism by which *C. difficile* spores germinate is not yet fully understood. The *C. difficile* spore germination pathway differs from that of other well-studied model organisms, such as *B. subtilis* or *C. perfingens*. Even though the underlying biology of a novel prosess warrants investigation, it is important for us to understand the *C. difficile* spore germination pathway in order to develop new theraputics that target the germination related proteins. These future anti - *C. difficile* compounds would not target other members of the colonic microbiota, and would ensure prevention of recurrence, which is a common problem with current CDI treatment options. Below, I will summarize my findings on the mechanisms of *C. difficile* spore germination in response to amino acids, along with the effect of the spore specific alanine racemase on germination, and propose potential future avenues for study.

6.1 Role of alanine racemase in *C. difficile* spore germination

Prior work by Fimlaid *et. al.*, showed that the Alr2 alanine racemase is one of the most highly expressed genes during sporulation (71). However a mutation in *alr2* does not affect *C. difficile* spore germination when measured in rich medium (71). To further investigate the role of the Alr2 in germination, I engineered an *alr2* mutation (TargeTron insertion mutation) and tested the germination phenotype in buffer supplemented with a fixed concentration of taurocholic acid (TA) and varying concentrations of either L-alanine or D-alanine . Interestingly, I found that the *alr2* mutation affected *C. difficile*

spore germination differently in response to L-alanine versus D-alanine. The results, and a model to explain these reults, are shown in Figure 23. In the spores lacking Alr2, L-alanine was more efficient germinant than D-alanine. The EC_{50} for L-alanine was 2.7 mM, while the EC_{50} for D-alanine was almost 10-fold higher at 24.2 mM. In the wildtype *C. difficile* spore, where Alr2 can interconvert, D-alanine and L-alanine, the EC_{50} for D-alanine decreased to 11.5 mM, and the EC_{50} for L-alanine was increased to 5.5 mM. These results suggest that the germination that was observed in response to D-alanine, was due to a response to both D-alanine and L-alanine (that was converted by Alr2). In addition, germination of WT spores in the presence of the L-alanine and TA, was effiecent (EC_{50} 5.5 mM) than the germination in response to D-alanine (Figure 23B). My results suggest that the germination of WT spores in response to L-alanine is due to both L-alanine as well as D-alanine. This is perhaps why there was no effect in germination in *alr2* mutants when observed in the rich medium (where all amino acid signals are available, especially glycine).

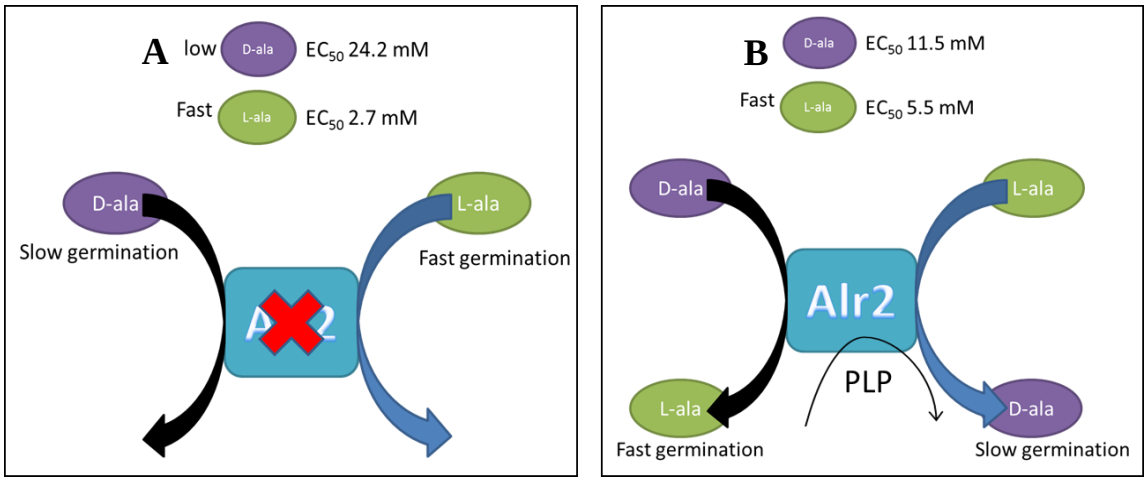

Figure 23. Proposed role of alanine racemase Alr2 during *C. difficile* spore germination. The Alr2 racemase is indicated by the blue square, the purple and blue ovals represent D-alanine and L-alanine respectively. The EC_{50} is the concentration of D-alanine or L-alanie where the rate of germination is 50 % the maximum rate. All germination assays were performed with 10 mM TA and were performed at 37 °C A) In *alr2* mutants, the alanine racemase does not convert L or D form of alanine, germination occurs only with in presence of D-alanine (Left) or L-alanine (Right). The EC_{50} values for the germination with both L-alanine and D-alanaine in absence of Alr2 is reported. B) In the WT, the alanine racemase (requires PLP as co-factor) is active, germination in presence of D-alanine also has L-alanine converted by Alr2 and vice-versa. The EC_{50} values in response to L-alanine and D-alanine in presence of active Alr2 is reported. The EC_{50} values are specific to UK1 strains, and might be different for other *C. difficile* strains.

