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Muricholic Acids Inhibit *Clostridium difficile* Spore Germination and Growth

Michael B. Francis, Charlotte A. Allen, Joseph A. Sorg*

Department of Biology, Texas A&M University, College Station, Texas, United States of America

Abstract

Infections caused by *Clostridium difficile* have increased steadily over the past several years. While studies on *C. difficile* virulence and physiology have been hindered, in the past, by lack of genetic approaches and suitable animal models, newly developed technologies and animal models allow these processes to be studied in detail. One such advance is the generation of a mouse-model of *C. difficile* infection. The development of this system is a major step forward in analyzing the genetic requirements for colonization and infection. While important, it is equally as important in understanding what differences exist between mice and humans. One of these differences is the natural bile acid composition. Bile acid-mediated spore germination is an important step in *C. difficile* colonization. Mice produce several different bile acids that are not found in humans. These muricholic acids have the potential to impact *C. difficile* spore germination. Here we find that the three muricholic acids (α-muricholic acid, β-muricholic acid and ω-muricholic acid) inhibit *C. difficile* spore germination and can impact the growth of vegetative cells. These results highlight an important difference between humans and mice and may have an impact on *C. difficile* virulence in the mouse-model of *C. difficile* infection.

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* E-mail: jsorg@bio.tamu.edu

Introduction

Clostridium difficile is an anaerobic, spore-forming bacteria that is the leading cause of antibiotic-associated diarrhea. As the costs associated with treatment continue to rise [1,2], much research has focused on understanding the normal course of infection within humans. One of the challenges in the study of *C. difficile* infections has been developing suitable animal models that adequately reproduce symptoms as presented in humans. Gnotobiotic neonatal piglets, rats, and germ-free mice have all been used to varying degrees of success [3,4,5,6]. The most widely used model has been the Syrian hamster model of *C. difficile* disease [7,8,9,10,11]. Antibiotic-treated hamsters are very sensitive to *C. difficile* infection with lethal disease presenting approximately 3 days after inoculation by *C. difficile* spores. While the hamster represents an excellent model of acute disease, hamsters typically succumb too quickly to disease to measure factors influencing colonization, representing only the full presentation of disease and not less severe symptoms when exposed to epidemic strains [12]. Such rapid progression of the disease and high mortality can also pose problems when attempting to study relapsing infection.

Several mouse models of infection have been developed [5,13,14,15,16,17]. Some of these models use heavy doses of antibiotics (e.g. kanamycin, gentamicin, colistin, metronidazole and vancomycin followed by clindamycin or cefoperazone followed by clindamycin) and then inoculation with *C. difficile* spores or vegetative cells [14,15]. These antibiotic regimens sensitize mice so that they respond to infection in a dose dependent manner (increasing disease severity with increasing number of dosed cells or spores). Further, antibiotic-treated mice can relapse after a course of antibiotic treatment, to cure the primary infection, and will express some resistance to reinfection when allowed to fully recover from disease [14]. These are important components of an animal model because relapse in humans represents one of the main challenges to current treatment regimens [18,19].

Because the mouse model is beginning to be a more widely accepted method of testing potential preventative therapies [20] and the genetic requirements for infection [13,21,22], it is important to understand what potential variability exists between the mouse model of infection and humans. One potential source of variability is the natural differences between mouse and human microbiota. The use of an antibiotic cocktail before infection is an attempt to impact these other microbes [23,24]. Another important source of variability is the differences in the natural fecal bile acid composition between mice [25,26] and humans [27] (and hamsters [28,29,30]).

In humans, bile acids are synthesized in the liver as either cholic acid (3α, 7α, 12α-trihydroxy-5β-cholanic acid) or chenodeoxycholic acid [3α, 7α,-dihydroxy-5β-cholanic acid (CDCA)] [27]. These bile acids are then conjugated with either taurine or glycine and, later, further modified by certain members of the colonic microbiota [27]. Previous work has shown that colony formation by *C. difficile* spores on rich medium occurs after exposure to cholic acid derivatives [31,32]. Subsequent work has shown that all cholic acid derivatives and some amino acids, commonly glycine, can stimulate the initiation of spore germination while CDCA-derivatives are competitive inhibitors of cholic acid-mediated germination [31,33,34,35,36]. In mice and rats, CDCA is a

component of bile, but there are two additional bile acids, α-muricholic acid (AMA) and β-muricholic acid (BMA), that are not present in humans [37]. A third muricholic acid, ω-muricholic acid (OMA), is an epimer of BMA and is produced by the normal microbiota. The effects of these compounds on *C. difficile* spore germination are unknown.

