Characterizing the Early Events of *Clostridium difficile* Spore Germination

by

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DEDICATION

To my parents, Ron and Deanna

For their love and endless support of all my “mad scientist” ideas

To Melissa

Your strength, love, and support has made this all possible

To Kayleigh

I hope this inspires you to dream and never stop pursuing those dreams

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ABSTRACT

Clostridium difficile is an anaerobic spore forming bacterium that is an important human pathogen and a leading cause of hospital acquired infectious diarrhea worldwide. *C. difficile* infections cause symptoms ranging from severe diarrhea, to pseudomembranous colitis, toxic megacolon, and can sometimes result in death. These symptoms are caused by the production of two large exotoxins (TcdA and TcdB), that attack the host intestinal epithelium. *C. difficile* infection is a toxin-mediated disease, however, transmission is dependent on the ability to produce spores. *C. difficile* spores are produced through a highly regulated process known as sporulation. Sporulation occurs when bacterial growth conditions are unfavorable which leads to the phosphorylation of the major transcription factor Spo0A. Spo0A phosphorylation leads to activation of a cascade of compartment specific transcription factors and the production of mature dormant spores. These spores allow the bacterium to survive in harsh environmental conditions such as oxygen, low pH, ethanol, desiccation, etc. *C. difficile* spores are the infectious particles; when a susceptible patient ingests a spore, it becomes reactivated in the small intestine through a process known as germination. Germination is the first pathogenic event that occurs after ingestion of the spore and thus, is required for disease. Germination occurs after specific environmental cues, known as germinants, signal to the spore that conditions are favorable for vegetative cell growth. These germinants are typically amino acids that interact with specific receptors found within the spore (GerA). Much is known about bacterial spore germination in Ba*cillus spp*., however, since the *C. difficile* genome does not

encode any orthologs of these receptors, *C. difficile* spores must germinate through a unique mechanism. Unlike *Bacillus*, *C. difficile* germinates in response to the combination of amino acids and bile salts with a unique pseudo-protease receptor, CspC. The amino acid receptor has yet to be discovered. The goals of this thesis are to: describe the earliest events of germination including the specific conditions that facilitate germination, describe the mechanism of bile salts amino acid induced germination, and characterize the role of calcium. In this work, I describe an additional germination pathway activated by bile salts and intestinal calcium. Data presented in this thesis indicate that germination of *C. difficile* spores is regulated within the gastrointestinal tract by pH and availability of specific germinants, limiting germination to the ileum. Bile salts, calcium, and amino acids synergize together to decrease the required concentrations to physiological levels. Here I show that this synergy allows *C. difficile* spores to overcome the effects of inhibitory bile salts by demonstrating that germination is affected by calcium levels in ileal contents from non-antibiotic treated mice. Overall, the data presented in this thesis contribute to our understanding of *C. difficile* germination. Importantly, these observations provide a plausible biological mechanism for why patients with impaired calcium absorption and increased risk of contracting a *C. difficile* infection. The findings presented here should lead to the development of novel therapeutics to prevent or improve the outcomes of *C. difficile* infections.

CHAPTER 1: INTRODUCTION

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Abstract

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

Introduction

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

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

C. difficile Pathogenesis: Toxin Expression And Secretion

There are approximately half a million cases of CDI, resulting in ~29,000 deaths in the United States annually (1). *C. difficile* pathogenesis is dependent on the production of at least one of two exotoxins TcdA and TcdB. Some strains also encode binary toxin, which may play a role in virulence and disease (13). These toxins target the host intestinal epithelium and immune cells to cause inflammation, tissue damage, and increased mucosal permeability, leading to the severe diarrhea typically associated with CDIs (14, 15). While pathogenic strains have been isolated that lack *tcdA*, all pathogenic strains isolated from humans encode *tcdB* (16, 17). The genes encoding TcdA and TcdB are found on a 19.6kb chromosomal region referred to as the pathogenicity locus (PaLoc) (18, 19). This locus also encodes several regulatory elements (TcdR, TcdC, and TcdE), which are believed to regulate expression, production, and secretion of the toxins (20-22). *tcdR* encodes an alternative sigma factor that is critical for initiating expression of *tcdA* and *tcdB* (23, 24). Several studies have independently verified TcdC as an anti-sigma factor that functions as a negative regulator of toxin production (25, 26). Interestingly, naturally occurring variants of TcdC play a role in "hyper-virulence" likely due to increased toxin production (25). The mechanisms of toxin secretion are currently unknown and neither TcdA nor TcdB possess known secretion signals. It is hypothesized that TcdE may play a role in secretion of the toxins since it shares homology with bacteriophage holin proteins that release phage from their bacterial hosts (27). Strains lacking the PaLoc have been identified and isolated from asymptomatic patients and are of interest for their potential to prevent colonization of toxigenic strains that cause CDIs. However, recent studies have shown that PaLoc negative strains can become toxigenic by acquiring PaLoc through horizontal gene transfer (28). While *C. difficile* infection is a toxin-mediated disease, its capacity to cause disease is dependent on the

ability to produce viable spores, which allows the organism to survive in the external environment until it infects a new host.

Bacterial Sporulation

Since *C. difficile* vegetative bacteria are highly sensitive to oxygen and transmission occurs through the fecal-oral route, the ability to produce viable, metabolically dormant spores is essential for colonization of a new host (29). Bacterial spores are produced in response to unfavorable growth conditions through a highly conserved process known as sporulation (30, 31). All *C. difficile* isolates sequenced to date encode Spo0A, a transcription factor often called the master regulator of sporulation (29, 32). Spo0A contains an N-terminal phosphorylation domain that is phosphorylated by Spo0A histidine kinases (*CD630_14920* and *Cd630_15790*) and a C terminal DNA binding domain (29). Upon phosphorylation, Spo0A initiates activation of a cascade of early sporulation genes by binding to the promoter regions of several genes including itself and *sigH* (33). Several studies have defined the *C. difficile* Spo0A regulon to also include several conserved early sporulation genes, such as those encoding SigF and SigE (32-34). SigF and SigE are responsible for controlling expression of genes involved in building the spore from both the outside and the inside, respectively. These global changes in gene expression lead to asymmetric cell division, resulting in formation of a large mother cell and smaller forespore (Figure 1.1) (35). Shortly after division the mother cell engulfs the forespore creating a unique architecture consisting of an inner membrane and an outer membrane. The forespore then undergoes several morphological changes due to expression of sigma factors specific to each compartment, SigG in the forespore and SigK in the mother cell (36). These changes lead to the eventual death of mother cell and release of the mature spore.

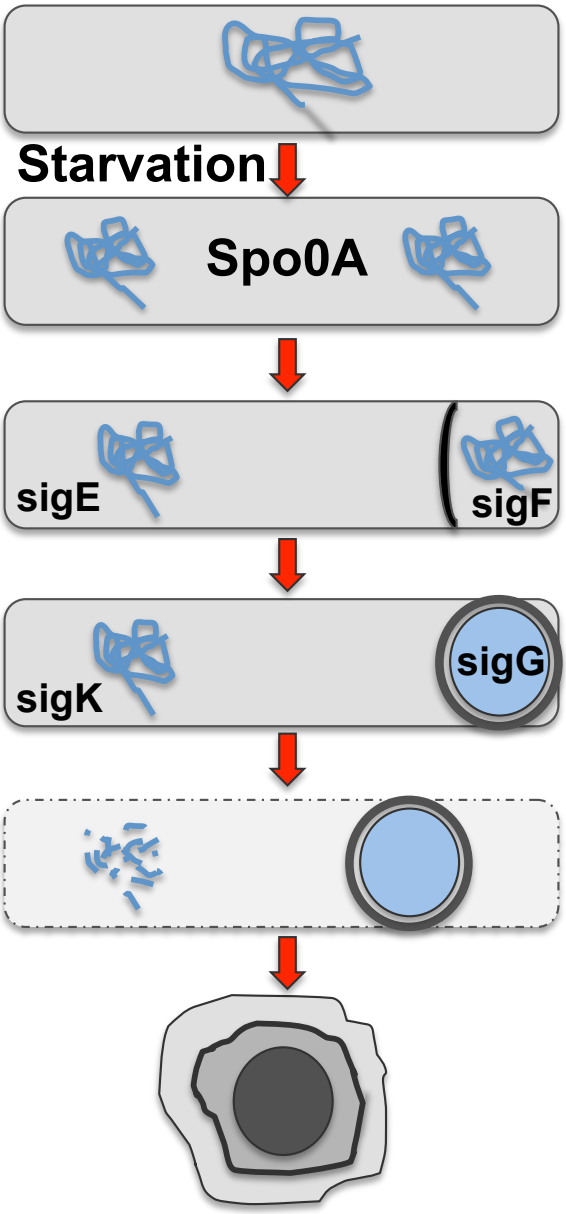

Figure 1.1 Model of bacterial sporulation
Bacterial sporulation is controlled by Spo0A, a transcription factor that is activated and phosphorylated due to nutrient deprivation (Starvation). Upon activation, Spo0A initiates a sporulation cascade where numerous other sigma factors are activated in sequential order. SigE and SigK are control expression of genes required for building the spore from the outside, spore coat, exosporium and uter membrane fractions. SigF and SigG control expression of genes responsible for building the spore from the inside, inner membrane and cortex fractions.

Spore Structure

The unique architectural design and structures present within bacterial spores contribute to their dormancy and extreme resistance properties and are, to a large extent, remarkably similar between the various bacterial species. For example, all bacterial spores have a partially dehydrated spore core containing up to 1M Calcium-Dipicolinic acid (Ca-DPA), a major contributor to a spore's heat resistance properties (Figure 1.2A) (37-39). Within the spore core, dormancy is maintained due to very low core water content. The DNA is supercoiled and bound with small, acid soluble proteins (SASPs) that are believed to block transcription and protect against DNA damage over long periods of time (40, 41). The spore core is surrounded by the inner membrane, which serves as a permeability barrier against damaging chemicals (42-44) (Figure 1.2B). Protecting the inner membrane is the germ cell wall, which will become the cell wall for the emerging *Bacillus* during outgrowth (Figure 1.2C) (45). Surrounding this is the spore cortex, a thick layer of modified peptidoglycan, where peptide side chains are removed from N-acetyl muramic acid, and a lactam ring is formed to make muramic acid-delta lactam (Figure 1.2D) (45, 46). This modification is essential for specific hydrolysis by cortex lytic enzymes (CLEs), a strategy that ensures CLE's degrade the cortex and not the germ cell wall during germination. The cortex layers are enclosed by the outer membrane/spore coat region, which contains the enzymes involved in cortex hydrolysis (Figure 1.2E) (47-49). The outermost layer is a highly permeable layer of carbohydrates known as the exosporium that likely plays a major role in host-pathogen interactions and spore persistence during recurrent CDIs (Figure 1.2F) (50-52). Collectively, these structures provide resistance to environmental stresses that this pathogen encounters during its unique lifecycle (11).

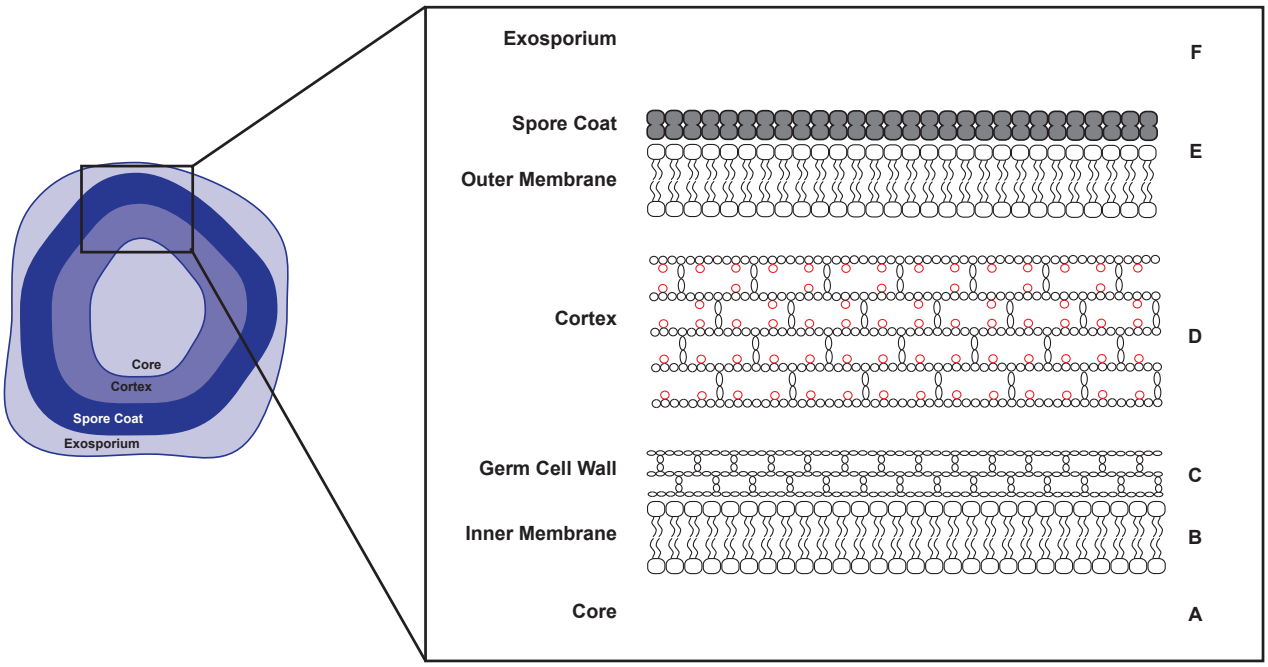

Figure 1.2 Anatomy of bacterial spores
Bacterial spores are composed of several layers including: a dehydrated spore core (A), an inner membrane (B), germ cell wall (C), the spore cortex (D), spore coat/outer membrane (E) and an exosporium (F). The spore cortex is a thick layer of modified peptidoglycan where ~50% of N-acetylmuramic acid sidechains are removed to produce muramic acid delta-lactam rings, which are depicted in red.

When a patient ingests *C. difficile* spores, they are able to endure the acidic stomach, and then transition to a metabolically active state (germinate) in the small intestine (53, 54). As *C. difficile* spores germinate they lose resistance properties, outgrow in vegetative cells that produce toxins, and colonize the large intestine, where the vast majority of all pathologies occur (55-58). Therefore, germination is an essential step that occurs prior to outgrowth, colonization, toxin production and the development of CDIs (59, 60). In this chapter, I compare and contrast well-examined *Bacillus* and *Clostridia* germination pathways and discuss the recent advances in understanding of *C. difficile* spore germination including: novel germination proteins, clinical significance of germination, discrepancies between current germination models, and the need for future investigation.

Sensing The Environment: Germinants & Receptors

Bacterial spore germination occurs within minutes in response to specific environmental cues, named germinants, believed to serve as indicators of conditions favorable for vegetative growth. Nutrient germinants are small molecules, such as sugars, amino acids, ions, and nucleotides that induce the irreversible reactivation of spores into metabolically active bacilli via interaction with specific germination receptors (61, 62). Typically, germinant receptors are found on the spore inner membrane, and the most extensively studied are the tri-cistronic *gerA* family of germination receptors (46, 54, 61, 63-65). The *gerA* family of receptors is highly conserved among spore formers including *Clostridia, e.g.,* the *Clostridium perfringens* genome encodes two GerA-type receptors, *gerA* and *gerK* (63, 66, 67). The *C. difficile* genome, however, contains no GerA-family ortholog. Instead, *C. difficile* senses the external environment with a unique pseudoprotease known as CspC (68). One model organism of the pathogenic

Bacillus spp., *B. anthracis*, encodes up to seven *gerA* type loci: *gerA*, *gerH*, *gerK*, *gerL, gerS, gerX,* and *gerY* (69).

Multiple distinct germination pathways have been identified in both *B. anthracis* and *Clostridium difficile,* each requiring specific germinant-receptor interactions. For example, there are five distinct germination pathways for *B. anthracis* (Table 1) (69, 70). The Alanine pathway (Ala), requires extremely high (non-physiological) concentrations of L-alanine (>30mM) and the combination of GerK and GerL receptors (Figure 1.3) (71, 72). At physiologically relevant concentrations, L-alanine can coordinate with L-proline (AP pathway) or aromatic amino acids such as L-histidine (AEA pathway) (71). In addition, several amino acids can cooperate with the purine ribo-nucleoside inosine (the most potent co-germinant) to make up the amino acid inosine dependent pathways (AAID-1 & 2) (Table 1) (71).

A

Bacillus anthracis	Germinants	Receptors
Alanine	L-alanine >30mM	GerK, GerL
Alanine-Proline	L-alanine, L-proline	GerK, GerL
Aromatic amino acid Enhanced Alanine	L-alanine, L-tyrosine L-histidine, or L-tryptophan	GerS, GerL
Amino Acid Inosine Dependent Pathway 1	inosine, L-alanine, L-valine, L-serine, L-proline, or L-methionine	GerH, GerL, GerK
Amino Acid Inosine Dependent Pathway 2	inosine, L-tyrosine, L-histidine, L-phenylalanine, or L-tryptophan	GerS, GerH

B

Clostridium difficile	Germinants	Receptors
Bile Salts-Amino Acids	Taurocholate, glycine, L-histidine, L-serine, or L-alanine	CspC, ?
Bile Salts-Alanine Racemase dep. D-amino acid	Taurocholate, D-Alanine or D-serine	CspC , ?

Table 1.1 Germination pathways of *B. anthracis* and *C. difficile*

For all *B. anthracis* pathways, germinants pass through the outer layers of the spore using dedicated channels that consist of multi-meric proteins from the GerP family (GerPABCDEF) (Figure 1.3A) (73-75). Then, receptor complexes on the spore inner membrane bind to their cognate germinants and initiate the release of monovalent cations and Ca-DPA (Figure 1.3B-D) (38, 42, 76, 77). Ca-DPA is released from the spore core and binds to the cortex lytic enzyme CwlJ, activating this enzyme and initiating hydrolysis of the cortex layer. Enzymatic degradation of the cortex is believed to lead to full core rehydration and initiation of outgrowth (Figure 1.3E,F) (78).

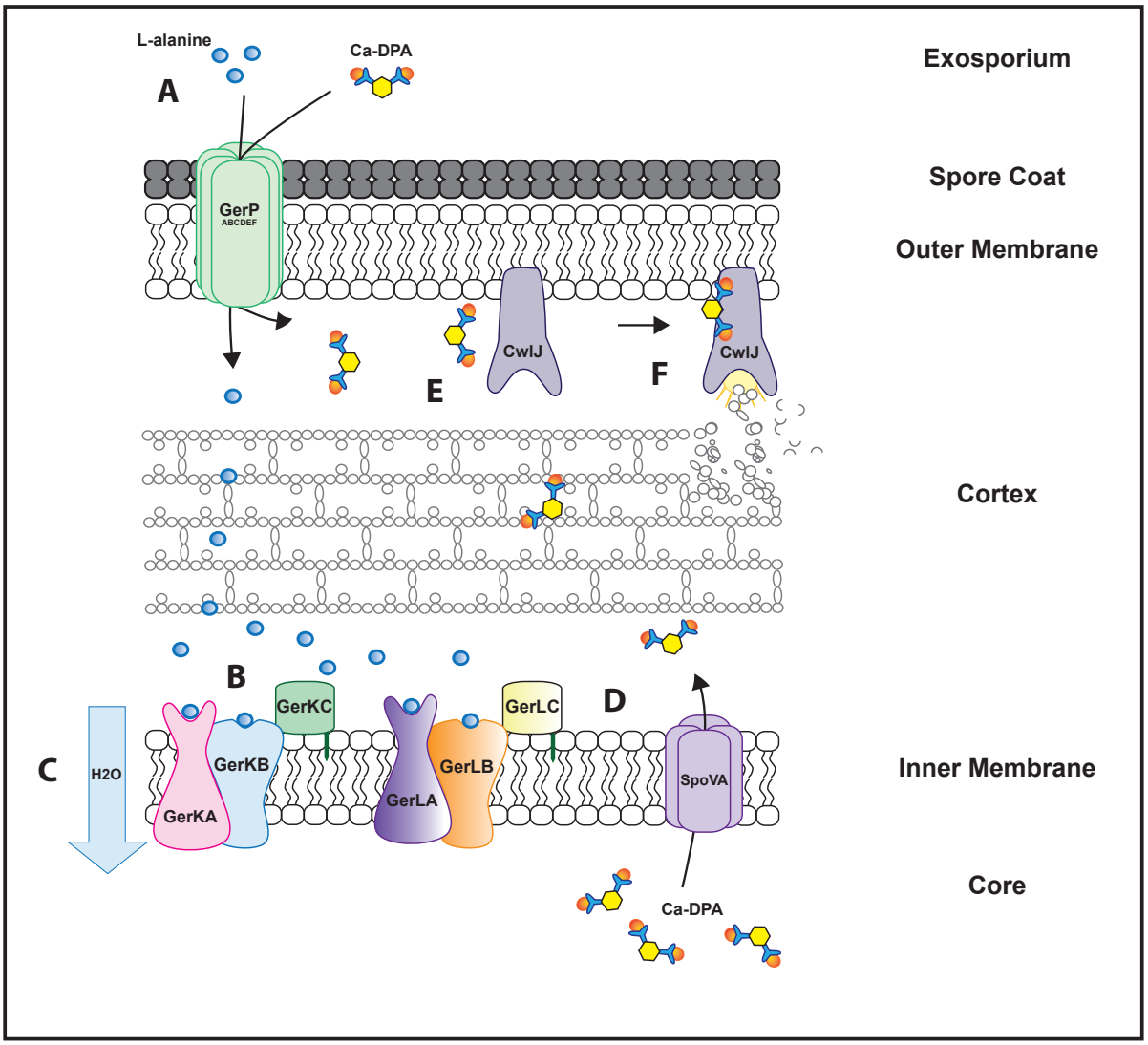

Figure 1.3 *Bacillus anthracis* L-alanine germination model
Nutrients enter the spore through $GerP_{ABCDEF}$ complex (A) which facilitates movement of L-alanine through the spore coat and outer membrane to the spore inner membrane where it binds to GerK and/or GerL germination receptors (B). These germinant/receptor interactions lead to slight core rehydration (C) and release of Ca-DPA from the spore core (D). Ca-DPA travels through the cortex (or can be added exogenously) and binds to CwlJ (E). This binding activates CwlJ initiating hydrolysis of the cortex peptidoglycan (F). This leads to full core rehydration and spore outgrowth.

Unlike *B. anthracis,* which germinates when exposed to the nutrient germinants associated with phagocytes*, C. difficile* germinates in the gut in response to a combination of nutrients and bile salts present in the gastrointestinal tract, specifically the bile salt cholate (79-84). Most derivatives of cholate can trigger spore germination, however taurocholate is the most effective germinant *in vitro* (85-87). Bile salts are detected directly by a unique, non-canonical receptor, CspC, which is located in the spore coat/outer membrane (68). These bile salts are not sufficient for efficient *C. difficile* germination; a co-germinant, such as amino acids, is essential for the initiation of germination signaling (88-90). The receptors for co-germinant molecules remain unknown. Glycine is the most efficient amino acid co-germinant for *C. difficile* spores, but can also be substituted for by other amino acids such as L-alanine, L-histidine, and L-serine (88, 91-93).

C. difficile can respond to a number of amino acids, although always in conjunction with bile salts, in what I describe as the bile salts amino acid pathway (Table 1). In addition, *C. difficile* maintains a unique germination pathway referred to here as the alanine-racemase dependent D-amino acid pathway (Table 1). Alanine racemases typically convert L-alanine to D-alanine (and vice versa). In *Bacillus,* D-alanine acts as a competitive inhibitor of L-alanine-dependent germination pathways. While D-alanine does not inhibit germination of *C. difficile* spores, a recent study found that the alanine racemase Alr2 is required for the epimerization of D-alanine, and D-serine, which can facilitate germination along with taurocholate (93).

While there are several environment-sensing pathways that can initiate spore germination, each initiates a signaling cascade that leads to activation of SleC, the spore CLE essential for germination. SleC activation initiates degradation of the cortex, resulting in full

core rehydration and release of Ca^{-}DPA from the spore core (39, 90, 93-95). The specific binding partners for amino acids during these early stages of germination, and any functional mechanisms linking them to initiation of cortex hydrolysis, remain to be elucidated and are an area of active investigation.

Initiation Of Cortex Hydrolysis: cspBAC Operon

In order to maintain spore dormancy, CLEs remain inactive until they receive a specific signal downstream of germinant/receptor interactions. In *Bacillus*, after sensing the environment is suitable for bacterial growth, Ca-DPA is released from the spore core, where it serves as a cofactor binding to and activating the CLE CwlJ (Figure 1.3D,E) (78). *B. anthracis* maintains four cortex lytic enzymes that contribute to spore germination: CwlJ1, CwlJ2, SleL, and SleB (96). The *C. difficile* genome encodes a SleB/CwlJ-like ortholog, but it does not contribute to germination. Instead, cortex hydrolysis is facilitated by the CLE SleC which has significant homology to CLEs from *C. perfringens* (97). In *C. perfringens,* activation of SleC occurs when the Csp family of subtilisin-like proteases (CspABC) (Figure 1.4A) cleave the prodomain from SleC initiating cortex hydrolysis (98, 99).

Subtilisin-like-proteases contain a highly conserved catalytic triad consisting of a serine-histidine-aspartate motif (Figure 1.4A) (100). The *C. difficile* genome encodes an operon of subtilisin-like proteases, *cspBAC*, that is essential for germination and has high homology to those encoded by *C. perfringens* (Figure 1.4B) (94). Expression of the *cspBAC* operon is controlled by SigE and these proteins are found in the spore coat/outer membrane fraction of mature spores (32, 94, 99, 101, 102). The operon contains two genes, *cspBA* and *cspC.* CspBA is expressed as a fusion protein during sporulation, but is ultimately processed by the YabG

protease into two proteins, CspB and CspA, that are incorporated into mature spores (Figure 1.4B) (101, 103). CspB cleaves the prodomain from pro-SleC, initiating cortex hydrolysis (94). The identity of the specific signal that activates CspB to facilitate *C. difficile* germination remains unknown. Since subtilisin-like proteases are calcium sensitive, one hypothesis is that CspB binds calcium and that this binding is required for CspB activity (90, 104-108). However, another study did not identify any calcium ions associated with the *C. perfringens* CspB (94).

The other portion of the CspBA fusion protein, CspA, is required for incorporation of the germinant receptor, CspC, into the mature spore (101, 103). While CspA is required for *C. difficile* germination, the precise mechanism of action and its interactions with CspC remain to be elucidated. One confounding factor is that both CspA and CspC have mutations in their catalytic triad, rendering them catalytically inactive (68, 101, 103). To provide additional insight on the *C. difficile* Csp catalytic domains, CspBAC structures were predicted using I-Tasser, and aligned with the *C. perfringens* CspB crystal structure (Figure 1.4) (109). According to these predictions, the catalytic sites of CspBAC are structurally similar to that of *C. perfringens* CspB, however only CspB has an intact D-H-S motif: CspA has D-Q-A motif and CspC has a D-T-G motif (Figure 1.4C) (103). These mutations are expected to render the proteases catalytically null, which is likely since these "pseudo" proteases do not auto-process their pro-domains like CspB or other subtilisins (94, 101).

