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Re-examining the germination phenotypes of several *Clostridium difficile* strains suggests another role for the CspC germinant receptor.

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Running Title: Re-examining *C. difficile* spore germination

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Abstract

Clostridium difficile spore germination is essential for colonization and disease. The signals that initiate *C. difficile* spore germination are a combination of taurocholic acid (a bile acid) and glycine. Interestingly, the chenodeoxycholic acid-class (CDCA) bile acids competitively inhibit taurocholic acid-mediated germination suggesting that compounds that inhibit spore germination could be developed into drugs that prophylactically prevent *C. difficile* infection or reduce recurring disease. However, a recent report called into question the utility of such a strategy to prevent infection by describing *C. difficile* strains that germinated in the apparent absence of bile acids or germinated in the presence of the CDCA inhibitor. Because the mechanisms of *C. difficile* spore germination are beginning to be elucidated, the mechanism of germination in these particular strains could yield important information on how *C. difficile* spores initiate germination. Therefore, we quantified the interaction of these strains with taurocholic acid and CDCA, the rates of spore germination, the release of DPA from the spore core and the abundance of the germinant receptor complex (CspC, CspB and SleC). We find that strains previously observed to germinate in the absence of taurocholic acid demonstrate more potent $EC_{50,TA}$ values towards the germinant and are still inhibited by CDCA, possibly explaining the previous observations. By comparing the germination kinetics and the abundance of proteins in the germinant receptor complex, we revise our original model for CspC-mediated activation of spore germination and propose that CspC may activate spore germination and then inhibit downstream processes.

Importance

Clostridium difficile forms metabolically dormant spores that persist in the healthcare environment. In susceptible hosts, *C. difficile* spores germinate in response to certain bile acids and glycine. Blocking germination by *C. difficile* spores is an attractive strategy to prevent the initiation of disease or to block recurring infection. However, certain *C. difficile* strains have been identified whose spores germinate in the absence of bile acids or are not blocked by known inhibitors of *C. difficile* spore germination (calling into question the utility of such strategies). Here, we further investigate these strains and re-establish that bile acid activators and inhibitors of germination affect these strains and use this data to suggest another role for the *C. difficile* bile acid germinant receptor.

Introduction

Spore formation and germination by *Peptoclostridium difficile* spores (1) (referred herein as *Clostridium difficile* for simplicity) are significant hurdles for overcoming the transmission of this pathogen within the hospital environment. Due to the strict anaerobic nature of *C. difficile* vegetative cells, spores are thought to be the main reservoir for transmission within the healthcare setting (2, 3). Prior antibiotic treatment is the greatest risk factor for *C. difficile* infection (4). Broad-spectrum antibiotics alter the balance of the colonic microbiota allowing *C. difficile* an opportunity to colonize the newly generated niche (5-7). Once in a host, *C. difficile* spores germinate to form the toxin-producing vegetative cells that colonize a host's colonic environment. In susceptible hosts, *C. difficile* vegetative cells secrete two toxins that damage the colonic epithelium and lead to the primary symptoms of disease (8, 9). *C. difficile* infections are commonly treated with more antibiotics (*e.g.*, metronidazole, vancomycin or fidaxomicin) (10).

58 After disease symptoms are alleviated and the antibiotics are discontinued, patients frequently
59 relapse with recurring disease due to germination by spores that remain in the colon or that re-
60 infect the host from the surrounding environment (10). Because germination by the spore form is
61 required for pathogenesis, compounds that prevent spore germination could be an attractive way
62 to prevent the primary or recurring infections (11-13).

63 Endospores are dormant forms of bacteria and are formed by vegetative cells in response
64 to environmental stress (14, 15). In *Bacillus subtilis*, sporulation is a tightly regulated process
65 and involves the formation of a forespore within a mother cell (16). The forespore and mother
66 cell communicate through a cascade of sigma factor activation and waves of
67 transcription/translation (16). The end result of this developmental program is a dormant spore
68 consisting of a DNA-containing core where much of the water has been replaced by dipicolinic
69 acid as a calcium chelate (CaDPA) (17). Surrounding the spore core is an inner spore membrane,
70 a thin germ-cell peptidoglycan layer, a thick cortex peptidoglycan layer, an outer spore
71 membrane and layers of coat protein (15). The coat proteins, cortex and the contents of the
72 desiccated core help protect the spore from environmental stressors (*e.g.*, heat, radiation,
73 chemicals and antibiotics) and keep the spore in a metabolically dormant state (15, 17).

74 Endospore germination has been extensively studied in *B. subtilis* (15). In *B. subtilis*,
75 spore germination can be initiated when L-alanine (a germinant) interacts with the GerAA-AB-
76 AC germinant receptor which is embedded within the inner spore membrane (15). This
77 interaction triggers the release of the large depot of CaDPA, presumably through the SpoVA
78 membrane channel (15). This results in the partial hydration of the spore's core. As CaDPA
79 passes through the cortex layer, CaDPA activates the CwlJ cortex hydrolase and the actions of

80 the CwlJ and SleB hydrolases degrade the spore cortex resulting in core expansion and full
81 rehydration of the spore core (15).