Germination by *C. difficile* spores must be the first step in colonization [20,38]. The toxins necessary for disease are not found within the spore or deposited on the outer layers during spore formation [39]. To generate active infection in the hamster model of *C. difficile* disease, approximately 100 spores will result in a lethal infection (LD_{100}) while in the mouse model, significantly more spores are required to generate lethal disease ($\sim 10^8$) [20]. Interestingly, when vegetative cells are used to inoculate antibiotic-treated mice, fewer cells are needed ($\sim 10^5$), suggesting the efficiency of *in vivo* germination by *C. difficile* spores may be affected differently in the mouse than in the hamster [14,20,40].

Here, we investigate how muricholic acids affect *C. difficile* spore germination and growth using two *C. difficile* isolates; UK1– an epidemic ribotype 027 isolate [34,41] and M68– a ribotype 017 that readily colonizes mice [13,17]. We find that all three muricholic acids can inhibit *C. difficile* spore germination with apparent affinities similar to what is observed for CDCA and that these compounds are also growth inhibitory.

Materials and Methods

C. difficile Growth Conditions

C. difficile strain UK1 [34,38,41,42] and strain M68 [13,43] were grown in BHIS medium (Brain Heart Infusion supplemented with 5 g/L yeast extract and 0.1% L-cysteine) at 37°C in an anaerobic environment (85% nitrogen, 10% hydrogen and 5% carbon dioxide).

C. difficile Spore Preparations

Spores of *C. difficile* UK1 and *C. difficile* M68 were prepared as described previously [34,42,43]. Briefly, *C. difficile* UK1 or M68 were streaked on BHIS agar medium and incubated for 4 days under anaerobic conditions at 37°C. Plates were then removed from the chamber and cell matter was scraped and diluted into 1 mL of water. Tubes were then left to incubate overnight at 4°C to aid in the release of spores from the mother cell. The next day, cell matter was resuspended and centrifuged at 14,000×g for 1 minute. Tubes were decanted and resuspended in 1 mL of water. After 5 washes, the pellets from several tubes were combined in 2 mL water and layered on top of 8 mL of 50% sucrose. Spores were separated from vegetative cells and cell debris by centrifugation for 20 minutes at 4,000×g. All liquid was then removed from the tube. The pellet, containing the purified spores, was resuspended in 1 mL of water. The purified spores were washed in water as described above. When examined by phase-contrast microscopy, the remaining pellet appeared to be composed >99.9% phase-bright spores.

Germination of *C. difficile* Spores

Purified spores were heat activated for 30 min at 65°C and placed on ice, as described previously [33,34,42,44,45]. Heat-activated spores were then diluted into 990 μL BHIS supplemented with 0 mM, 2 mM, 5 mM, 10 mM, 20 mM or 50 mM taurocholate. When testing muricholic acids or CDCA, bile compound was added to tubes before the addition of spores. The initiation of germination was followed by monitoring absorbance at 600 nm. The ratio of the A_{600} at time× (T_x) to the A_{600} at time zero (T_0) was plotted against time. Germination rates, and apparent affinities, were determined using the slopes of the linear portions of the germination plots, as described previously [34,42,45]. Data are reported as the averages from three independent experiments with one standard deviation from the mean. For clarity, only every fourth data point is plotted. CDCA, AMA, BMA and OMA were dissolved at 100 mM in 100% ethanol. AMA, BMA and OMA were purchased from Steraloids, Inc (Newport, RI).