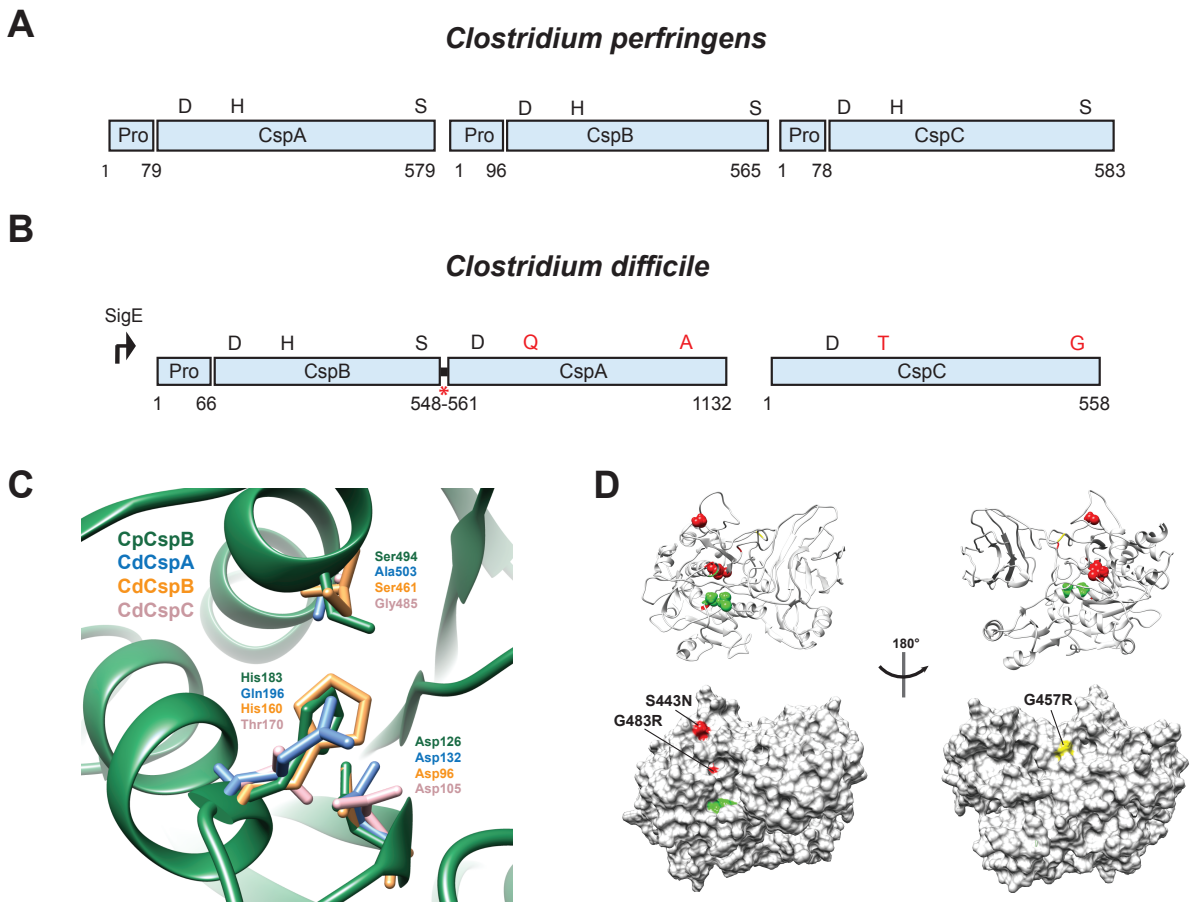

Figure 1.4 CspBAC operon: Structural map of conserved catalytic triad
Schematic of Csp proteases in *C. perfringens* (A) and *C. difficile* (B). Intact catalytic residues are in black and mutated residues are red. The red * indicates the YabG cleavage site. Superposition of the *C. difficile* Csp mutated catalytic residues (model from ITASSER structure prediction) with the *C. perfringens* CspB (PDB 4i0W) (Adams et al., PMID 18215316, 20360767) (C) (94). Map of the mutations reported in Francis et al. on CspC (ITASSER structure prediction) showing both internal mutations in ribbon diagram (top) and mutations on the protein surface (bottom) (D) (68). Catalytic triad residues are highlighted in green, mutated residues that results in a loss of germination are highlighted in red, and the G457R mutation is highlighted in yellow.

The final protein encoded by the *cspBAC* operon, CspC, is a pseudo-protease that is required for germination (68). CspC first was identified using a chemical mutagenesis screen to identify proteins required for germination (68). While this unbiased approach would be expected to identify several proteins involved in germination, the only mutations effecting germination reported were in *cspB* and *cspC*. Multiple point mutations in CspC were identified that disrupt germination, presumably by destabilizing the protein as described below. One of these point mutations (G457R) was shown to alter receptor specificity, allowing germination in response to chenodeoxycholate, a bile salt that typically inhibits *C. difficile* germination (80, 110). However, it is unclear if chenodeoxycholate is inducing germination or if these spores are now able to germinate in the absence of taurocholate. In any case, this observation suggests that CspC is directly involved in bile salt recognition and, therefore, can be characterized as a new class (non-GerA-family) of germination receptor(s). In order to understand how these mutations may be affecting CspC function, I mapped the locations of each mutated residue on the predicted protein structure (Figure 1.4D) (111). The majority of the point mutations that disrupted germination were found on internal glycine residues that may have affected CspC protein folding, creating a nonfunctional protein and mutant that behaves like a *cspC* deletion mutant. The point mutation at G457R is located on the surface of the protein and could potentially alter a residue that interacts with bile salts. Since CspC has no proteolytic activity, the events that that lead to activation of CspB after bile salts interact with CspC remain unknown.

The Role Of Dipicolinic-Acid DPA In Germination

Ca-DPA is a major component of bacterial spores making up approximately 15% of the dry weight of the spore (112). DPA is synthesized by the mother cell during sporulation, is

transported across the outer membrane of the forespore by the nucleoside transporter SpoVV (recently characterized in *Bacillus*), and is then transported across the forespore inner membrane and into the core by SpoVA (37, 39, 113, 114). Inside the spore core, Ca-DPA displaces water creating a dehydrated core and contributing to the heat resistance of the spore (37, 39, 114). In *Bacillus spp.,* Ca-DPA is released after germinant/receptor interactions and acts as a signaling molecule, initiating cortex hydrolysis by binding to and activating the CwlJ.

In *C. difficile*, the signaling events that trigger cortex hydrolysis remain unknown. In fact, Ca-DPA release does not occur until after cortex hydrolysis (115). Ca-DPA is eventually released by a mechanosensing mechanism whereby the core swells after cortex hydrolysis and DPA is released to relieve hypo-osmotic stress (95). *C. difficile* spores that are deficient for DPA biosynthesis retain the ability to germinate in rich media.

Other Proteins Involved In *C. difficile* Spore Germination

A few novel sporulation/germination proteins have been identified in the course of studying *C. difficile* germination, including GerG, GerS, and Cd630_32980 (90, 116, 117). The expression of these genes is controlled by the mother-cell sigma factor SigE (32). GerS is a novel lipoprotein regulator that is highly expressed during sporulation and localizes to the spore coat/outer membrane fraction of mature spores (117). Although SleC is activated in GerS mutants, these strains are unable to initiate cortex hydrolysis in response to any combination of co-germinants (90, 117). While the exact role of GerS remains unclear, it is hypothesized that GerS regulates the activity of SleC by playing a role in modification of the cortex allowing SleC to recognize it and initiate hydrolysis, similar to the role of CwlD cortex modification in *Bacillus* (118, 119).

GerG is a putative gel forming protein that is exclusively found in *C. difficile* isolates that is required for *C. difficile* germination. GerG mutants generate spores that lack key Csp proteins, likely leading to defects in initiating cortex hydrolysis. It is hypothesized that GerG functions by facilitating transport of Csp proteins across the outer forespore membrane into the cortex region during sporulation (116). Interestingly, the central repeat of GerG that is predicted to be required for gel formation is not required for germination.

Clinical Significance Of Germination

Since spores are the infectious form of *C. difficile,* and CDIs are mediated by toxin producing vegetative cells, germination is necessary for the disease process to progress. In fact, many of the germination and sporulation proteins described above are required for *C. difficile* colonization and pathogenesis (68, 94, 97, 116, 117). Assessment of over 100 clinical isolates of *C. difficile* has also indicated a role for tight control of germination in human disease severity (120). Strains that were unable to germinate in response to bile salts alone, but rather required the presence of a co-germinant, were more likely to have caused severe disease in the individuals that they were isolated from (120).

The importance of germination in the pathogenesis of many spore-forming bacterial species raises the possibility of generating novel therapeutics that target this pathway. The dependence of *C. difficile* germination for specific molecules, including bile salts and glycine, has led to speculation that these molecules could be used to alter germination *in vivo*. In this context, many have focused on the idea of blocking germination in order to prevent outgrowth and the production of virulence factors. Indeed, if germination were completely inhibited, *C. difficile* spores would not convert to vegetative bacilli and, therefore, would not be produce

toxins or cause disease. Since spores are inherently antibiotic resistant, this strategy could prevent disease in the short term, but since it does not specifically induce clearance of the spores from the body, could increase the likelihood that the individual would experience a recurrence of CDI. Recurrence is a major problem in CDI, with 20-30% of individuals experiencing at least one bout of recurrent disease (121, 122). One recent study showed that this recurrence could be due to either reinfection of a susceptible individual or reactivation of latent spores within the individual's gastrointestinal tract (122). Knowledge of germination pathways and mechanisms could be exploited to activate spores within a host to prevent recurrence. If spores within the host are forced to germinate during antibiotic therapy for a CDI, these would be killed by the antibiotic and unable to cause subsequent recurrence. Since bile salts are absorbed rapidly in the intestines, this strategy of forcing germination during antibiotic treatment will likely require use of methods to target specific areas within the gastrointestinal tract.

Clostridium difficile Germination Model Prior To This Thesis

C. difficile is an important human pathogen resulting in severe gastrointestinal infection. Clinical manifestations of CDI are mediated by toxins (TcdA, TcdB, & CDT) that target the intestinal epithelium causing cell death and inflammation (14, 15). While these toxins are essential for *C. difficile* pathogenesis, the ability of this organism to transfer between hosts and, therefore, cause disease is dependent on its ability to produce spores. During infection these spores germinate in response to bile salts and amino acids in the gastrointestinal tract. Very little is known regarding the mechanisms controlling germination in this pathogen because *C. difficile* genomes do not encode the GerA family of receptors commonly found in other spore forming bacteria. While it is known that bile salts and amino acids are **required** co-germinants, the

mechanism(s) involved in amino acid induced germination have yet to be elucidated. A recent publication from Francis, et al suggests that Tc interacts with CspC, however the downstream signal transduction cascade that leads to activation of cortex hydrolysis and germination remains unknown (Figure 1.5A) (68). It is likely that taurocholate binding to CspC leads to one of two outcomes: 1) activation of CspB or 2) opening of a channel to allow co-germinants access into the spore. The mechanism by which the germinants gain entry to the spore is currently unknown. In *B. anthracis*, this process occurs through the GerP family of proteins, however, orthologs of these proteins are not encoded in the *C. difficile* genome (73-75, 123). Glycine must gain entry into the spore and interact with its receptor to induce germination. Kinetic analysis of *C. difficile* germination in response to taurocholate and glycine provides evidence of the existence of receptors for these molecules (92). Prior to the studies conducted as part of this thesis, the model of *C. difficile* germination was as follows. First, taurocholate binds to CspC (Figure 1.5A) and glycine interacts with an unknown receptor (Figure 1.5B). These germinant-receptor interactions lead to the activation of CspB through an unknown mechanism (Figure 1.5C). CspB cleaves the pro-domain from SleC, activating it (Figure 1.5C), initiating cortex hydrolysis (Figure 1.5D) and subsequent rehydration of the spore core (Figure 1.5E). Ca-DPA is then released from the spore core (Figure 1.5F) and this release leads to metabolic activation and outgrowth of the vegetative bacillus. In this work, it is my aim to contribute to our understanding of *C. diffiicle* germination and pathogenesis by characterizing: 1) the mechanism of *C. difficile* germination in response to glycine, 2) novel proteins involved in this process, and 3) the mechanism of CLE activation.

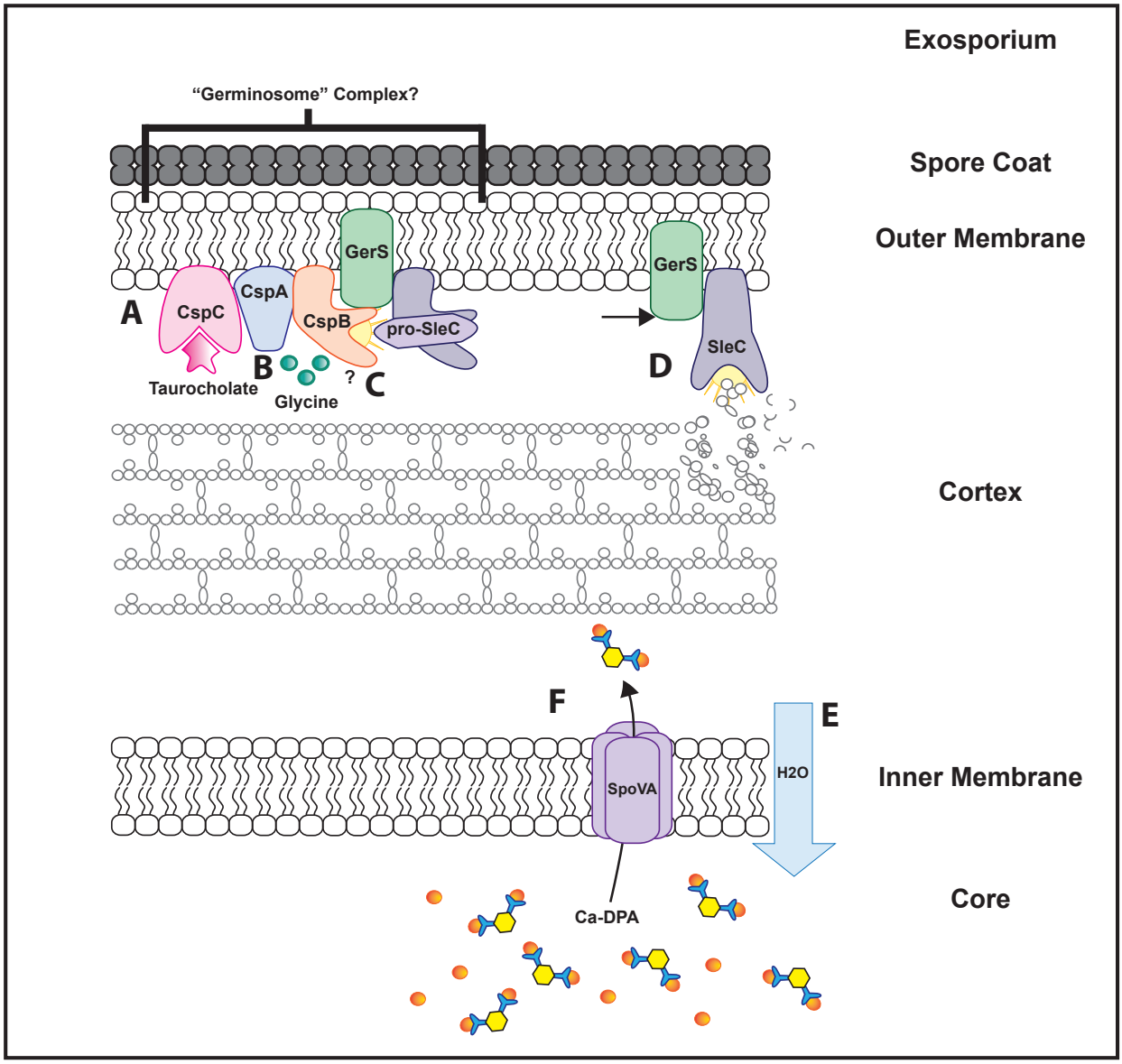

Figure 1.5 Previous model of *C. difficile* spore germination
Taurocholate binds to CspC (A). Glycine interacts with an unknown receptor (B). CspB becomes activated and cleaves the pro-domain from SleC (C). Active SleC degrades the cortex (D), which leads to full core rehydration (E), release of CaDPA (F), and outgrowth out of the spore.

Chapter Outlines

In Chapter 2, *Intestinal Calcium and Bile Salts Facilitate Germination of Clostridium difficile Spores*, I discovered that calcium induces spore germination *in vitro* and calcium found within the gastrointestinal tract plays a major role in inducing germination of *C. difficile* spores.

In Chapter 3, *Germinant Synergy Facilitates Clostridium difficile Spore Germination Under Physiological Conditions*, I discovered that calcium and glycine synergize to induce germination with bile salts decreasing the required concentrations of germinants to physiological levels.

In Chapter 4, I summarize the findings of each chapter, discuss implications of the data and how it affects the field, and propose future directions.

CHAPTER 2: INTESTINAL CALCIUM AND BILE SALTS FACILITATE GERMINATION OF *CLOSTRIDIUM DIFFICILE* SPORES

Modified from:
Kochan TJ, Somers MJ, Kaiser AM, Shoshiev MS, Hagan AK, et al. (2017) Intestinal calcium and bile salts facilitate germination of *Clostridium difficile* spores. PLOS Pathogens 13(7): e1006443. https://doi.org/10.1371/journal.ppat.1006443

Abstract

Clostridium difficile (*C. difficile*) is an anaerobic gram-positive pathogen that is the leading cause of nosocomial bacterial infection globally. *C. difficile* infection (CDI) typically occurs after ingestion of infectious spores by a patient that has been treated with broad-spectrum antibiotics. While CDI is a toxin-mediated disease, transmission and pathogenesis are dependent on the ability to produce viable spores. These spores must become metabolically active (germinate) in order to cause disease. *C. difficile* spore germination occurs when spores encounter bile salts and other co-germinants within the small intestine, however, the germination signaling cascade is unclear. In this chapter, I describe a signaling role for Ca^{2+} during *C. difficile* spore germination and provide direct evidence that intestinal Ca^{2+} coordinates with bile salts to stimulate germination. Endogenous Ca^{2+} (released from within the spore) and a putative AAA+ ATPase, encoded by *Cd630_32980*, are both essential for taurocholate-glycine induced germination in the absence of exogenous Ca^{2+}. However, environmental Ca^{2+} replaces glycine as a co-germinant and circumvents the need for endogenous Ca^{2+} fluxes. *Cd630_32980* is dispensable for colonization in a murine model of *C. difficile* infection and *ex vivo* germination

in mouse ileal contents. Calcium-depletion of the ileal contents prevented mutant spore germination and reduced WT spore germination by 90%, indicating that Ca^{2+} present within the gastrointestinal tract plays a critical role in *C. difficile* germination, colonization, and pathogenesis. These data provide a biological mechanism that may explain why individuals with inefficient intestinal calcium absorption (*e.g.,* vitamin D deficiency, proton pump inhibitor use) are more prone to CDI and suggest that modulating free intestinal calcium is a potential strategy to curb the incidence of CDI.

Introduction

The anaerobic spore-forming pathogen *Clostridium difficile* (*C. difficile*) is the leading cause of infectious nosocomial diarrhea, with 500,000 infections and 29,000 deaths in the U.S. annually (1). *C. difficile* infection (CDI) typically occurs after antibiotic therapy disrupts the indigenous gut microbiota, allowing *C. difficile* colonization. Symptoms of CDI include diarrhea, pseudomembranous colitis, and toxic megacolon. Two *C. difficile* toxins, toxin A (TcdA) and toxin B (Tcd) are the primary cause of these pathologies causing epithelial cell death and inflammation (55). While CDI symptoms are toxin-mediated, transmission and initiation of disease depend on the production of viable, metabolically dormant spores. *C. difficile* spores have a dehydrated core that contains cytoplasmic macromolecules (e.g. DNA, ribosomes) and 0.8-1M calcium-dipicolinic acid (Ca-DPA), which is biosynthesized during sporulation and required for the heat resistance of bacterial spores (37, 38). The spore core is surrounded by an inner membrane, a thick cortex of modified peptidoglycan, an outer membrane, a proteinaceous coat, and an outermost exosporium layer of proteins, lipids, and carbohydrates (124). Collectively, these layers protect spores from harsh environmental conditions such as acidic pH,

extreme temperature, and desiccation.

Bacterial spores become metabolically active, *i.e.,* germinate, upon sensing specific small molecules, called germinants, in the environment. In the related *Bacillus spp.*, spores contain numerous well-characterized germinant receptors on the inner membrane. These receptors interact with combinations of germinants including nucleotides and amino acids to initiate germination (125). This process has been extensively studied in *Bacillus*, however, all sequenced *C. difficile* genomes lack the germinant receptors found in other spore-forming bacteria suggesting that the mechanism of germination in *C. difficile* is unique (65). It is known that *C. difficile* germinates in response to co-germinants that include a combination of amino acids and bile salts; glycine and taurocholate (Tc) are the most efficient germinant combination (88, 126). It has been shown that Tc binds to CspC during germination (68, 117), however, the receptors involved in the recognition of glycine or other amino acids have not been identified (92).

In *Bacillus spp.*, germinant-receptor interactions induce slight hydration of the core causing a rapid release of monovalent cations (*e.g.,* Na^+, K^+, H^+) (42), followed by the release of Ca-DPA (114), and subsequent activation of cortex lytic enzymes (CLEs). CLEs degrade the cortex, initiating full core hydration and outgrowth of the vegetative bacteria. In *C. difficile,* SleC is the sole CLE that is essential for germination (127). It is expressed as a zymogen that is activated by the subtilisin-like protease, CspB (94, 98). It is not known how CspC binding to Tc leads to the activation of CspB. CspC may directly interact with CspB, however, Tc-CspC interactions might also facilitate access of co-germinants to their receptors to initiate signaling to activate CspB. The mechanism by which co-germinants permeate the spore coat is not currently known, since the *C. difficile* genome does not contain homologues to the GerP proteins that

perform this function in *Bacillus anthracis* (73). Therefore, the signaling cascade leading to CspB activation is an important gap in the current knowledge of *C. difficile* germination. In this work, I provide direct evidence that calcium ions are a germination signal to activate CspB and can be derived from either endogenous or exogenous sources. I demonstrate that intestinal calcium is a key molecule for efficient germination in a murine model. This chapter provides novel insight into the Ca^{2+} signaling pathways controlling *C. difficile* germination and a biological mechanism that may help explain why inefficient intestinal Ca^{2+} absorption increases susceptibility to CDI.

Results

Exogenous Calcium Circumvents The Glycine Requirement For Efficient *C. difficile* Germination

The germination signal leading to CspB activation and cortex hydrolysis by SleC is currently unknown. In *Bacillus spp*., Ca-DPA released from the core functions as the germination signal to activate cortex hydrolysis. The addition of exogenous Ca-DPA induces spore germination by direct activation of the CLE CwlJ (78), circumventing the need for germinants or germinant receptors. To determine if exogenous Ca-DPA can induce *C. difficile* spore germination, spores of three toxigenic *C. difficile* strains were incubated with 60mM Ca-DPA. While none germinated in Ca-DPA alone (128), all three strains germinated efficiently (measured by loss of OD_{600}) in Ca-DPA supplemented with 0.2% Tc (Figure 2.1 A-C). All three strains also germinated as expected in response to our positive control, Tc-Gly (Figure 2.1 D-F). However, two recent studies have demonstrated that cortex hydrolysis occurs prior to (115) or in the absence of DPA release (39), indicating that DPA is not essential for CLE activation. This

led to our hypothesis that calcium ions serve as a germination signal in *C. difficile*. To test this hypothesis, Cd630 spores were incubated with Tc and either $CaCl_2$, DPA, or Ca-DPA. As before, Tc-CaDPA induced full germination of *C. difficile* spores, however, Tc-$CaCl_2$ also induced full germination of *C. difficile* spores in the absence of exogenous DPA (Figure 2.1 G). Additionally, Tc-$CaCl_2$-treated spores lost heat resistance properties of dormant spores and released internal stores of DPA (Figure 2.1 I, J). In contrast, *B. anthracis* spores germinated as expected in response to Ca-DPA but were unable to germinate in response to $CaCl_2$ alone (Figure 2.1 H). I hypothesized that exogenous Ca^{2+} (or Ca-DPA) initiates cortex hydrolysis through SleC activation. To test this hypothesis, SleC activation was determined by western blot following incubation of Cd630 spores in PBS plus Tc, glycine, Tc-Gly, $CaCl_2$, Tc-$CaCl_2$, DPA, Ca-DPA, or Tc-CaDPA. While pro-SleC was present in all samples, activated SleC was only detected in samples incubated with Tc-Gly, Tc-$CaCl_2$, or Tc-CaDPA (Figure 2.1 K). These results indicate that SleC activation requires both Tc and an additional signal (*i.e.* glycine, calcium, or exogenous Ca-DPA) and that calcium induces germination through SleC activation. Since exogenous Tc-$CaCl_2$ induced germination in *C. difficile,* and spores have large stores of internal calcium (129), these data suggest that calcium functions as a co-germinant, and can come from either exogenous or endogenous sources. Because both PBS only (untreated) and Tc only treated spores have identical germination phenotypes (Figure 2.1 D-F, I, K), Tc alone treatment was used as a negative control for remaining experiments.

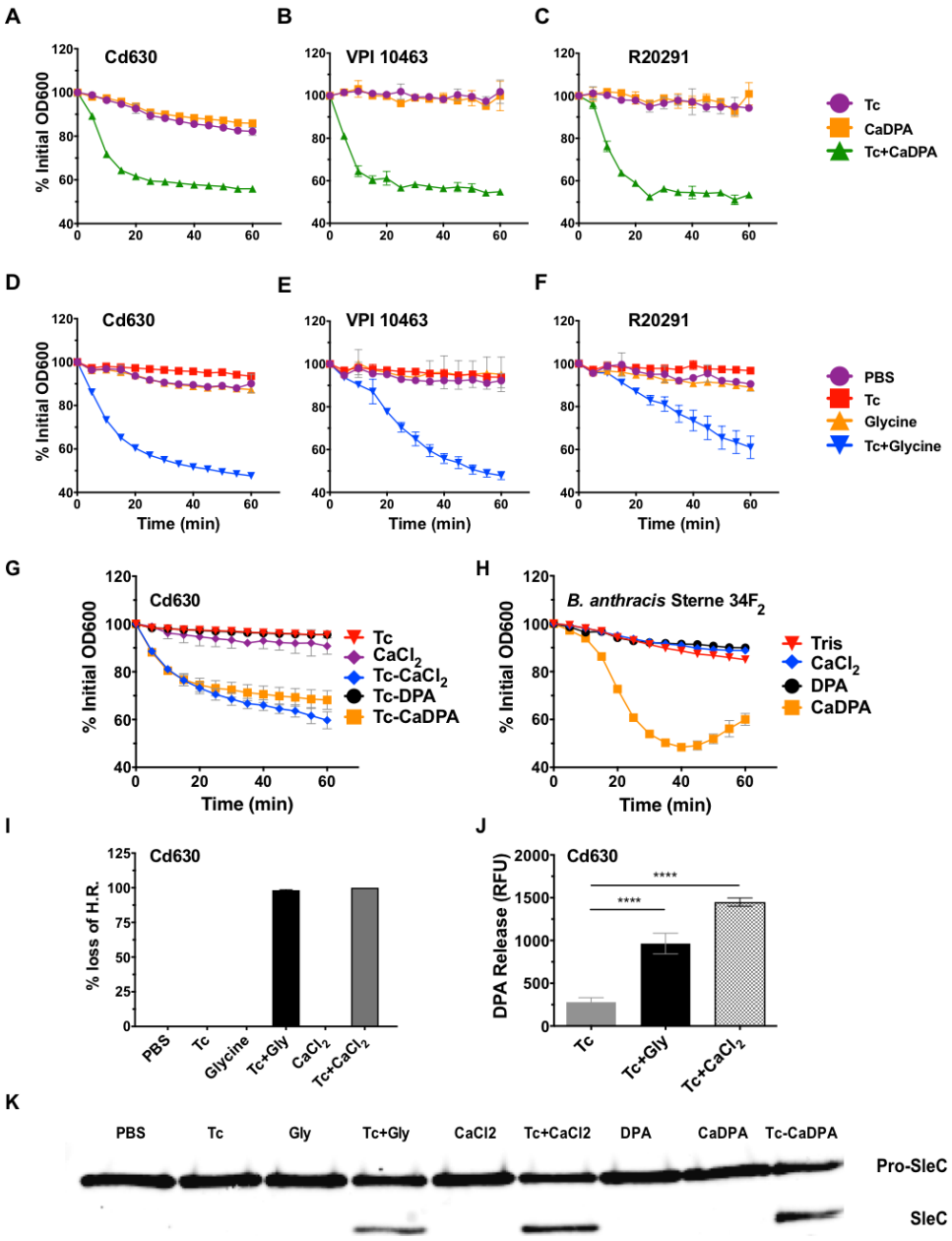

Figure 2.1 Exogenous calcium induces *C. difficile* germination in concert with taurocholate. Cd630, VPI 10463, or R20291 spores were incubated with the indicated combinations of 0.2% Tc, 60 mM Ca-DPA, 50 mM glycine, 60 mM $CaCl_2$, or 60 mM DPA (A-G, I, J). Activation of SleC was assessed by western blot analyses. Cd630 spores were incubated for 15 minutes at 37°C with the indicated combinations of 1% Tc, 50 mM glycine, 60 mM $CaCl_2$, 60 mM DPA, or 60 mM Ca-DPA. Spores were subsequently lysed and assayed for levels of pro-SleC and SleC (K). *Bacillus anthracis* strain Sterne $34F_2$ spores were incubated with the indicated combinations of either 60 mM $CaCl_2$, 60 mM DPA, or 60 mM CaDPA (H). Germination was measured either by tracking the loss of OD_{600} over time (A-H), measuring loss of heat resistance at 37°C after 1 hour (I), or measuring release of DPA at 37°C after 1 hour (J). Germination assays were performed in triplicate. Germination assays and western blots are representative of three independent spore preps. Error bars are mean plus or minus SD. Statistical analysis was performed using one-way ANOVA. (****) $p<0.0001$

To determine if other cations could stimulate *C. difficile* germination pathways, Cd630 spores were incubated with Tc and either $CaCl_2$, $Ca(NO^3)_2$, $Ca(C_2H_3O_2)_2$, $MgCl_2$, NaCl, $ZnCl_2$, KCl, or LiCl. In addition to Tc-$CaCl_2$, Tc-$Ca(NO^3)_2$, and Tc-$Ca(C_2H_3O_2)_2$ induced germination (~40% drop in OD_{600}, which is considered ~100% germination), while Tc-$MgCl_2$ induced minor germination (15% drop in OD_{600}, Figure 2.2 A). No other divalent cations were able to induce germination. *B. anthracis* spores were incubated with DPA and the cations listed above but were only able to germinate with Ca-DPA (Figure 2.2 B). These data show that of the numerous cations tested only Ca^{2+} induced efficient germination in *C. difficile*.

