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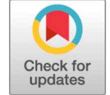

Germinant Synergy Facilitates *Clostridium difficile* Spore Germination under Physiological Conditions

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ABSTRACT *Clostridium difficile* is a Gram-positive obligate anaerobe that forms spores in order to survive for long periods in the unfavorable environment outside 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 (GI) tract through a process known as germination. In this work, we 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 to 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 concentrations of nutrients required to initiate an essential step of pathogenesis.

IMPORTANCE *Clostridium difficile* is an anaerobic spore-forming human pathogen that is the leading cause of nosocomial infectious diarrhea worldwide. Germination of infectious spores is the first step in the development of a *C. difficile* infection (CDI) after ingestion and passage through the stomach. This study investigates the specific conditions that facilitate *C. difficile* spore germination, including the following: location within the gastrointestinal (GI) tract, pH, temperature, and germinant concentration. The germinants that have been identified in culture include combinations of bile salts and amino acids or bile salts and calcium, but *in vitro*, these function at concentrations that far exceed normal physiological ranges normally found in the mammalian GI tract. In this work, we 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 are also able to overcome the effects of inhibitory bile salts. In addition, we 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.

KEYWORDS *Clostridium difficile*, germination, spore

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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 *C. difficile* infection (CDI) (1, 2). *C. difficile* produces spores during conditions unfavorable for vegetative cell growth through a process known as sporulation (3). Spores are metabolically dormant and resistant to numerous harsh environmental conditions that are unsuitable for vegetative cell growth (4). Bacterial 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 (5–8). 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 (9, 10). 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 (3, 8, 11, 12). *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 (13, 14). 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 (15). 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 (GI) tract with a unique pseudoprotease receptor, CspC, that is localized, not to the inner membrane, but to the spore coat (16, 17). It has been proposed that downstream signaling occurring after CspC-bile salt interactions leads to proteolytic activation of the SleC cortex hydrolase, but the molecular details underlying these events remain largely unknown (17–19).

In *C. difficile*, three distinct germination pathways have been described (5, 13, 14, 20, 21). The bile salt amino acid pathway requires relatively high, nonphysiological concentrations (millimolar) of both a bile salt and amino acid (13, 22). Taurocholate (Tc) and glycine are the most effective combination, but other amino acids and bile salt combinations have been identified as viable cogerminant pairs (20). The amino acid concentrations required can be decreased to micromolar levels with the addition of increased amounts of bile salts and vice versa (20). In addition, *C. difficile* spores can germinate in response to bile salts in combination with divalent cations (in the absence of amino acids) (14). Calcium is the most effective cation in inducing germination through the bile salt divalent cation pathway, though magnesium can also induce this process (14). *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 are then able to induce germination in conjunction with bile salts (21). This pathway is described as the alanine racemase-dependent D-amino acid pathway (5). 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 by 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 (18, 23–25). Several previous studies have analyzed *C. difficile* germination and outgrowth in gastrointestinal contents, in this work, we 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 cogerminants play in this process?

RESULTS

***Clostridium difficile* spores germinate in the mouse ileum.** 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 through the gastrointestinal tract. Our data show that *C. difficile*

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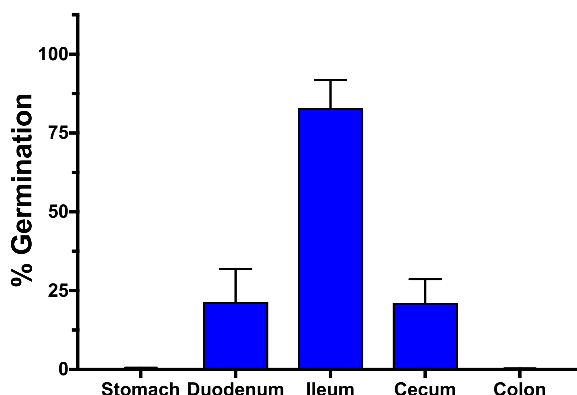

FIG 1 *C. difficile* spores germinate efficiently in the mouse ileum. *C. difficile* VPI 10463 spores were incubated in various GI contents from antibiotic-treated mice ($n = 5$) for 1 h at 37°C. Data are presented as percent germination, which was determined by differential plating (CFU on BHIS/total CFU on BHIS-Tc × 100). One hundred percent germination is the amount of CFU when plated on BHIS+Tc. Statistical analyses were done using two-way analysis of variance (ANOVA), and germination in ileal contents was significantly different from all other conditions ($P < 0.0001$).

spores (strain VPI 10463) were not able to germinate in stomach contents (Fig. 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 (Fig. 1). However, 70 to 90% spore germination was observed from contents sampled from the ileum (Fig. 1). These data suggest that spore germination occurs in the small intestine, specifically in the ileum, prior to colonization and infection of the large intestine. The lack of robust germination in duodenal contents is an unexpected finding and may be due to slightly acidic pH within the duodenum (see Fig. S1A in the supplemental material) (26, 27). On the basis on these data, we hypothesize that the acidity in the duodenum may be nonconducive for germination and thus, pH plays a role in regulating germination within the small intestine.