6.2 Hierarchy of amino acid co-germinant recognition by *C. difficile* spores

While studying D-alanine as a co-germinant, I found that the temperature at which the germination assays were conducted played a significant role in the germination of *C. difficile* spores. At room temperature, D-alanine did not contribute to germination, but at 37 °C D-alanine was recognized as a co-germinant even though the efficiency of germination was lower compaired to that of L-alanine. This is a first time I showed that along with D-alanine, other D-forms of amino acids such D-serine and D-lysine, could

contribute to *C. difficile* spore germination when tested at 37 °C (but not at 25 °C). Based on the EC_{50} value calculated at 37 °C I ranked the soluble amino acids as co-germinants (Table 4 and Table 5). My results suggest that the putative amino acid germinant receptor is very promiscuous.

6.3 Updated model of *C. difficile* spore germination

When I made SRQS deletion mutation in *cspA* of *C. difficile*, I observed that the spores no longer required amino acids for germination and the spores germinated in response to TA alone. Based on my results, I hypothesized that CspA is the amino acid germinant receptor, and proposed a model for the role of amino acids during *C. difficile* spore germination (Figure 19). In my previous working model I proposed that CspC, CspB, CspA and proSleC were all forming a germinosome complex, and were localized in the spore cortex (Figure 3). In my current, updated model, I propose that only CspC, CspB and CspA but not proSleC are in a complex. In this complex, the protease activity of CspB is inhibited by CspA and CspC. In the presence of germinants, the bile acid germinant receptor, CspC, binds to TA and the amino acid receptor germinant, CspA binds to glycine and both CspC and CspA fall off the complex freeing the CspB protease. Free CspB is then able to convert proSleC to SleC which then degrades the cortex layer and leads to the release of DPA. Based on the EC_{50} values of different amino acid during spore germination, I hypothesize that CspA can recognize different amino acids with varying efficiencies. Inhibition of germination by chenodeoxychoic acid (CDCA) could be explained if the presence of CDCA prevents CspC from binding TA (62) and the presence amino acid alone cannot free CspB from the complex.

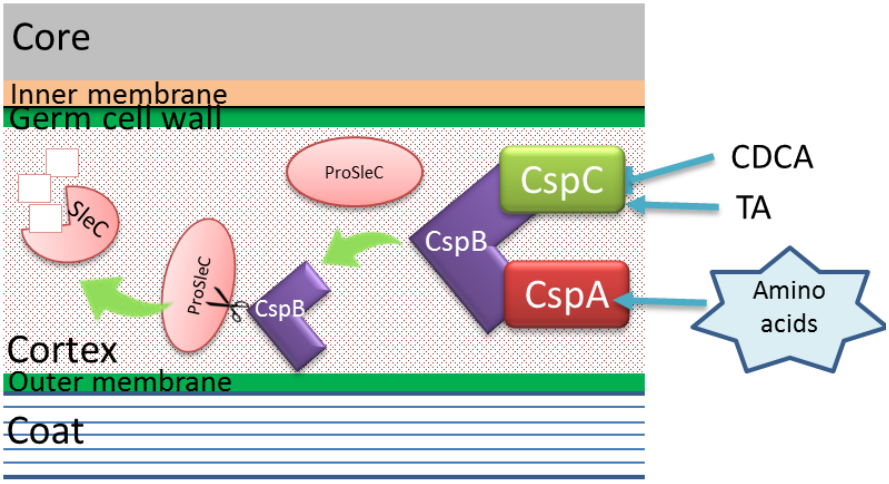

Figure 24. Updated model for *C. difficile* spore germination pathway. CspB activity is inhibited by CspC and CspA. Once CspC binds TA and CspA binds amino acids such as glycine, both proteins fall off the complex and CspB becomes free to cleave proSleC into active SleC. Activate SleC then degrades the cortex allowing spores to release DPA from the core in exchange for water molecules. The CspC protein is unable to leave the complex when CDCA is present and CspB remains inactive.

6.4 Future directions

Apart from the alanine racemase, other amino acid racemases present in the spore (*e.g.*, proline racemase) could also contribute to the germination. Whether the other amino acid racemases also play a role in *C. difficile* spore germination need to be determined.

One way to test the model shown in Figure 24, is by purified CspA, CspC, and CspB proteins in *E. coli* and determine if they form a complex in vitro by using functional assay looking for cleavage of proSleC. If we can sucessfully show that they form a complex, we can also test if the complex is altered by TA, glycine or both TA and glycine.

Another way to test my proposed model is to pull down the CspC and CspA proteins from the spore and identify co-precipitated protein(s).