82 The receptors with which spore germinants interact are conserved between most of the
83 studied spore-forming bacteria (*e.g.*, *B. subtilis*, *B. anthracis* and *C. perfringens*) while the
84 germinants which activate spore germination vary between organisms (but germinants are
85 generally amino acids, nucleotides, ions or sugars) (18). *C. difficile* spore germination is initiated
86 by certain bile acids (presumably a host signal) and glycine (presumably a nutrient signal) (14,
87 19-21). Bile acids are small, steroid acids that are released by the gall bladder into the digestive
88 tract to aid in the absorption of fats and cholesterol (22). Two families of bile acids are
89 synthesized by the liver: cholic acid-derivatives and chenodeoxycholic acid-derivatives (CDCA).
90 Each of these families is conjugated with either a taurine [taurocholic acid (TA) or
91 taurochenodeoxycholic acid] or a glycine (glycocholic acid or glycochenodeoxycholic acid) (22).
92 *C. difficile* spore germination is stimulated by cholic acid while CDCA-derivatives inhibit cholic
93 acid-mediated germination (11, 19, 23, 24). Based on previous studies, *C. difficile* spores have an
94 EC_{50} value (the concentration that achieves half-maximum germination rate) in the low
95 millimolar range for TA and the high micromolar range for CDCA, suggesting that *C. difficile*
96 spores may have a tighter interaction with inhibitors of germination than with the activators of
97 germination (11, 23-25).

98 Though the signals that stimulate spore germination have been studied for some time,
99 only recently was the receptor with which the bile acids interact identified (13). *C. difficile* does
100 not encode the classical, membrane-embedded, *ger*-type, germinant receptor (26). Instead, *C.*
101 *difficile* spore germination proceeds through a novel germination pathway involving direct
102 stimulation of cortex hydrolysis and subsequent release of CaDPA from the spore core (opposite

103 to what is observed for germinant receptor-mediated germination in *B. subtilis* or *Clostridium*
104 *perfringens*) (13-15, 27, 28). In our working model, *C. difficile* spore germination is initiated
105 when the germination-specific, pseudoprotease, CspC, interacts with the cholic acid-class of bile
106 acids (13). Activated CspC transmits the bile acid activating signal to the CspB protease which
107 then activates the spore cortex lytic enzyme SleC (deposited in the spore as a zymogen) (13, 29).
108 Activated SleC then begins to degrade the spore cortex and CaDPA is subsequently released
109 from the spore core (27, 28).

110 Based on the model described above, all *C. difficile* isolates should have the requirement
111 for bile acids to stimulate germination. Indeed, spores derived from most clinical isolates require
112 taurocholic acid (TA) to initiate spore germination in rich medium (30, 31). However, a few
113 isolates have been reported not to require TA as a spore germinant or not to be inhibited the
114 CDCA anti-germinant (31). If these strains have no requirement for TA or are not inhibited by
115 CDCA, by what mechanism would they be activated for germination (*i.e.*, would the
116 mechanisms for germination in these strains be different from what has been described
117 previously)?

118 Here we aimed to re-examine the germination of spores derived from *C. difficile* strains
119 that were reported previously to germinate in rich medium alone or germinate in the presence of
120 a known inhibitor of *C. difficile* spore germination, CDCA (31), and compare this germination to
121 that of spores that clearly require TA for germination (11, 25, 27, 32). Upon reinvestigation of
122 these strains, we find that strains previously thought not to be inhibited by CDCA are actually
123 inhibited by CDCA and interact with CDCA with similar inhibitor constant values as other
124 strains. However, these strains do exhibit greater $EC_{50,TA}$ values, possibly explaining previous
125 observations (31). We further characterize these strains by determining the abundance of the

126 germinant receptor complex and show that the levels of CspC, CspB and SleC differ between
127 strains. By comparing the generated kinetic data with the abundance of spore proteins, we revise
128 our model for the mechanism of initiating *C. difficile* spore germination. Based on our prior
129 genetic data and the data herein, we propose that CspC acts to initiate spore germination in the
130 presence of TA but then to inhibit downstream processes until another signal is received.

131

132 **Materials and Methods**

133 **Strains and growth conditions**

134 *C. difficile* strains (Table S1) were grown in a Model B, Coy Laboratories anaerobic chamber
135 (10% H_2, 5% CO_2, 85% N_2) at 37 °C in Brain Heart Infusion supplemented with 5 g / L yeast
136 extract and 0.1% L-cysteine (BHIS), as described previously (13, 27, 32, 33). *Escherichia coli*
137 routinely was grown at 37 °C in LB medium or in 2xTY medium (5 g NaCl, 10 g yeast extract,
138 16 g tryptone / L) for protein expression (see below). Antibiotics were supplemented as needed
139 (100 µg / mL ampicillin).