***C. difficile* spores are acid resistant and able to germinate when pH neutralizes.** Since *C. difficile* spore germination involves critical biochemical processes that are dependent on the activity of at least two enzymes (CspB and SleC), we 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 (20). We confirmed that germination efficiency is improved at temperatures ranging from 37 to 40°C (data not shown). However, since temperature is consistent across the gastrointestinal tract, it is unlikely that temperature is a key environmental regulator of *C. difficile* spore germination during infection.

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 brain heart infusion broth plus taurocholate (BHIS+Tc) adjusted to a range of different pHs (as indicated) for 1 h at 37°C, and germination was measured by an optical density (OD) assay. Similar to findings from previous studies, *C. difficile* spores exhibited efficient germination when incubated at pH values between 6.5 and 8.5 but germinated poorly at a pH of 9.5 or not at all below 5.5 or above 10.5 (Fig. 2A) (28, 29). These data were confirmed by observing the zymogen processing and activation of SleC by Western blotting. Activation of SleC was observed at pH between 6.5 and 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 (Fig. 2C). These data show that highly acidic (or basic) pH is able to inhibit *C. difficile* spore germination.

In the context of an *in vivo* infection, however, *C. difficile* spores pass through the stomach and then the ileum where the majority of germination occurs (Fig. 1). We hypothesize that acidic pH, such as those conditions in the stomach, will inhibit

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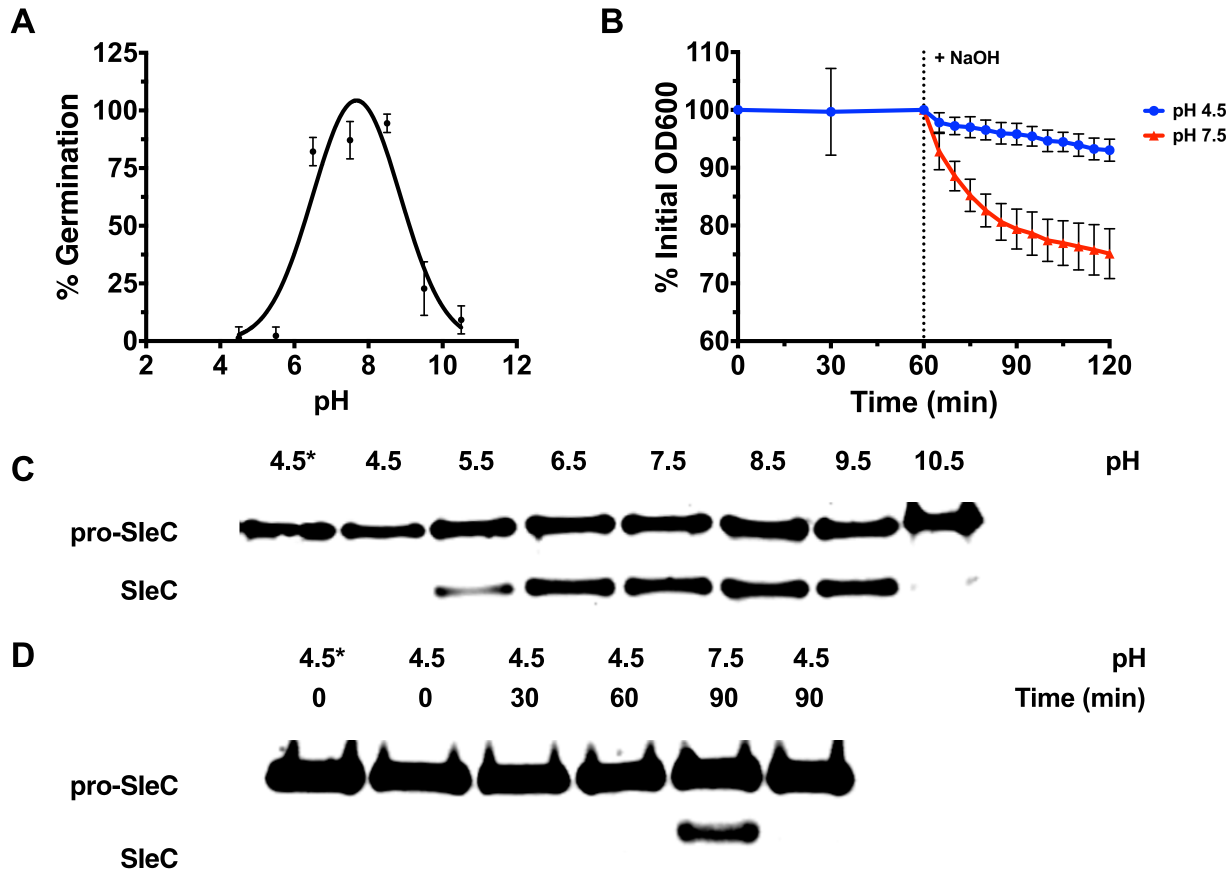