Minimum Inhibitory Concentration

C. difficile, from an actively growing plate, was grown overnight in 5 mL liquid BHIS under anaerobic conditions. The next day, 25 mL BHIS medium was inoculated with 0.25 mL of the overnight *C. difficile* culture and then incubated until an OD_{600} of 0.45. One hundred twenty five-microliters of this culture then added to 50 mL of ice cold reduced BHIS and kept on ice. Microtiter plates containing BHIS and serially diluted compound were previously prepared and placed in anaerobic chamber to reduce. 10 μL of chilled cells were then added to wells and incubated for 24 hours at 37°C. After 24 hours, plates were removed from the anaerobic chamber and growth measured using a BioRad Xmark plate reader.

Statistical significance. Experiments were performed in triplicate and data represent the average of the three independent experiments. Statistical significance between UK1 and M68 was determined using the Student's T-test.

Results

Structures of Muricholic Acids

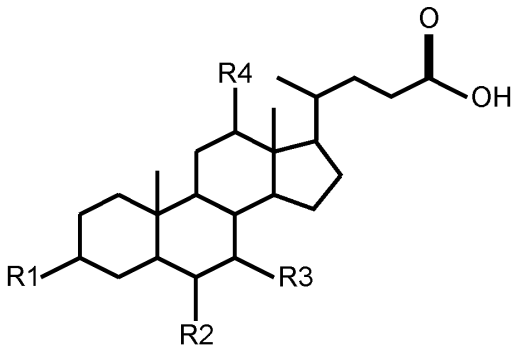
Mice synthesize three bile acids not found in humans. Two of these compounds are synthesized directly by the mouse; AMA (3α, 6β, 7α-trihydroxy-5β-cholanic acid) and BMA (3α, 6β, 7β-trihydroxy-5β-cholanic acid) (Figure 1) [25,26]. The third muricholic acid, OMA (3α, 6α, 7β-trihydroxy-5β-cholanic acid) is produced by oxidation of the 6β-hydroxyl of β-muricholic acid followed by reduction of the compound to a 6α-hydroxyl group (Figure 1) by members of the mouse colonic microbiota [46,47].

AMA and BMA contain a 6β-hydroxyl group while OMA contains a 6α-hydroxyl group (Figure 1). The conformational effect of this 6-hydroxyl group is untested on *C. difficile* spore germination because bile acids normally found in the human gut lack the 6-hydroxyl group. As shown in Figure 1, all three muricholic acids lack a 12α-hydroxyl group, suggesting they might act as inhibitors of *C. difficile* spore germination [34].

Muricholic Acids Inhibit *C. difficile* Spore Germination

To understand how these compounds affect germination, *C. difficile* spores were assayed for germination in the presence or absence of muricholic acids. As positive and negative controls, respectively, the initiation of spore germination was followed in the presence of taurocholic acid, a known *C. difficile* spore germinant [31,32] or in the presence of taurocholic acid and CDCA, a known inhibitor of *C. difficile* spore germination [33,34].

Purified *C. difficile* UK1 spores were suspended in BHIS medium and different taurocholic acid concentrations (Figure 2A). As described previously, the rate of germination increased with increasing taurocholic acid concentration [34,42,45]. The addition of 1 mM CDCA had an inhibitory effect on germination (Figure 2B). The addition of 1 mM AMA resulted in a clear reduction of the ability of *C. difficile* spores to germinate in response to TA (Figure 2C). The effect of this inhibition of germination was quantified by applying Michaelis-Menten kinetics to the germination plots to generate apparent K_m values. While not traditional



	R - group			
Bile Acid	**R1**	**R2**	**R3**	**R4**
Cholic Acid	αOH	-	αOH	αOH
Deoxycholic Acid	αOH	-	-	αOH
Chenodeoxycholic Acid	αOH	-	αOH	-
Lithocholic Acid	αOH	-	-	-
α - Muricholic Acid	αOH	βOH	αOH	-
β - Muricholic Acid	αOH	βOH	βOH	-
ω - Muricholic Acid	αOH	αOH	βOH	-