140 **Molecular Biology**

141 The *C. difficile* UK1 *sleC* gene was amplified with Phusion DNA polymerase (New England
142 Biolabs, Beverly, MA) using primers 5'pET_SleC
143 (TTTTGTTTAACTTTAAGAAGGAGATATACATATGCAAGATGGTTTCTTAACAGTAAG
144 C) and 3'pET_SleC
145 (ATCTCAGTGGTGGTGGTGGTGGTGCTCGAGAATTAAAGGATTTAAAGAAGCTATT).
146 The resulting PCR product was inserted into pET22b between the XhoI and NdeI restriction sites

using Gibson Assembly (34) and transformed into *E. coli* DH5α to generate pKS08. To construct a strain producing CspB for recombinant protein expression, the *cspB* gene was amplified with Phusion DNA polymerase from *C. difficile* UK1 genomic DNA with primers 5'CO_cspB (GTTTAACTTTAAGAAGGAGATATACATATGATTATCATTAATTACGAACTGATTGTG AAG) and 3'CO_cspB_CPD (TTCCATCCGCGAGCTCGCTTTTATTAATGCTGCG). To aid in solubility and allow for the generation of an un-tagged CspB protein, the cysteine protease domain from the *Vibrio cholerae* MARTX protease (CPD) was fused to the 3'end of *cspB* (35). *cpd* was amplified from the p*cspC-CO-Δ50CPD* plasmid using primers 5'CPD_cspB (CGCAGCATTAATAAAAGCGAGCTCGCGGATGGAA) and 3'CPD_pET (ATCTCAGTGGTGGTGGTGGTGGTGCTCGAGACCTTGCGCGTCCCA). The resulting *cspB* and *cpd* PCR products were inserted into pET22b between restriction sites XhoI and NdeI using Gibson Assembly (34) and the resulting plasmid was named pKS02. The *C. difficile cspC* gene was codon optimized for expression in *E. coli* (Eurofins Genomics, Ebersberg, Germany). The resulting gene was sub-cloned into pET22b-CPD (35). The sequences of all inserts were verified.

Spore preparation

Spores were generated and purified as described previously (13, 25, 27, 32). Briefly, *C. difficile* strains were streaked onto 10 – 15 plates of BHIS agar medium. After 4 days, the growth from each plate was scraped into 1 mL sterile water and incubated overnight at 4 °C. The following day, the growth suspension was washed 5 times in sterile water. During each wash step, the layer of white cell debris was removed. After washing, the spores/cell debris were suspended in 25% (w/v) HistoDenz (Sigma Aldrich, St. Louis, MO.) and layered onto a 50% (w/v) HistoDenz solution. The resulting gradient was centrifuged at 18,900 x g for 30 minutes. During

centrifugation, spores migrate through the 50% HistoDenz while the less-dense vegetative cells and cell debris remain at the interface. After centrifugation, the solution is removed and the pellet, containing the purified spores, was washed 5 times in sterile water before being resuspended in a final volume of 1 mL. Purified spores were examined microscopically and were found to be >99.9% pure and phase-bright (dormant).

Germination

To quantify the interaction between the bile acids and *C. difficile* spores, purified spores were heat-activated at 65 °C for 30 min and then placed on ice. Ten microliters of the heat-activated spores were added to a final OD_{600} of 0.5 in 990 µL BHIS medium alone or medium supplemented with the indicated concentrations of bile acids and germination was monitored at 600 nm for 15 minutes in a PerkinElmer (Waltham, MA) Lambda25 UV/Vis Spectrophotometer. The data points at OD_{600} (T_x) were normalized to the starting OD_{600} value (T_0). Germination rates and EC_{50} values were calculated using the slopes of the linear portions of the germination plots, as described previously (11, 25). The data shown in Figures 1 and 2 are a representative of three independent experiments (the data cannot be averaged between experiments due to differences in time points generated by the PerkinElmer spectrophotometer. For transparency, all germination plots are included Figures S1 – S3.). Rates and EC_{50} values were individually calculated from each germination experiment and are reported as averages with the standard error of the mean.

The release of CaDPA from germinating *C. difficile* spores was monitored in real time using terbium fluorescence (36). An opaque, 96-well plate was prepared with 125 µL of 10 mM Tris (pH 7.5), 150 mM NaCl, 800 µM $TbCl_3$ and 100 mM glycine alone or supplemented with

10 mM TA, as described previously (27). Heat-activated spores were then sedimented for 1 min at 14,000 x g and resuspended in an equal volume of water to remove any CaDPA that may have been released due to auto germinating spores. A 5-µL sample of a spore suspension (OD_{600} = 60) was added to each well, and the CaDPA release was monitored in a Molecular Devices (Sunnydale, CA) Spectramax M3 fluorescence plate reader. CaDPA release was monitored with the following settings: excitation = 270 nm; emission = 545 nm; cutoff = 420 nm. The CaDPA release data are reported as the average of 3 independent experiments and error bars represent the standard error of the mean.