FIG 2 pH regulates *C. difficile* spore germination *in vitro*. (A) *C. difficile* VPI 10463 spores were incubated at 37°C in BHIS+Tc at various pH values for 1 h, and germination was monitored by decrease in OD_{600}. (C) VPI 10463 spores were incubated at 37°C in BHIS+Tc at various pH values for 15 min, and SleC activation was observed by Western blotting. (B) VPI 10463 spores were incubated at 37°C with 0.2% Tc in BHIS at a pH of 4.5 for 1 h, and optical density was tracked every 30 min. After 1 h, 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 decreases in the OD at 37°C over the course of 1 h. (D) For observing SleC activation, VPI 10463 spores were incubated as described above, and samples were taken every 30 min and prepared for Western blotting. Germination (loss of OD) assays are the results from three independent spore preparations. Western blots are representative of three independent spore preps. In panels C and D, samples labeled with an asterisk indicate that the sample did not receive taurocholate.

germination even in the presence of germinants and that these spores maintain the capacity to germinate once the pH is neutralized in the ileum. To test this hypothesis *in vitro*, *C. difficile* spores were exposed to acidic conditions and germinants (BHIS+Tc [pH 4.5]) for 60 min. Following this incubation, a portion of the spores were removed, and the solution was neutralized (pH changed to ~7.5). No measurable germination was observed during the 1-h pretreatment with BHIS+Tc at a pH of 4.5; however, a rapid drop in optical density, a result of spore germination, was detected within the first hour after neutralization of the pH (Fig. 2B). In comparison, a minimal drop in optical density was detected for spores that remained at pH 4.5 (Fig. 2B). Zymogen processing of SleC was also assessed in spores from these samples. SleC activation was detected only in samples that were adjusted to a pH of 7.5 (Fig. 2D). Taken together, these data suggest that CspB activity is sensitive to alterations in pH. It remains possible that events upstream of CspB activation (such as germinant sensing) or conformational dynamics of SleC are sensitive to pH. Regardless, germination is inhibited by acidic pH, and these data suggest that pH may regulate germination along the gastrointestinal tract.

pH regulates germination within the small intestine. Only 25% of *C. difficile* spores are able to germinate in the duodenum, despite the presence of sufficient levels of germinants, as both bile salts and calcium are secreted into the duodenum through the bile duct (Fig. 1). In humans, the pH rises from ~2.5 in the stomach to 4.5 to 5.5 in

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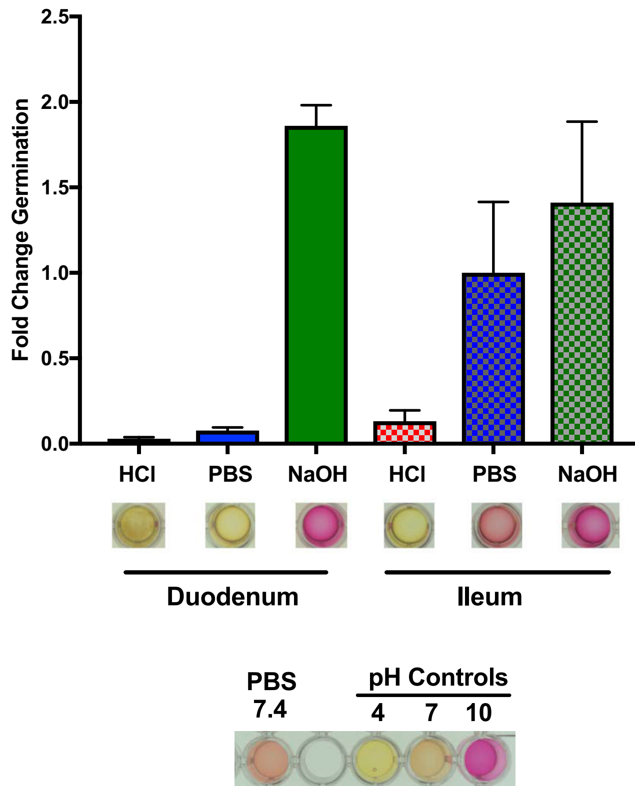

FIG 3 pH regulates *C. difficile* spore germination in small intestinal contents. Duodenal contents and ileal contents were harvested from mice treated with antibiotics ($n = 5$), and the contents were then solubilized in PBS. Supernatants were aliquoted into three groups: unadjusted (PBS), adjusted with acid (HCl), or adjusted with base (NaOH) before incubation with VPI 10463 spores for 30 min. Germination was measured by differential plating as described in Materials and Methods. Data are presented as the fold change in percent germination compared to percent germination in unadjusted ileal contents. pH was estimated using phenol red (pH < 6.8 [yellow] and pH > 8.2 [pink]).