Figure 1. Structures of common muricholic acids. The primary bile acids (cholic acid, chenodeoxycholic acid, α-muricholic acid and β-muricholic acid) are listed. Deoxycholic acid and ω-muricholic acid are secondary bile acids and are products of the normal microbiota.
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enzyme kinetics, these types of analyses have aided in the identification of the requirements for spore germination and for novel inhibitors of spore germination [34,36,42,44,45,48,49,50]. Analysis of the Lineweaver-Burk plot of *C. difficile* UK1 spore germination in taurocholic acid alone (Figure 2D) yielded an apparent K_m value similar to what has been previously reported (Table 1) [34,42]. When analyzing germination by *C. difficile* UK1 spores in the presence of different muricholic acids, it was immediately obvious that these compounds were germination-inhibitory. From the germination plots, we determined the rates of germination and used this data to generate apparent inhibitory constants (K_i) for each inhibitor tested (Table 1). Comparing the muricholic acids to CDCA, BMA and OMA yielded apparent inhibition constants similar to CDCA while AMA proved to be least efficient at inhibiting germination (Table 1). The difference between CDCA and AMA can be observed by comparing the ability of *C. difficile* spores to germinate in BHIS medium supplemented with 2 mM TA. Addition of 1 mM CDCA had a greater effect than did the addition of 1 mM AMA.

Germination of *C. difficile* M68 in medium with taurocholic acid was similar to *C. difficile* UK1. *C. difficile* M68 rapidly germinated in medium supplemented with taurocholic acid (Figure 3A) and was inhibited when 1 mM AMA was added to the germination solution (Figure 3B). However, by analyzing the kinetics of *C. difficile* M68 spore germination, we observed that the data from this strain produced non-linear Lineweaver-Burk plots (Figure 3C), a phenomenon observed for some other *C. difficile* strains [45]. The Hill plot (Figure 3D) was used to generate the apparent K_m. This value was then used to determine the apparent K_i under each condition tested. When *C. difficile* M68 spores were germinated in the presence of CDCA, germination was strongly inhibited (Table 1). The inhibition of germination by BMA was similar to the inhibition observed for *C. difficile* UK1 and, again, AMA was the least efficient at inhibiting spore germination (Table 1). OMA was a more potent inhibitor of *C. difficile* M68 spore germination than *C. difficile* UK1 spore germination (p-value <0.05).

Minimum Inhibitory Concentration of Muricholic Bile Acids

Previously, we demonstrated that CDCA and deoxycholic acid inhibited *C. difficile* growth [31]. In antibiotic-treated mice, the levels of deoxycholic acid are likely to be very low because it is a product of the 7α-dehydroxylation of cholic acid by the normal microbiota [27]. However, cholic acid, CDCA, AMA and BMA will be present and could affect *C. difficile* growth. To quantify the effects of these compounds on *C. difficile* growth, we determined the MIC. Serial, 2-fold dilutions of bile acids in growth media were used determine the MIC for each bile acid (Table 2). *C. difficile* strain UK1 did not grow in the presence of CDCA or AMA or BMA, at a concentration of 1 mM or above. OMA was less toxic to the strain; a concentration of 2 mM was necessary to inhibit growth. In stark contrast, the MIC of cholic acid for *C. difficile* UK1 was 10 mM, a concentration not found in the colon. We observed slightly different results when analyzing the MIC of these bile acids for *C. difficile* M68 growth. This strain was more resistant to the toxic effects of AMA and BMA while equally as sensitive to CDCA, cholic acid and deoxycholic acid (Table 2). BMA is more prevalent in the gut of rats and mice than is CDCA [25,51], suggesting that a strain which is more resistant to the toxic effects of BMA (e.g. *C. difficile* M68) might be able to better colonize mice.

Discussion

In the laboratory setting, certain combinations of bile acids and amino acids are the most effective conditions for measuring *C. difficile* spore germination [31,32,35]. While cholic acid derivatives can stimulate *C. difficile* spore germination [31], CDCA-derivatives inhibit cholic acid-mediate germination by *C. difficile* spores [33,34]. Compared to humans, mice produce a low level of CDCA but produce other bile acids (AMA and BMA), in greater abundance. These muricholic acids may have an impact on how *C. difficile* spores germinate *in vivo*. Here, we found that AMA, BMA and OMA (a microbial product) inhibit taurocholic acid-mediated spore germination with BMA and OMA being the most potent germination-inhibiting muricholic acids (Table 1). These results are consistent with our previous work that has shown the 12-hydroxyl group to be an important determinant of whether a compound functions as a germinant or inhibitor of germination [31,33,34]. One difference observed between the germination of *C. difficile* UK1 spores and *C. difficile* M68 spores was the non-linear double-reciprocal plot for germination by *C. difficile* M68 spores. As seen in other strains, *C. difficile* M68 may bind taurocholic acid cooperatively [45]. With the recent identification of the molecular target of bile acids on the *C. difficile* spore, this hypothesis could be tested outright [38].