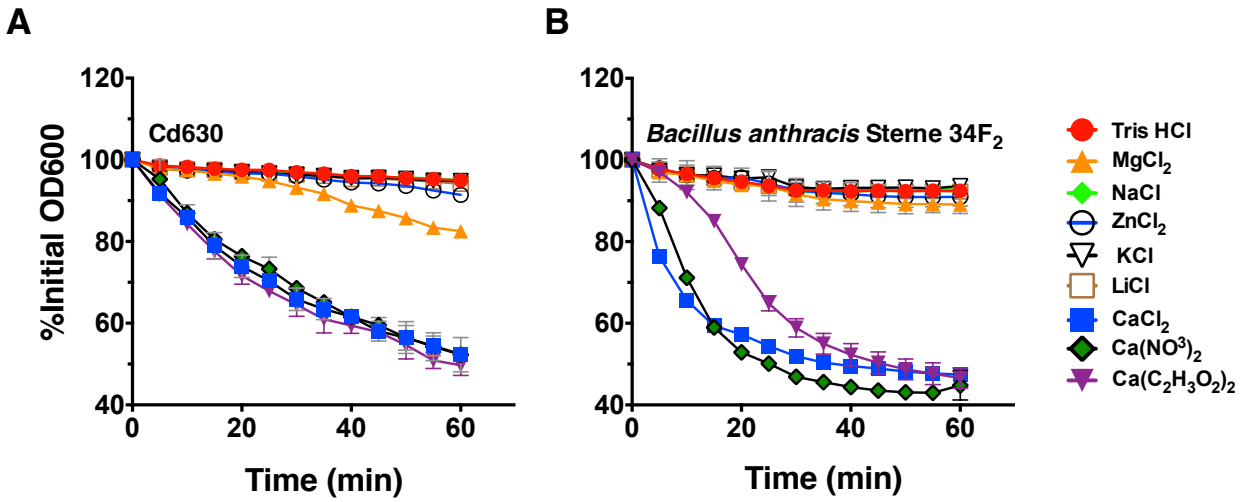

Figure 2.2 Cation induction of spore germination.
Cd630 spores were incubated with 0.2% Tc and 60 mM of the indicated cation (A). *Bacillus anthracis* (Sterne $34F_2$) spores were incubated with 60 mM DPA and 60 mM of the indicated cation (B). Germination was measured by tracking loss of optical density at 600 nm at 37°C over the course of an hour. Germination assays were performed in triplicate and are representative of 3 independent spore preps. Error bars are mean plus or minus SD.

Calcium Released From *C. difficile* Spores Is Essential For Germination In The Absence Of Environmental Ca^{2+}

Since exogenous calcium is sufficient to induce germination (in the presence of Tc), I sought to understand the role of endogenous calcium in this process. To determine if endogenous calcium is required for Tc-Gly induced germination, Cd630 spores were incubated with Tc, glycine, and the calcium-specific chelator EGTA. EGTA inhibited Tc-Gly induced germination at all concentrations tested (Figure 2.3 A), but was restored to wildtype levels with the addition of excess $CaCl_2$ (Figure 2.3 B). These data show that endogenous calcium is essential for *C. difficile* germination in the presence of the co-germinants, Tc-Gly. I hypothesize that calcium is functioning as an enzymatic cofactor for CspB and EGTA treatment will inhibit the activation of SleC. To test this hypothesis, Cd630 spores were incubated with Tc, Tc-Gly, or Tc-Gly-EGTA. As expected, no SleC processing was observed with Tc alone and SleC activation was observed in Tc-Gly treated spores (Figure 2.3 D). However, EGTA completely inhibited SleC activation. These data support the hypothesis that calcium is functioning as signal to activate CspB, possibly by functioning as an enzymatic cofactor.

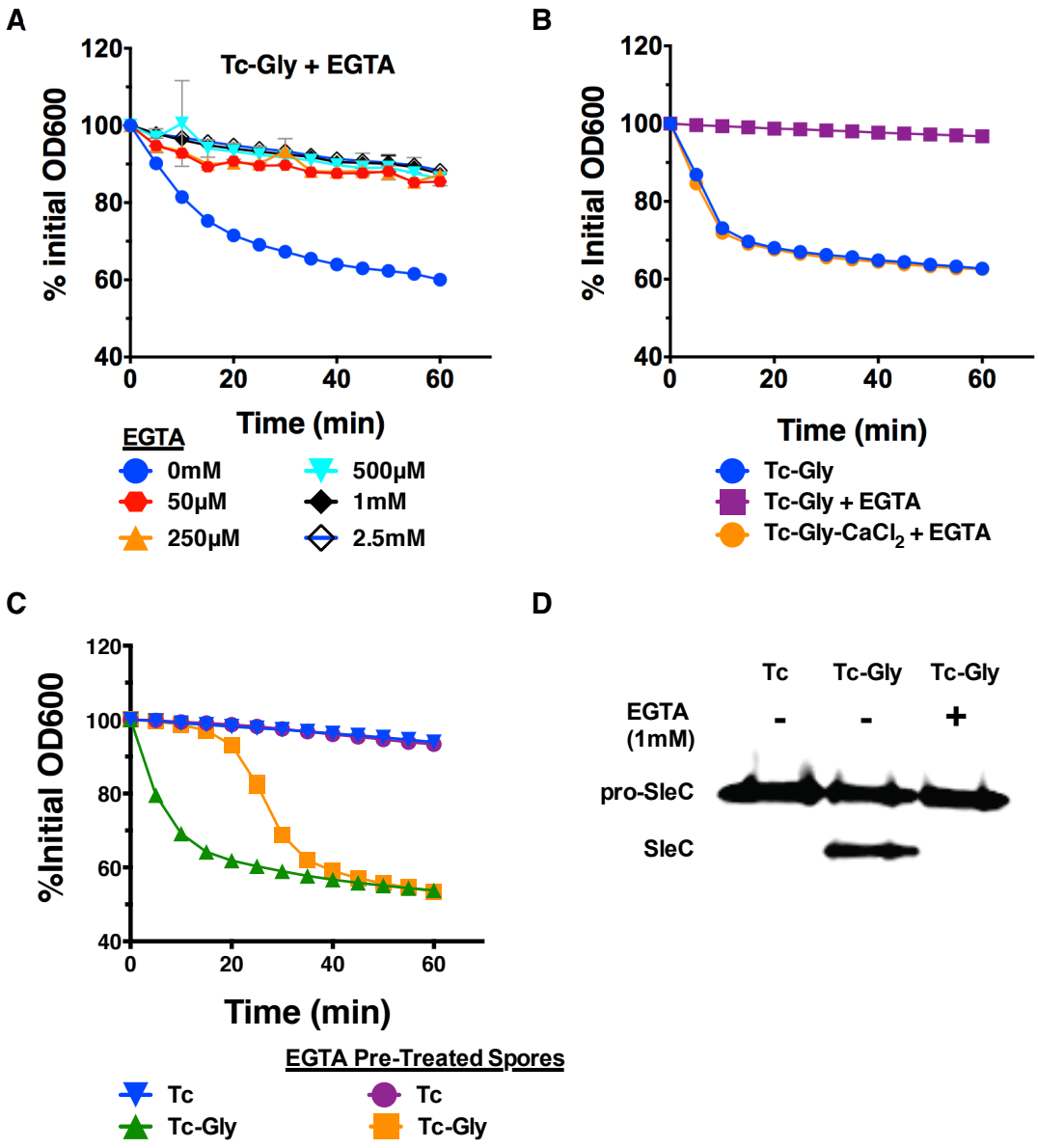

Figure 2.3 Calcium from within the spore is essential for germination in response to Tc-Gly. Cd630 spores were incubated with 0.2% Tc, 50mM glycine and different concentrations of EGTA (A). Cd630 spores were incubated with the indicated combinations of 0.2% Tc, 50 mM glycine, 1 mM $CaCl_2$, and 50 µM EGTA (B). EGTA (1mM) pretreated Cd630 spores and non-treated Cd630 spores were incubated with the indicated combinations of chelex-treated germinants, Tc (0.2%) and glycine (50mM) (C). Activation of SleC was assessed by western blot. Cd630 spores were incubated 37°C for 15 minutes with 1% Tc and the indicated combinations of 50mM glycine and 1mM EGTA (D). Germination was tracked by loss of OD at 37°C over the course of one hour. Germination assays were performed in triplicate. Germination assays and western blots are representative of 3 independent spore preps. Error bars are mean plus or minus SD.

Since EGTA treatment inhibited Tc-Gly germination at concentrations as low as 50 μM, I hypothesized that spores may have Ca^{2+} in the spore coat/cortex layers. In order to test this hypothesis, I first chelex-treated Tc, glycine, and PBS to ensure there is no contaminating calcium, and then pretreated Cd630 spores with 1mM EGTA. These spores were washed 3 times with chelex-treated PBS and then incubated with chelex-treated Tc-Gly. EGTA pre-treated spores displayed ~20 min delay in germination as compared to untreated spores (Figure 2.3 C). These data suggest that *C. difficile* spore outer layers contain small amounts of calcium (<50 μM) that when removed, delay germination until enough calcium is released from the spore core to activate SleC.

I hypothesized that endogenous calcium is transported out of the spore core and initiates cortex hydrolysis through the actions of calcium-dependent enzymes. In order to test this hypothesis, Cd630 spores were incubated with either 1mM Phenamil, an ion channel inhibitor (130), or 0.5mM Chlorpromazine (CPZ), an inhibitor of Ca^{2+}-enzyme interactions(131) and either Tc-Gly or Tc-$CaCl_2$. Phenamil-inhibited Tc-Gly-induced germination (~60% reduction) but not Tc-$CaCl_2$-induced germination (>95% germination) (S2 Fig). These data suggest that Tc-$CaCl_2$ induces germination independent of endogenous Ca^{2+} while Tc-Gly-induced germination requires Ca^{2+} efflux from the core. CPZ delayed both Tc-Gly and Tc-$CaCl_2$ induced germination with an overall reduction of 30-50% (Figure 2.4), indicating that calcium-dependent enzyme activation is required for efficient *C. difficile* germination. While the concentrations of CPZ and Phenamil are similar to that of other known spore inhibitors (130), I interpret these data cautiously given that they are higher than those used to inhibit eukaryotic cell targets. This difference in effective concentration could be due to low spore permeation.

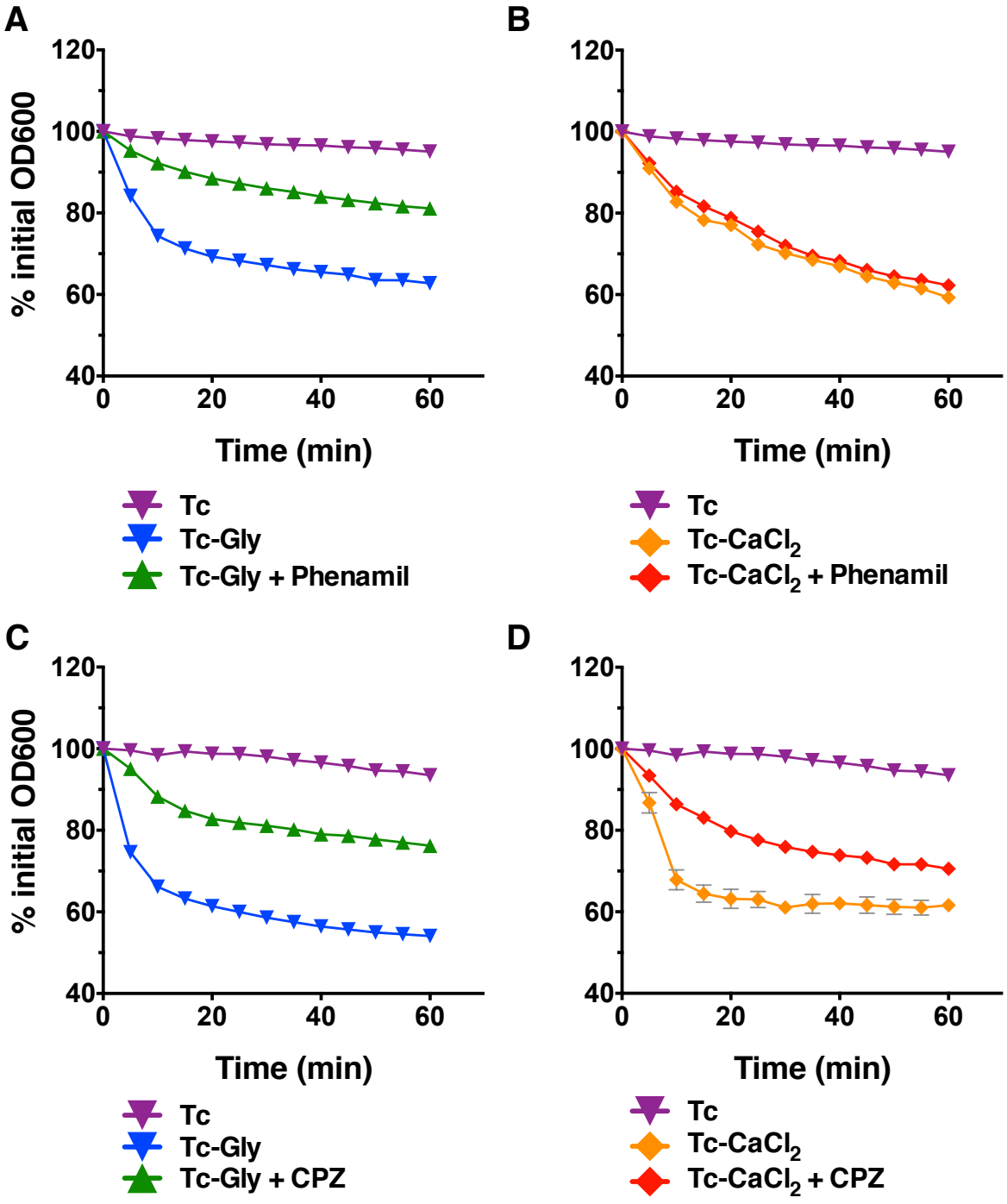

Figure 2.4 Chemical inhibitors of spore germination
Cd630 spores were incubated with the indicated combinations of 0.2% Tc, 50 mM glycine, 60mM $CaCl_2$, and 1 mM Phenamil (A,B) or 0.5 mM CPZ (C,D). Germination was measured by tracking loss of optical density at 600 nm at 37°C over the course of an hour. Germination assays were performed in triplicate and are representative of 3 independent spore preps. Error bars are mean plus or minus SD.

Tc-$CaCl_2$ Induces Germination Through A Similar Pathway As Tc-Gly

Since Tc-$CaCl_2$ induced germination through the activation of SleC (Figure 2.1 K), I hypothesized that this germinant combination signals through a similar pathway as Tc-Gly. To test this hypothesis, clean, unmarked deletions of genes essential for Tc-Gly-induced germination (*cspC, cspB, gerS, sleC*) were generated and germination kinetics were measured in response to Tc alone, Tc-Gly, or Tc-$CaCl_2$ (Figure 2.5). None of the mutants germinated in response to Tc-Gly, confirming previously published reports of their importance for *C. difficile* germination (Figure 2.5 A) (68, 94, 117). In response to Tc-$CaCl_2$, Δ*cspC* and Δ*sleC* spores did not germinate, but Δ*cspB* and Δ*gerS* spores exhibited low, but appreciable, levels of germination (~15% drop in OD_{600}), possibly due to pro-SleC activity in the presence of high calcium (Figure 2.5 B). As expected, zymogen processing of SleC was detected in Cd630 spores incubated with 1% Tc and all concentrations of either glycine or $CaCl_2$ (Figure 2.5 C-D). In contrast to a previous report (*14*), only minimal SleC activation was observed in Δ*gerS* spores incubated with either Tc-Gly or Tc-$CaCl_2$ (Figure 2.5 C-D). No zymogen processing was detected under any condition for Δ*cspB,* and Δ*cspC* spores (Figure 2.5 C-D). These results indicate that both Tc-Gly and Tc-$CaCl_2$ use the same set of enzymes in facilitating spore germination.

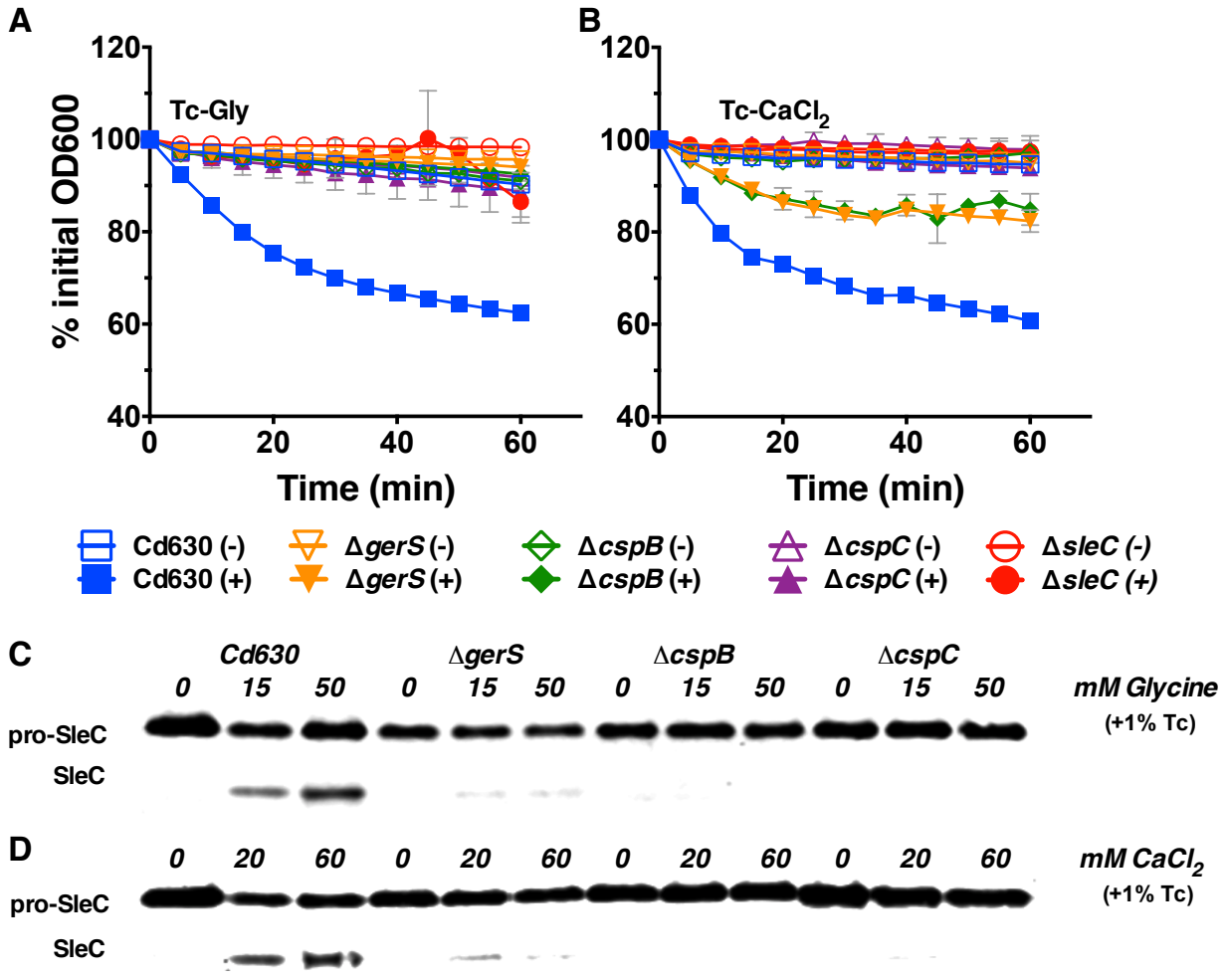

Figure 2.5 Tc-$CaCl_2$ induced germination occurs through the same pathway as Tc-Gly.
Cd630, *Δ*gerS, *Δ*cspB, *Δ*cspC, and *Δ*sleC spores were incubated with 0.2% Tc and with (+) or without (-) the indicated co-cogerminant: 50 mM glycine (a) or 60 mM $CaCl_2$ (b). Germination was measured by loss of OD_{600} at 37°C over the course of one hour. (a-b). Western blot of SleC from WT, *Δ*gerS, *Δ*cspB, or *Δ*cspC spores incubated at 37°C for 15 minutes with 1% Tc and the indicated concentrations of glycine (c) or $CaCl_2$ (d). Germination assays were performed in triplicate. Germination assays and western blots are representative of 3 independent spore preps. Error bars are mean plus or minus SD.

Cd630_32980 Is Required For Tc-Gly But Not Tc-$CaCl_2$ Induced Germination

Thus far I have shown that both Tc-Gly and Tc-$CaCl_2$ induce germination through activation of SleC and that endogenous calcium transport is required for Tc-Gly germination but not Tc-$CaCl_2$. I next sought to identify the step in the germination pathway where these mechanisms diverge. *Cd630_32980* is a gene identified as being highly expressed during sporulation (32) and predicted to be essential for *C. difficile* sporulation (132). Our independent bioinformatics analysis of these data comparing the transposon depth of coverage of dormant spores to that of vegetative cells after germination suggested that this gene might also be essential for Tc-Gly germination. To test this hypothesis, I constructed a clean, unmarked deletion (Δ*32980*) and purified spores (albeit at a lower yield than Cd630). I next tested Δ*32980* spores for germination in 0.2% Tc with increasing concentrations of either glycine (0-50mM) or $CaCl_2$ (0-60mM). Cd630 spores germinated in a dose-dependent manner in response to Tc-Gly (Figure 2.6 A) but Δ*32980* spores did not germinate, regardless of the glycine concentration (Figure 2.6 B). In contrast, Δ*32980* spores germinated in a dose-dependent manner in response to Tc-$CaCl_2$, albeit at somewhat lower efficiencies than Cd630 at calcium concentrations ≤30mM (Figure 2.6 C,D). Germination in Δ*32980* spores was restored by complementation of Cd630_32980 *in trans* (Figure 2.7 A). Spores lacking Cd630_32980 exhibited minimal SleC activation in response to Tc-Gly, while retaining full Cd630 levels in response to Tc-$CaCl_2$ (Figure 2.6 E,F). These results suggest that Cd630_32980 is vital for Tc-Gly induced germination but not Tc-$CaCl_2$.

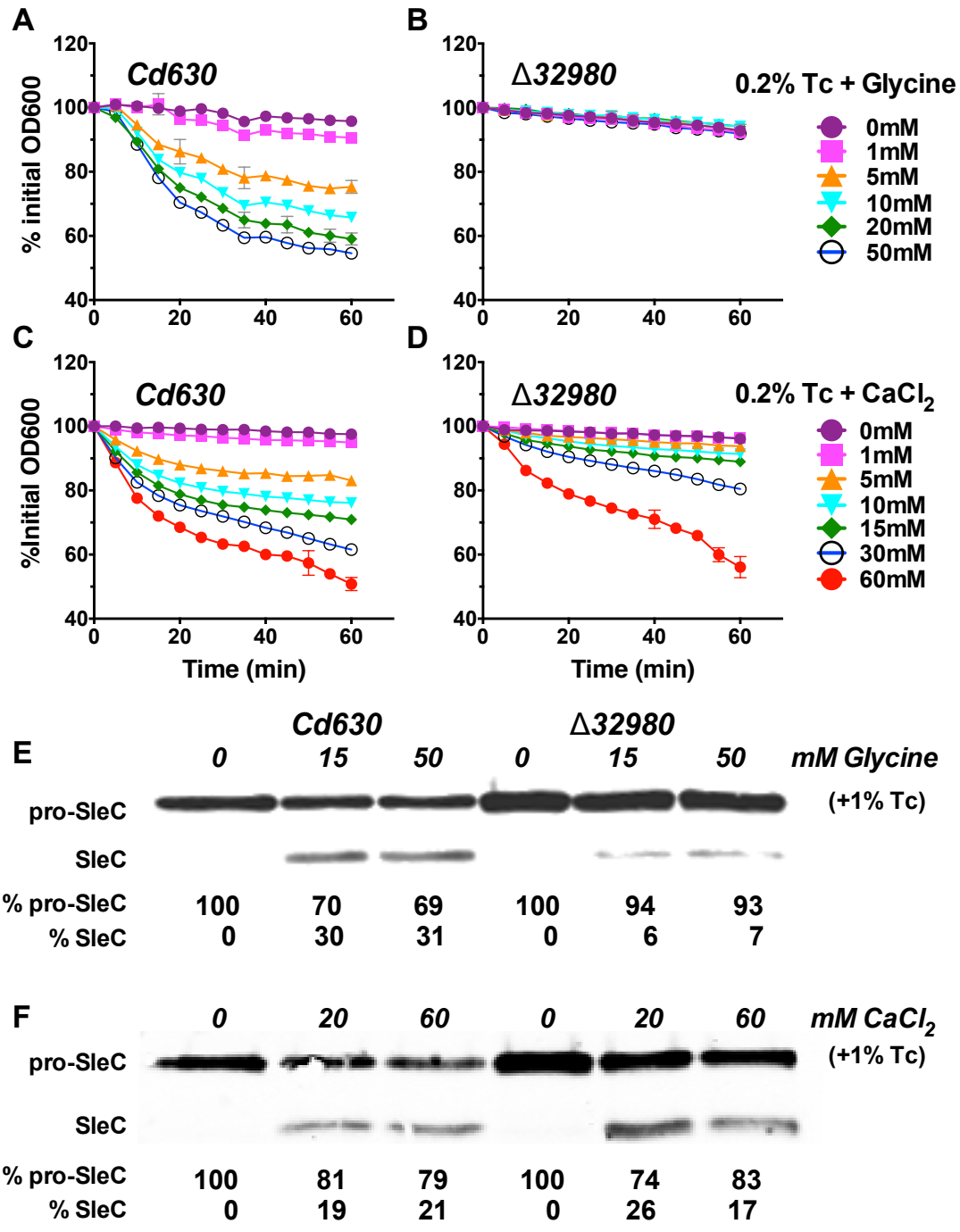

Figure 2.6 *Cd630_32980* is required for Tc-Gly induced germination.
WT and Δ*32980* spores were incubated with 0.2% Tc and the indicated concentrations of glycine (a-b) or $CaCl_2$ (c-d) with germination was measured by loss of OD_{600} at 37°C over the course of an hour. Western blot assessing SleC activation from WT and Δ*32980* spores incubated at 37°C for 15 minutes with 1% Tc and the indicated concentrations of glycine (e) or $CaCl_2$ (f). %pro-SleC and % SleC relative densities were calculated for each lane using ImageJ by determining the ratio of each band to the total density for the two bands combined. Blots are representative of 3 experiments. Germination assays were performed in triplicate and are representative of 3 independent spore preps. Error bars are mean plus or minus SD.