Total DPA content of the spores was determined by suspending $1x10^5$ spores in buffer and incubating at 95 °C for 30 minutes. The spores were cooled and sedimented for 2 minutes at 14,000 x g. The amount of released DPA was determined as above using terbium fluorescence.

Protein purification

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

phytic acid at room temperature for 20 minutes with shaking (4 cleavage reactions were sufficient to cleave the respective proteins from the CPD tag). The volume of the phytic acid cleavings was equal to the Ni-beads bed volume. The resulting proteins were analyzed for their purity SDS-PAGE and Coomassie staining and quantified by NanoDrop.

Protein extraction and Western Blotting

NuPAGE soluble proteins (which include CspB, CspC and SleC) were extracted from $2x10^9$/mL purified spores as described previously (37). A separate spore extract from $2x10^9$ *C. difficile* JSC11 (*cspBA::ermB*) spores or *C. difficile* CAA5 (*sleC::ermB*) spores was also generated. Standard amounts of NuPAGE-solubilized recombinant proteins (generated above) were added to a 10% SDS polyacrylamide gel (a standard curve was generated on every gel run). Equal volume of extract from mutant spores was added to the wells containing the recombinant protein standard to allow for equal transfer efficiencies between recombinant protein-containing wells and extract wells. Proteins were separated by SDS-PAGE. After separation, the gels were transferred overnight at 30v to a low fluorescence polyvinylidene difluoride (PVDF) membrane (Bio-Rad). Subsequently, the membrane was blocked for 1 hour at room temperature with 10% dried, skimmed milk in tris-buffered saline (TBS). Each membrane was washed thrice for 15 min each with TBS containing 0.1 % (v/v) Tween 20 (TBST). The membrane was then incubated at room temperature with either rabbit anti-CspC, anti-CspB or anti-SleC antibodies. After incubation with the primary antibody, membranes were washed as above in TBST. The membranes were then labeled with Alexa Fluor 555®-labeled donkey anti-rabbit IgG (Life Technologies) in the dark for 2 hours at room temperature. The membranes were again washed as above but in the dark. Finally, the fluorescent signal on the membranes were detected by scanning on a GE Typhoon Scanner using the Cy3 setting (appropriate wavelength for Alexa

237 Fluor 555®). The resulting scans were quantified using ImageQuantTL 7.0, image analysis
238 software provided by GE.

239 **Statistical Analyses**

240 Statistical analysis between the calculated $EC_{50,TA}$ or inhibitor constant (CDCA) values was
241 accomplished with a One-Way Analysis of Variance (ANOVA) with Tukey's test for multiple
242 comparisons. A 99% confidence interval was set for significance ($p\text{-val} < 0.01$)

243

244 **Results**

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

246 There have been recent reports describing heterogeneity of spore germination between *C.*
247 *difficile* strains (30, 31). Some of these strains appeared to germinate in rich medium in the
248 absence of the TA germinant. Other strains were not inhibited by CDCA (31). This calls into
249 question the impact bile acids may have on *C. difficile* infection and the utility of
250 germination/antigermination-based therapies. While there are undoubtedly differences in
251 germination responses between *C. difficile* strains, the lack of inhibition by CDCA and
252 germination in the absence of TA warranted further investigation.

253 Germination by *C. difficile* strains UK1 and M68 have been described previously by our
254 lab and spore germination by these strains is activated by TA and inhibited by CDCA (25). We
255 also analyzed germination by *C. difficile* strains 5108111, DH1834, DH1858, CD2315
256 (generously provided by Dr. Nigel Minton) and M120. Of these, spores derived from *C. difficile*
257 strains 5108111, DH1834 and CD2315 were reported not to be inhibited by CDCA (spore

germination by DH1858 was inhibited by CDCA) (31). To quantitate the differences in germination responses between isolates, we determined the EC_{50} values for taurocholic acid and spores from different *C. difficile* strains. These types of assays have been useful to determine the potency of activators and inhibitors of germination (11, 24, 25, 38-40). Though not traditional enzyme kinetics (spore germination is a multi-enzyme process), these studies can provide a quantitative measure for the interaction of the bile acids and *C. difficile* spores. *C. difficile* spores were suspended in BHIS medium alone or in medium supplemented with increasing TA concentrations (2 mM, 5 mM, 10 mM, 20 mM or 50 mM) and germination was monitored at OD_{600}. These TA concentrations are routinely used by our laboratory to assign EC_{50} values for bile acids. As shown in Figure 1 and Figure S1, BHIS medium alone did not stimulate germination of the 7 strains tested, suggesting a requirement for TA to initiate spore germination in rich medium. However, when supplemented with increasing concentrations of TA, *C. difficile* UK1, M68 and M120 (Figure 1A, 1F and 1G, respectively) exhibited a concentration-dependent increase in the rate of germination. Interestingly, *C. difficile* strains 5108111, CD2315, DH1834 and DH1858 achieved near maximum germination rates at the lowest tested TA concentration (BHIS + 2 mM TA) (Figure 1B – 1E; Figure S1B – S1E).

