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Chenodeoxycholate Is an Inhibitor of *Clostridium difficile* Spore Germination▽

Joseph A. Sorg and Abraham L. Sonenshein*

Department of Molecular Biology and Microbiology, Tufts University, School of Medicine, Boston, Massachusetts

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Some cholate derivatives that are normal components of bile can act with glycine to induce the germination of *Clostridium difficile* spores, but at least one bile component, chenodeoxycholate, does not induce germination. Here we show that chenodeoxycholate inhibits the germination of *C. difficile* spores in response to cholate and taurocholate.

The anaerobic human pathogen *Clostridium difficile* must be in the spore form to survive for extended periods of time outside the colonic environment (6). Spores are also the form of the organism most likely to be ingested by a host. To cause disease, however, *C. difficile* spores must germinate in the gastrointestinal tract and reach the anaerobic environment of the colon, where they can grow out as vegetative bacteria (2). The vegetative form produces two toxins that damage the colonic epithelium and lead to *C. difficile*-associated diseases, such as diarrhea, pseudomembranous colitis, and toxic megacolon (4, 15). Extending the work of Wilson and colleagues (17, 18), we have shown that certain bile salts and glycine act as cogerminants for *C. difficile* spores (13). Primary bile salts produced by the liver are composed mainly of cholate (CA) and chenodeoxycholate (CDCA) derivatives conjugated with either taurine or glycine (11). Since CA derivatives are found in the relatively aerobic proximal ileum (9), we reasoned that *C. difficile* might benefit if its germination were inhibited until the spores reached the anaerobic environment of the large intestine.

Inhibitors of germination are typically structurally similar to the germinant whose activities they inhibit. For example, L-alanine-mediated germination of *Bacillus subtilis* spores is inhibited by D-alanine (16) and 6-thioguanosine inhibits inosine-mediated germination in *Bacillus anthracis* (1, 16). Since CA and CDCA are structurally similar but CA induces the germination of *C. difficile* spores (13) and CDCA does not, we tested whether CDCA could act as an inhibitor of germination. *C. difficile* strain CD196 (10) spores were produced and their concentration determined as described previously (13). After the vegetative bacteria were killed by incubation at 60°C for 20 min, spores were incubated in water containing various concentrations and combinations of bile salts for 10 min. Here we took advantage of the finding by Wilson et al. that *C. difficile* spores germinate very inefficiently on rich medium plates lacking bile salts (18) unless they are preincubated with bile salts (13, 17). After incubation, spores were serially diluted and plated on brain heart infusion agar supplemented with 5 g yeast extract per liter–0.1% L-cysteine (BHIS) (Difco) in the absence of any bile salt (BHIS contains enough glycine to act as a cogerminant). After overnight growth at 37°C, colonies were enumerated. As a positive or negative control, spores were plated on BHIS containing 0.1% taurocholate (TA) [BHIS(TA)] or on BHIS agar alone, respectively. Preincubation of spores with 0.1% TA in water resulted in the recovery of approximately 0.5% of the total number of spores as colonies compared to results for spores plated directly on BHIS(TA). These results are similar to our previous findings that spores germinate and grow out as colonies more efficiently on agar medium containing TA (13). As reported previously, 0.1% CDCA poorly stimulated colony formation by *C. difficile* spores (13), yielding only 0.006% spore recovery (Fig. 1A). When TA and CDCA were combined, both at 0.1%, colony formation by *C. difficile* spores was reduced 21-fold to 0.024% compared to the effect of TA alone. This result indicates that CDCA blocks TA-stimulated colony formation and suggests that CDCA may be an inhibitor of *C. difficile* spore germination. Increasing the ratio of TA to CDCA suppressed the inhibitory effect of CDCA, increasing colony formation by spores (Fig. 1A). Thus, CDCA seems to block colony formation by competing with TA.