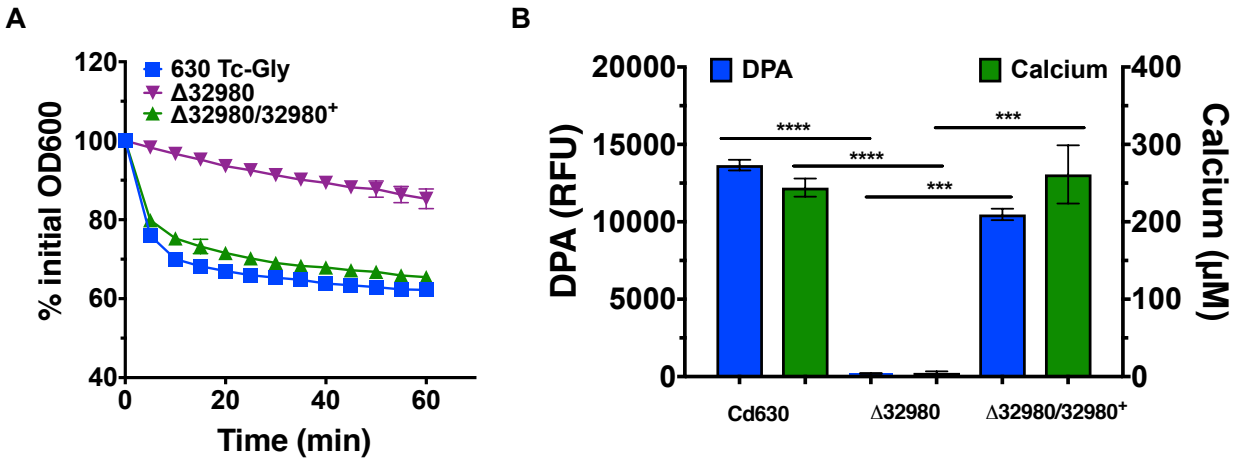

Figure 2.7 Complementation of Cd630:*Δ*32980.
Cd630, Δ*32980*, or Δ*32980*/*32980*$^{+}$ spores were incubated at 37°C with 0.2% Tc and 50 mM Glycine (A). Germination was tracked by loss of optical density. Time-points were taken every 5 minutes for one hour. Cd630, Δ*32980*, or Δ*32980*/*32980*$^{+}$ spores were incubated at 100°C for 20 minutes and total DPA content was measured using Terbium fluorescence and calcium content was measured using a colorimetric assay (B). DPA release units are displayed as relative fluorescent units. Calcium concentrations were determined using a standard curve. Germination assays were performed in triplicate and are representative of 3 independent spore preps. Error bars are mean plus or minus SD. Statistical significance was calculated using one-way ANOVA (****) $p<0.0001$ (***) $p<0.001$.

Cd630_32980 expression occurs in the mother cell and is controlled by the sporulation sigma factor SigE. *Cd630_32980* encodes a AAA+ ATPase that is associated with a putative type-4 secretion system predicted to be essential for sporulation (132). I hypothesized that Cd630_32980 may be involved in transport of nutrients (including DPA) from the mother cell, across the outer forespore membrane into the spore during sporulation. To test this hypothesis, I measured the total amount of DPA that had been packaged into mature spores using a terbium fluorescence assay following a 30-minute boiling step to release internal stores of DPA. Δ*32980* spores contained <1% of the DPA content found in Cd630 spores but was rescued by expressing *Cd630_32980 in trans* (S3 Fig). These data indicate that Cd630_32980 is essential for the proper packaging of DPA in the spore. In addition, these data may explain why spore yields were low, as spores low in DPA are less dense and do not pellet readily during spore purification in 50% histodenz (see methods). Previous studies have demonstrated that Ca^{2+} and DPA are packaged in a 1:1 ratio and spores with less DPA have less internal stores of calcium (129, 133). To measure levels of calcium in mature spores I used a calcium colorimetric assay following a 30-minute boiling step to release internal stores of calcium. Δ*32980* spores contained ~5 μM calcium compared to ~250 μM calcium in either Cd630 or *Cd630_32980* complemented spores (Figure 2.7 B). Taken with these data, our results suggest that DPA is essential for packaging of calcium into the *C. difficile* spore core and this calcium is essential for Tc-Gly germination. The role of Cd630_32980 in sporulation was assessed in Appendix A.

Calcium And Glycine Synergize With Bile Salts To Induce *C. difficile* Germination

Since Tc, glycine, and calcium are present within the host intestine I hypothesize that they function together to induce *C. difficile* germination. To test this hypothesis, I treated Cd630 spores with suboptimal concentrations of Tc (0.05%), glycine (5mM), or calcium (5mM). At these concentrations, Cd630 spores do not germinate in response to Tc-Gly or Tc-$CaCl_2$. However, they germinated (~30% drop in OD) in response to the combination of Tc, glycine, and calcium (Figure 2.8), indicating that glycine and calcium can synergize to induce *C. difficile* germination in the presence of Tc. Because complex growth media (e.g., BHIS, etc.) are commonly used as a germination media, and they typically contain both amino acids and calcium, I hypothesized that *C. difficile* germination in BHIS+Tc is due to the synergy between calcium and glycine. To test this hypothesis, I measured the concentration of calcium present in BHIS (0.4mM), calcium-depleted BHIS (CDP; 0mM), and calcium-replete BHIS (CRP; 1mM) (Figure 2.9 C) prior to measuring Cd630 or *B. anthracis* Sterne 34F_2 spore germination in each medium. Cd630 spores germinated in BHIS+Tc and CRP+Tc but did not germinate in CDP+Tc (Figure 2.9 A,B). This indicates that the amino acid concentrations found in BHIS are insufficient for *C. difficile* germination in the absence of calcium. In addition, the concentration of calcium in BHIS (0.4mM) is insufficient to induce germination on its own (Figure 2.6 C) suggesting that germination in BHIS+Tc is due to calcium-glycine synergy. In contrast, *B. anthracis* spores germinated fully in both BHIS and CDP (Figure 2.9 A). These data indicate that *C. difficile* germination in BHIS is due to the combination of available amino acids and calcium while *B. anthracis* (which is not responsive to calcium, Figure 2.1 H) germinates in response to amino acids and other nutrients available. Interestingly, Δ*32980* spores germinate slightly

(~20% drop in OD) in BHIS+Tc but not in CDP+Tc (Figure 2.9 D). This indicates that calcium present in BHIS is sufficient to induce slight germination in a strain that is lacking DPA as also reported by Donney et al. (39). In accordance with these data, I propose that within the host intestine, (where Tc, glycine, and calcium are all present) these stimuli function to decrease the concentrations of individual germinants required for *C. difficile* germination and thus colonization within the host.

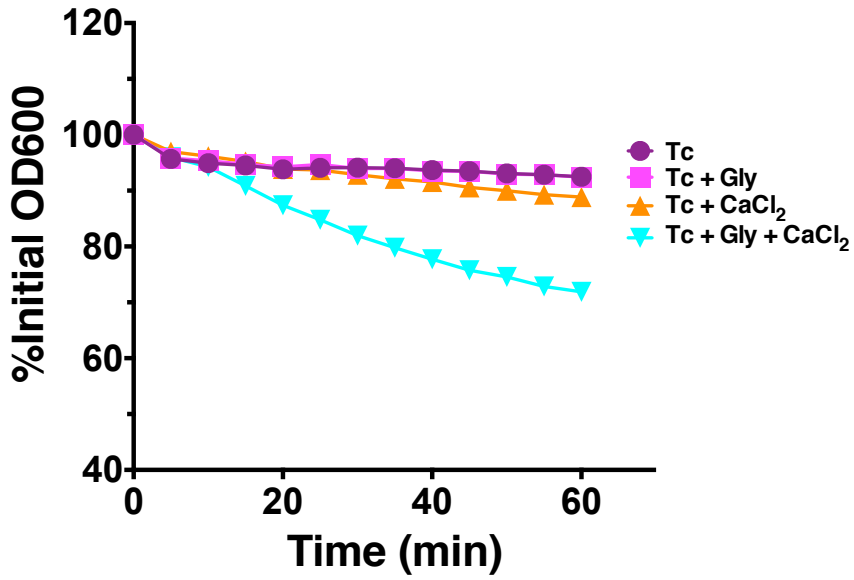

Figure 2.8 Calcium and glycine synergize with bile salts to induce *C. difficile* germination
Cd630 spores were incubated at 37°C with suboptimal concentrations of Tc (0.05%), Glycine (5 mM), or Calcium (5 mM). Germination was tracked by loss of optical density. Time-points were taken every 5 minutes for one hour. Germination assays were performed in triplicate and are representative of 3 independent spore preps. Error bars are mean plus or minus SD.

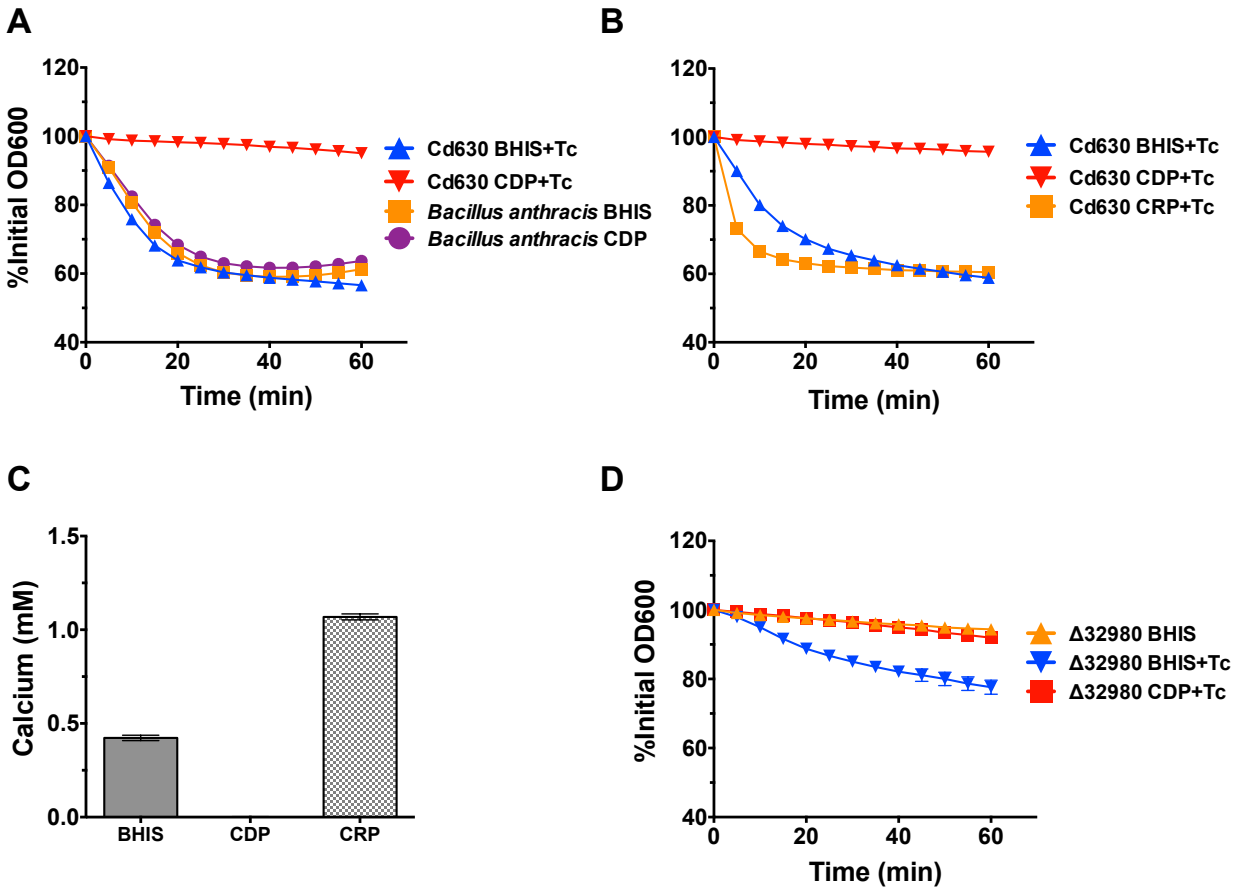

Figure 2.9 Germination in BHIS is due to Calcium/Glycine synergy.
Sterne 34F2 spores were incubated in BHIS or Calcium Deplete BHIS (CDP) (A). Cd630 spores were incubated in BHIS, CDP, or CRP supplemented with 0.2% Tc (A, B). Calcium concentrations of BHIS, CDP, or Calcium Replete BHIS (CRP) were measured using a calcium colorimetric assay. Calcium concentrations were calculated using a calcium standard curve (C). Δ*32980* spores were incubated at 37°C in BHIS, CDP, or CRP supplemented with 0.2% Tc (D). Germination was measured by loss of optical density. Time-points were taken at 37°C every 5 minutes for one hour (A, B, D). Germination assays were performed in triplicate and are representative of 3 independent spore preps. Error bars are mean plus or minus SD.

Intestinal Calcium Plays A Key Role During *in vivo* Germination In A Murine Model Of CDI

Since bile salts, glycine, and calcium each play a role in *C. difficile* germination and synergize to increase germination levels at low concentrations, I hypothesize that dietary calcium (800-1300 mg/day)(134) coordinates with Tc (0.03%) (8), in the host intestines. In order to test this hypothesis, mice were pre-sensitized to *C. difficile* colonization with antibiotic therapy (135) (see methods) and inoculated with either Cd630 or Δ*32980* spores (which is deficient for endogenous calcium). Mice infected with either strain exhibited similar levels of *C. difficile* in the stool (Figure 2.10 A) indicating that Cd630_32980, endogenous calcium, and therefore Tc-Gly-induced germination, are not essential for *in vivo* germination in our murine model. These data, in combination with our *in vitro* findings, suggest that intestinal calcium plays a role in *C. difficile* germination bypassing the requirement for glycine. To directly test if intestinal calcium plays a role in *C. difficile* spore germination, ileal contents were collected from antibiotic-treated, non-infected mice, calcium was depleted, and *ex vivo* germination assays were performed. In mouse ileal contents, that were found to contain ~15mM calcium, 100% of both Cd630 and Δ*32980* spores germinated within one hour (Figure 2.10 B). However, when calcium levels were depleted using chelex resin (Figure 2.10 C), only 10% of WT and no Δ*32980* spores germinated (Figure 2.10 B). Complete germination was restored through the addition of supplemental calcium (~15mM), for both Cd630 and Δ*32980* spores (Figure 2.10 B). These data indicate that calcium in the intestines is required for efficient *C. difficile* spore germination. Taken together, these data show that intestinal calcium is a key molecule involved in *C. difficile* germination and imply that modulation of intestinal calcium represents a means to decrease germination, colonization, and pathogenesis.

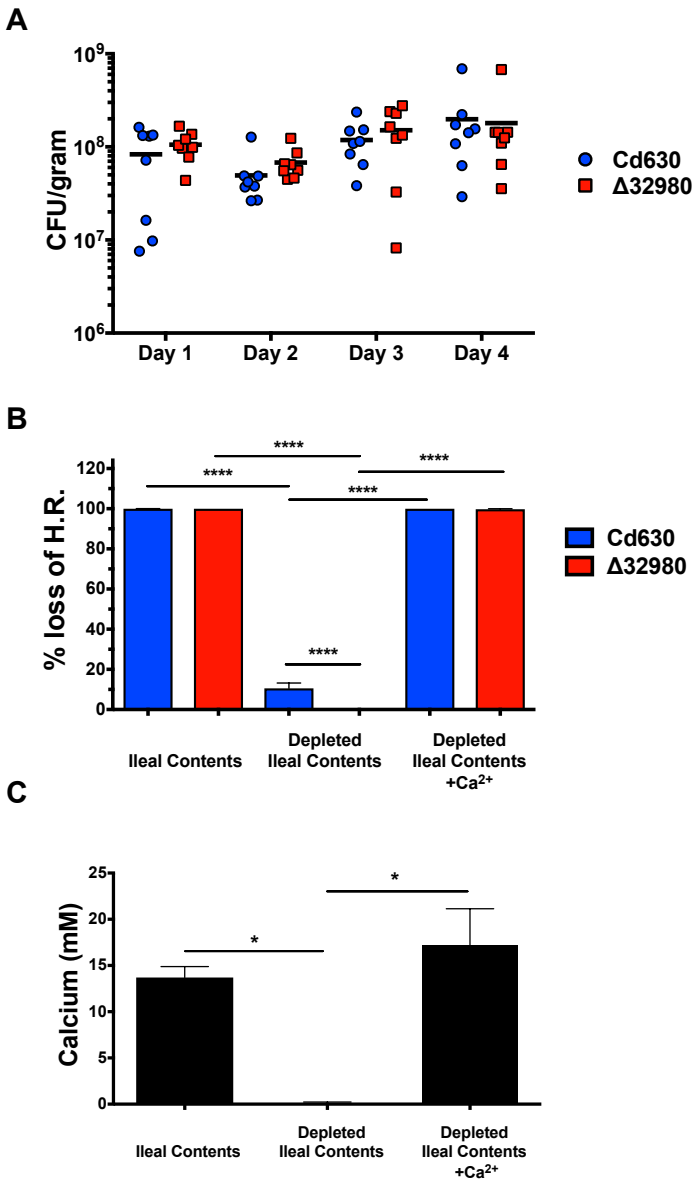

Figure 2.10 Tc-Gly induced germination is dispensable for in vivo germination of *C. difficile* spores. Cefoperazone-treated C57BL/6 mice (n=8) were infected with 1500 spores of WT or Δ*32980* by oral gavage. Colonization levels were assessed daily and are presented in total CFU per gram of feces. (a) Multiple t tests were performed and p= 0.23 for each time point tested (a). Ileal contents were collected from Cefoperazone treated C57BL/6 mice (n=3) and ex vivo germination assays were performed. $1x10^3$ spores of Cd630 or Δ*32980* were incubated for one hour at 37°C in ileal contents, calcium depleted ileal contents or calcium depleted ileal contents treated with 15 mM $CaCl_2$. Samples were then incubated at 65°C for 20 min and then plated on BHIS-Tc plates. Data are presented as % loss of Heat Resistance (b). Free calcium levels of ileal contents, calcium depleted ileal contents or calcium depleted ileal contents treated with 15 mM $CaCl_2$ were measured using a calcium colorimetric assay. Levels of calcium (mM) were determined using a standard curve (c). Assays were performed in triplicate using ileal contents from three mice. Error bars are mean plus or minus SD. Statistical significance was calculated using Two-way ANOVA . (*) $p<0.05$ (****) $p<0.0001$

Discussion

The unconventional mechanism of *C. difficile* spore germination has remained elusive due to the absence of known germinant receptor orthologues. In this work, I describe a central role for calcium ions in *C. difficile* germination. Our data shows that *C. difficile* spores germinate in response to a combination of bile salts and intestinal calcium. Amino acid concentrations within the mouse gastrointestinal tract are inadequate to support high levels of germination independent of intestinal calcium. This is the first report of intestinal calcium playing a vital role in *C. difficile* spore germination. I also provide evidence that endogenous calcium ions released from the spore core in response to Tc-Gly can serve as a germination signal by activating cortex hydrolysis. The putative AAA+ ATPase encoded by *Cd630_32980* is essential for proper packaging of DPA during sporulation as well as germination in response to Tc-Gly (but not Tc-$CaCl_2$). Despite the lack of glycine-induced germination, Δ*32980* spores retain the ability to germinate in *ex vivo* ileal contents and colonize the mouse gastrointestinal tract to levels identical to that of Cd630. In calcium-depleted ileal contents, no mutant spores germinated and Cd630 spores had a 90% reduction in germination. Our data supports the hypothesis that *C. difficile* germination *in vivo* occurs due to synergistic effects between bile salts, glycine, and calcium. The data presented here suggest that the role of glycine in *C. difficile* germination is to facilitate calcium release from the spore core (Figure 2.11) and that this mechanism can be circumvented with the addition of exogenous calcium. In short, *C. difficile* spore germination requires calcium that can be provided by either environmental or endogenous sources. Collectively, these data suggest that restricting free intestinal calcium in susceptible patients is a potential prophylactic treatment to inhibit human CDI.

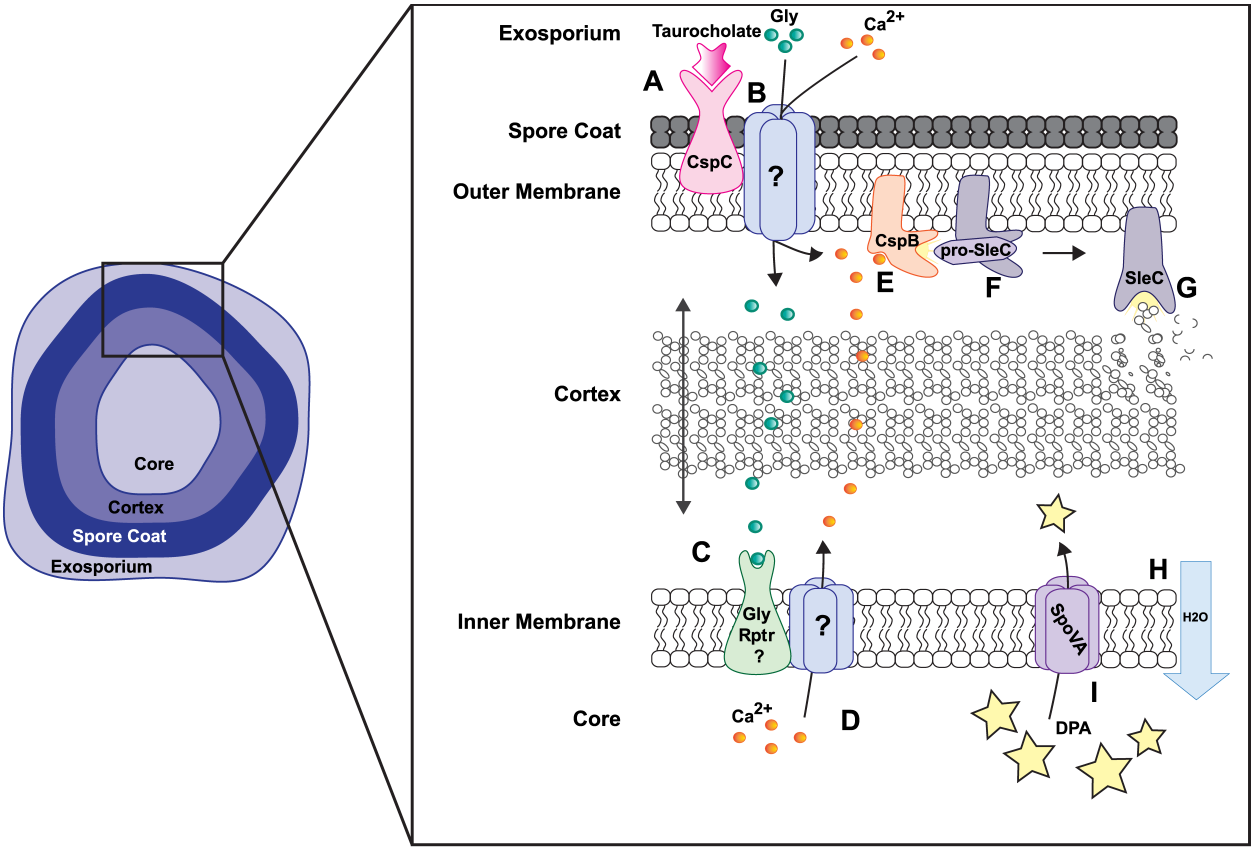

Figure 2.11 Proposed model for the role of calcium in *C. difficile* germination.
Tc binds to CspC (A) facilitating movement of glycine or calcium through the spore coat and outer membrane (B). Glycine then interacts with an unknown receptor (C) inducing the release of Ca^{2+} from the spore core (D). Ca^{2+} from the environment or the spore core activates CspB (E), which processes pro-SleC (F) subsequently initiating cortex hydrolysis (G). This leads to full core rehydration (H), complete release of DPA (I) and spore outgrowth.

While recent studies have begun to elucidate *C. difficile* germination, the mechanisms responsible for initiating these critical events are not completely understood. The subtilisin-like CspBAC family of proteases, a novel lipoprotein regulator GerS, and the cortex lytic enzyme SleC have been identified as key regulators in *C. difficile* germination (68, 94, 117, 127). Each of these proteins are essential for germination in response to Tc-Gly and, as described here, Tc-$CaCl_2$. The identical role for these proteins in both germination mechanisms indicates that Tc-$CaCl_2$ triggers germination upstream of CspB and downstream of the unidentified glycine receptor (Figure 2.11 B). In our proposed model, Tc binds to CspC, (Figure 2.11 A) allowing environmental calcium and/or amino acids to penetrate the spore coat (Figure 2.11 B). While there are two hypotheses for the function of CspC—directly activating CspB or facilitating germinant entry into the spore—I prefer the latter explanation but do not want to discount the possibility that CspC functions to activate CspB. Our data support the hypothesis that glycine interacts with an unknown receptor (Figure 2.11 C) and induces the release of calcium ions from the spore core (Figure 2.11 D). Calcium released from within the spore, and/or calcium from the environment, then activates the subtilisin protease, CspB (Figure 2.11 E), which induces SleC zymogen processing and activation (Figure 2.11 F). Activated SleC then hydrolyzes the spore cortex, leading to full-core rehydration, DPA release through SpoVA channels (39), and spore outgrowth (Figure 2.11 H,I).

Since exogenous $CaCl_2$ initiates cortex hydrolysis through CspB and SleC activation (Figure 2.1 K & 3D), and EGTA treatment inhibits SleC activation (Figure 2.3 D), I hypothesize that calcium is a necessary cofactor for CspB activity. Calcium and the activity of calcium dependent enzymes are required for *C. difficile* germination as demonstrated by EGTA calcium chelation and CPZ treatment studies (Figure 2.3 A-C, 2.4 C-D) demonstrating a requirement for

calcium. EGTA pre-treatment and Phenamil treatment studies indicate that calcium efflux from the spore core is required for Tc-Gly induced germination (Figure 2.4 A,B). Exogenous Tc-calcium induced germination at a maximal rate of 5.2% germination/min and the concentration of calcium required to reach half of the maximal germination rate is 12.6mM (extrapolated (71) from Figure 2.6 C). The concentration of exogenous calcium required to induce germination then is much higher than the concentration of EGTA (50 μM) needed to chelate endogenous calcium and inhibit Tc-Gly germination. This discrepancy could be partially explained by low permeability of the spore coat to exogenous calcium thus, greater concentrations of exogenous calcium are required. I also speculate that endogenous calcium, following release from the spore core, could participate in a positive feedback loop inducing release of more calcium until a threshold concentration is achieved and CspB is activated. Very low concentrations of EGTA could interrupt this positive feedback loop. These data support our model that calcium is vital for SleC activation and thus *C. difficile* germination.