the duodenum and eventually reaches ~7.4 in the ileum (26, 27). We hypothesize that *C. difficile* spores germinate poorly within mouse duodenal contents because the pH of these contents is too acidic and if pH is neutralized, then germination would increase. To test this, small intestinal contents were harvested from antibiotic-treated mice and resuspended in phosphate-buffered saline (PBS) (pH 7.4). The pH of these samples was estimated by phenol red indicator and adjusted by adding 0.5 μl of NaOH or HCl before *ex vivo* germination assays were performed (Fig. S1B). Contents harvested from the duodenum had an acidic pH (<6.8), and ileal contents were neutral (~7.4). The levels of germination in unadjusted duodenal contents or acidic duodenal contents were 0.07× and 0.02× that of unadjusted ileal contents, respectively (Fig. 3). Adding NaOH to duodenal contents improved germination levels to ~1.8× that of unadjusted ileal contents and ~20× that of unadjusted duodenal contents (Fig. 3). In addition, adding HCl to ileal contents reduced the levels of germination to 0.13× that of unadjusted ileal contents (Fig. 3). These data show that duodenal contents are too acidic to support efficient germination of *C. difficile* spores, and once the pH is neutralized (as in the ileum), germination is able to proceed. Taken together, these data suggest that pH regulates germination along the gastrointestinal tract allowing for germination only within the ileum.

***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 (14, 23, 24). 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, the rate of germination of *C. difficile* spores,

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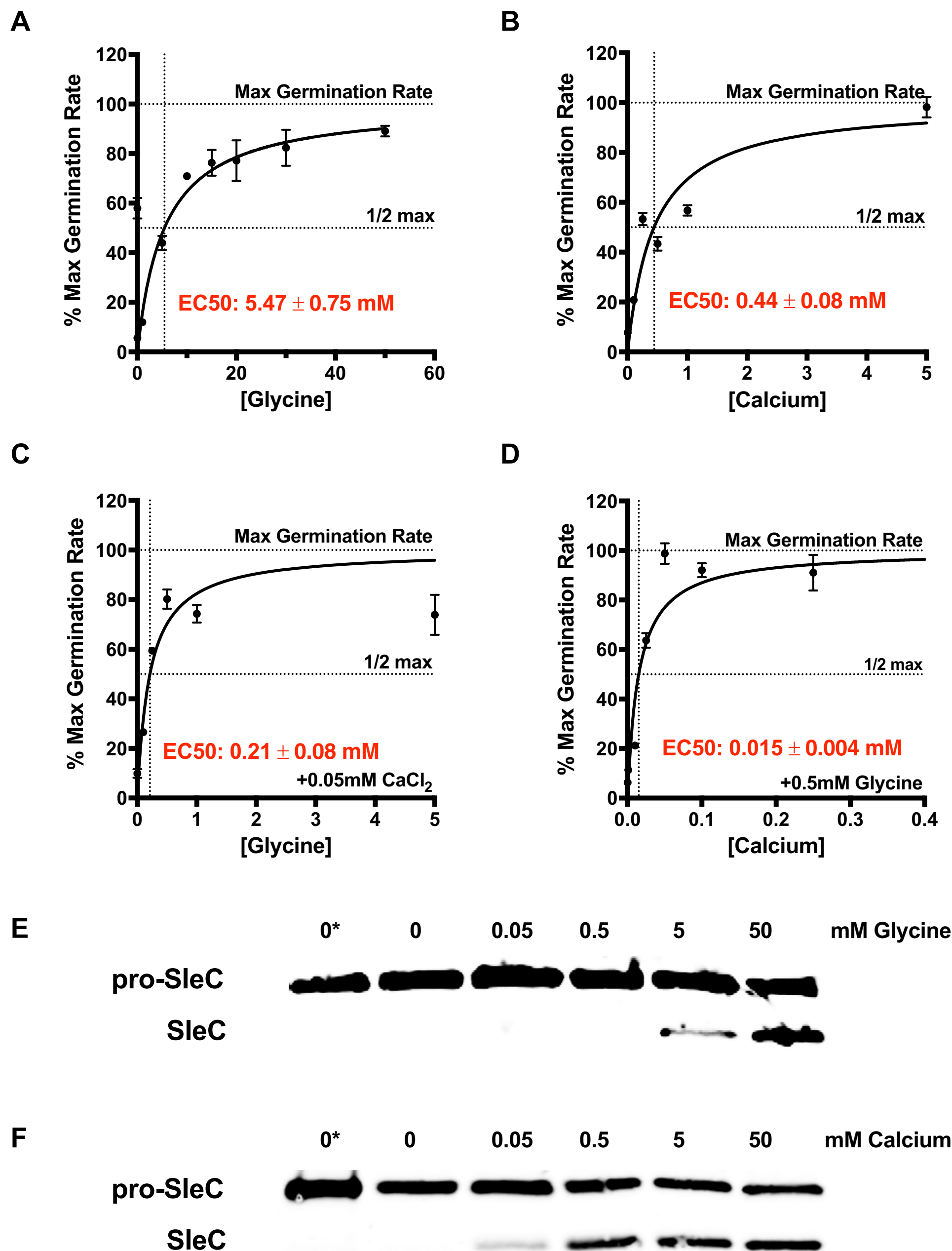