Because these strains germinated at near maximum rates in the lowest tested TA concentration, we modified our germination conditions to determine if these strains are capable of germinating at even lower TA concentrations (Figure S2). When germination by *C. difficile* strains 5108111, CD2315, DH1834 and DH1858 were tested at lower TA concentrations, we observed dose-dependent increases in germination rates. From the newly generated germination plots, we generated rate curves and $EC_{50,TA}$ values (Table 1). Spores from *C. difficile* UK1 and *C. difficile* M68 generated $EC_{50,TA}$ values similar to those described previously (25). *C. difficile*

DH1834, DH1858 and M120 had similar $EC_{50,TA}$ values (3.0, 3.03 and 2.37 respectively) and these values are similar to UK1 and M68. Interestingly, compared to the other tested strains, *C. difficile* 5108111 and CD2315 had a statistically significant increased EC_{50} values for TA (Table 1) suggesting that spores derived from these strains require less TA to activate spore germination.

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

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

Inhibition constants were generated from the curves in Figure 2/S3 and the calculated $EC_{50,TA}$ (Table 1). *C. difficile* UK1 spores and *C. difficile* M68 spores generated similar inhibitor constant values as previously described (Table 1) (25) and all strains generated values near or below 0.5 mM. Spores derived from *C. difficile* 5108111 had the weakest interaction with CDCA (0.52 mM; p-val < 0.01) when compared to the other strains. These results suggest that

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CDCA inhibits germination by spores of *C. difficile* strains previously thought not to be affected by the inhibitor.

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

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

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

Previously, germination by *C. difficile* strains 5108111, DH1834, DH1858 and CD2315 occurred slowly but in the absence of the TA germinant (31). To further investigate the phenomenon, we utilized an assay which measures the release of CaDPA from the spore core during germination (CaDPA release is a requirement for spore germination). Because this assay is (i) based on the detection of fluorescent CaDPA-Tb^{3+} complexes and not OD and (ii) each well is mixed prior to analysis, this assay eliminates any chance of spores settling/clumping/localizing to the center of a well during the 4 hour incubation. *C. difficile* spores were suspended in germination buffer supplemented with glycine only (non-germinating conditions) or TA and glycine (germination-inducing conditions) and Tb^{3+} fluorescence was monitored for 4 hours (Figure S7). Spores from all *C. difficile* strains tested released CaDPA which resulted in Tb^{3+} fluorescence when suspended in both TA and glycine. Spores suspended in glycine alone did not release CaDPA during the 4 hour assay. These results suggest that these strains require TA to induce germination in combination with glycine and have a low frequency of autogermination.

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

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To further characterize the differences between these strains, we analyzed the amount of CaDPA present within the core of each strain and normalized this to the amount found in *C. difficile* UK1 (Figure 3H). For all strains, $1x10^5$ spores were boiled in buffer, sedimented and the amount of CaDPA determined using Tb^{3+} fluorescence. As shown in Figure 3H, the amount of CaDPA present in *C. difficile* spores varied between strains (though only by approximately 2-fold in the lowest DPA-containing strain, *C. difficile* DH1834).

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

To begin to understand how different *C. difficile* isolates display different EC_{50} or inhibitor constant values for bile acids, we analyzed the abundance of CspB, CspC and SleC in purified *C. difficile* spores. Purified *C. difficile* spores were extracted with NuPAGE buffer, as described previously (37). We found that by boiling spores in NuPAGE buffer, the amount of remaining CspB, CspC or SleC in the NuPAGE insoluble fraction (containing unbroken spores) to be below the limit of detection. Known amounts of the recombinant CspC, CspB or SleC protein and spore extracts were separated by SDS-PAGE and detected using rabbit polyclonal antisera followed by Alexa Fluor® 555-conjugated donkey anti-rabbit IgG. The standard curves generated from the signals on each SDS-PAGE were linear (Figure 4). From the signal intensities of the extracted CspB (Figure 4A), CspC (Figure 4B) and SleC (Figure 4C), the volume loaded onto the SDS-PAGE, the molecular weights of each protein and the amount of spores extracted, we were able to determine the average abundance of each protein in a *C. difficile* spore (Table 2).

The abundance of these proteins varied between *C. difficile* isolates. For example, *C. difficile* UK1 had approximately 2,000 CspC molecules per spore while *C. difficile* M68 had over

3,800. Interestingly, *C. difficile* CD2315 and *C. difficile* M120 had a very low abundance of CspB (435 and 633 molecules per spore, respectively). As described previously for *C. perfringens*, the average amount of SleC per spore was greater than that of both CspC and CspB but also varied between strains (Table 2) (41).