Clinical and epidemiological studies have demonstrated a correlation between risk of CDI and patients who have defects in calcium absorption. For example, patients taking proton pump inhibitors (PPIs) have an elevated risk of contracting CDI (136-139). PPIs are commonly prescribed to ICU patients for numerous reasons (*e.g.,* peptic ulcer diseases, upper gastrointestinal bleeding, stress ulcer prophylaxis), many of these patients may also be on antibiotics. A known side effect of this treatment is decreased calcium absorption in the small intestine resulting in increased luminal concentrations of calcium (140, 141). In light of our results, it is possible that elevated levels of intestinal Ca^{2+} in these individuals allows for more efficient germination of *C. difficile* in the small intestines and therefore increased risk of infection. Additionally, a potential correlation between age, dietary calcium, and CDI exists as

elderly individuals exhibit decreased absorption of calcium and have a significantly higher incidence and severity of CDI. Finally, patients deficient in vitamin D, which is required for calcium absorption from the gut, are five times more likely to contract CDI (142, 143). While the classical role of vitamin D is to facilitate calcium absorption in the small intestines (144, 145), it also plays a role in innate immunity and the regulation of mucosal immunity (146, 147). A deficiency in vitamin D could increase the risk of *C. difficile* colonization through elevated levels of available germinant (calcium) in the intestines and an inefficient immune response leading to severe disease.

The *C. difficile* germination mechanism presented here, together with clinical correlations regarding calcium absorption, suggests an exploitable target for the development of new therapeutic strategies. Decreasing intestinal calcium levels in at-risk patients (*e.g.,* by vitamin D supplementation to improve calcium absorption, (143)) represents a novel prophylactic approach for influencing establishment, outcome, or recurrence of CDIs. In contrast, increasing intestinal calcium levels during CDI treatment could facilitate germination of resident spores, rendering them susceptible to antibiotic treatment, and thus decreasing both spore dissemination and incidence of recurrent CDI. In light of our findings, future studies are warranted to determine the clinical benefits of modulating intestinal calcium levels to improve CDI outcomes.

Materials and Methods

Bacterial Strains And Growth Conditions

C. difficile strains used in this study are described in Table 2.1 *C. difficile* was grown in an anaerobic chamber (10% hydrogen, 5% CO_2, 85% N_2) (Coy Lab Products, MI) at 37°C in brain-heart infusion broth (BD Life Sciences) supplemented with 0.5% yeast extract (BD Life

Sciences) and 0.1% cysteine (Sigma-Aldrich) (BHIS). *Escherichia coli* strains were grown at 37°C in Luria-Bertani (LB) broth (BD Life Sciences) or LB agar (Fisher Scientific) supplemented with the appropriate antibiotics. All antibiotics were purchased from Sigma-Aldrich and used at the following concentrations: for *C. difficile,* thiamphenicol (15 μg/mL); for *E. coli,* ampicillin (50 μg/mL), chloramphenicol (25 μg/mL). Conjugations are plated on BHIS plates supplemented with cefoxitin (8μg/mL), D-cycloserine (250 μg/mL), and thiamphenicol (*15* μg/mL) (CCT). Secondary crossovers were selected by plating on chemically defined media plates (CDMM) supplemented with fluorocytosine (50 μg/mL) (148).

Table 2.1 Bacterial strains

Strain #	Strain	Relevant Genotype	Source
***C. difficile* strains**			
	Cd630		ATCC BAA-1382
	R20291		(149)
	VPI 10463		ATCC 43255
94	Δ*cspC*	Cd630:*Δ22460*	This Study
121	Δ*cspB*	Cd630:*Δ13550*	This Study
85	*ΔgerS*	Cd630:*Δ34640*	This Study
107	*ΔsleC*	Cd630:*Δ05510*	This Study
97	*Δ32980*	Cd630:*Δ32980*	This Study
144	*Δ32980:32980*$^{+}$	Cd630:*Δ32980* + pMTL-83151+32980^{+} Thi^{R}	This Study
***E.coli* strains**			
SD46	HB101	pRk24 Amp^{R}	(148)
84	HB101	pMTL-SC7215-cspC Cam^{R} pRk24 Amp^{R}	This Study
120	HB101	pMTL-SC7215-cspB Cam^{R} pRk24 Amp^{R}	This Study
106	HB101	pMTL-SC7215-sleC Cam^{R} pRk24 Amp^{R}	This Study
71	HB101	pMTL-SC7215-gerS Cam^{R} pRk24 Amp^{R}	This Study
152	HB101	pMTL-SC7215-32980 Cam^{R} pRk24 Amp^{R}	This Study
148	HB101	pMTL-83151+32980^{+}32980 Cam^{R} pRk24 Amp^{R}	This Study
***B. anthracis* strains**			
	Sterne 34F2		(150)
Plasmids			
	pMTL-SC7215	Cam^{R}	(148)
	pMTL-83151	Cam^{R}	(148)
	pMTL-83151+32980^{+}	Cam^{R}	This Study

Spore Production And Purification

Spores were generated as follows: *C. difficile* was allowed to grow overnight at 30°C in Columbia broth (BD Life Sciences) and 2mL of culture were added to 38 mL of Clospore sporulation media (151) then incubated at 37°C for 6 days. Spores were collected by centrifugation at 4,000 RPM and washed 3 times with sterile distilled, deionized water (ddH_2O, Millipore). To remove vegetative cells and debris, spore pellets were re-suspended in 50% Histodenz (Sigma-Aldrich) and centrifuged at 13,200 RPM (117). Supernatant was discarded and spore pellets washed 3 times with sterile ddH_20. Spore purity was >95% as confirmed by phase contrast microscopy.

Cloning And Construction Of *C. difficile* Mutants

Clean unmarked deletions in *C. difficile* were created using a protocol modified from *Cartman et al.* (148). Briefly, 1,000 bp fragments of DNA flanking the target gene were Gibson cloned into the NotI site of plasmid pMTL-SC7215 (Gibson Assembly Master Mix, New England Biosciences). Plasmids were conjugated into Cd630 via the *E. coli* strain HB101 which harbors the conjugative plasmid pRk24 (148). Conjugations were plated on BHIS for 24 hours to allow transfer of the plasmid. Bacterial growth was scraped off and plated on CCT for 2 days and colonies were picked and re-streaked for isolation. Primary insertions were confirmed via PCR using primers described in Table 2.2. A pure culture of an isolate with a confirmed primary insertion was then plated on BHIS without selection overnight to allow for secondary crossover events to occur. Secondary crossovers were selected by plating on CDMM supplemented with fluorocytosine, selecting against colonies carrying the original knockout plasmid. Single colonies were picked and screened for deletion of targeted genes by PCR.

Table 2.2 Primers

Primer	Sequence	Description
1	AATTTTTTTATCAGGAAACAGCTATGACCGCCCTATGTACTGTTCTTTTTCTCTG	P1 Forward Primer to amplify 1000bp upstream cspC
2	GGATTTATGGAAAAAACTCTATAGATAAGAACCTATGTAA	P2 Reverse Primer to amplify 1000bp upstream cspC
3	TCTTATCTATAGAGTTTTTTCCATAAATCCCTCCTATCTT	P3 Forward Primer to amplify 1000bp downstream cspC
4	GTAATCATGGTCATATGGATACAGCGGCCTGCATCTCAGTATGCATATAAAAAA	P4 Reverse Primer to amplify 1000bp downstream cspC
5	AAAAGTATTACTTCTTGGTCTTAAATGTGCTATCGTT	P5 Forward primer to amplify cspC
6	ATAATAAAACTCGGCAAGATAGATGATTTTTATAATAGTG	P6 Reverse primer to amplify cspC
7	AATTTTTTTATCAGGAAACAGCTATGACCGTCTTGATAAATTAAGAGCTAAATTG	P1 Forward Primer to amplify 1000bp upstream cspB
8	TCCCTCCTATCTTAATAAAATGTATTAGACTATATAATTT	P2 Reverse Primer to amplify 1000bp upstream cspB
9	GTCTAATACATTTTATTAAGATAGGAGGGATTTATGGAAA	P3 Forward Primer to amplify 1000bp downstream cspB
10	GTAATCATGGTCATATGGATACAGCGGCCAACTTCACCTGAAGGAGATATTATC	P4 Reverse Primer to amplify 1000bp downstream cspB
11	AAGACACTTATAGTTAATAGGAGCTGGATAATTATGTT	P5 Forward primer to amplify cspB
12	TTCCTCTATATATATAAAAGTCTGGAGAATACCTTATATCA	P6 Reverse primer to amplify cspB
13	TTTGTATAAGGATTGGAAAATACCA	P1 Forward Primer to amplify 1000bp upstream SleC
14	GCAAGATTTAATTTAAAGCTTGATTTAGAACATAAACTCAAAT	P2 Reverse Primer to amplify 1000bp upstream SleC
15	AATTAAATCTTGCATCAAATCACCCTTTCTTTAAATGAAT	P3 Forward Primer to amplify 1000bp downstream SleC
16	TGTGTGTCATTTATTATACTTTAAT	P4 Reverse Primer to amplify 1000bp downstream SleC
17	GTATTTATAAATATGAAAAAAGGCGATTTTATATGG	P5 Forward primer to amplify SleC
18	CCTTTGGTAATTCTGTCAAATCTATAATCCC	P6 Reverse primer to amplify SleC
19	AATTTTTTTATCAGGAAACAGCTATGACCGCTAACCATTATTTGATCCATACATA	P1 Forward Primer to amplify 1000bp upstream gerS
20	TGTATTATGTTCTTGAGAAACTAAGGGGGACTAAAGACAT	P2 Reverse Primer to amplify 1000bp upstream gerS
21	TCCCCCTTAGTTTCTCAAGAACATAATACATACTATGGTC	P3 Forward Primer to amplify 1000bp downstream gerS
22	GTAATCATGGTCATATGGATACAGCGGCCTAAATAATAATGTGTATTATCGATA	P4 Reverse Primer to amplify 1000bp downstream gerS
23	TCTTTTTCTGAGTTATATAGAACTGAAAAGCAGC	P5 Forward primer to amplify gerS
24	TGCTTAGTTGTGTAGTATAATAAATAATATAATAGATTTGTATATAATATTC	P6 Reverse primer to amplify gerS
25	AATTTTTTTATCAGGAAACAGCTATGACCGATACGTTTATAAGTTTCCTTCCATATTAGCGCCTCCT	P1 Forward Primer to amplify 1000bp upstream 32980
26	CTACAGCTCCAGTGATTCCTATAGC	P2 Reverse Primer to amplify 1000bp upstream 32980
27	TGCTTATTTTCATTGGATAATTGGT	P3 Forward Primer to amplify 1000bp downstream 32980

28	GTAATCATGGTCATATGGATACAGCGGCC GCGCTAATATGGAAGGAAACTTATAAACGTATATGTTT AAAATT	P4 Reverse Primer to amplify 1000bp downstream 32980
29	CCTTTAGTTCTCCATCTACCCTCATTCTCA	P5 Forward primer to amplify 32980
30	CTAAAATTATACCTCCAATTACAGGACCACTCAT	P6 Reverse primer to amplify 32980
31	AAATTTTATAAAATAGTTTTATCTACAATTTTTTTATCA GGA	Not diagnostic primer
32	TAGTggatccTTATTCTACCTCTACCATAAGCATCT	Forward primer to amplify 500 bp upstream 32980
33	ATACTcTcgagGGATATTCTGCAATTAATATTTTAAACAG TATAGTTA	Reverse primer to amplify 32980
34	ATAggATCCATTTTTGTTTCATGATAACACTCCAATCTT	Forward primer to complement 32980
35	CATctcgagTAAACATATACGTTTATAAGTTTTCCTCA	Reverse primer to complement 32980

Loss Of OD Germination Assay

Germination was measured by tracking the loss of optical density at 600 nm over time at 37° C in a Spectramax M2 microplate reader (Molecular Devices). Loss of OD following full rehydration of the core is a known indicator of spore germination (68, 115, 152). Purified spores were added to phosphate buffer saline (PBS, Invitrogen) with the indicated germinants at a starting OD of ~0.5. For Ca-DPA or $CaCl_2$ induced germination, spores were added to 50 mM Tris-HCl to a pH of 7.4 (Sigma-Aldrich) plus the indicated germinants. The OD_{600} was taken every 5 minutes for one hour with the results reported as percent initial OD_{600}. Assays were performed in triplicate.

Germination Inhibitor Studies

Germination inhibitors, (Phenamil, EGTA, or Chlorpromazine) were purchased from Sigma-Aldrich. EGTA was solubilized in 100 mM Tris-HCl, pH 7.4. Phenamil was re-suspended in 100% DMSO. All germination assays in which Phenamil was used were conducted in 10% DMSO. Chlorpromazine is freely soluble in water.

Loss Of Heat Resistance Assay

Germination was also measured by loss of heat resistance after one hour. $1x10^3$ Cd630 spores were incubated with 0.2% Tc (Sigma-Aldrich) and 50 mM glycine (Sigma-Aldrich) or 60 mM $CaCl_2$ (Sigma-Aldrich) at 37° C. After one hour, samples were heat treated at 65°C for 20 minutes, serially diluted in PBS, and plated on BHIS+Tc plates. Non-heat-treated samples (total spores) were also plated on BHIS+Tc. Data are reported as a percentage of the total spores that lost the heat resistance properties of dormant spores.

Western Blot Analysis

Zymogen processing of SleC was detected by western blot using an anti-SleC antibody (94) graciously provided by Dr. Aimee Shen, Tufts University. Here, $1x10^6$ spores were added to the indicated germination mixture and incubated at 37°C for 15 minutes. Spores were then pelleted and re-suspended in 100 µL EBB lysis buffer (9M urea, 2M thiourea, 4% SDS, and 10% β-mercaptoethanol) prior to the addition of 10 µL of 4x loading buffer to the protein lysates(94). Proteins were separated on a 4-12% SDS-PAGE gel and transferred to a 0.22 µm nitrocellulose membrane (Whatman). Membranes were blocked for one hour in Odyssey blocking buffer (LI-COR) then probed for one hour at room temperature with an anti-SleC antibody (1:5000). Membranes were washed 3 times for 10 minutes in TBS-T. Goat anti-rabbit IR800 secondary antibodies (LI-COR) were added at a 1:20,000 dilution and incubated at room temperature for an hour. The membranes were washed a minimum of 3 times with TBS-T before LICOR detection (Odyssey).

Monitoring DPA Release Assay

DPA release from the spore core was measured using terbium fluorescence (115). Cd630 spores were incubated with 0.2% Tc and 50mM glycine in PBS or 60mM $CaCl_2$ in Tris-HCl at 37°C for 1 hour. Germinant solutions were supplemented with 800µM $TbCl_3$ (Sigma Aldrich) to measure Ca-DPA release in real-time using a spectramax M2 microplate reader (Molecular Devices) (excitation 270 nm, emission 545 nm, cutoff 420 nm). Data is presented as relative fluorescent units. For measuring total amounts of DPA packaged into spores, $1x10^8$ Cd630 spores were incubated at 100°C for 30 minutes. Boiled samples were supplemented with 800µM $TbCl_3$ and DPA release was measured.

Murine Model Of *Clostridium difficile* Colitis

8-week old C57BL/6 mice were given cefoperazone (Sigma-Aldrich) (0.5 mg/ml) in sterile drinking water for five days which was refreshed every other day (135). Mice were then switched to regular drinking water, allowed to recover for 2 days prior to *C. difficile* infection. For *ex vivo* germination assays, uninfected mice (n=3) were euthanized, and at the time of necropsy ileal contents were collected, and frozen at -80°C until further analysis. For *in vivo* infections, groups of mice (n=8) were inoculated by oral gavage with 50µL of water containing approximately 1500 spores as determined by phase contrast microscopy. Feces were collected daily for 4 days and samples weighed, serially diluted, and plated for total CFU per gram of feces (spores + vegetative cells). Samples from day one were also heat treated and assayed for total heat resistant CFU (spores). Mice were placed on a standard diet of Prolab Isopro RMH 3000 (LabDiet, St. Louis, MO) containing 1.1% calcium. C57BL/6 mice consume an average of ~4 g of chow per day (153).

Ex vivo Germination Assays

Mouse ileal contents from uninfected antibiotic treated mice were weighed and diluted 1:1 in PBS. Samples were then freeze-thawed three times to release any available nutrients and centrifuged at 13,200 RPMs for 2 minutes and supernatants were collected. ~$1x10^3$ Cd630 or Δ*32980* spores were added to PBS, diluted ileal contents, calcium depleted ileal contents, or calcium replete ileal contents and incubated at 37°C. After one hour, samples were heat treated at 65°C for 20 minutes, serially diluted in PBS, and plated on BHIS+Tc plates. Non-heat-treated samples (total spores) were also plated on BHIS+Tc. Data are reported as a percentage of the total spores that lost the heat resistance properties of dormant spores.

Calcium Depletion

Calcium was depleted by incubating BHIS or ileal contents for 2 hours with 0.1g/mL chelex 100 resin (BioRad) and then removing the resin by centrifugation for 2 minutes at 13,200 RPMs and collecting the supernatant. Calcium-replete BHIS was made by adding 1mM $CaCl_2$ to calcium-depleted BHIS. Calcium-replete ileal contents were made by adding 15mM $CaCl_2$ to calcium-depleted ileal contents.

Calcium Colorimetric Assay

Calcium levels were measured using a calcium colorimetric assay purchased from Sigma-Aldrich. Ileal contents were diluted 1:200 in PBS to fit into the linear range of the assay. Briefly, 90μL of the chromogenic reagent was added to each well. 50μL of either sample or diluted calcium standard were added to each well. 60μL of calcium assay buffer are then added to each well and samples were incubated for 5-10 minutes at room temperature and absorbance was measured at 575nm in a Spectramax M2 microplate reader (Molecular Devices). Each sample was measured in triplicate.

Ethics Statement

C. difficile mouse infections were performed at the US Food & Drug administration. All animal procedures were approved by the CBER Animal Care and Use Committee (Protocol #2015-08) in accordance with the principles outlined in the Guide for the Care and Use of Laboratory Animals by the Institute for Laboratory Animal Resources, National Research Council. All experiments were performed in an Association for Assessment and Accreditation of Laboratory Animal Care International approved facility. I have calculated that 8 mice per group

are required for power analysis (assuming 80% power) for the desired P value of 0.05, a standard deviation of <10% and a failure rate of <10%.

CHAPTER 3: GERMINANT SYNERGY FACILITATES *CLOSTRIDIUM DIFFICILE SPORE* GERMINATION UNDER PHYSIOLOGICAL CONDITIONS

Modified from:
Kochan TJ, Shoshiev MS, Somers MJ, Plotnick YM, et al. (Under Review) Germinant Synergy Facilitates *Clostridium difficile* Spore Germination Under Physiological Conditions

Abstract:

Clostridium difficile is Gram-positive obligate anaerobe that forms spores in order to survive for long periods in the unfavorable environment outside of a host. *C. difficile* is the leading cause of nosocomial infectious diarrhea worldwide. *C. difficile* infection (CDI) arises after a patient treated with broad spectrum antibiotics ingests infectious spores. The first step in *C. difficile* pathogenesis is the metabolic reactivation of dormant spores within the gastrointestinal tract through a process known as germination. In this work, I aim to elucidate the specific conditions and the location within the GI tract that facilitate this process. Our data suggest that *C. difficile* germination occurs through a two-step biochemical process that is regulated by pH and bile salts, amino acids, and calcium present within the GI tract. Maximal germination occurs at a pH ranging from 6.5-8.5 in the terminal small intestine prior to bile salt and calcium reabsorption by the host. Germination can be initiated by lower concentrations of germinants when spores are incubated with a combination of bile salts, calcium, and amino acids, and this synergy is dependent on the availability of calcium. The synergy described here

allows germination to proceed in the presence of inhibitory bile salts and at physiological concentrations of germinants, effectively decreasing the concentration of nutrients required to initiate an essential step of pathogenesis.

Introduction

The life-cycle of *Clostridium difficile*, like other *Clostridium* and *Bacillus spp.,* consists of transitions between two unique cell morphologies: active vegetative cells and dormant spores. *C. difficile* spores are the infectious morphotype which, upon ingestion by a susceptible host, initiate a *C. difficile* infection (CDI) (2, 3). *C. difficile* produces spores during nutrient deprivation through a process known as sporulation (124). Spores are metabolically dormant and resistant to numerous harsh environmental conditions that are unsuitable for vegetative cell growth (11). Spores can remain dormant for hundreds of years (or more), but they can reactivate within minutes when spores encounter and sense environmental conditions that are suitable for vegetative growth, a process known as germination (12, 60, 77, 154). Germination has been well-studied in a variety of pathogenic spore-forming species since it is the first step in pathogenesis following ingestion of spores (53, 54). The environmental signals, or germinants, that initiate germination vary widely among bacterial species, but they are most commonly small molecule nutrients such as amino acids, sugars or nucleosides (60-62, 124). *C. difficile* is unique among spore-forming bacteria in that it requires a combination of bile salts and either amino acids or divalent cations to initiate germination (88, 90). In most species, germinants are thought to interact with receptors found on the spore inner membrane that initiate a series of biophysical and biochemical changes that facilitate the transition from spore to a metabolically active vegetative bacterium (69). However, unlike other spore formers, orthologs of these inner

membrane receptors (*e.g.*, *gerA*) have not been identified in any sequenced *C. difficile* genomes. Instead, *C. difficile* senses bile salts in the gastrointestinal tract with a unique pseudo-protease receptor, CspC, that is localized, not to the inner membrane, but to the spore coat (68, 117). It has been proposed that downstream signaling occurring after CspC-bile salt interactions leads to proteolytic activation of the cortex hydrolase, SleC, but the molecular details underlying these events remains largely unknown (68, 95, 115).

In *C. difficile*, three distinct germination pathways have been described (88-90, 93, 154). The bile salt amino acid pathway requires relatively high, non-physiological concentrations (millimolar) of both a bile salt and amino acid (88, 92). Taurocholate (Tc) and glycine are the most effective combination, but other amino acids and bile salt combinations have been identified as viable co-germinant pairs (89). The amino acid concentrations required can be decreased to micromolar levels with addition of increased amounts of bile salts and *vice versa* (89). In addition, *C. difficile* spores can germinate in response to bile salts in combination with divalent cations (in the absence of amino acids) (90). Calcium is the most effective cation in inducing germination through the bile salt divalent cation pathway, though magnesium can also induce this process (90). *C. difficile* also maintains an additional unique germination pathway that involves an alanine racemase, Alr2, which allows spores to germinate in response to D-alanine or D-serine by epimerizing them into L-forms which then are able to induce germination in conjunction with bile salts (93). This pathway is described as the alanine racemase-dependent D-amino acid pathway (154). For all the various germination pathways, interactions between bile salts and CspC are a key requirement but the identities of the amino acid and divalent cation receptors remain unknown. While *C. difficile* germination can be activated with a variety of different germinants, all pathways ultimately initiate activation of the same enzymes, CspB and

SleC, resulting in biochemical modifications to the spore that initiate core hydration, release of calcium dipicolinic acid (DPA), replication, and outgrowth out of the spore (39, 94, 115, 127). Several previous studies have analyzed *C. difficile* germination and outgrowth in gastrointestinal contents, in this work, I aim to build on prior work by answering three key questions: how is germination regulated in the gastrointestinal tract, where are *C. difficile* spores sensing as the optimal growth environment, and what role do the various co-germinants play in this process.

Results

Clostridium difficile Spores Germinate In The Mouse Ileum Independent Of Antibiotic Treatment

In order to determine potential locations of efficient *C. difficile* spore germination along the murine gastrointestinal tract, *ex vivo* germination studies were performed using contents isolated from various locations throughout the gastrointestinal tract. Prior to GI content sample collection C57BL/6 mice were either pretreated with or without cefoperazone in order to establish if exposure to antibiotics, a predisposing factor for contracting CDIs, influences germination.

Our data shows that *C. difficile* spores (strain - VPI 10463) were not able to germinate in stomach contents (Figure 3.1). Likely, this is due to low pH and/or the lack of bile salts in the stomach. Minimal germination was observed in spores incubated in contents sampled from the duodenum, cecum, or colon (Figure 3.1). However, 70-90% spore germination was observed from contents sampled from the ileum (Figure 3.1). There were no significant differences in levels of germination found between contents sampled from antibiotic-treated and untreated animals (although there is a slight trend showing enhanced germination with antibiotic treatment)

(Figure 3.1). These data suggest that spore germination occurs in the ileum and that it is independent of microbiota population shifts caused by exposure to antibiotics. The lack of robust germination in the duodenal contents is an unexpected finding and may be due to slightly acidic pH within the duodenum (155, 156). Based on these data we hypothesize that the acidity in the duodenum may be non-conducive for germination and thus pH plays a role in regulating germination within the small intestine.

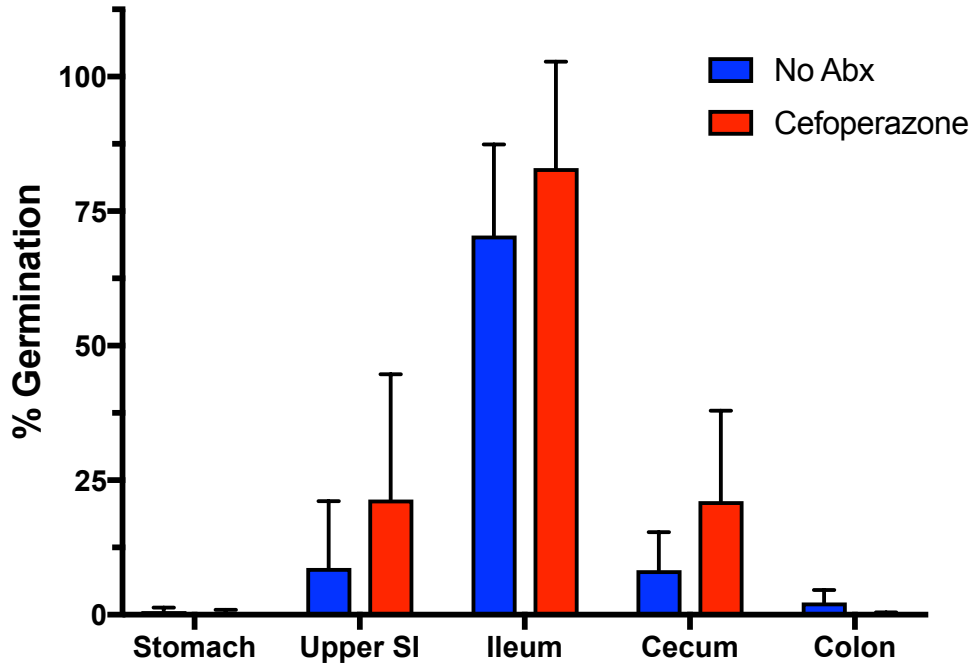

Figure 3.1 *C. difficile* spore germinate in the mouse ileum independent of antibiotic treatment
VPI 10463 spores were incubated in various GI contents from antibiotic and non antibiotic treated mice (n=5) for 1 hour at 37 °C. Data is presented as %Germination which was determined by differential plating (CFU on BHIS/Total CFU on BHIS-Tc x 100). Statistical analyses were done using two-way ANOVA and germination in Ileal contents was significantly different from all other conditions ($p<0.0001$). No significant differences were observed between +/- antibiotics.

Effect Of Temperature And pH On *C. difficile* Spore Germination

Since *C. difficile* spore germination involves critical biochemical processes that are dependent on the activity of at least two enzymes (CspB and SleC), I hypothesized that factors such as temperature and pH could regulate germination in the gastrointestinal tract. One recent study showed that spore germination is sensitive to temperature, with optimal germination occurring at 37°C (89). In order to test the effect of temperature on the germination of *C. difficile*, spores were incubated in rich media with bile salts for 1 hour at various temperatures and germination was assessed by loss of OD. *C. difficile* spores germinated at all temperatures within 1 hour with ~75% germination at 25°C (room temperature) and ~100% germination at 37°C (temperature of the GI tract) or 40°C (temperature of person with fever) (Figure 3.2A). These data show that germination efficiency is improved at temperatures ranging from 37-40°C. However, since temperature is consistent across the gastrointestinal tract, it is unlikely that temperature is an environmental regulator of *C. difficile* spore germination.