FIG 4 *C. difficile* germination rates are dependent on concentrations of germinant. (A and B) *C. difficile* VPI 10463 spores were incubated with 0.2% Tc and various concentrations of either glycine (A) or $CaCl_2$ (B), and rates of release of DPA were measured and presented as percent maximal germination rate. (C) VPI 10463 spores were incubated with 0.2% Tc, 50 μM $CaCl_2$, and the indicated concentrations of glycine, germination rates were observed by DPA release, and EC50 values were calculated. (D) VPI 10463 spores were also incubated with 0.2% Tc, 500 μM glycine, and the indicated concentrations of calcium, germination rates were observed by DPA release, and EC50 values were calculated. SleC activation was observed by Western blotting, VPI 10463 spores were incubated in Tris-HCl plus 1% Tc, and the indicated concentrations of glycine (D) or $CaCl_2$ (E). In panels D and E, samples labeled with an asterisk indicate that the sample did not receive taurocholate.

is also dependent on the concentrations of germinants present. In order to investigate the effect of specific germinants and cogerminants on *C. difficile* germination kinetics, DPA release assays were performed, and the concentration of germinant required to reach 50% of the maximal germination rate (EC50) was calculated using Michaelis-Menten-like kinetics (Fig. 4) (20). All dose-response assays were performed using 0.2% Tc and different concentrations of the indicated cogerminants (Fig. 4).

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Glycine has been shown to be the most effective amino acid cogerminant, and this is confirmed in our analyses; here glycine was found to have an EC50 of 5.47 mM (20) (Fig. 4A). This absolute value differs somewhat from that previously reported likely due to strain-to-strain variation (VPI 10463 versus UK1) and variations in concentrations of Tc (2 mM/0.1% versus 10 mM/0.5%) used (20). Interestingly, calcium was extremely efficient at inducing germination with an EC50 of 0.44 mM in the presence of Tc but in the absence of any amino acids (Fig. 4B). Dose-response analysis was also performed by observing the activation of SleC by Western blotting. Spores were incubated for 15 min at 37°C with 1% Tc and the indicated concentrations of glycine or calcium. SleC activation was observed at concentrations as low as 5 mM for glycine (Fig. 4E) and 0.05 mM for calcium (Fig. 4F).

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 10 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% (14). 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, we 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 (14).

In order to quantify this "synergy" observed with addition of glycine and calcium in combination, we performed DPA release assays and calculated EC50s for both glycine and calcium when the other was present at 10% of its EC50 (50 μM calcium or 500 μM glycine). The EC50 for glycine was reduced from 5.5 mM to 0.210 mM when calcium (50 μM) was present (Fig. 4C). The EC50 for calcium also decreased from 440 μM to 15 μM when glycine (500 μM) was present (Fig. 4D). 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 (14, 30).

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, we hypothesize that calcium will also improve the effectiveness of other, less efficient, amino acid cogerminants. 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 10 mM/0.5% concentration of Tc), only glycine (1 or 10 mM) was able to induce DPA release (Fig. 5A to D) (20). However, when 50 μM $CaCl_2$ was added simultaneously with Tc and amino acids, DPA release was observed at near-maximal levels for spores incubated with 10 mM concentrations of glycine, L-alanine, and L-serine (Fig. 5A to C, illed blue symbols). L-histidine also exhibited appreciable, though significantly lower, levels of DPA release at 10 mM plus calcium with Tc (Fig. 5D). 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.

Glycine does not have robust synergy with other amino acids. Since calcium is able to synergize with multiple amino acids, we 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, 500 μM glycine (10% of the EC50), and the indicated concentration of $CaCl_2$, L-alanine, L-serine, or L-histidine and assayed for DPA release. Spores incubated with 0.1 mM $CaCl_2$ had a >5-fold increase in the amount of DPA released when incubated simultaneously with 500 μM glycine (Fig. S2, purple filled symbols). However, when spores were incubated simultaneously with 1 or 10 mM L-alanine, L-serine, or L-histidine and 500 μM glycine in the absence of calcium, no DPA release was observed (Fig. S2B to D). These data indicate that while calcium can synergize with a variety of amino