I found that the processing of CspBA by YabG is critical for the activity of CspA in the complex. To test if the YabG protease directly processes CspBA and preproSleC, we could recombinantly express and purify YabG, full length CspBA, and preproSleC protein from *E.coli*. Then, we could mix CspBA or preproSleC with YabG. Under this condition, if we observe processing of CspBA or preproSleC, we would conclude that YabG directly processes CspBA into CspB and CspA and preproSleC into proSleC. If we do not observe the processing of CspBA or preproSleC then that would suggest that there might be another protein that is required in addition to YabG for the processing of these proteins.

If we find that YabG directly processes CspBA and preproSleC in the spore, we could then identify the YabG processing site in CspBA or preproSleC. For this experiment we would sequence the YabG-processed CspA or proSleC forms using Edman sequencing and observe if both of these proteins have similar target sequence. We could then use the identified target sequence(s) to find other protein sequences in the *C. difficile* spore that might be targeted by YabG.

Another way to identify target proteins of the YabG protease is to run both YabG mutant spore extract and WT spore extract and separate the proteins using SDS-PAGE. By identifying the differences in the two spore extracts, we can cut the gel fragments and identify the sequence using mass spectometry. The results might give us possible target proteins of YabG.

Finally, to show if caclium works similarly to glycine during germination or works through a separate pathway similar to what is seen in most *Bacillus* species, we can test if germination is affected in our *cspA* mutant spores. Since, calcium can also be affecting the activity of CspB or SleC in the spore, when we purify recombinant CspB and proSleC we can test the effect of calcium and see if it can bypass the requirement of both TA and glycine *in vitro* and CapB can cleave proSleC to SleC.

Identifying the amino acid receptor was very important for our understanding of the *C. difficile* spore germination pathway. Future work could develop new therapeutics that only target these germinant receptors (CspC and CspA) to prevent the *in vivo* germination of *C. difficile* spores while having little impact on the rest of the colonic microbiome.

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APPENDIX A SUPPLEMENTAL FIGURES

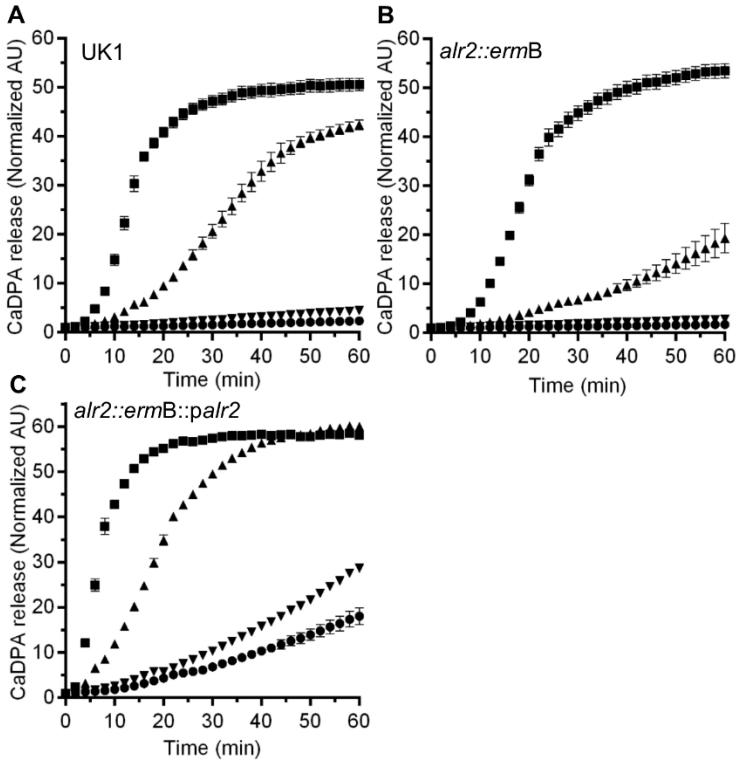

Figure S1. Germination by *C. difficile* spores in defined medium at 25 °C. Purified spores from (A) wildtype *C. difficile* UK1 or (B) *C. difficile* RS07 (*alr2::ermB*) or (C) *C. difficile* RS07 pRS89 (p*alr2*) were suspended germination buffer supplemented with TA alone (●), or supplemented with glycine (■) or L-alanine (▲) or D-alanine (▼). CaDPA release from the germinating spores was monitored at 25 °C as an increase in Tb^{3+} fluorescence over time. Data points represent the average from three independent experiments and error bars represent the standard deviation of the mean.

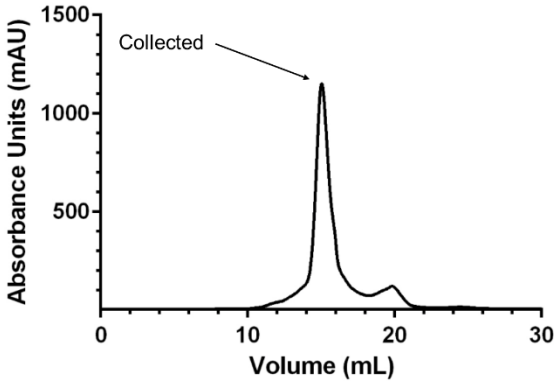

Figure S2. Purity of recombinantly expressed Alr2. Ni-affinity-purified Alr2 was concentrated and separated on a Sephedex G200 size exclusion column. The peak at ~15 minutes was collected and is Alr2.