Total bile acid levels in the distal small intestine have been estimated to be between 1 mM to 2 mM in concentration [52]. This is in the range of the concentrations which inhibit *C. difficile* growth for the individual bile acids tested (Table 2); variations in pH may affect the toxicity of each bile acid [53]. Comparing these concentrations to the apparent K_i values determined for AMA and BMA, they are approximately 3× to 8× greater, respectively (Table 1). That is, in an antibiotic-treated mouse, the levels of AMA and BMA might prevent efficient *C. difficile* spore germination, possibly explaining why such greater numbers of spores, compared to vegetative cells, are required to colonize a

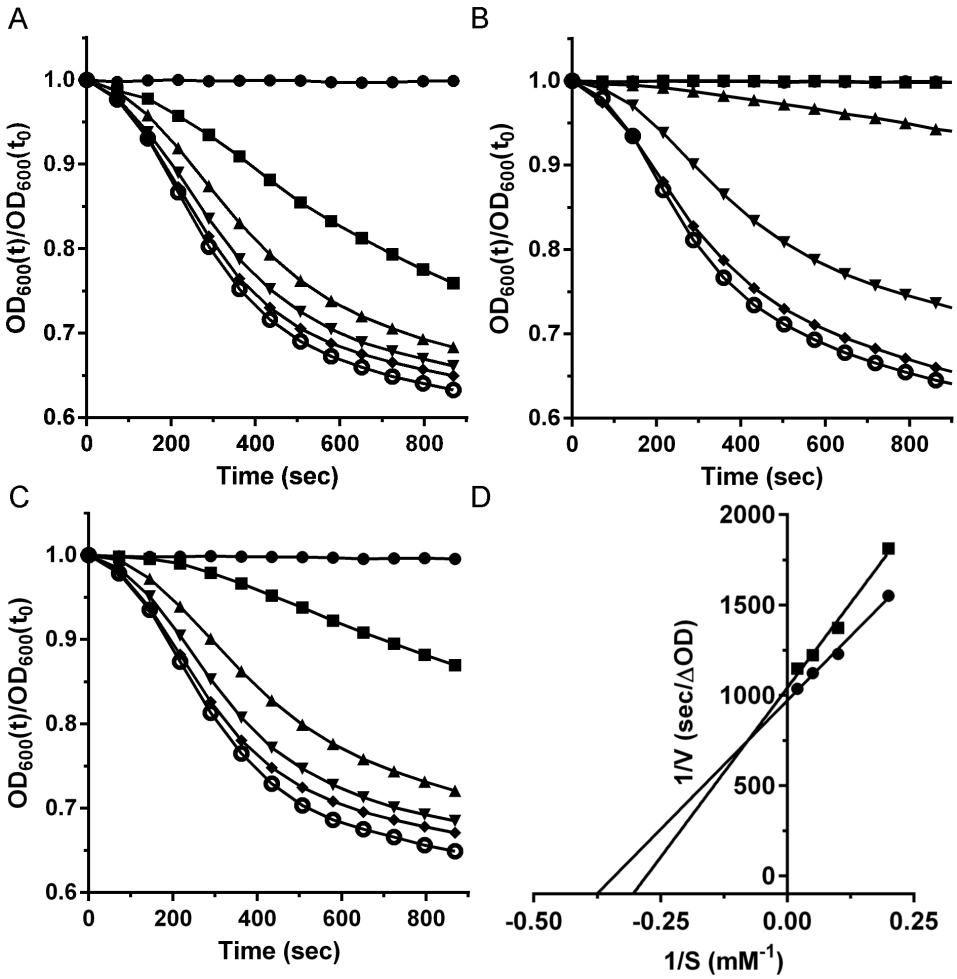

Figure 2. α-muricholic acid inhibits germination by *C. difficile* UK1 spores. (A) Germination of *Clostridium difficile* UK1 spores in complex medium supplemented with taurocholic acid (TA) or (B) medium supplemented with TA and 1 mM CDCA or (C) medium supplemented with TA and 1 mM α-muricholic acid. ● 0 mM TA, ■ 2 mM TA, ▲ 5 mM TA, ▼ 10 mM TA, ♦ 20 mM TA or ○ 50 mM TA. (D) The inverse rate (1/*v* [sec/OD_{600}]), versus the inverse taurocholate concentration (1/*S* [mM^{-1}], was plotted. Apparent K_m values for TA alone (●) and in the presence of α-muricholic acid (■) were determined from the linear best fit.
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mouse [20]. It is also important to note that most mice used as a model for *C. difficile* infection would likely contain reduced levels of OMA because its formation requires the presence of mouse gut microbes [46,47] which are likely 'collateral damage' during a routine course of broad-spectrum antibiotics.