Prior work has suggested that much of the OD change observed during spore germination is due to the release of CaDPA from the spore core (42). During *C. difficile* spore germination, this event is dependent on the hydrolysis of the spore cortex by SleC (27). Because SleC is deposited into the spore as a zymogen, SleC must be activated by CspB in order to stimulate germination. In our original model for bile acid mediated spore germination, CspB is activated by the CspC germinant receptor. Because the abundance of CspC varied between isolates, we determined if the maximum rate of spore germination correlated with the abundance of CspC. Thus, for each strain we calculated the maximum rate of germination from the germination curves used to calculate the $EC_{50,TA}$ (Table 3). Surprisingly, there was a slight trend for an inverse correlation of the abundance of CspC and rate ($R^2 = 0.32$; Figure 5A), suggesting that CspC may have an inhibitory effect on spore germination.

Because SleC-mediated cortex hydrolysis is required for CaDPA release during *C. difficile* spore germination, if the above trend is true we should observe a positive correlation between the ratio of SleC to CspC and germination rate (more SleC molecules to CspC molecules should result in an increased rate of germination). As shown in Figure 5B, we observed a strong correlation ($R^2 = 0.81$) between SleC/CspC and germination rate, again suggesting that CspC has an inhibitory effect on spore germination.

Discussion

391 Spore germination is an important step in the lifecycle of any spore-forming organism.
392 The metabolically dormant spores must sense when conditions again become favorable for
393 growth and quickly respond to the germinants used by that bacterium. Germination in an
394 environment unfavorable for growth could prevent the outgrowth of a vegetative cell from the
395 germinated spore or prevent the vegetative growth of that bacterium. Thus the signals that
396 stimulate germination (germinants) can vary between organisms depending on their growth
397 niche. For example, *C. difficile* vegetative cells are not normally found growing outside of a host,
398 thus it would be logical for *C. difficile* spores to respond to a molecule that is host-specific. In
399 fact, *C. difficile* spore germination is activated by cholic acid-class bile acids (*e.g.*, TA) and is
400 inhibited by chenodeoxycholic acid-class bile acids (*e.g.*, CDCA) (11, 14, 19, 23). Though
401 essential, TA is not sufficient to stimulate *C. difficile* spore germination. Glycine is also required,
402 presumably as a measure of the nutrient status of the surrounding environment (19, 43, 44).

403 Until recently, most of the studies on *C. difficile* spore germination have relied on
404 identifying germinants and their interactions with bile acids, bile acid analogs, amino acids and
405 amino acid analogs (11, 19, 23-25, 39, 45, 46). Only recently have the mechanisms of *C. difficile*
406 spore germination been studied, largely due to breakthroughs in genetics (47-51). These
407 breakthroughs allowed us to determine that the germination-specific, pseudoprotease, CspC, is
408 the bile acid germinant receptor.

409 The germination-specific proteases have largely been studied in *C. perfringens* (41, 52-
410 54). In *C. perfringens* the CspA, CspB and CspC proteins are all catalytically active proteases
411 that can cleave the cortex hydrolase, SleC, to an active form. In *C. difficile*, the *cspB* and *cspA*
412 genes have been translationally fused and yield CspBA upon translation (26, 29). The CspBA
413 protein undergoes interdomain processing by the YabG protease to generate CspB and CspA

414 (55). We proposed a model whereby activated CspC transmits the signal to the CspB protease
415 which then activates SleC. In support of this model, we found that CaDPA release from the core
416 is dependent on hydrolysis of the spore cortex (27).

417 If SleC activation occurs prior to CaDPA release from the spore core and if SleC-
418 mediated cortex hydrolysis is essential for germination to begin, all *C. difficile* strains should
419 have the requirement for bile acids to initiate germination (unless some *C. difficile* strains
420 germinate using yet another novel mechanism for spore germination). However, some *C. difficile*
421 strains were reported to have no apparent requirement for TA-mediated germination or whose
422 germination was not inhibited by CDCA (31). If germination in these strains was not influenced
423 by bile acids, by what mechanism(s) were they germinating?