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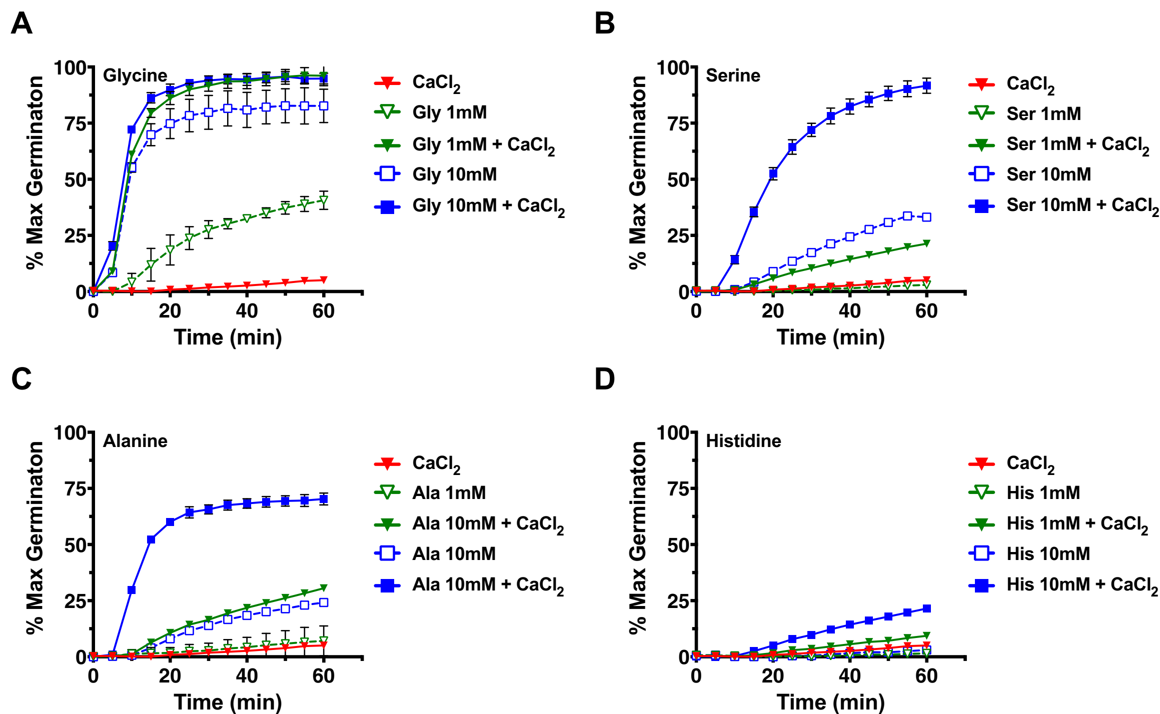

FIG 5 Calcium synergizes with other amino acids to improve *C. difficile* germination efficiency. (A to D) *C. difficile* VPI 10463 spores were incubated with 0.2% Tc, 50 μM $CaCl_2$, and either 1 or 10 mM concentration of glycine (A), serine (B), alanine (C), or histidine (D), and release of DPA was measured over the course of an hour. Data from three independent experiments and two spore preparations were normalized and presented as percent maximal germination.

acids, glycine does not have robust synergy with other amino acids. On the basis of these data, we conclude that by adding combinations of amino acids, the germination response will be additive and may be the result of different amino acids being able to interact with the same receptor with variable efficiency.

In addition, we hypothesized that glycine might synergize with other divalent cations. We previously showed that in addition to calcium, magnesium is able to induce slight germination when present at fairly high concentrations (50 mM) (14). In order to test this hypothesis, *C. difficile* spores were incubated simultaneously with 0.2% Tc, 500 μM glycine, and 30 mM concentrations of either zinc, lithium, potassium, or magnesium. Of the cations tested, only magnesium was able to induce appreciable germination at 30 mM, and this germination was enhanced by the addition of 500 μM glycine (Fig. S3A to 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 either amino acids might compete for a single binding site on a single receptor, while the cations interact with another site of either the same receptor or that different germinants bind altogether different receptors from one another.

Germinant synergy overcomes chenodeoxycholate inhibition. While taurocholate is the most effective bile salt at inducing *C. difficile* germination, most derivates of cholate are also effective. However, chenodeoxycholate (CDCA), and several secondary bile salts, can function as competitive inhibitors of germination and outgrowth (31). Because of this, it has been hypothesized that inhibitory bile salts block germination by directly interfering with taurocholate-CspC interactions (32). On the basis of the results presented above, we hypothesized that calcium-glycine synergy can overcome CDCA inhibition of germination. In order to test this hypothesis, we incubated *C. difficile* spores with 20 mM glycine and various concentrations of taurocholate in the presence and absence of 0.25 mM CDCA, and germination was measured by DPA release. CDCA decreased germination to less than 25% at physiological concentrations of Tc (0.05%) compared to ~50% in the absence of CDCA; however, this inhibition was overcome