Significant changes in pH are expected along the length of the GI tract. In order to assess the role of pH in germination, *C. difficile* spores were incubated in BHIS+Tc adjusted to a range of different pHs (as indicated) for 1 hour at 37°C, and germination was measured by loss of OD assay. Similar to findings from previous studies, *C. difficile* spores exhibited efficient germination when incubated at pH between 6.5 and 8.5, but germinated poorly at a pH 9.5 and not at all below 5.5 or above 10.5 (Figure 3.2B) (157, 158). These data were confirmed by observing the zymogen processing and activation of SleC by western blot. Activation of SleC was observed at pH between 6.5-9.5, no activation of SleC was observed at a pH of 4.5 or 10.5 and only slight activation at a pH of 5.5 (Figure 3.2C). Taken together, these data suggest that either CspB is sensitive to alterations in pH or the events upstream to CspB activation are

sensitive to pH (such as germinant sensing). In addition, the optimal pH for SleC activation and *C. difficile* germination is between 6.5 and 8.5.

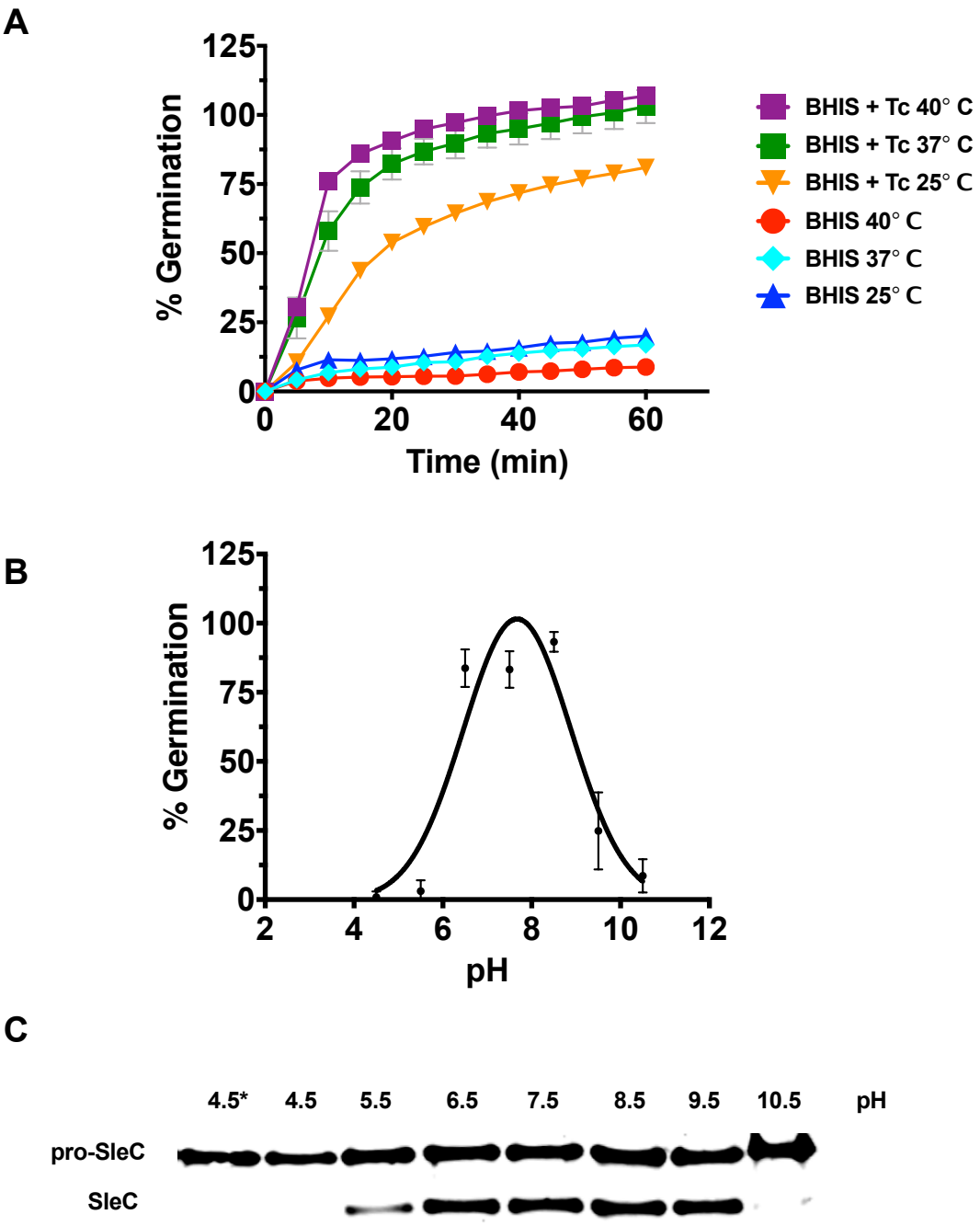

Figure 3.2 Temperature and pH regulate *C. difficile* spore germination *in vitro*
VPI 10463 spores were incubated at the indicated temperatures in BHIS+Tc and OD600 was monitored every 5 minutes for 1 hour (A). VPI 10463 spores were incubated at 37 °C in BHIS+Tc at various pH's for 1 hour and germination was monitored by loss of OD 600 (B). VPI10463 spores were incubated at 37 °C in BHIS+Tc at various pH's for 15 minutes and SleC activation was observed by Western blot (C). Loss of OD assays are the results from 3 independent spore preps and presented as % Germination as described in methods. Western blots are representative of 3 independent spore preps. Samples labeled with * indicates that this sample did not receive taurocholate (C).

C. difficile spores are acid resistant and able to germinate when pH neutralizes. While the conditions in the ileum appear suitable for efficient *C. difficile* spore germination, these spores must first survive and pass through the highly-acidic stomach. Additionally, in duodenal contents, only 25% of *C. difficile* spores are able to germinate even though germinants are present since both bile salts and calcium are secreted into the duodenum through the bile duct. The pH rises from ~2.5 in the stomach to 4.5-5.5 in the duodenum and eventually reaches ~7.4 in the ileum (155, 156). I hypothesize that acidic pH is inhibiting spore germination in the duodenum (even though germinants are present), and then these spores maintain the ability to germinate once the pH is neutralized in the ileum. To test this hypothesis *in vitro*, *C. difficile* spores were exposed to acidic conditions, in the presence of germinants, (BHIS+Tc, pH 4.5) for 60 minutes. Following this incubation, a portion of the spores were removed and the pH was neutralized. No measurable germination was observed during the 1 hour pre-treatment with BHIS+Tc at a pH of 4.5, however a large drop in optical density was observed (~30%) following neutralization of the pH to ~7.5. In comparison minimal drop in OD occurred for spores that remained at a pH of 4.5 germinated in the same time frame (Figure 3.3A). In addition, spore germination was assessed by observing the zymogen processing of SleC. SleC activation was only detected in samples that were adjusted to a pH of 7.5 (Figure 3.3B). Taken together, these data suggest that CspB is inactive at acidic pH, but is not permanently damaged. Once pH approaches its optimal range (6.5-8.5), CspB can again process SleC allowing germination to proceed.

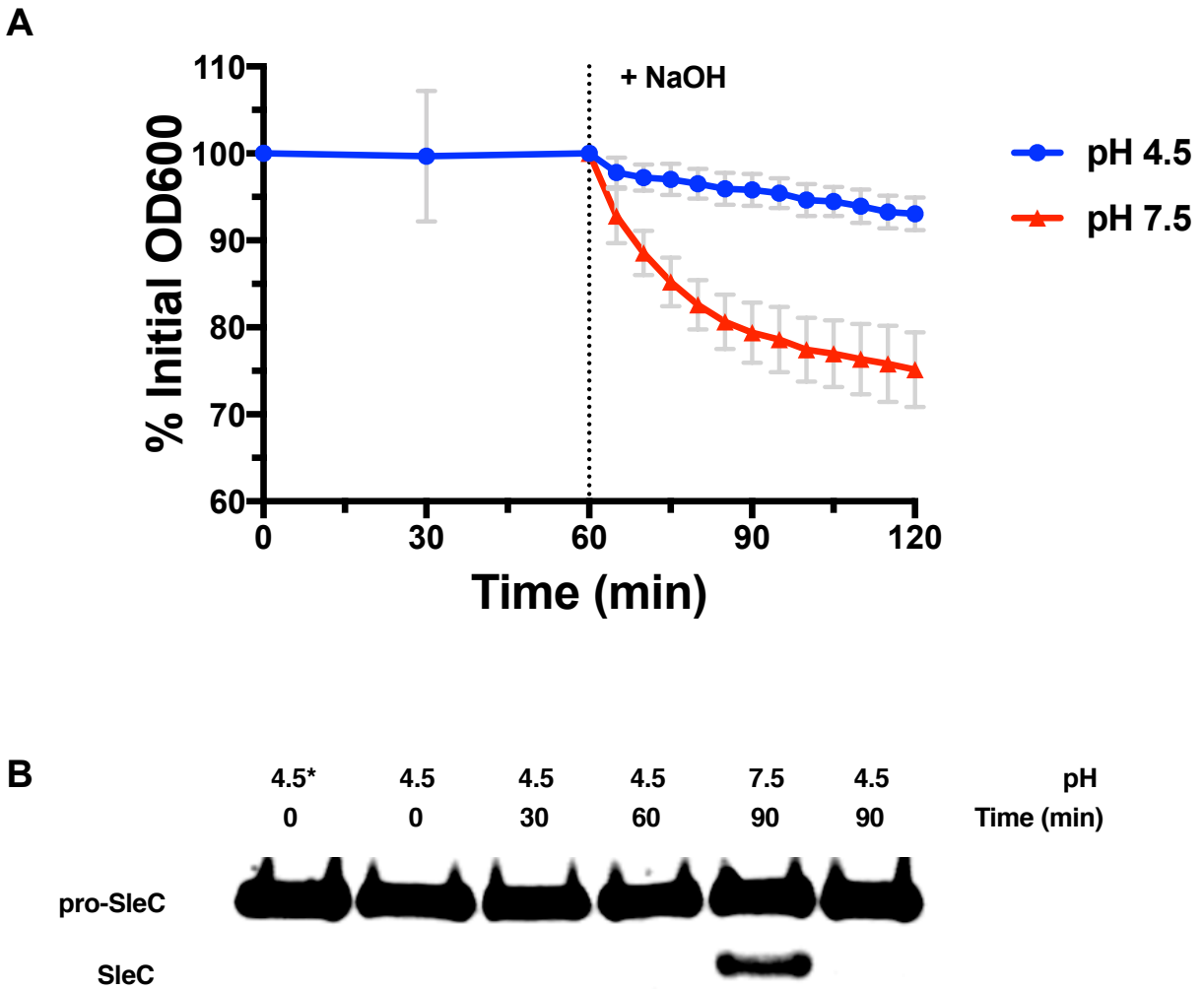

Figure 3.3 Effect of acid pretreatment on germination of *C. difficile* spores
VPI 10463 spores were incubated at 37°C with 0.2% Tc in BHIS at a pH of 4.5 for one hour and optical density was tracked every 30 minutes. After 1 hour, the sample was split into two and the pH of one sample was adjusted to 7.5, and the other remained at 4.5. Germination was tracked via loss of OD assay at 37°C over the course of one hour (A). For observing SleC activation, VPI 10463 spores were incubated as above, and samples were taken every 30 minutes and prepared for western blot (B). Loss of OD assays are the results of 3 experiments with 3 independent spore preps. Western blots are representative of 3 independent spore preps. Samples labeled with * indicates that this sample did not receive taurocholate (B).

C. difficile Spore Germination Kinetics

C. difficile germination occurs through a biochemical process in which CspB activation leads to SleC activation, cortex hydrolysis, and outgrowth of the spore. Our data presented here suggest that the activity of these enzymes is sensitive to alterations in both pH and temperature. However, the enzymatic activity, and thus rate of germination of *C. difficile* spores, is also dependent on the concentrations of germinants present. In order to investigate the effect of specific germinants and co-germinants on *C. difficile* germination kinetics, DPA release assays were performed and the concentration of germinant required to reach 50% of the max germination rate (EC50) was calculated using Michaelis Menton-like kinetics (89). All dose response assays were performed using 0.2% Tc and different concentrations of the indicated co-germinants.

Glycine has been shown to be the most effective amino acid co-germinant and this is confirmed in our analyses; here glycine was found to have an EC50 of 5.47mM (Figure 3.4A). This absolute value differs somewhat from that previously reported likely due to strain to strain variation (VPI 10463 vs UK1) and variations in concentrations of Tc (2mM vs 10mM) used (89). Interestingly, calcium was extremely efficient at inducing germination with an EC50 of 0.44mM in the presence of Tc, but the absence of any amino acids (Figure 3.4B). Dose response analysis was also performed by observing the activation of SleC by western blot. Spores were incubated for 15 minutes at 37°C with 1% Tc and the indicated concentrations of glycine or calcium. SleC activation was observed at concentrations as low as 5mM for glycine (Figure 3.4C) and 0.05mM for calcium (Figure 3.4D).

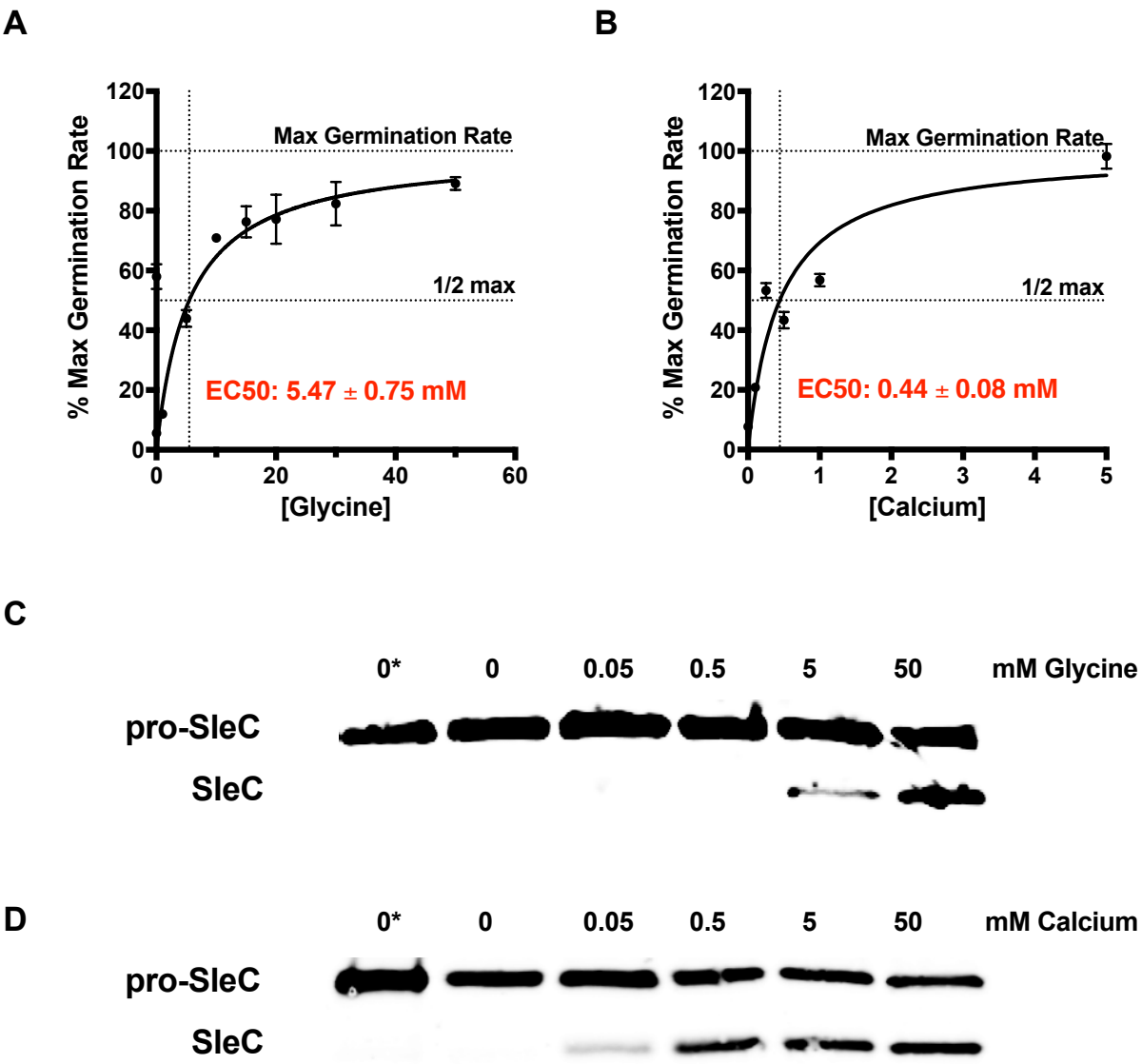

Figure 3.4 *C. difficile* germination rates are dependent on concentrations of germinant
VPI 10463 spores were incubated with 0.2% Tc and various concentrations of either glycine (A) or $CaCl_2$ (B) and rates of release of DPA were measured and presented as % Max Germination Rate. SleC activation was observed by western blot, VPI 10463 spores were incubated in Tris-HCl + 1%Tc and the indicated concentrations of glycine (C) or $CaCl_2$ (D). Samples labeled with * indicates that this sample did not receive taurocholate (C).

Collectively, these germination kinetic data suggest that while both glycine and calcium are very effective at inducing *C. difficile* germination in the presence of bile salts, calcium is about ten times more efficient. Recent work from our group reported that depletion of calcium from mouse ileal contents led to a reduction in spore germination by ~90% (90). However, in the small intestine, bile salts, calcium and amino acids are all found and likely playing a role in inducing germination *in vivo*. Indeed, I previously reported that the limiting, physiological concentrations of taurocholate, glycine and calcium are unable to induce germination on their own, but when added as a trinary mix *C. difficile* spores were able to germinate efficiently (90).

In order to quantify this "synergy" observed with addition of glycine and calcium in combination, I performed DPA release assays and calculated EC50s for both glycine and calcium when the other was present at 10% of its EC50, (50μM calcium or 500μM glycine). The EC50 for glycine was reduced from 5.5mM to 0.210mM when calcium (50μM) was present (Figure 3.5A-B). The EC50 for calcium also decreased from 440μM to 15μM when glycine (500μM) was present (Figure 3.5C-D). Taken together, these data show that there is robust synergy between bile salts, glycine and calcium, which allows spores to germinate at much lower, physiologically-relevant, concentrations of germinants.

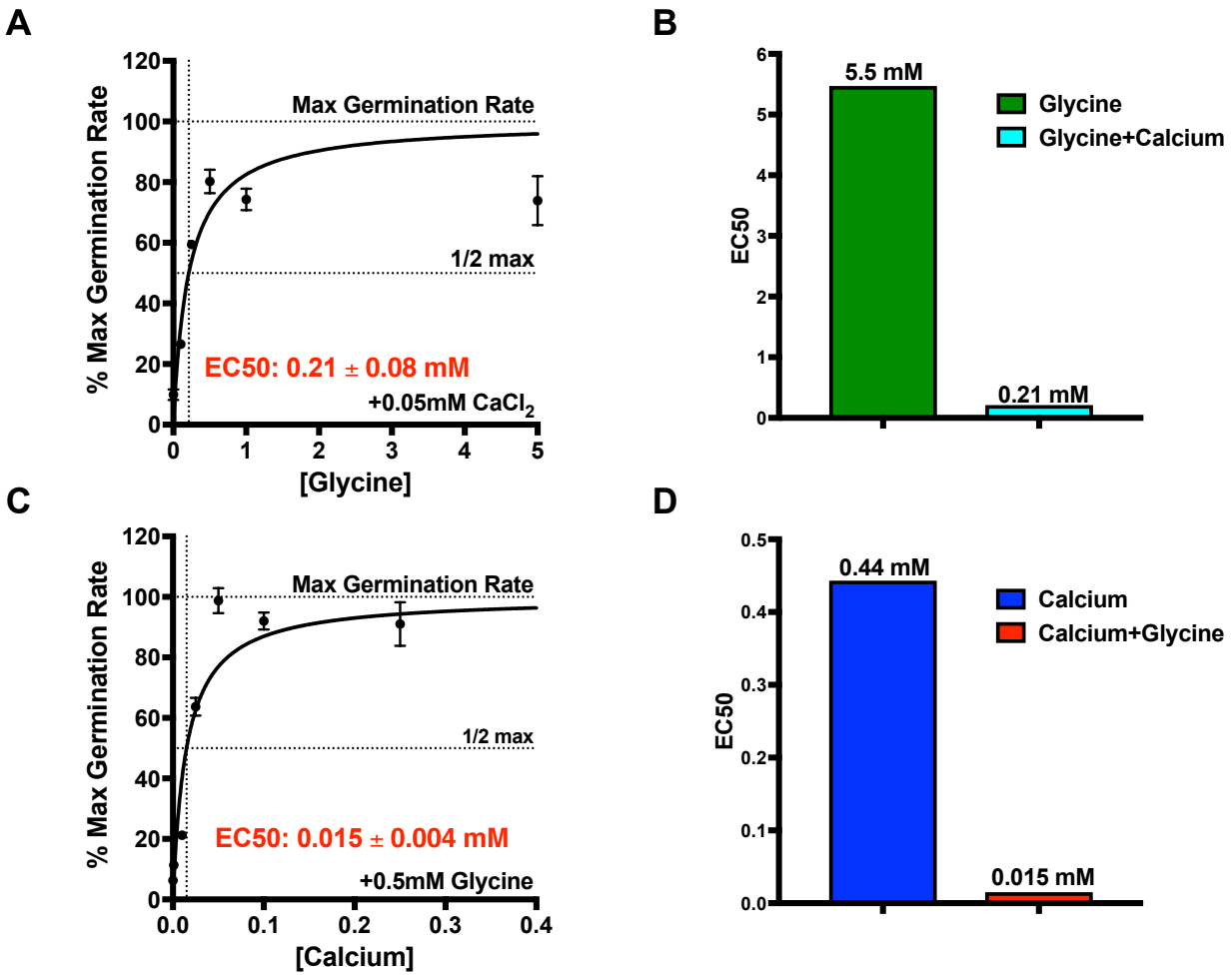

Figure 3.5 Glycine and calcium synergize to improve *C. difficile* germination efficiency
VPI 10463 spores were incubated with 0.2% Tc, 50 µM $CaCl_2$ and the indicated concentrations of glycine and germination rates were observed by DPA release (A) and EC50 values were calculated (B). VPI 10463 spores were also incubated with 0.2% Tc, 500 µM glycine and the indicated concentrations of calcium and germination rates were observed by DPA release (C) and EC50 values were calculated (D).

Calcium Synergy With Other Amino Acid Germinants

Since calcium and glycine (in the presence of Tc) have robust synergy and decrease the required concentrations of each, I hypothesize that calcium will also improve the effectiveness of other, less efficient, amino acid co-germinants. In order to test this hypothesis, *C. difficile* spores were incubated with 0.2% taurocholate and the indicated concentrations of L-serine, glycine, L-histidine, or L-alanine. While previous studies have shown that these amino acids induce germination (with *C. difficile* strain UK1 and 10mM concentrations of Tc), only glycine (1 or 10mM) was able to induce DPA release (Figure 3.6A-D, red lines) (89). However, when 50μM $CaCl_2$ was added simultaneously with Tc and amino acids, DPA release was observed at near maximal levels for spores incubated with 10mM concentrations of glycine, L-alanine, and L-serine (Figure 3.6A-C, filled blue symbols). L-histidine also exhibited appreciable, though significantly lower, levels of DPA release at 10mM plus calcium with Tc (Figure 3.6D). These data show that calcium is able to synergize with numerous amino acids and improve their effectiveness in inducing germination of *C. difficile* spores in concert with bile salts.

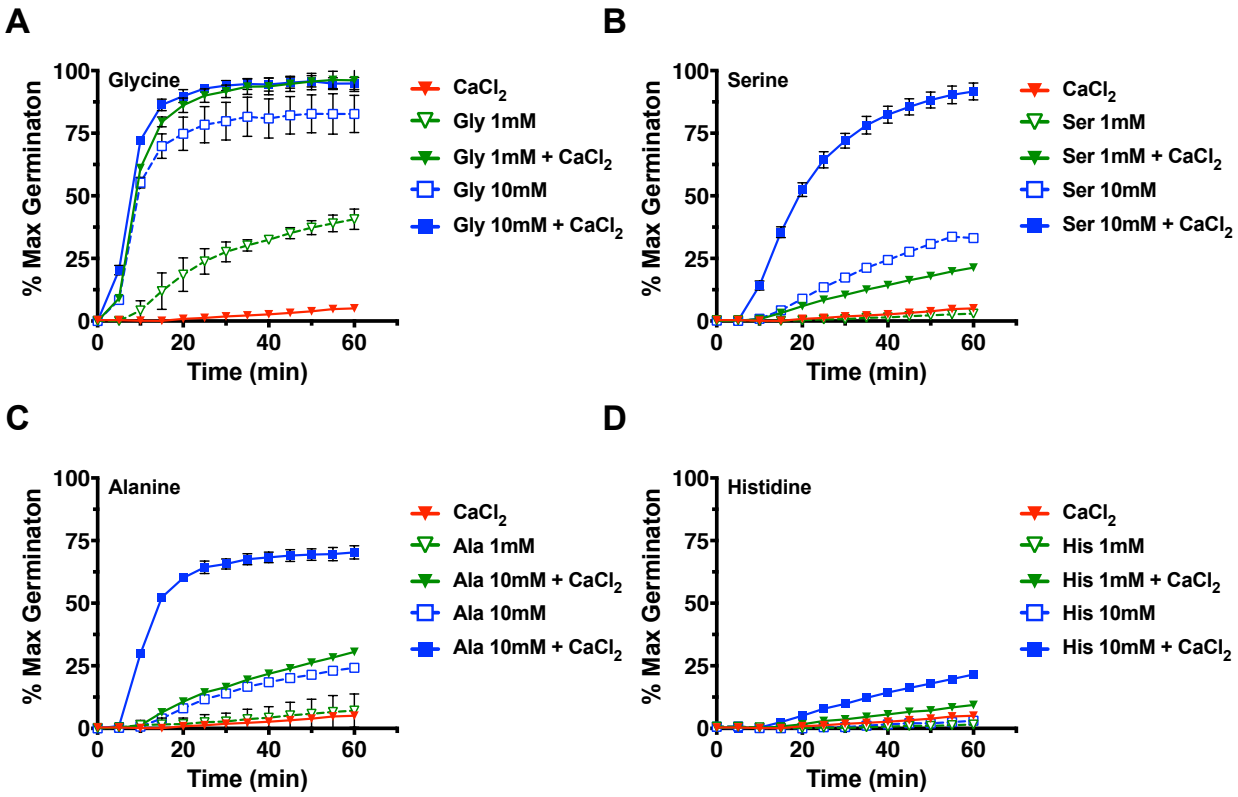

Figure 3.6 Calcium synergizes with other amino acids to improve *C. difficile* germination efficiency VPI 10463 spores were incubated with 0.2% Tc, 50 µM $CaCl_2$ and either 1 or 10mM concentrations of the Glycine (A), Serine (B), Alanine (C), or Histidine (D) and release of DPA was measured over the course of an hour. Data from three independent experiments and two spore preps was normalized and presented as % Maximal Germination.

Glycine Does Not Have Robust Synergy With Other Amino Acids

Since calcium is able to synergize with multiple amino acids, I next questioned whether the addition of glycine in combination with other amino acids in the presence of Tc would also induce robust synergy. In order to test this hypothesis, *C. difficile* spores were incubated with Tc, 500μM glycine (10% of the EC50) and the indicated concentrations of $CaCl_2$, L-alanine, L-serine, or L-histidine and assayed for DPA release. Spores incubated with 0.1mM $CaCl_2$ had a >5 fold increase in amount of DPA released when incubated simultaneously with 500μM glycine (Figure 3.7A). However, when spores were incubated simultaneously with 1 or 10mM L-alanine, L-serine, or L-histidine and 500μM glycine in the absence of calcium, no DPA release was observed (Figure 3.7B-D). These data indicate that while calcium can synergize with a variety of amino acids, glycine is unable to synergize with other amino acids. Based on these data, I conclude that glycine is more effective at stimulating germination than other amino acids the lack of enhanced germination response with combinations of amino acids may be the result of different amino acids interacting with the same receptor or pathway.