424 To characterize the germination phenotypes in these strains, we determined the the
425 $EC_{50,TA}$ and inhibitor constant values for these strains and strains previously shown to be
426 activated by TA and inhibited by CDCA. As suggested previously, the germination
427 characteristics of these strains varied and did not correlate with ribotype (31). Importantly,
428 though, we did observe that some strains have increased $EC_{50,TA}$ values while another had a
429 reduced inhibitor constant for CDCA (Table 1). These strains' increased potency towards TA
430 could possibly explain previous observations (31). Spores derived from strains 5108111,
431 DH1834, DH1858 and CD2315 were found previously to lose OD_{600} in rich medium in the
432 absence of apparent TA (bile acids can be found in blood and, thus, animal products). Moreover,
433 the authors found that germination of spores derived from strains 5108111, DH1834 and
434 CD2315 was not inhibited by the CDCA anti-germinant. Importantly, those strains whose
435 germination was not inhibited by CDCA had increased $EC_{50,TA}$ values. Because the prior work
436 used only one concentration of TA and CDCA [0.1% (1.8 mM) and 2 mM, respectively], to test

437 the effects of CDCA as an inhibitor, it is likely that these strains' increased $EC_{50,TA}$ overcame the
438 inhibitory effect of CDCA (31). By quantifying the interactions between spores derived from
439 these strains and TA /CDCA, we re-establish the importance of bile acids in promoting and
440 inhibiting germination in these strains.

441 The differences in $EC_{50,TA}$ and inhibitor constants were not consistent between ribotypes.
442 A growing body of evidence suggests that toxin production, sporulation and germination are not
443 correlated with ribotype (30, 31, 56). In support of this observation, the calculated $EC_{50,TA}$ and
444 inhibitor constant values were not attributed to specific substitutions within the CspB, CspA or
445 CspC protein sequences and substitutions were ribotype-specific. Of the three proteins, CspB had
446 the fewest substitutions. Because CspB is required to cleave pro-SleC, CspB is likely under
447 evolutionary pressure to perform this function. Interestingly, CspC and CspA had 26 and 42
448 substitutions, respectively. The many substitutions in CspA could suggest that of the Csp
449 proteins, it is under the least amount of selective pressure. Though the role of CspA is unknown,
450 the presence of CspA is important for controlling the levels of the germinant receptor, CspC
451 (55). Only one strain, *C. difficile* CD2315, encoded an extra substitution in the CspC protein
452 sequence that was not present in the other 078 ribotype, P317L. Curiously, *C. difficile* CD2315 is
453 the only strain that germinated in buffered TA without the need for the glycine co-germinant
454 (though glycine still enhanced germination in this strain suggesting the spore still responds to
455 glycine). Clearly, more studies are needed to characterize the effects of these substitutions on *C.*
456 *difficile* spore germination.

457 We further characterized these strains by determining the average per spore abundance of
458 CspB, CspC and SleC (we could not generate a standard curve for CspA – it is insoluble when
459 recombinantly expressed). As expected, the number of molecules per spore varied between

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isolates and did not correlate with ribotype (Table 2). The abundance of each of these proteins is greater than the amount of germinant receptors found in *B. subtilis* (57). However, the increased abundance of CspB and SleC found in the *C. difficile* spore, compared to the *B. subtilis* germinant receptors, is consistent with a recent publication describing the amount of these proteins in *C. perfringens* (41). And, as seen in *C. perfringens*, the amount of SleC was greater than the amount of CspB. The amount of CspC in the *C. difficile* spore was similar to CspB but varied (Table 2).

By determining the kinetics of spore germination for several *C. difficile* strains, we were positioned to determine if the kinetic properties of germination could be attributed to differences in the abundance of germination-specific proteins (CspB, CspC and SleC). In *B. subtilis*, overexpression of the GerAA germinant receptor results in an increase in the germination rate (as measured by the decrease in OD_{600} and by the release of CaDPA) (58). Prior work has shown that much of the OD_{600} change that occurs during germination is due to the release of CaDPA from the spore core (42). In *C. difficile*, CaDPA release is dependent on SleC-mediated cortex hydrolysis (27, 28). Thus, we hypothesized that an increased abundance of SleC would correlate with an increased rate of germination. Also, because SleC activity is dependent on activation by CspB, an increased abundance of CspB could result in more active SleC during germination and, thus, an increased rate of germination. However, we did not observe any correlation between the rate of spore germination and the abundance of the SleC hydrolase or CspB protease. One possible explanation for this could be that the abundance of both SleC and CspB in the *C. difficile* spores are far past saturating levels and any alteration in the observed levels would have minimal effects on the rate of germination. Surprisingly, we observed a trend for an inversed

correlation between the rate of germination and CspC abundance [the more CspC molecules/spore the slower the rate of germination (Figure 5A)].

If this correlation is true, we predicted that more SleC molecules would be needed to overcome the potential inhibitory effect CspC would have on *C. difficile* spore germination. Indeed, we observed a strong correlation between SleC/CspC and the rate of germination (Figure 5B; $R^2 = 0.81$). These results suggest that, instead of the activator function we originally hypothesized, that CspC may be acting to inhibit *C. difficile* spore germination.