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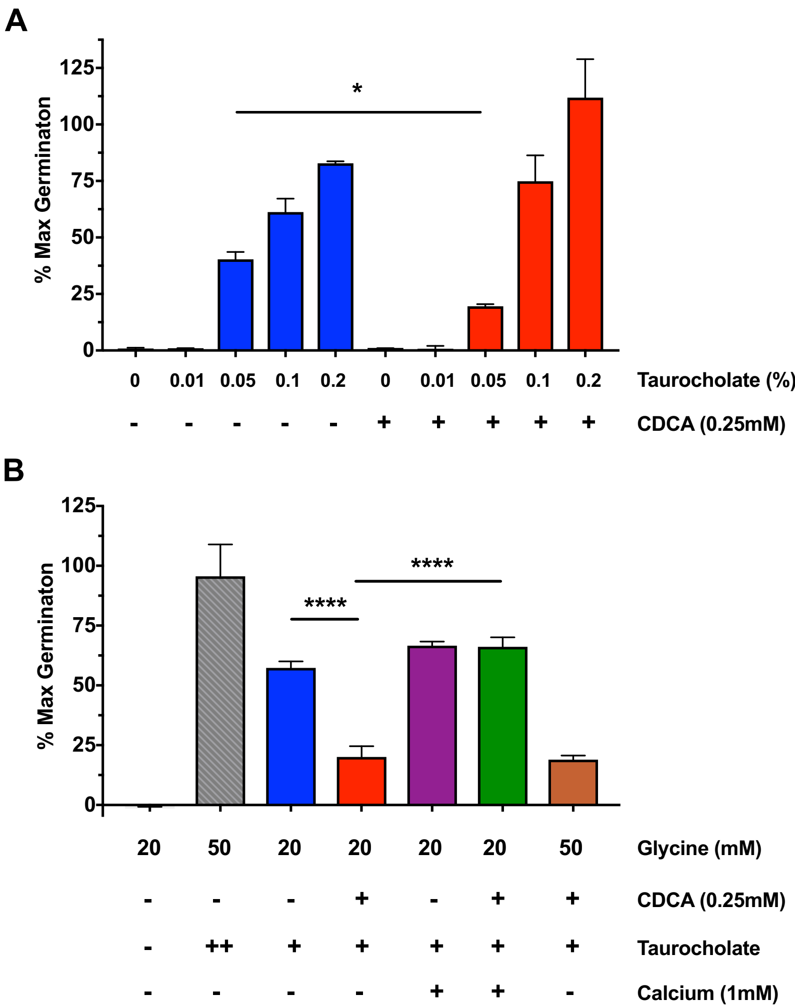

FIG 6 Germinant synergy overcomes chenodeoxycholate inhibition of germination. (A) *C. difficile* VPI 10463 spores were incubated with 20 mM glycine in the presence (+) or absence (−) of 250 μM CDCA and the indicated concentrations of taurocholate, and release of DPA was measured after 60 min. (B) VPI 10463 spores were incubated with taurocholate (0.05% [+] or 0.2% [++]) and the indicated concentrations of either glycine, calcium, or CDCA, and release of DPA was measured after 60 min. Data from three independent experiments and two spore preparations were normalized and presented as percent maximal germination. Data were analyzed by one-way ANOVA, and values that are significantly different are indicated by a bar and asterisks as follows: *, $P = 0.0269$; ****, $P < 0.001$.

when Tc concentrations were increased (Fig. 6A). In order to test whether the addition of excess glycine or calcium could overcome CDCA treatment, *C. difficile* spores incubated with 0.05% Tc, 0.25 mM CDCA, and the indicated combinations of 20 mM or 50 mM glycine or 1 mM $CaCl_2$, and germination was measured by DPA release. Again, CDCA decreased germination with 20 mM glycine to <25% of maximal germination; however, CDCA-mediated inhibition was overcome with the addition of 1 mM $CaCl_2$, restoring germination to >60% (Fig. 6B, 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 50 mM (Fig. 6B, brown bar). Taken together, these data suggest that calcium-glycine synergy decreases the necessary concentrations of cogerminants required to overcome inhibitory bile salts present within the gastrointestinal tract.

DISCUSSION

In this study, we investigated some of the specific environmental conditions that facilitate *C. difficile* spore germination that we hypothesized matched those encoun-

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tered *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 (30, 32–35). These studies hypothesize that exposure to antibiotics may influence, albeit indirectly, spore germination and therefore patient susceptibility to contracting CDIs. Several studies from the Young and Theriot groups have shown that while germination may be enhanced after antibiotic treatment, it is always supported in murine ileal contents (32, 33, 36). On the basis of our findings in Fig. 6, we conclude that it is possible 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. This suggests that while there is an apparent effect 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 larger role in the large intestine where secondary bile salts would inhibit growth of vegetative *C. difficile* rather than inhibiting germination.

Interestingly, 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 (Fig. 1). In humans, the pH changes rapidly from the stomach pH of ~2 to pH ~4.5 to 5.5 in the duodenum; however, our data show that *C. difficile* spores are not germinating in the duodenum because the contents are too acidic to support efficient germination and when the pH is increased artificially, efficient spore germination is able to proceed (Fig. 3). While it is known that *C. difficile* spores are acid resistant, data in Fig. 2 indicate that they are not able to germinate under acidic conditions even when all the nutrients necessary to facilitate this process are present. Importantly, 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 (Fig. 2B). Taken together, these data suggest that alterations in pH have a profound effect on *C. difficile* spore germination by altering the spore enzymatic activity (Fig. 2 and 3). Additionally, although efficient spore germination shows a degree of temperature dependency, the temperature inside a mammal is constant, and it is unlikely that temperature is an environmental signal controlling the regulation of germination within the gastrointestinal tract.

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 (37). The use of PPIs 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 (38–40). PPIs, by design, increase the pH of the upper gastrointestinal tract, including the stomach and the proximal duodenum (41). 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 effects could lead to increased germination efficiency within the small intestine and alterations in the microbiota that allow *C. difficile* colonization at lower infectious doses.