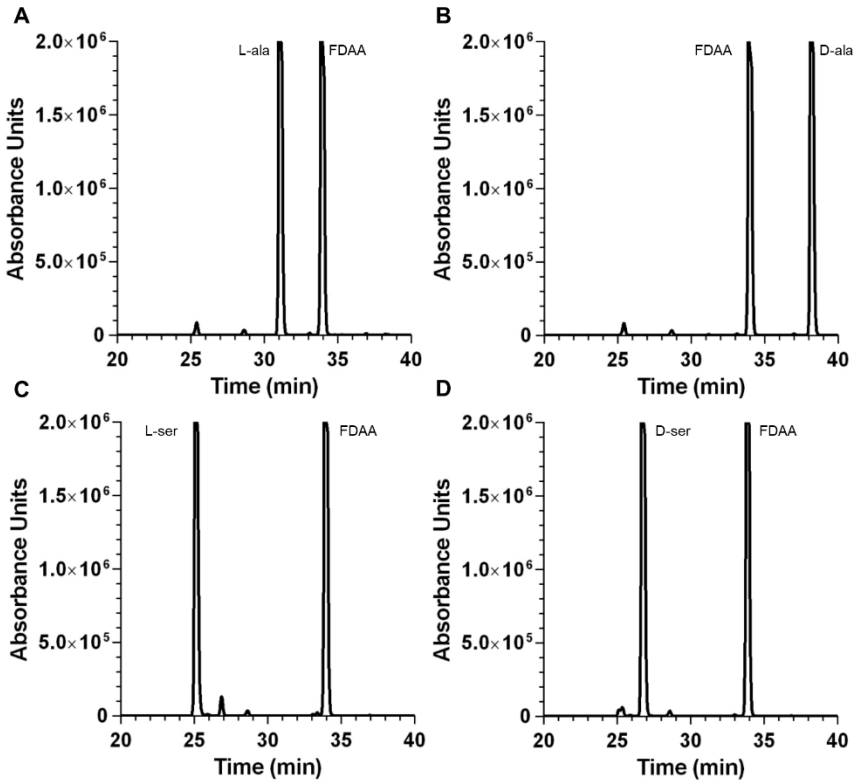

Figure S3. Purity of L / D alanine and serine used for germination, conversion and ITC. (A) L-alanine, (B) D-alanine, (C) L-serine, or (D) D-serine were labeled with FDAA and separated by HPLC. The amino acids used were >98 % pure and there is minimal detection of the opposite enantiomer in the labeling and HPLC analysis.

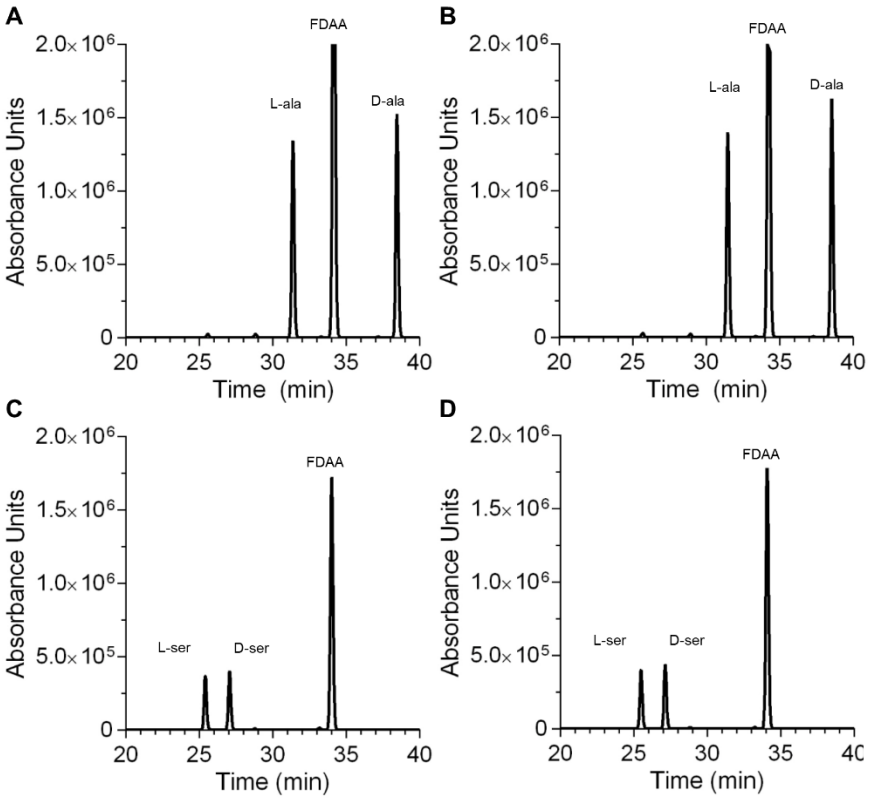

Figure S4. L / D conversion of alanine and serine during ITC. After completion of the ITC binding for (A) L-alanine or (B) D-alanine or (C) L-serine or (D) D-serine, the samples in the ITC cell were labeled with FDAA as described in the material and methods. The reacted samples were separated by HPLC. The HPLC retention times of each amino acid were determined using labeled amino acid standards.