CA and other cholate derivatives (e.g., TA, glycocholate, and deoxycholate [DCA]) are also germinants for *C. difficile* spores (13, 17). To test if CDCA prevents colony formation induced by CA, spores were preexposed to 0.1% CA with and without CDCA. Exposure to CA alone resulted in approximately 1% spore recovery, whereas exposure to 0.1% CA and 0.1% CDCA together led to a decrease in colony formation to 0.075% (Fig. 1B). The effect of CDCA on CA-mediated colony formation was relieved by increasing the concentration of CA to 1.0%, raising colony formation to 2.6% (Fig. 1B). These results indicate that CDCA blocks colony formation induced by CA, as well as that induced by TA, and may be an inhibitor of germination by *C. difficile* spores that acts competitively in both cases.

Spore germination per se is classically measured as a decrease in the optical density of a spore suspension occurring concomitantly with a release of Ca^{2+}-dipicolinate from the spore core, rehydration of the core, and degradation of the cortex (8, 12). As determined by this assay, TA is the most effective bile salt for inducing rapid germination (13). To test if CDCA is an inhibitor of germination as opposed to an

* Corresponding author. Mailing address: Department of Molecular Biology and Microbiology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111. Phone: (617) 636-6761. Fax: (617) 636-0337. E-mail: linc.sonenshein@tufts.edu.

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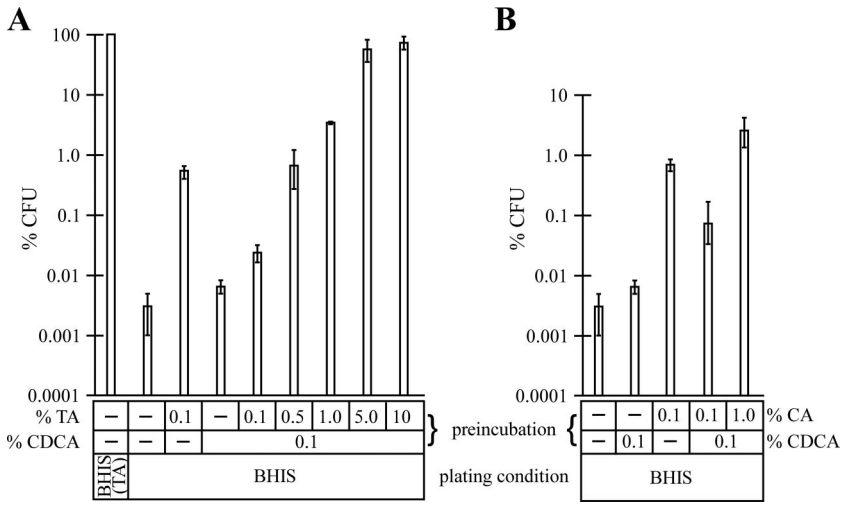

FIG. 1. CDCA inhibits colony formation by *C. difficile* spores in response to TA and CA. (A) Spores were prepared and preincubated with TA or CDCA or both in water for 10 min before serial dilution and plating on BHIS agar in the absence of TA. Spores plated on BHIS(TA) served as a positive control for 100% colony formation (CFU). Based on comparisons of total spore counts obtained by microscopy and by colony formation on BHIS(TA) plates, the efficiency of colony formation on BHIS(TA) was estimated at 83%. (B) Spores were prepared as described for panel A and exposed to CA or CDCA or both. Values shown are the averages for three independent experiments, and error bars represent one standard deviation from the mean.

inhibitor of some other step between germination and colony formation, spores were purified as described previously (13). Spores did not germinate in BHIS medium alone or when this medium was supplemented with 0.1% CDCA (Fig. 2). When *C. difficile* spores were suspended in BHIS containing 0.1% TA, the optical density of the suspension rapidly decreased, indicating that the spores were germinating. However, the optical density of the spores suspended in BHIS with 0.1% TA plus 0.1% CDCA did not decrease over time, indicating that CDCA inhibited TA-mediated germination (Fig. 2). When the concentration of TA was increased from 0.1% to 1.0% in the presence of 0.1% CDCA, spores were able to germinate (Fig. 2). After overnight incubation in BHIS with 0.1% TA plus 0.1% CDCA, 84% of the spores remained phase bright, while only 11% of spores remained phase bright in BHIS with 1.0% TA plus 0.1% CDCA, indicating that CDCA blocks germination at a very early step. Thus, CDCA is an inhibitor of germination by *C. difficile* spores that functions by competing with TA and possibly with CA.