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 (Fig. 4 and 5). Glycine was previously known to be the most effective amino acid cogerminant, and although our data confirm this observation, they also show that calcium is approximately 10 times more effective than glycine at equivalent concentrations (Fig. 4). However, for all germination pathways, maximal germination rates are achieved using nonphysiological concentrations of

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germinants (14, 20, 21). We 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 (14). Here, we 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, noninducing concentrations (50 μM), it improves the effectiveness of amino acids, effectively decreasing the concentrations of all germinants required to facilitate *C. difficile* germination. Interestingly, when glycine is added at noninducing concentrations (500 μM), 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. carry genes that encode numerous *gerA* type germination receptors, each recognizing a specific amino acid or amino acid combination (15). These findings suggest that calcium and amino acids may be interacting with different proteins, but the amino acids could also be interacting with the same receptor with variable efficiency. It is also just as likely that calcium and glycine are interacting with the same receptor, and calcium acts as a cofactor that improves the effectiveness of this receptor. Regardless, taurocholate and calcium/glycine (both together and separately) induce germination through the activation of SleC. This combined pathway is likely the most physiologically relevant pathway given that bile salts, amino acids, and calcium are all present in the ileum at concentrations that are too low to induce germination without synergy. These observations raise questions for the future. The identity of the specific protein(s) with which the amino acids or calcium is interacting remains to be elucidated. In addition, the signaling cascade that occurs after these germinant-receptor interactions that lead 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 the biochemical modifications of the spore (14). Further understanding of the biology of spores 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 medium (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 (16, 42). Briefly, *C. difficile* VPI 10463 spores were grown overnight in BHIS broth and then plated onto 70:30 plates for 4 days (42). Spores and cellular debris were scraped off the plates and resuspended in phosphate-buffered saline (PBS). Samples were spun down, PBS was removed, and spores were purified with 50% histodenz as described previously.

***Ex vivo* germination assays.** Intestinal contents were isolated from C57BL/6J mice following treatment with cefoperazone (MP Biomedicals) (0.5 mg/ml) in sterile drinking water for 5 days. Intestinal contents were resuspended in PBS (pH 7.4) at 1 ml PBS per 1 mg of contents. Samples were vortexed, and solid material was removed by centrifugation at maximum speed for 10 min. Aliquots of supernatant (45 μl) were left unadjusted or adjusted with 0.5 μl of 1 M HCl or 3 N NaOH. Supernatants were added to 5 μl of a pretitered spore stock and incubated for 30 min (unless otherwise indicated) in an anaerobic chamber. Following this incubation, samples were serially diluted and plated on BHIS plates with cefoxitin (8 μg/ml), D-cycloserine (250 μg/ml), and kanamycin (50 μg/ml) without taurocholate (Tc). 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 plus 0.1% Tc, which was defined as 100% germination. pH was estimated by adding 5 μl of phenol red (0.05%) to 45-μl aliquots of adjusted or unadjusted supernatants.

Germination assay (loss of OD). The loss of optical density (OD) was tracked at 600 nm over a 1-h 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 (14, 15, 43). Purified spores were added to BHIS+Tc (0.2%) at the indicated pHs at a starting OD of ~0.2. The assays were performed in triplicate using three independent spore preparations. The optical density at 600 nm (OD_{600}) measurements were taken every 5 min for 1 h. For results presented as percent germination, a drop in OD to 55% of its initial OD represents approximately 100% spore germination.

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Western blot analysis. SleC activation was observed by Western blotting as described previously (14, 16, 23). Briefly, 1×10^6 spores were added to the indicated germinant mixtures plus 1% taurocholate and incubated at 37°C for the indicated time points. Spores were pelleted, and then resuspended in 40 μl of EBB lysis buffer (23). Samples were run on a 4 to 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 with an IR800 Li-Cor secondary antibody. The membrane was then washed three times with Tris-buffered saline with Tween 20 (TBS-T) before Li-Cor (Odyssey) detection.

DPA release assay. Dipicolinic acid (DPA) release was measured by terbium fluorescence (14, 18, 20, 21). Solutions of germinants were incubated in 100 mM Tris-HCl (pH 7.4) at 37°C with 800 μM $TbCl_3$ and placed in an M2 microplate reader (Molecular Devices) (excitation wavelength, 270 nm; emission wavelength, 545 nm; cutoff wavelength, 420 nm). Readings were taken every 5 min for an hour. The concentrations of germinant required to reach 50% of the maximal germination rate (EC50 values) were calculated from the slopes of germination rates plotted against time. Data were analyzed with Michaelis-Menten kinetics, and V_{max} was calculated. V_{max} was set at the maximal germination rate, and data were normalized as a percentage of the maximal rate. Each curve was performed in triplicate and are the results of biological duplicates. When data are presented as percent maximal germination, this is defined as the amount of DPA released in response to our positive control (0.2% Tc and 50 mM glycine) for each individual experiment.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mSphere.00335-18.

FIG S1, PDF file, 1.5 MB.

FIG S2, PDF file, 0.1 MB.

FIG S3, PDF file, 0.1 MB.

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