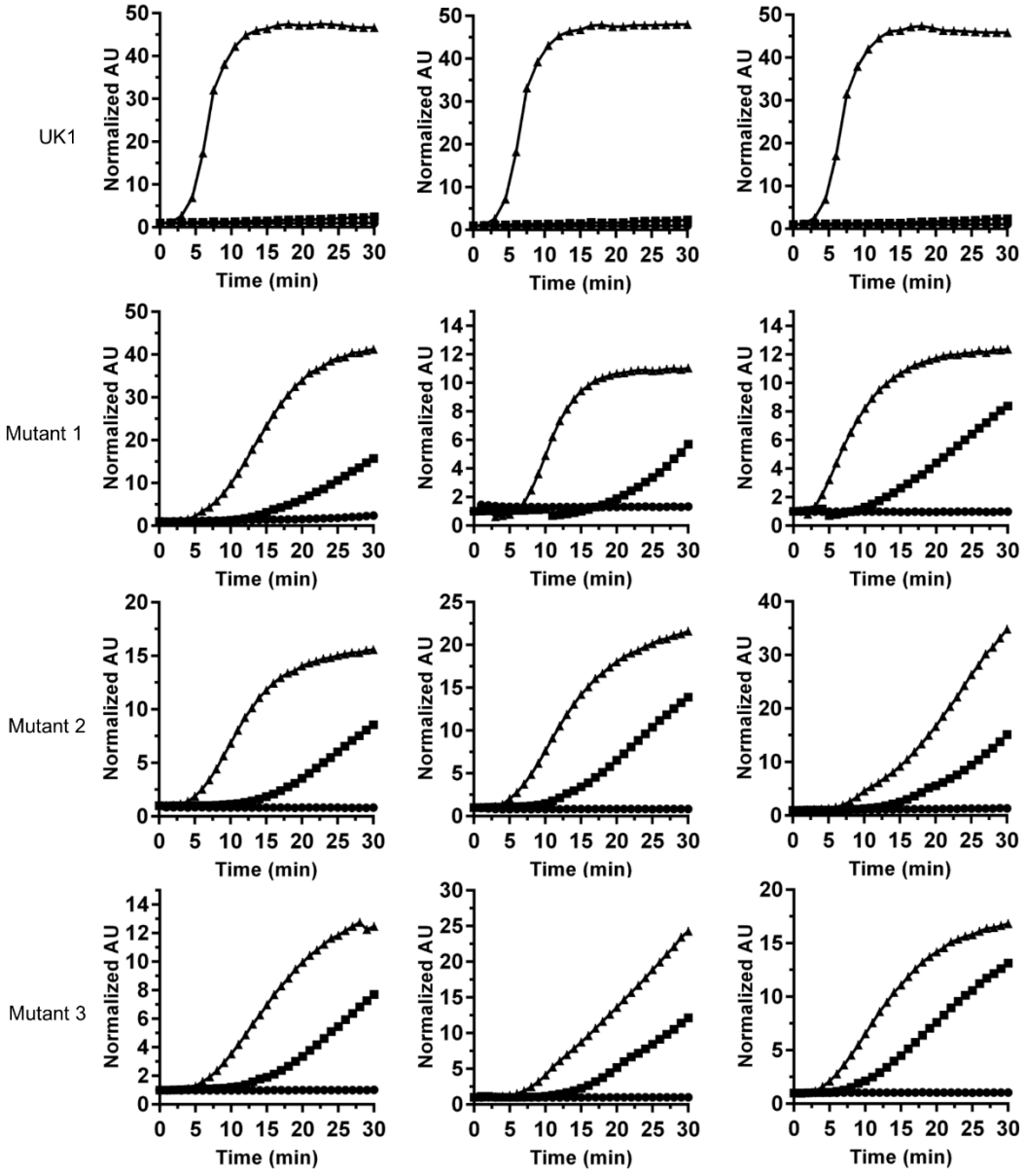

Figure S5. Full data set of the germination plots in Figure 10 The germination phenotype of wild type *C. difficile* UK1 and mutant spores were screened by measuring CaDPA release in presence of (●) 30 mM glycine, (■) 10 mM TA or (▲) 10 mM TA and 30 mM glycine.