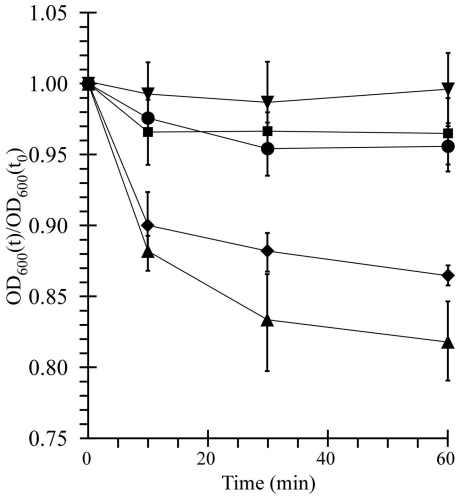

FIG. 2. CDCA inhibits germination of *Clostridium difficile* spores. Spores were prepared as described previously (13). *C. difficile* spores were suspended in BHIS alone (●), BHIS plus 0.1% CDCA (▼), BHIS plus 0.1% TA (◆), BHIS plus 0.1% TA–0.1% CDCA (■), or BHIS plus 1.0% TA–0.1% CDCA (▲). The ratio of the OD_{600} at the various time points to the OD_{600} at time zero is plotted versus time. Data points are the averages of three independent experiments, and error bars represent one standard deviation from the mean.

We previously suggested a role for bile salts in determining the ability of *C. difficile* to colonize and cause disease (13). In this model, germination of *C. difficile* spores depends on interaction with glycine and certain bile salts. We show here that the primary bile salt CDCA inhibits germination of *C. difficile* spores. As mentioned above, germination inhibitors are commonly structurally related to the germinant they inhibit. The structures of CA derivatives and CDCA derivatives are very similar; they differ only insofar as CDCA lacks the 12α hydroxyl group (11).

CDCA and CA derivatives are present in approximately equal concentrations in the cecum (5). Under such conditions, CDCA would compete with CA derivatives for binding to putative germinant receptors on *C. difficile* spores. Mekhjian and colleagues measured the colonic absorption rates of CDCA, CA, and DCA that were introduced into the cecum and collected at the distal colon (7). They found that CDCA was absorbed by the colon at 10 times the rate for CA (7). Thus, when spores reach the distal large intestine, they encounter a decreased ratio of CDCA to CA. Such a change in ratio might allow CA derivatives to act as effective germinants. Thus, *C. difficile* spores would not be expected to germinate until they reach the colon, which also provides the anaerobic environment required for *C. difficile* growth.

The colonic microflora, which is known to protect the host against *C. difficile* infection, plays a significant role in the

metabolism of bile salts (3, 11). Many different species express on their cell surfaces bile salt hydrolases that serve to remove the conjugated tauryl or glycyl groups from primary bile salts (11). After deconjugation, CA and CDCA are further metabolized by a small percentage of the bacterial species in the cecum to the secondary bile salts deoxycholate and lithocholate, respectively (11, 14). Deoxycholate is an inhibitor of *C. difficile* growth (13, 17). CDCA inhibits both germination and growth (13). The use of CDCA either as prophylaxis or as a therapy for *C. difficile*-associated disease might be helpful for patients who are undergoing antibiotic regimens or who are colonized by this bacterium. For example, when an antibiotic that is known to be associated with an increased risk of inciting *C. difficile*-associated disease is administered, the coadministration of CDCA might protect that individual from colonization by *C. difficile* through inhibiting spore germination. Alternatively, administering CDCA to individuals who are already being given vancomycin or metronidazole for *C. difficile*-associated disease may have the benefit of preventing spore germination and further vegetative growth (13) after antibiotic therapy is stopped. This strategy may reduce the already significant risk of a relapse.

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