Table 1. Bile acid effects on *C. difficile* spore germination.

Strain	UK1	M68
	K_m (mM)	K_m (mM)
Taurocholic Acid	3.2±0.5	3.5±0.5
	K_i (mM)	K_i (mM)
Chenodeoxycholic Acid	0.22±0.07	0.12±0.02
α-Muricholic Acid	0.62±0.09	0.59±0.05
β-Muricholic Acid	0.27±0.12	0.26±0.02
ω-Muricholic Acid	0.29±0.03	0.20±0.01*

K_i = [inhibitor]/[($K_{m,TA}$ with inhibitor)/(($K_{m,TA}$ without inhibitor) −1)].
*$p<0.05$.
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Antibiotics can affect host functions. That is, treating mice with antibiotics could lead to alterations in the bile acid spectrum and increase or decrease the availability of activators or inhibitors of *C. difficile* spore germination. Treatment of mice with antibiotics has been shown to increase hepatic bile acid synthesis [54]. Specifically, the authors identified that small intestine, lumenal concentrations of taurocholic acid, tauro-β-muricholic acid and taurochenodeoxycholic acid were more abundant in antibiotic-treated C57/BL6 mice than in vehicle-only controls [54]; the authors did not measure the levels of AMA. Thus, upon antibiotic exposure, an increase in the abundance of germination-inhibiting bile acids could contribute to an environment which is more resistant to *C. difficile* spore germination.

Some *C. difficile* strains have been shown to stably colonize mice and enter a 'contagious' state, where disease is limited but spore shedding is maintained, while other strains are cleared by the host [13,17]. The mechanisms by which some *C. difficile* strains are able to stably colonize a host while others do not, is unclear. While the answer is likely to be multifactorial, an increased resistance to bile