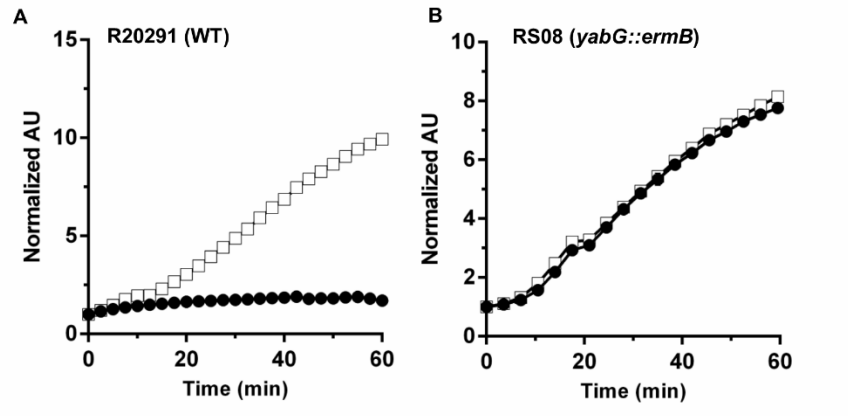

Figure S6. *C. difficile yabG::ermB* spores do not respond to L-alanine. Spores purified from (A) *C. difficile* R20291 or (B) *C. difficile* RS08 strains were suspended in buffer supplemented with 250 µM Tb^{3+} and (●) 10 mM TA or (□) 10 mM TA and 30 mM L-alanine and CaDPA release was monitored.

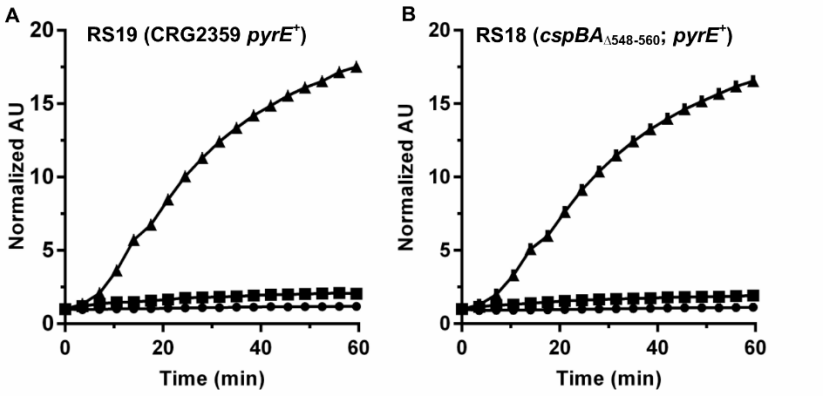

Figure S7. CaDPA release for *C. difficile* CRG2359 *pyrE*$^+$ (RS19) and *cspBA*$_{\Delta 548\text{-}560}$ (RS18). CaDPA release from spores purified from *C. difficile* CRG2359 with restored *pyrE* and RS18 (*cspBA*$_{\Delta 548\text{-}560}$; restored *pyrE*) strains was analyzed by suspending the spores in buffer supplemented with 250 µM Tb^{3+} and (●) 30 mM glycine or (■) 10 mM TA or (▲) 10 mM TA and 30 mM glycine.