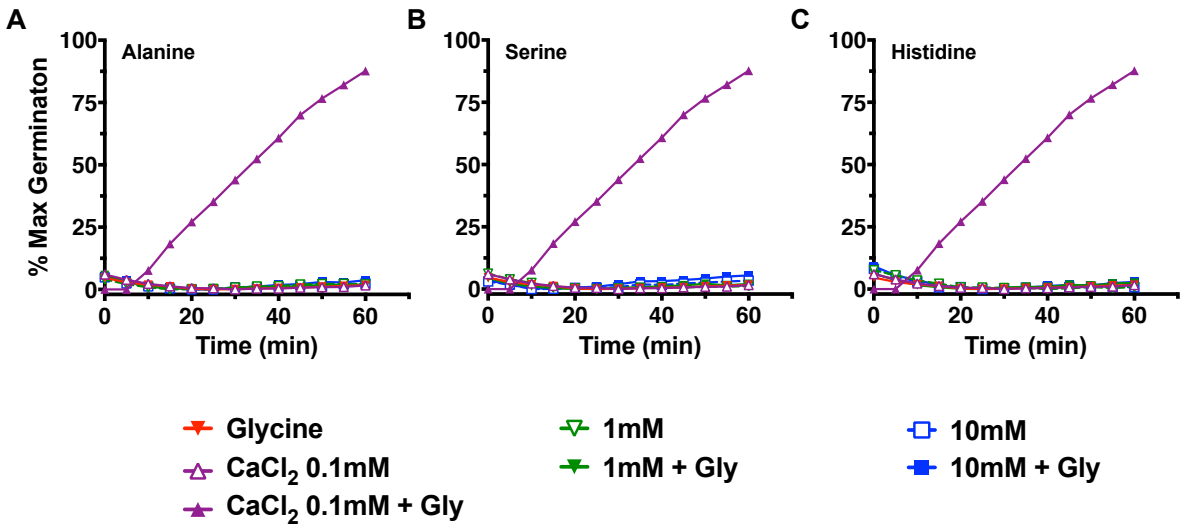

Figure 3.7 Glycine does not synergize with other amino acids
VPI 10463 spores were incubated with 0.2% Tc, 500 µM Glycine and either 1 or 10mM concentrations of Serine (A), Alanine (B), or Histidine (C) and release of DPA was measured over the course of an hour. Spores were also incubated with 0.1mM $CaCl_2$ as a positive control (A-C). Data from three independent experiments and two spore preps was normalized and presented as % Maximal Germination.

Given the ability of a variety of amino acids to stimulate germination, I wondered whether divalent cations other than calcium might create a similar synergy with glycine. Consistent with this hypothesis, I had previously shown that magnesium is able to induce germination when added at fairly high concentrations (50mM)(90). In order to more carefully determine the role of other divalent cations, *C. difficile* spores were incubated simultaneously with 0.2% Tc, 500μM glycine, and 30mM concentrations of either zinc, lithium, potassium, or magnesium. Of the cations tested, only magnesium was able to induce appreciable germination at 30mM and this germination was enhanced with the addition of 500μM glycine (Figure 3.8 A-D). Taken together, these data show that while glycine does not synergize with other amino acids, it does synergize with calcium or magnesium. This suggests that amino acids may identify the same receptor and then compete with each other while the cations interact with another site of either the same receptor or another protein altogether.

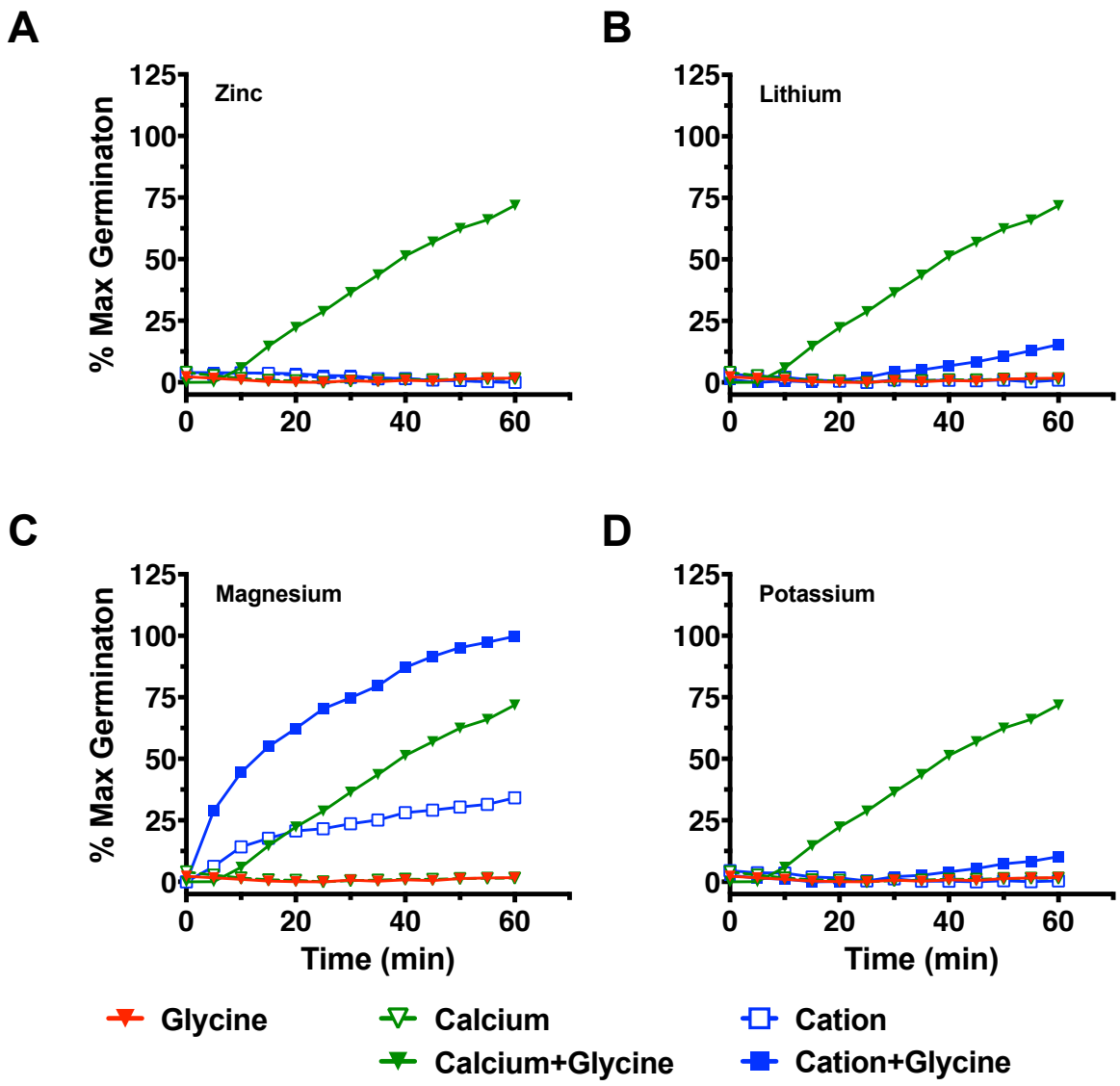

Figure 3.8 Glycine synergizes with magnesium
VPI 10463 spores were incubated with 0.2% Tc, 500 µM Glycine and 30mM concentrations of the $ZnCl_2$ (A), LiCl (B), $MgCl_2$ (C), KCl (D), (A), Alanine (B), or Histidine (C) and release of DPA was measured over the course of an hour. Spores were also incubated with 0.1mM $CaCl_2$ as a positive control (A-D). Data from three independent experiments and two spore preps was normalized and presented as % Maximal Germination.

Germinant Synergy Overcomes Chenodeoxycholate Inhibition

While taurocholate is the most effective bile salt at inducing *C. difficile* germination, most derivatives of cholate are also effective. However, chenodeoxycholate (CDCA), and several secondary bile salts, can function as competitive inhibitors of germination and outgrowth (110). Because of this, it has been hypothesized that inhibitory bile salts block germination by directly interfering with taurocholate-CspC interactions(8). Based on the results presented above, I hypothesized that calcium-glycine synergy can overcome CDCA inhibition of germination. In order to test this hypothesis, I incubated *C. difficile* spores with 20mM glycine and various concentrations of taurocholate in the presence and absence of 0.25mM CDCA and germination was measured by DPA release. CDCA decreased germination to less than 20% of maximal germination compared to ~50% germination at physiological concentrations of Tc (0.05%), however, this inhibition was overcome when Tc concentrations were increased to 0.1% (Figure 3.9A). In order to test if the addition of excess glycine or calcium could overcome CDCA treatment, *C. difficile* spores incubated with 0.05% Tc, 0.25mM CDCA, and the indicated combinations of 20mM-50mM glycine or 1mM $CaCl_2$ and germination was measured by DPA release. Again, CDCA decreased germination with 20mM glycine to <25% of maximal germination; however, CDCA mediated inhibition was overcome with the addition of 1mM $CaCl_2$, restoring germination to >60% (Figure 3.9B, green bar). Increased levels of glycine did not prevent CDCA inhibition as no differences in germination were observed in the presence of CDCA when concentrations of glycine were increased as high as 50mM (Figure 3.9B, brown bar). Taken together, these data suggest that calcium-glycine synergy decreases the necessary concentrations of co-germinants required to overcome inhibitory bile salts present within the gastrointestinal tract.

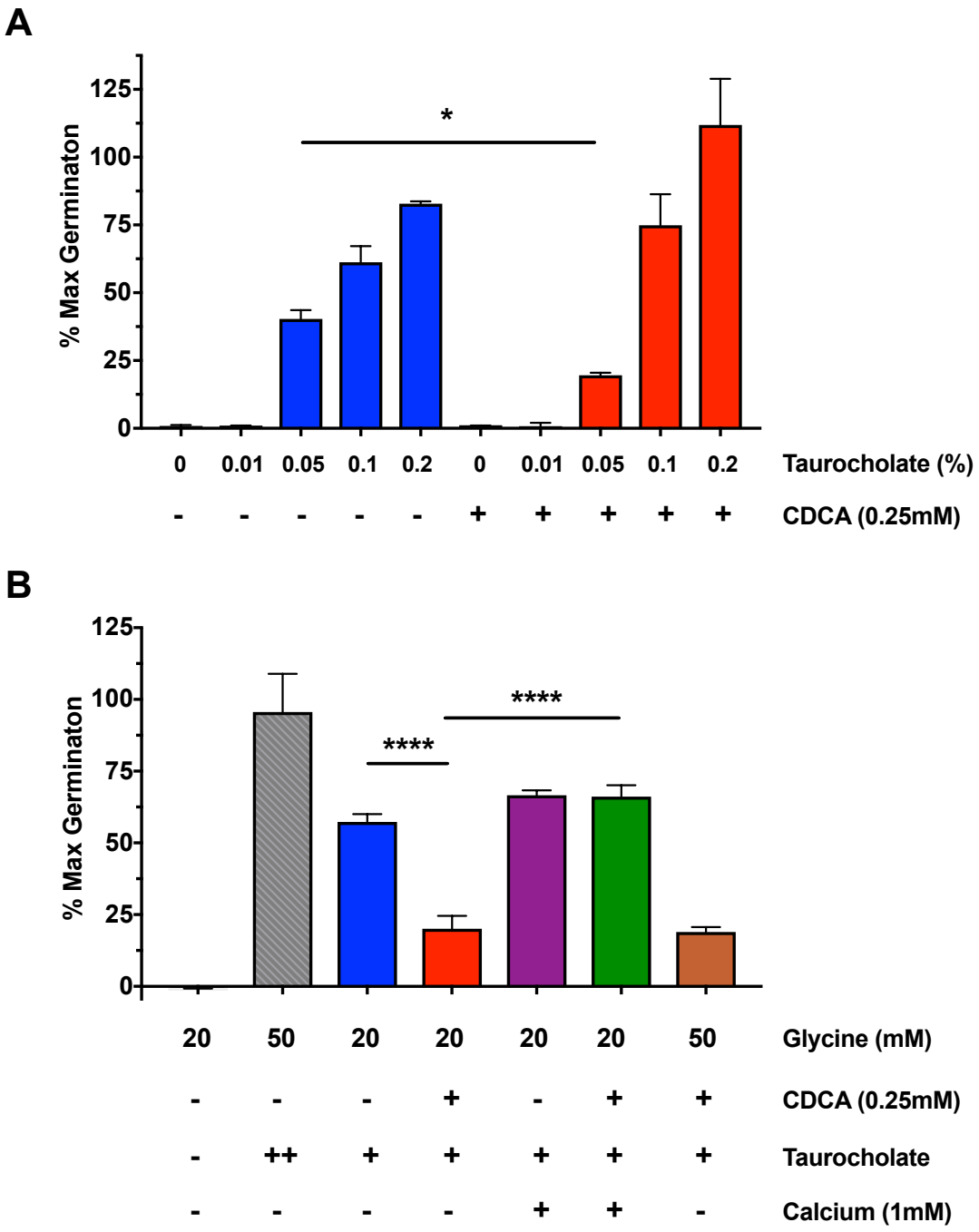

Figure 3.9 Germinant synergy overcomes chenodeoxycholate inhibition of germination
VPI 10463 spores were incubated with and without 250 μM CDCA and 20 mM Glycine and the indicated concentrations of Tc and release of DPA was measured after 60 minutes (A). VPI 10463 spores were incubated with Tc (+ =0.05%, ++ =0.2%) and the indicted concentrations of either glycine, calcium or CDCA and release of DPA was measured after 60 minutes (B). Data from three independent experiments and two spore preps was normalized and presented as % Maximal Germination. Data was analyzed with one-way ANOVA and $p=0.0269$ (A) and $p<0.001$ (B).

Discussion

In this chapter, I investigated some of the specific environmental conditions that facilitate *C. difficile* spore germination that I hypothesized matched those encountered *in vivo*. Several groups have shown independently that conversion of primary bile salts (germination activators) into secondary bile salts (germination inhibitors) occurs in the gut and that this metabolism is performed by members of the local microbiota which, in turn, are subject to population shifts upon exposure to antibiotics (8, 10, 159-161). These studies hypothesize that exposure to antibiotics may influence, albeit indirectly, spore germination and therefore patient susceptibility to contracting CDIs. Similar to the findings of *Theriot et al.*, our data indicates that *C. difficile* spores germinate in ileal contents *ex vivo* regardless of whether the animal was exposed to antibiotic pre-treatment (Figure 3.1) (8). Based on our findings in Figure 3.9, I conclude that *C. difficile* spores are able to germinate in ileal contents independent of antibiotic treatment by overcoming the effects of inhibitory bile salts with germinant synergy. While our data do not show a statistically significant difference between antibiotic treatment and untreated animals, I do observe a trend of increased germination after cefoperazone treatment similar to other studies that have shown enhanced germination after antibiotic treatment (9, 10). This suggests that while there is an apparent affect of inhibitory bile salts within the small intestine, inhibition of germination may not be a major mechanism for colonization resistance provided by the normal gut microbiota. Bile salt metabolism may be playing a role in the large intestine where secondary bile salts would inhibit growth of vegetative *C. difficile* rather than inhibiting germination.

The duodenum does not support high levels of spore germination even though all of the necessary signals bile salts, amino acids, and calcium, are present (Figure 3.1). The pH changes

rapidly from the stomach pH of ~2 to pH ~4.5-5.5 in the duodenum, however, our *in vitro* data suggest that acidic pH, not the absence of determinants, inhibits *C. difficile* germination in the in the duodenum (Figure 3.1, B.1). While it is known that *C. difficile* spores are acid-resistant, data in Figure 2 indicates that they are not able to germinate under acidic conditions even when all the nutrients necessary to facilitate this process are present. While acid treatment inhibits SleC activation and thus germination, the enzymes involved in this process are not irreversibly damaged, and can function once the pH has been neutralized (Figure 3.3A-B). Taken together, these data suggest that alterations in temperature and pH have a profound effect on *C. difficile* spore germination by altering the spore enzymatic activity (Figure 3.2, 3.3). Additionally, although efficient spore germination shows a degree of temperature dependency, the temperature inside of a mammal is constant and it is unlikely that temperature is an environmental signal controlling the regulation of germination within the gastrointestinal tract. Modifications to the pH of intestinal contents and the role of acid regulation of germination are further discussed in Appendix B.

Acidic regulation of *C. difficile* spore germination throughout the gastrointestinal tract has some implications clinically. While prior antibiotic therapy is associated with the largest risk of contracting a CDI, proton-pump inhibitor (PPI) use has been identified as an independent risk factor associated with the development of a CDI (162). PPIs use has become exceptionally common for both inpatient and outpatient settings for the treatment of numerous conditions related to upper gastrointestinal complications, stress ulcer prophylaxis, and functional dyspepsia (163-165). PPIs, by design, increase the pH of the upper gastrointestinal tract including the stomach and the proximal duodenum (166). This increase in pH in the proximal duodenum could lead to *C. difficile* spores germinating earlier than usual and increasing the exposure dose,

and therefore risk, of vegetative cells colonizing the large intestine. In addition, PPI use has been shown to affect the composition of the intestinal microbiota and causes malabsorption of calcium in the small intestine. These side affects could lead to increased germination efficiency within the small intestine and alterations in the microbiota that allow *C. difficile* colonization.

The concentration of each germinant has a profound effect on *C. difficile* germination kinetics, especially when synergies between types of germinants are taken into consideration. In the presence of bile salts, increasing concentrations of glycine or calcium increased spore germination rates (Figure 3.4). Glycine was previously known to be the most effective amino acid co-germinant, and although our data confirms this observation, they also show that calcium is approximately ten times more effective than glycine at equivalent concentrations (Figure 3.4). However, for all germination pathways, maximal germination rates are achieved using non-physiological concentrations of germinants (89, 90, 93). I recently showed that there is a 90% reduction in germination in *ex vivo* mouse ileal contents following calcium depletion, suggesting that the amino acid concentrations found in the gastrointestinal tract are not sufficient to induce germination on their own (90). Here, I investigated the extent to which calcium synergizes with amino acids to decrease the required concentrations of all germinants. When calcium is added at extremely low, non-inducing concentrations (50μm), it synergizes with amino acids effectively decreasing the concentrations of all germinants required to facilitate *C. difficile* germination. When glycine is added at non-inducing concentrations (500μm), it improves the effectiveness of calcium and magnesium, but not other amino acids. This is a surprising finding given that in *Bacillus* spp., various amino acids are able to synergize, likely because *Bacillus* spp. encode for numerous *gerA* type germination receptors, each recognizing a specific amino acid or amino acid combination (69). These findings suggest that calcium and amino acids are interacting with

different proteins, but the amino acids are interacting with the same receptor with glycine being the best at inducing germination. It is also just as likely that calcium and glycine are interacting with the same receptor, and calcium acts as a co-factor that increases germination kinetics initiated by this receptor. Regardless, calcium and glycine (both together and separately) in the presence of Tc induce germination through the activation of SleC. This combined pathway is likely the most physiologically relevant one given that bile salts, amino acids, and calcium are all present in the ileum at concentrations that are too low to induce germination alone. These observations raise questions that could be answered by future studies. The identity of the specific protein(s) with which the amino acids or calcium are interacting remains to be elucidated. In addition, the signaling cascade that occurs after germinant/receptor binding that leads to CspB activation remains unclear. A recent study from our group suggested a possible signaling cascade whereby glycine facilitates release of the spore's intracellular stores of calcium in order to activate CspB and initiate cortex hydrolysis (90). Further understanding of the spore biology and factors that influence *C. difficile* spore germination may improve the types of treatment available to patients suffering from recurrent CDIs.

Materials And Methods

Bacterial Strains And Growth Conditions

C. difficile strain VPI 10463 was grown anaerobically (10% hydrogen, 5% CO_2, and 85% N_2) (Coy Laboratory Products, MI) at 37°C in brain heart infusion media (BD Life sciences) supplemented with 5% yeast extract (BD Life Sciences) and 0.1% cysteine (Sigma-Aldrich).

Spore Production And Purification

C. difficile spores were produced as described previously (117, 167). Briefly, VPI 10463 spores were grown overnight in BHIS broth and then plated onto 70:30 plates for 4 days. Spores and cellular debris were scraped off of the plates and re-suspended in PBS. Samples were spun down, PBS removed, and spores were purified with 50% histodenz as described previously .

***Ex vivo* Germination Assays**

Intestinal contents were isolated from C57Bl/6 mice either before antibiotic treatment or following cefoperazone (MP Biomedicals) (0.5 mg/ml) in sterile drinking water for five days. Contents were re-suspended in either water or PBS at the indicated pHs. Samples were vortexed and solid material was removed by centrifugation at maximum speed for 10 minutes. Supernatants were added to pre-titered spore stocks and incubated for 30 minutes at 37C in an anaerobic chamber. Following this incubation, samples were serially diluted and plated on BHIS plates with cefoxitin (8μg/mL), D-cycloserine (250 μg/mL), and kanamycin (50μg/mL) without taurocholate. Antibiotics were added to kill off host commensal bacteria present in the intestinal contents. Only germinated vegetative bacilli can outgrow on these plates due to the lack of bile salts. Samples were compared to spores from the same stock germinated in BHIS+0.1% Tc, which was defined as 100% germination.

Loss Of OD Germination Assay

The loss of optical density was tracked at 600nm over a one-hour period in a 37°C Spectramax M2 microplate reader (Molecular Devices) as a measure of spore germination. Full rehydration of the core, which precedes loss in optical density, is a known indicator of spore

germination (69, 71, 90). Purified spores were added to BHIS+Tc (0.2%) at the indicated pH's at a starting OD of ~0.2. The assays were performed in triplicate using three independent spore preps. The OD_{600} measurements were taken every 5 minutes for one hour. A drop in OD to 55% of its initial OD represents approximately 100% spore germination. The results are reported as % Germination.

Western Blot Analysis

SleC activation was observed by western blot as described previously (90, 94, 117). Briefly, $1x10^6$ spores were added to the indicated germinant mixtures + 1% taurocholate and incubated at 37°C for the indicated time points. Spores were pelleted, and then resuspended in 40μL of EBB lysis buffer. Samples were run on a 4-12% BIS-Tris gel (Bio-Rad) and then transferred to a 0.22 μm nitrocellulose membrane (Thermo-Fisher). The membrane was probed with an anti-SleC antibody and then IR800 Li-Cor secondary antibody. The membrane was then washed three times with TBS-T before LICOR (Odyssey) detection.

DPA Release Assay

DPA release was measured by terbium fluorescence (89, 90, 93, 115). Solutions of germinants were incubated in 100mM Tris-HCl at 37°C with 800 μM $TbCl_3$ and placed in an M2 microplate reader (Molecular Devices) (excitation 270 nm, emission 545 nm, cutoff 420 nm). Readings were taken every 5 minutes for an hour. EC50 values were calculated from slopes of germination rates plotted against time. Data was analyzed with Michaelis Menton Kinetics and Vmax was calculated. Vmax was set to Max germination rate and data was normalized as a percentage of the max rate. Each curve was performed in triplicate and are from 2 independent

spore preps. When data is presented as %Max Germination, this is defined as the amount of DPA released in response to our positive control (0.2% Tc and 50mM glycine) for each individual experiment. For chenodeoxycholate inhibitory experiments, chenodeoxycholate was solubilized in 100% DMSO, all samples were treated with 5% DMSO as a vehicle control.

CHAPTER 4: DISCUSSION

Chapter 2 Overview

The anaerobic, spore-forming bacterium *Clostridium difficile* is a prominent pathogen in hospitals worldwide and the leading cause of nosocomial diarrhea. Numerous risk factors are associated with *C. difficile* infections (CDIs) including: use of antibiotics, advanced age, vitamin D deficiency, and use of proton pump inhibitors. Antibiotic use disrupts the intestinal microbiota allowing *C. difficile* to colonize, however, why these other risk factors increase CDI incidence is unclear. Notably, deficient intestinal calcium absorption (*i.e.*, increased intraluminal calcium levels) is associated with several of these risk factors. In this chapter, I investigate the role of calcium in *C. difficile* spore germination. *C. difficile* spores are the infectious particles and they must become metabolically active (germinate) to cause disease. Here, I show that calcium is required for *C. difficile* germination, specifically activating the key step of cortex hydrolysis, and that this calcium can be derived from either within the spore or from the environment. I also demonstrate that intestinal calcium is required for efficient spore germination *in vivo*, suggesting that intestinal concentrations of other co-germinants are insufficient to induce *C. difficile* germination. Collectively, these data provide a mechanism that explains the strong clinical correlations between increased intestinal calcium levels and risk of CDI.

Bile-salt Divalent Cation Germination Pathway

A major finding described in Chapter II is the discovery of a novel germination pathway. In addition to the amino acid-dependent germination pathways, the divalent cations, calcium and magnesium, can also function as co-germinants in what I will refer to as the bile salt-divalent cation pathway (90). This pathway does not require any amino acids or other typical nutrient germinants. Instead, sufficient levels of calcium (along with bile salts) circumvent the need for any amino acid co-germinants (Figure 2.1-2.2). Calcium induces germination through activation of key germination enzymes, CspB-SleC (Figure 2.1). In addition, all of the genes previously shown to be required for Tc-gly germination are also required for Tc-$CaCl_2$ induced germination (Figure 2.5). There is synergy between the calcium and glycine pathways, where ten-fold lower concentrations of each can induce efficient spore germination when provided together in combination with taurocholate (Figure 2.8) (90). This is likely the most physiologically relevant germination pathway, as bile salts, calcium, and glycine are all present in the host gastrointestinal tract. In fact, removal of intestinal calcium from mouse ileal contents, decreased germination by 90% indicating that intestinal calcium is a key signal in inducing germination in the gastrointestinal tract (90).

The Role Of Intra-Spore Calcium In *C. difficile* Germination

In addition to intestinal calcium, I also investigated the role of calcium found within the spore. There are large quantities of calcium stored within the spore core in the form of Ca-DPA. Several groups have shown that Ca-DPA is not required for *C. difficile* germination, however, my data shows that DPA deficient spores are also deficient in calcium and that germination of these spores only occurs in rich media, a process that is facilitated by the presence of exogenous calcium (Figure 2.9) (39, 90). Data presented in chapter II also shows that calcium packaged

within the spore is required for germination through the bile salt-amino acid pathway. Germination of *C. difficile* spores was significantly diminished in the presence of the chelating agent egtazic acid, which has high affinity for calcium (Figure 2.3) (90). Germination was blocked upstream of SleC activation suggesting that calcium is required for CspB activity (Figure 2.3). Calcium is also transported from the spore core during taurocholate-glycine induced germination (90). This has been shown through studies in which calcium from outer layers was depleted and using ion-channel inhibitors that presumably block calcium release (Figure 2.3, 2.4). How calcium is released from the spore core independent of DPA is currently unknown. It is possible that this is the result of increased solubility of calcium salts. Given that calcium can be up to 100x more soluble than DPA, only a slight core rehydration may be necessary to solubilize and release calcium independent of DPA. Determining specifically how calcium affects CspB enzymatic activity and if calcium transport is an important part of the germination mechanism or if it is an artifact of calcium chelation are subjects of ongoing investigation.

In addition to describing the role of calcium, I also identified a protein that is essential for Tc-gly germination but not Tc-$CaCl_2$. Cd630_32980 encodes a AAA+ ATPase that is highly induced during sporulation (32). As an ATPase, this protein likely functions during sporulation, because ATP is not required for germination (168). Cd630_32980 mutants are deficient for both calcium and DPA and, as a result, are non-responsive to Tc-Gly germination, but can germinate in response to Tc-Ca (Figure 2.6, 2.7) (90). Since spores lacking Cd630_32980 are deficient for DPA, I hypothesize that Cd630_32980 is involved in transport of Ca-DPA across the forespore outer membrane.