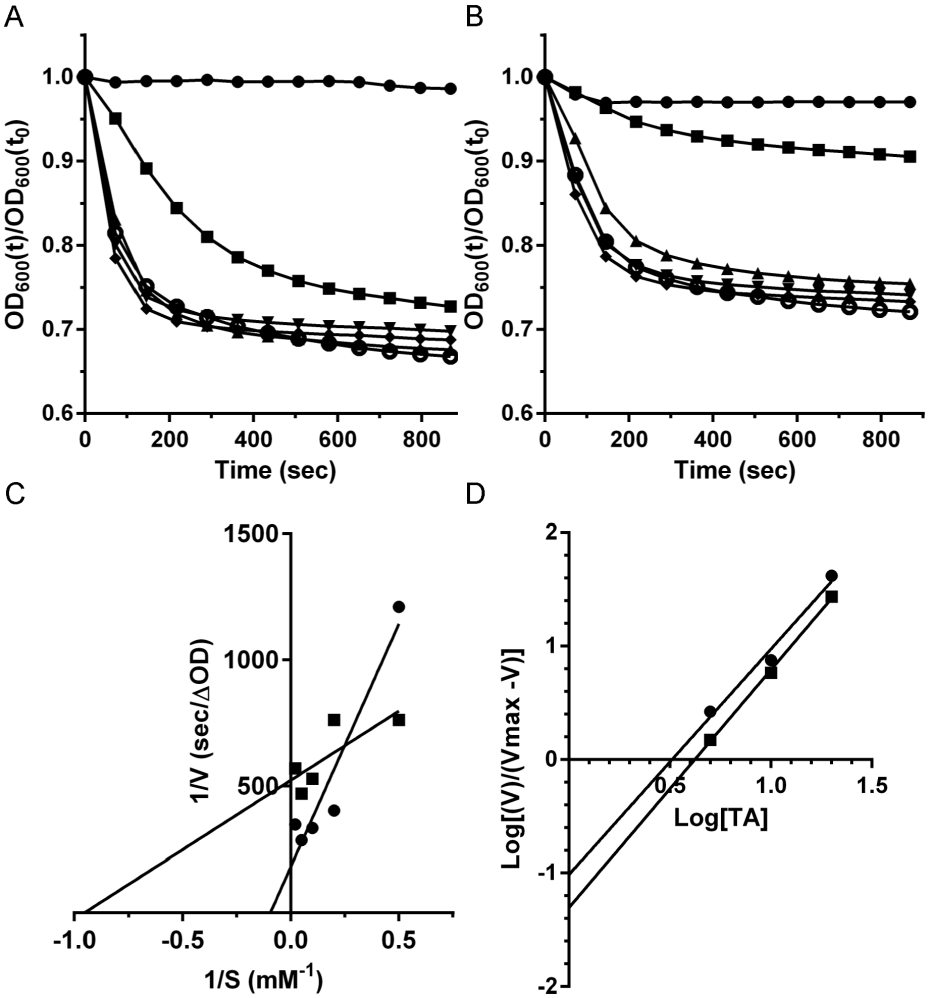

Figure 3. α-muricholic acid inhibits germination by *C. difficile* M68 spores. (A) Germination of *Clostridium difficile* M68 spores in complex medium supplemented with taurocholic acid (TA) or (B) medium supplemented with TA and 1 mM α-muricholic acid. ● 0 mM TA, ■ 2 mM TA, ▲ 5 mM TA, ▼ 10 mM TA, ♦ 20 mM TA or ○ 50 mM TA. (C) The inverse rate (1/*v* [sec/OD_{600}]), versus the inverse taurocholate concentration (1/*S* [mM^{-1}], was plotted. Apparent K_m values for TA alone (●) and in the presence of α-muricholic acid (■) were determined from the linear best fit of the plotted data. (D) Hill Plot was generated to determine the apparent K_m values for each condition.
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acids could contribute to a strain's ability to persist within a host. *C. difficile* M68 is a strain that can enter a supershedder state after the cessation of antibiotic treatment [13]. We find *C. difficile* M68 to be more resistant to bile acid toxicity than is *C. difficile* UK1 and this increased resistance may aid *C. difficile* M68 in maintaining active colonization.

Table 2. Minimum inhibitory concentration of bile acids for *C. difficile* strains.

Strain	UK1 (mM)	M68 (mM)
Cholic Acid	10.0±0.0	10.0±0.0
Deoxycholic Acid	1.0±0.0	1.0±0.0
Chenodeoxycholic Acid	1.0±0.0	1.0±0.0
α-Muricholic Acid*	1.0±0.0	2.0±0.0
β-Muricholic Acid*	1.0±0.0	2.0±0.0
ω-Muricholic Acid	2.0±0.0	2.0±0.0

The MIC did not vary between experiments.
*$p<0.01$.
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Muricholic acids might provide a level of protection to mice from *C. difficile* infection that is not seen in other models of *C. difficile* disease. While our results suggest that particular bile acids may inhibit *C. difficile* spore germination or vegetative growth *in vitro*, it is unclear if AMA or BMA could substitute for each other in preventing *in vivo* spore germination. Clearly, BMA is a more potent inhibitor of *in vitro* spore germination than is AMA. But, given the vast repertoire of mouse lines and genetic approaches, testing the ability of *C. difficile* to colonize mice that have had introduced mutations into specific steps in the bile acid/muricholic acid synthesis pathway would allow the determination of which bile acids are relevant for stimulating or inhibiting *in vivo* spore germination and vegetative growth.

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Author Contributions

Conceived and designed the experiments: MBF CAA JAS. Performed the experiments: MBF CAA. Analyzed the data: MBF CAA JAS. Wrote the paper: MBF JAS.

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