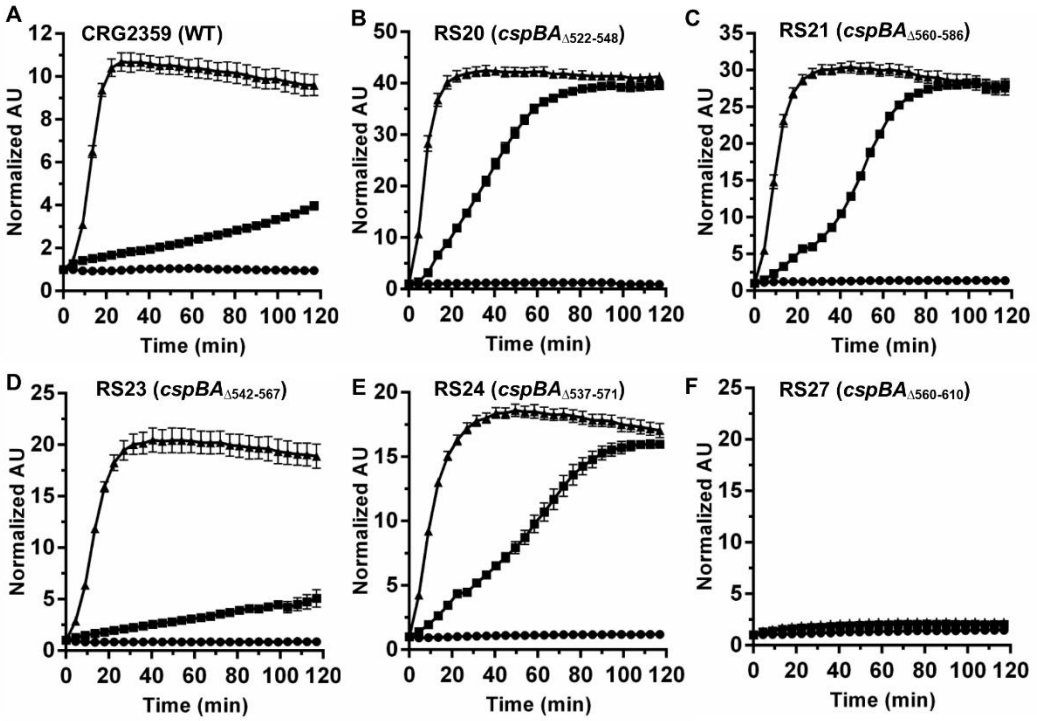

Figure S8. Monitoring CaDPA release from spores with defined deletions in *cspBA*. CaDPA release during spore germination of the indicated strain was monitored by suspending spores in buffer supplemented with Tb^{3+} and (●) 30 mM glycine or (■) 10 mM TA or (▲) 10 mM TA and 30 mM glycine. (A) CRG2359, (B) RS20 (*cspBA*$_{\Delta 522\text{-}548}$), (C) RS21 (*cspBA*$_{\Delta 560\text{-}586}$), (D) RS23 (*cspBA*$_{\Delta 542\text{-}567}$), (E) RS24 (*cspBA*$_{\Delta 537\text{-}571}$) and (F) RS27 (*cspBA*$_{\Delta 560\text{-}610}$). Data points represent the averages from three, independent experiments and error bars represent the standard error of the mean.

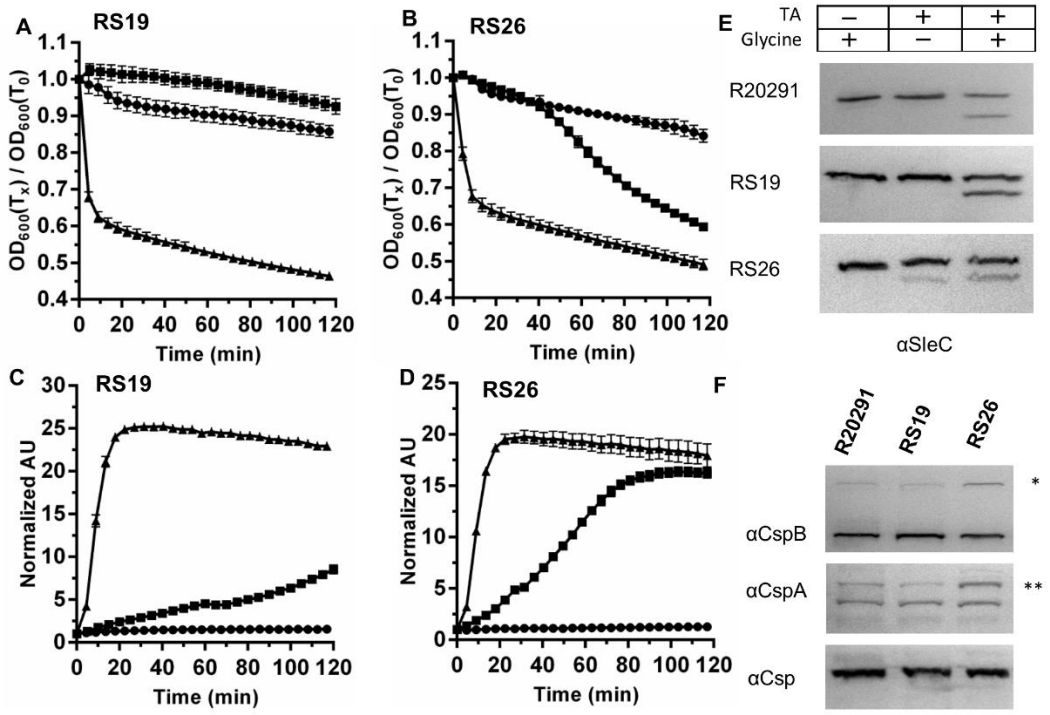

Figure S9. Comparing germination and western blots for spore purified from wild type and RS26. (A, B) Spores purified from *C. difficile* RS19 (CRG2359 with restored *pyrE*) and RS26 (*cspBA*$_{\Delta 560\text{-}586}$; restored *pyrE*) strains were suspended in buffer supplemented with (●) 30 mM glycine or (■) 10 mM TA or (▲) 10 mM TA and 30 mM glycine and OD_{600} was monitored over time. (C, D) CaDPA release from the indicated strains was determined as in A & B with buffer also supplemented with 250 µM Tb^{3+}. (E) The activation of SleC from spores purified from R20291, RS19 and RS26 in presence of buffered 30 mM glycine or 10 mM TA or both 10 mM TA and 30 mM glycine was determined after incubation for 2 hours at 37 °C. (F) Equal numbers of spores derived from R20291, RS19 or RS26 were extracted, separated by SDS-PAGE and transferred to PVDF membranes for immunoblotting of CspB, CspA and CspC (* full length CspBA form, ** alternatively processed CspA).