If true, how would CspC function during germination? Our prior genetic data suggests that CspC transmits the bile acid signal to begin the germination process, though the mechanism is unclear (13). Recently, GerS was identified and recognized to play a role during germination (59). GerS is a protein that is anchored to the inner leaflet of the outer spore membrane (though the lipidation of GerS is not required for it to function during germination). Interestingly, *C. difficile gerS*$^-$ strains have a defect in germination but still cleave SleC to the active hydrolase form. This suggested that SleC activity, somehow, is inhibited in this strain (59). Based on the correlation between CspC and SleC abundance and our kinetic data, we hypothesize that CspC activates CspB but inhibits SleC activity in the absence of GerS. Investigating the biochemistry of this germinant receptor complex could lead to a greater understanding of the mechanism of initiating *C. difficile* spore germination.

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Table 1. Quantifying the interaction of *C. difficile* spores with bile acids.

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

The apparent $EC_{50,TA}$ and inhibitor constant values were calculated from the germination curves as described previously (11, 25, 32).

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

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

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

Table 2. Number of Csp molecules per *C. difficile* spore

Strain	Average CspB	Average CspC	Average SleC	Ratio SleC/CspC

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

The average number of molecules CspB, CspC and SleC were calculated as described in Figure 4. The numbers represent the averages of three independent extractions ± the standard deviation from the mean.

Table 3. Rates of *C. difficile* spore germination

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

The rates of spore germination were calculated using the linear portion of the germination curves, as described previously (11, 25, 32). Values represent the mean from three independent experiments ± the standard deviation from the mean.

Figure 1. Bile acid-mediated spore germination of several *C. difficile* strains. Purified *C. difficile* spores were suspended in BHIS medium alone (●) or medium supplemented with 2 mM (■), 5 mM (▲), 10 mM (▼), 20 mM (♦) or 50 mM (○) TA. Germination was monitored at OD_{600} as described previously. (A) *C. difficile* UK1, (B) *C. difficile* 5108111, (C) *C. difficile* CD2315, (D) *C. difficile* DH1834, (E) *C. difficile* DH1858, (F) *C. difficile* M68, (G) *C. difficile* M120. A

539 representative sample from 3 independent experiments is shown. For transparency, all plots are
540 shown in Figure S1.

541

542 **Figure 2. Chenodeoxycholic acid inhibits *C. difficile* spore germination.** Purified *C. difficile*
543 spores were suspended in BHIS medium supplemented with 1 mM CDCA (●) or medium
544 supplemented with 2 mM (■), 5 mM (▲), 10 mM (▼), 20 mM (♦) or 50 mM (○) TA and 1 mM
545 CDCA. Germination was monitored at OD_{600} as described previously. (A) *C. difficile* UK1, (B)
546 *C. difficile* 5108111, (C) *C. difficile* CD2315, (D) *C. difficile* DH1834, (E) *C. difficile* DH1858,
547 (F) *C. difficile* M68, (G) *C. difficile* M120. A representative sample from 3 independent
548 experiments is shown. For transparency, all plots are shown in Figure S3.

549

550 **Figure 3. DPA release by several *C. difficile* strains.** Purified *C. difficile* spores were
551 suspended in HEPES buffer (●) or buffer supplemented with 10 mM TA (■), 100 mM glycine
552 (▲) or 10 mM TA and 100 mM glycine (▼). DPA release during spore germination was
553 monitored using Tb^{3+} fluorescence, as described previously. (A) *C. difficile* UK1, (B) *C. difficile*
554 5108111, (C) *C. difficile* CD2315, (D) *C. difficile* DH1834, (E) *C. difficile* DH1858, (F) *C.*
555 *difficile* M68, (G) *C. difficile* M120. (H) Purified *C. difficile* spores were suspended in buffer and
556 incubated at 100 °C for 30 minutes. Total DPA content was normalized to the amount of DPA
557 found in *C. difficile* UK1. All data represent the average of three independent experiments and
558 error bars represent the standard deviation from the mean.

559

Figure 4. Quantifying the abundance of CspB, CspC and SleC in *C. difficile* spores. $1x10^9$ *C. difficile* UK1 spores were extracted with NuPAGE buffer, as described previously. NuPAGE-soluble protein from three independent extracts was loaded onto a 10% SDS polyacrylamide gel along with recombinantly expressed and purified protein, as a protein standard. Samples were separated and detected as described in the materials and methods. Signal intensities were quantified and used to generate a standard curve for (A) CspB, (B) CspC and (C) SleC. Signal intensities in the spore extract samples were quantified and the generated standard curve was used to quantify the abundance of the specified proteins in the spore extracts. Independent standard curves were generated for each strain and the results of the quantification can be found in Table 2. ** full length pre-pro-SleC. * pro-SleC.

Figure 5. Correlating the abundance of *C. difficile* proteins with the kinetics of spore germination. (A) The calculated average per spore abundance of CspC was plotted vs. the calculated rates of spore germination. (B) The ratio of SleC to CspC was plotted vs. the calculated rates of spore germination. For all plots, GraphPad Prism was used to generate the linear fit to the curves and the listed R^2 values represent the fit of the curve to the data.

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