Chapter 3 Overview

In Chapter III, I investigate the specific conditions that facilitate *C. difficile* spore germination including: location within the GI tract, pH, temperature, and germinant concentration. The germinants that have been identified include combinations of bile salts and amino acids or calcium but, *in vitro,* these function at concentrations that far exceed normal physiological ranges normally found in the mammalian GI tract. In this work, I describe and quantify a previously unreported synergy observed when bile salts, calcium, and amino acids are added together. These germinant cocktails improve germination efficiency by decreasing the required concentrations of germinants to physiologically-relevant levels. Combinations of multiple germinant types also are able to overcome the effects of inhibitory bile salts. In addition, I propose that the acidic conditions within the GI tract regulate *C. difficile* spore germination and could provide a biological explanation for why patients taking proton-pump inhibitors are associated with increased risk of developing a CDI.

Temperature And pH Regulation Of *C. difficile* Germination

C. difficile spore germination occurs through a two-step biochemical process that is dependent on CspB mediated cleavage of SleC. In this section I show that both temperature and pH regulate germination of *C. difficile* spores. Germination occurs at an optimal temperature of 37°C and a pH of 7.4 (Figure 3.2). When spores are treated with bile salts and nutrients at an acidic pH, they are unable to germinate, but retain the ability to germinate once the pH has been neutralized (Figure 3.3). These data suggest that pH is regulating germination across the gastrointestinal tract and could explain the lack of germination in the upper small intestine while the lower small intestine has near complete germination (Figure 3.1).

C. difficile Germination Kinetics

While *C. difficile* germination is regulated by pH and temperature, it is also dependent on the availability of germinants. Glycine has been known to be the most effective co-germinant, however, EC50s were calculated for both glycine (5.5mM) and calcium (0.44mM) and these data show that calcium is 10x more efficient than glycine, when comparing concentrations required for germination, both in DPA release and SleC activation (Figure 3.4). If calcium, glycine, and taurocholate are added together the concentrations required to induce germination decrease to physiological concentrations (μM) (Figure 3.5). This synergy also occurs between calcium and several other amino acids (Figure 3.6), but no synergy was measured with combinations of amino acids and taurocholate without calcium (Figure 3.7). In addition, synergy is observed when spores are incubated with the combination of glycine and magnesium (Figure 3.8). These observations suggest that the combination of amino acids and divalent cations found within the gastrointestinal tract synergize together to facilitate *C. difficile* germination.

Germinant Synergy Allows *C. difficile* Spores To Overcome The Effects Of Inhibitory Bile Salts

Several groups have shown that the microbiota metabolizes primary bile salts into secondary bile salts that are inhibitory for *C. difficile* spore germination and growth (8, 10, 159-161). These data suggest that antibiotic use will improve germination in the gastrointestinal tract by reducing the concentrations of secondary bile salts. However, my data presented in Chapter III shows that *C. difficile* spores germinate in mouse ileal contents independent of antibiotic treatment (Figure 3.1). These data suggest that within the ileum *C. difficile* spores are able to germinate even when inhibitory bile salts are present. In fact, when spores were incubated with

the combination of taurocholate, glycine, and calcium, they were able to overcome the inhibitory effects of CDCA (Figure 9). These data suggest that while antibiotic treatment does seem to improve germination *in vivo*, germinant synergy allows spores to overcome the effects of inhibitory bile salts and thus inhibition of germination by the microbiota does not seem to be a major mechanism of colonization resistance.

Conclusions And Implications Of These Data

The data presented in the chapters above has importance for the field of *C. difficile* germination as well as several clinical implications.

Clinical Implications

There have been several clinical and epidemiological studies assessing patient's risk of contracting a CDI infection. Broad-spectrum antibiotic use is a well-known risk factor, however, several studies have demonstrated that patients with defects in calcium absorption are also at increased risk of developing a CDI. For example, patients taking proton pump inhibitors (PPIs); PPIs are commonly prescribed in critically ill patients for numerous reasons (*e.g.,* peptic ulcer diseases, upper gastrointestinal bleeding, stress ulcer prophylaxis) (136-139). This type of treatment blocks acid production in the stomach, thus increasing the pH of the upper gastrointestinal tract. Given my studies on the effect of pH on germination, increasing the pH of the proximal intestines might allow *C. difficile* spores to germinate earlier, increasing the number of vegetative bacteria that are then able to colonize and cause disease in the large intestine. In addition to increasing the pH of the upper GI tract, a known side effect of PPI use is impaired calcium absorption in the small intestine resulting in increased luminal concentrations of calcium (140, 141). Based on data presented in chapter II, it is likely that elevated levels of luminal Ca^{2+}

in these patients allows for more efficient germination of *C. difficile* in the small intestines and therefore increased risk of colonization and infection. In addition, correlations between age, dietary calcium, and CDIs are described as elderly individuals exhibit decreased absorption of calcium and have a significantly higher incidence and severity of CDI. Finally, patients deficient in vitamin D are five times more likely to contract a CDI (142, 143). Vitamin D is well known to facilitate calcium absorption in the small intestines (144, 145), however, it also plays a role in regulation of mucosal immunity in the gut (146, 147). My work, demonstrating the important role that intraluminal calcium plays in spore germination, provides a potential explanation for why several groups of patients are more likely to acquire a CDI and why they may have more severe disease. A deficiency in vitamin D may increase the risk of *C. difficile* colonization through elevated levels of available germinant (calcium) in the intestines and an inefficient immune response leading to severe disease. Future studies are planned investigating the role of dietary calcium, PPI use and Vitamin D levels on germination and disease using the animal model. These studies could lead to changes in prescribing recommendations in hospitalized patients to reduce the risk and severity of CDI.

Lock And Key Germination Model

While significant progress has been made in describing the germination mechanisms and identification of novel germination proteins there are still major gaps that remain to be elucidated. The role of essential co-germinants and the downstream signaling events that lead to CspB activation are two major gaps discussed above. There are two hypotheses for the downstream events occurring after CspC binds taurocholate with some indirect data in support of each (Figure 4.1).

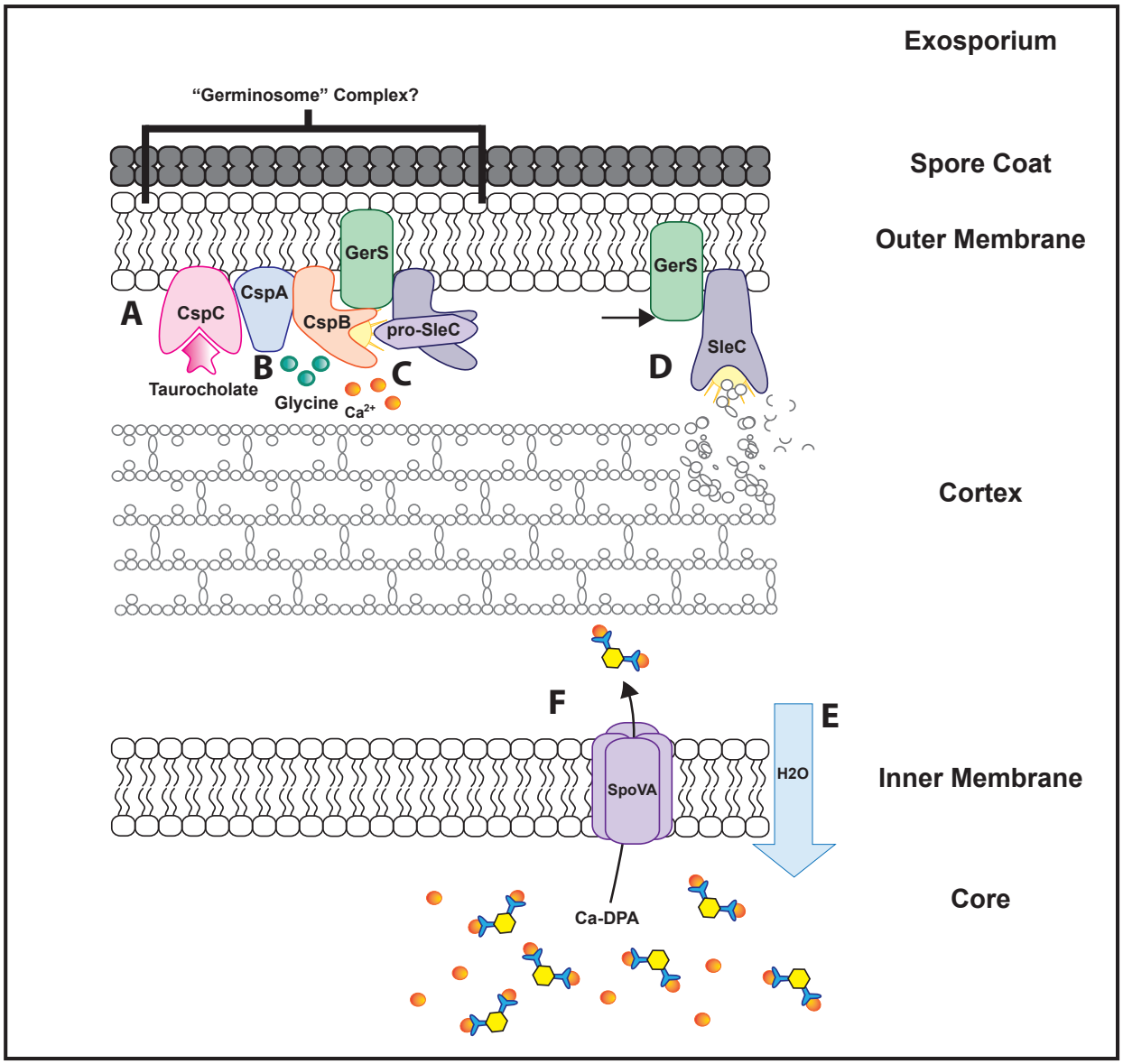

Figure 4.1 Protein-protein interaction model
Taurocholate binds to CspC (A). Glycine interacts with an unknown receptor (B). CspB becomes activated and cleaves the pro-domain from SleC (C). Active SleC degrades the cortex (D), which leads to full core rehydration (E), release of CaDPA (F), and outgrowth out of the spore.

In the original model (prior to this thesis), CspC is thought to directly activate CspB through some yet to be characterized protein-protein interactions (Figure 4.1). CspA is hypothesized to function as a chaperone that helps to maximize CspC incorporation into the mature spore. CspA expression also seems to be controlled, in part, by CspB. Since the packaging into the spores and activity of each protein encoded by the *cspBAC* operon appears dependent on the others, it is possible that they form a "germinosome complex," together with the cortex lytic enzyme, SleC. In this model, after taurocholate binding to CspC and amino acids or calcium interacting with the germinosome, CspC activates CspB and it cleaves the pro domain from pro-SleC, activating cortex hydrolysis.

In contrast, the "Lock and Key" model hypothesizes that taurocholate binding to CspC is a required event for facilitating passage of the other co-germinants, a chemical key provided by the host to open a path for glycine and calcium to access deeper into the spore (Figure 4.2). If so, CspC may serve a function for *C. difficile* similar to the one that the GerP complex serves for *B. anthracis*, with the addition of the key -- GerP does not require bile salts to unlock its channel.

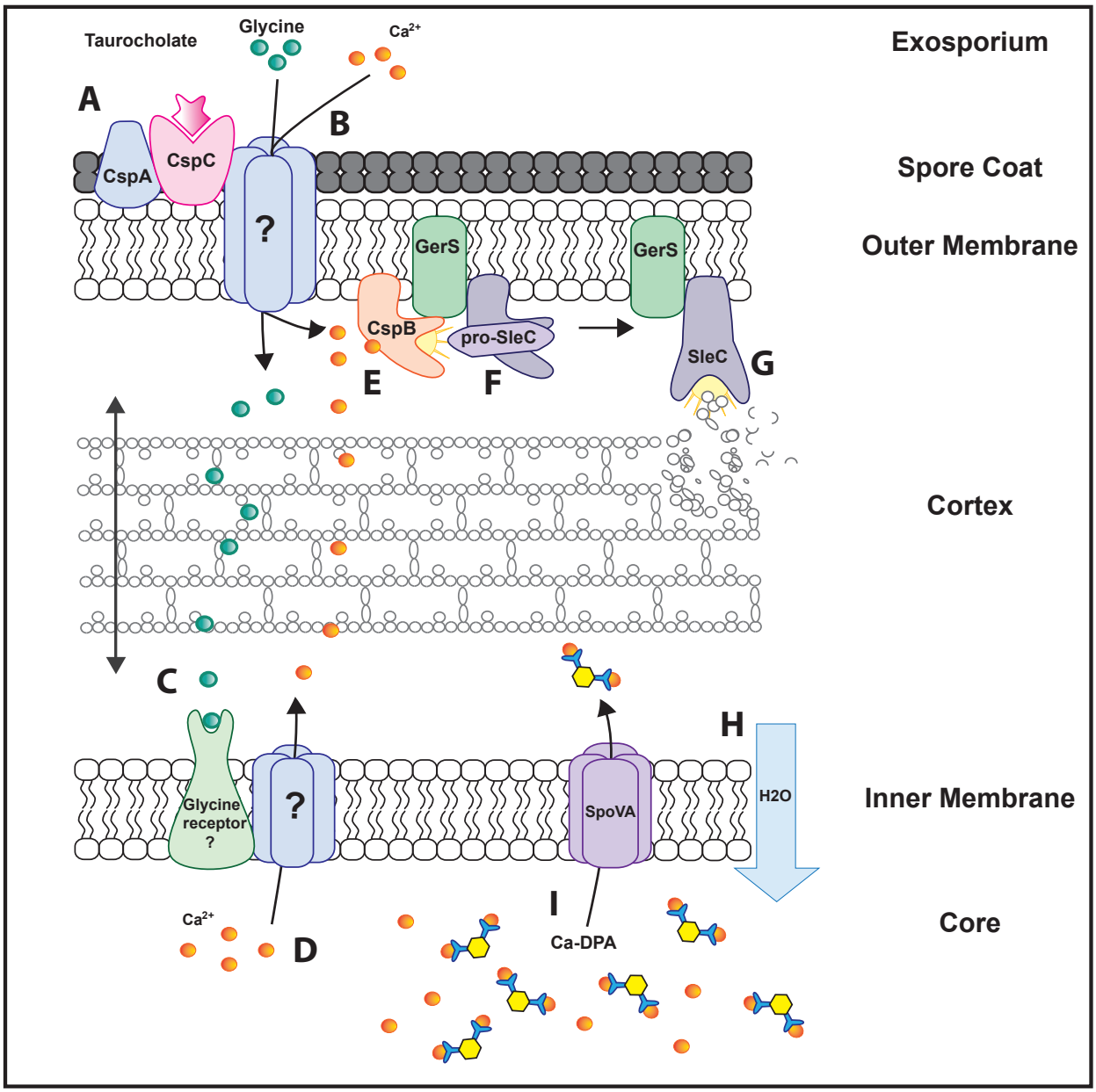

Figure 4.2 Lock and key germination model
Tc binds to CspC (A) facilitating movement of glycine or calcium through the spore coat and outer membrane (B). Glycine then interacts with an unknown receptor (C) inducing the release of Ca^{2+} from the spore core (D). Ca^{2+} from the environment or the spore core activates CspB (E), which processes pro-SleC (F) subsequently initiating cortex hydrolysis (G). This leads to full core rehydration (H), complete release of DPA (I) and spore outgrowth.

The latter model raises two major questions: How do germinants gain access into the spore and what role does calcium play during germination? In *Bacillus spp.* germinant access into the spore is facilitated by the GerP family of proteins (73), however, no orthologs have been identified in *C. difficile* genomes sequenced to date. Therefore, the relative permeability of the spore and mechanism by which germinants gain access to the spore's inner layers remain unknown. In this model, CspC is outward facing and when taurocholate binds, it facilitates access of co-germinants into the spore where they interact with their putative receptors. These receptors are likely found on the inner membrane where they facilitate slight hydration of the core and release of Ca^{2+} ions. Calcium from the spore core (or the external environment) then binds to and activates CspB and subsequently SleC leading to cortex hydrolysis.

This new model raises some important queries that need to be investigated: First, amino acid and calcium receptors need to be identified. The "Lock and Key" model suggests that calcium and amino acids are interacting with different proteins to facilitate germination. It also is possible that calcium may be improving the germination response of a single amino acid germinant receptor. Next, the interactions between bile salts and CspC, and the downstream signaling that occurs, need to be characterized. Several groups have independently verified that CspC is essential for germination, but only a single piece of data exists to suggest that CspC is the taurocholate receptor, the G457R mutation described in Figure 1.4 (68, 90, 94, 103)#2. In addition, the Lock and Key model assumes that there is a permeability barrier blocking molecules from entering the spore, as in *Bacillus spp*. Overall permeability of the spores should be assessed in the presence and absence of taurocholate. Finally, the localization of key germination proteins within the spore need to be more precisely defined. Several groups have shown independently through fractionation and immune-EM that these proteins reside in the

spore coat/outer membrane, however, many of them lack trans-membrane domains and it is unclear how they could be localized to these outer layers while having activity within the cortex layer (99, 117, 169). In addition, based on the germinant synergy described in chapter III, and that the gastrointestinal tract contains bile salts, amino acids, and divalent cations, germination cocktails including a variety of germinants should be tested to describe a standardized, optimal germination media.Future research into *C. difficile* germination should focus on addressing these important gaps in the literature. In addition to advancing the field of spore germination, understanding the specific mechanisms involved in *C. difficile* germination has broader impacts and is essential for the development of novel, well-targeted therapeutics.

APPENDICES

Appendix A: Cd630_32980 Functions During Sporulation

Sporulation Assay

In order to determine the role of Cd630_32980 in sporulation, sporulation assays were performed as described previously (167, 170). Briefly, *C. difficile* was grown O/N in BHIS supplemented with 0.1% Tc and 0.2% fructose. Overnight cultures were back diluted and grown to an OD600 of 0.5 and plated on 70:30 plates. Plates were incubated overnight and then a loopful of bacteria/spores was resuspended in BHIS and pipetted onto an agarose pad. Spores and vegetative cells were visualizd by phase contrast microscopy (Figure A.1 A-C).

Cd630_32980 Mutants Have A Kinetic Sporulation Defect

In order to access the role of Cd630_32980 in sporulation, sporulation assays were performed on wild type, Δspo0A, and Δ32980 strains. Wild type and Δ32980 both had similar levels of overall sporulation (~50% sporulation) (Figure A.1 D). However only ~3% Δ32980 spores were mature spores that had separated from the mother cell, compared to ~40% for the wild type (Figure A1E). Δspo0A vegetative cells were unable to produce any spores because Spo0A is the master regulator of sporulation (Figure A.1 B). Cd630_32980 were also found to be lacking any DPA (Figure 2.7A). Taken together, these data indicate that Cd630_32980 functions during sporulation, most likely as an ATPase providing energy for the transport of DPA across the outer forespore membrane.

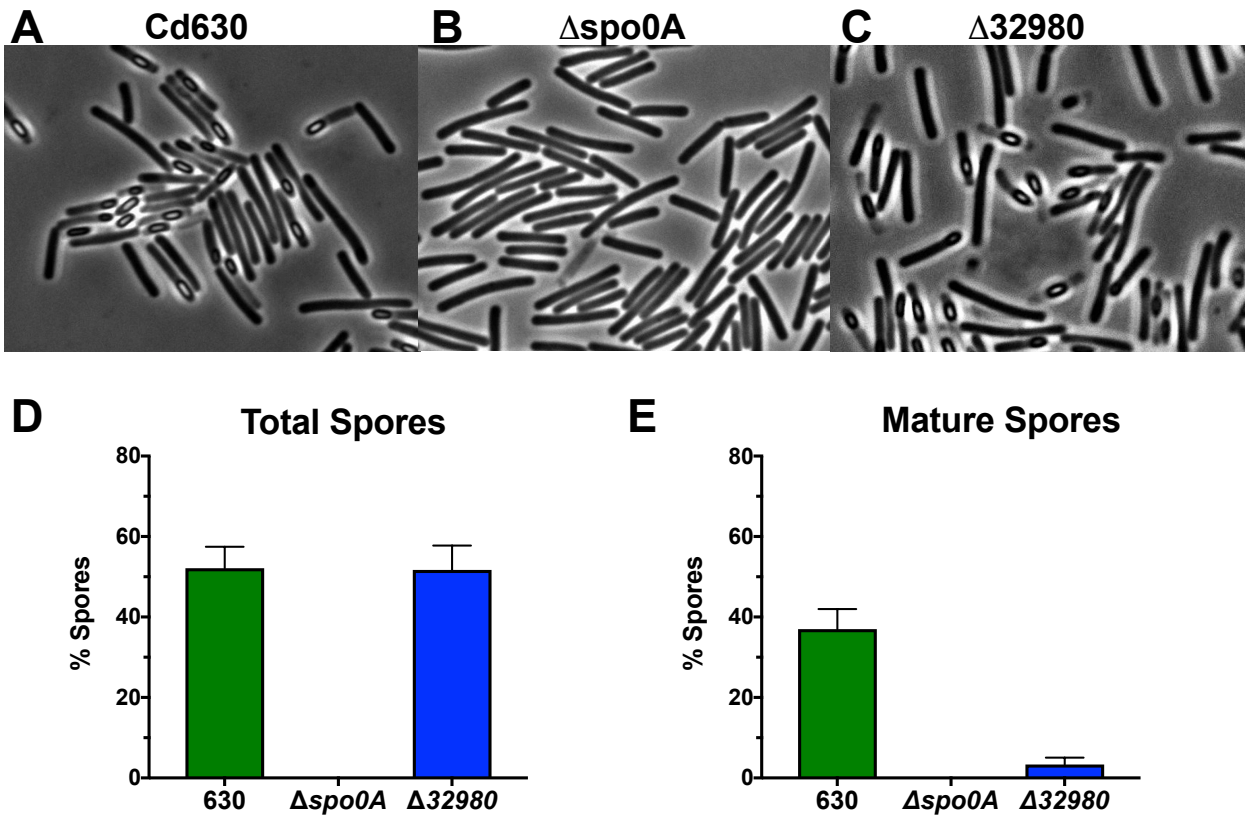

Figure A.1 Δ32980 spores have a kinetic defect in sporulation
Cd630, Δspo0A, and Δ32980 cells were visualized by phase contrast microscopy (A-C). Spores and vegetative cells were counted and are presented as % Spores. %Spores are defined as # of spores / spores+vegetative cells (D). Mature spores are defined as the percentage of spores that have completed sporulation and completed dissociated from the mother cell (E).

Appendix B: Acidity Within The Gastrointestinal Tract Regulates Germination

Adjusting The pH Of Contents From Mouse Ileum And Duodenum

Ileal contents and duodenal contents were harvested from antibiotic treated mice (n=5) as described above. These contents were re-suspended in PBS (pH 7.4) and then pH was measured using phenol red. The pH was adjusted by adding 0.5µl of either 1M HCl or 3N NaOH and pH was measured using phenol red. Each sample was then assayed for ex vivo germination as described in the methods above.

pH Regulates Germination Along Different Sections Of The Small Intestine

In order to test if the pH of the duodenum is too low for efficient spore germination, duodenal contents and ileal contents were harvest from antibiotic treated mice. Duodenal contents were found to be acidic (~pH 4) and ileal contents were found to be more neutral or slightly basic (pH 7.5-8.5). In order to test if the pH is regulating germination in small intestinal contents, the pH of duodenal and ileal contents was adjusted by adding either 1M HCl or 3N NaOH, pH was measured using phenol red, and *C. difficile* germination was measured within these contents (Figure B.1). In duodenal contents re-suspended in PBS or adjusted with HCl, the contents are too acidic (~pH 4) to facilitate efficient germination; 0.07 fold and 0.3 fold change compared to ileal contents re-suspended in PBS respectively (Figure B.1). However when 0.5µl of NaOH was added to duodenal contents, the pH became neutral or slightly basic and germination as vastly improved (1.86 fold change compared to ileal contents re-suspended in PBS) (Figure B.1). In addition, when 0.5µl of HCl was added to mouse ileal contents, the pH became acidic

and germination was reduced (0.13 fold change compared to ileal contents re-suspended in PBS) (Figure B.1). When NaOH was added to mouse ileal contents there was no change in germination. Together these data indicate that *C. difficile* spores germinate poorly in duodenal contents because of acidic pH and suggest that pH is regulating *C. difficile* spores germination along the gastrointestinal tract *in vivo*.

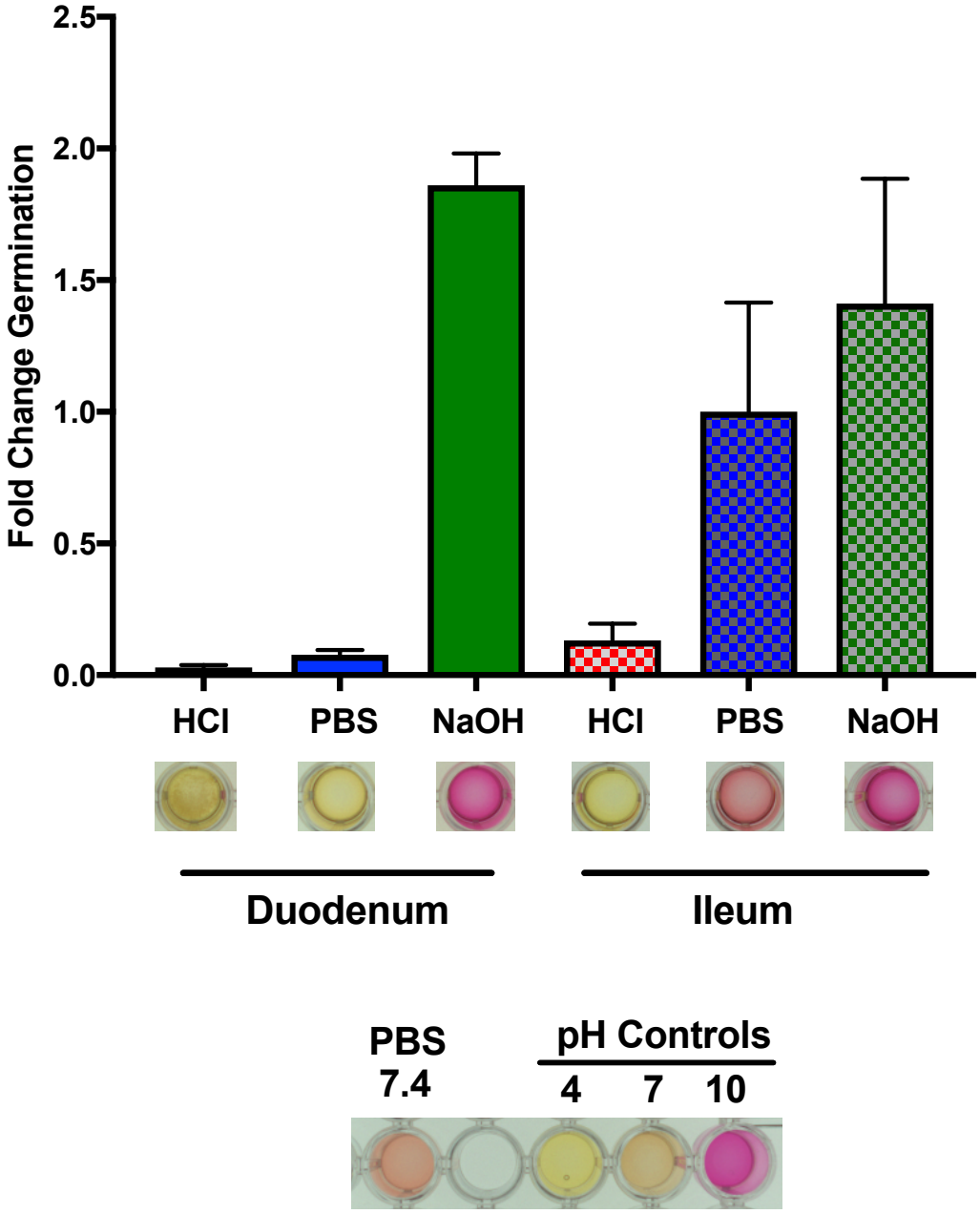

Figure B.1 Acidic pH regulates germination along the gastrointestinal tract
Ileal contents and duodenal contents were harvested from antibiotic treated mice. VPI 10463 spores were incubated for 1 hour at 37°C in duodenal or ileal contents that were re-suspended in either PBS, PBS + HCl, or PBS + NaOH.

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