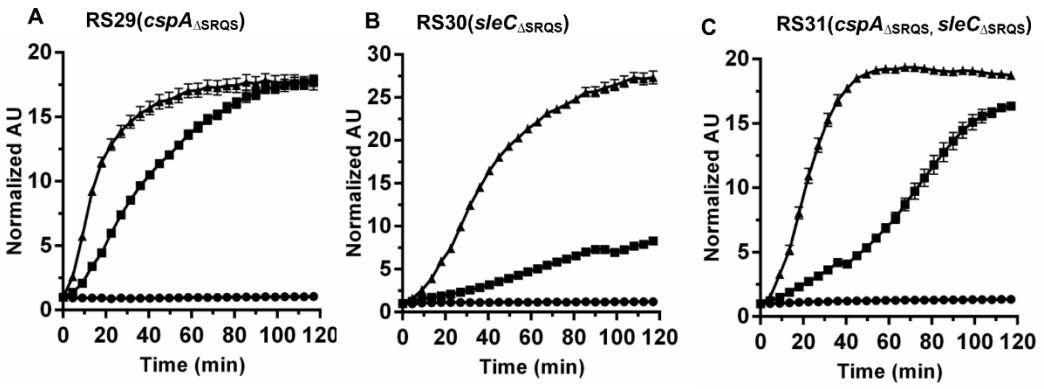

Figure S10. CaDPA release for SRQS deletion mutants. CaDPA release during spore germination of the indicated strain was monitored by suspending spores in buffer supplemented with Tb^{3+} and (●) 30 mM glycine or (■) 10 mM TA or (▲) 10 mM TA and 30 mM glycine. (A) RS29 (*cspA*$_{\Delta SRQS}$), (B) RS30 (*sleC*$_{\Delta SRQS}$), (C) RS31 (*cspA*$_{\Delta SRQS}$; *sleC*$_{\Delta SRQS}$). Data points represent the averages from three, independent experiments and error bars represent the standard error of the mean.

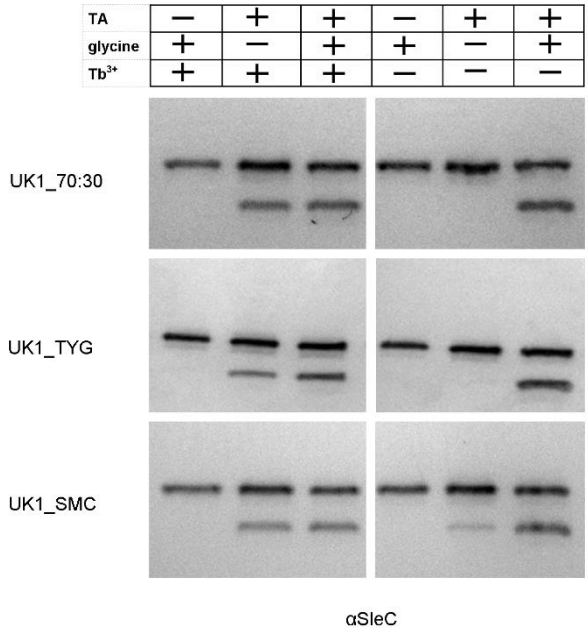

Figure S11. SleC activation with/without terbium for UK1 spores. Equal numbers of *C. difficile* UK1 spores prepared in with 70:30, SMC or TYG medium were incubated with or without 250 µM terbium in 30 mM glycine or 10 mM TA or 10 mM TA and 30 mM glycine for 2 hours and soluble proteins were extracted with NuPAGE buffer and separated by SDS-PAGE followed by immunoblotting with SleC antisera to observe SleC activation.

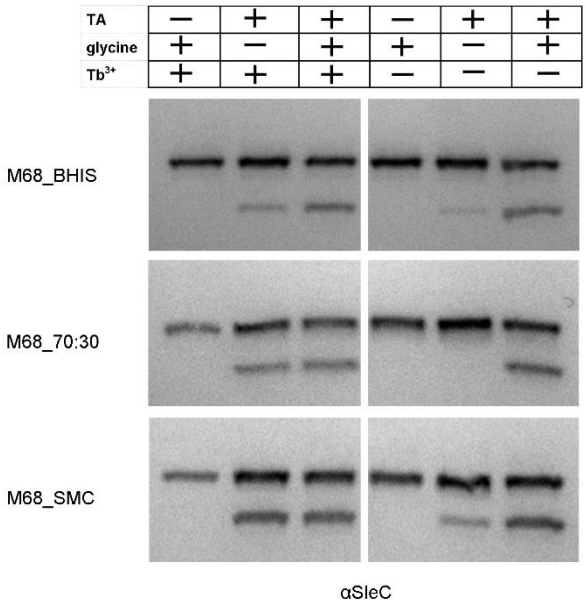

Figure S12. SleC activation with/without terbium for M68 spores. Equal numbers of *C. difficile* M68 spores prepared in with BHIS, 70:30 or SMC medium were incubated with or without 250 µM terbium in 30 mM glycine or 10 mM TA or 10 mM TA and 30 mM glycine for 2 hours and soluble proteins were extracted with NuPAGE buffer and separated by SDS-PAGE followed by immunoblotting with SleC antisera to observe SleC.