



An Aniline-Substituted Bile Salt Analog Protects both Mice and Hamsters from Multiple *Clostridioides difficile* Strains

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ABSTRACT *Clostridioides difficile* infection (CDI) is the major identifiable cause of antibiotic-associated diarrhea. The emergence of hypervirulent *C. difficile* strains has led to increases in both hospital- and community-acquired CDI. Furthermore, the rate of CDI relapse from hypervirulent strains can reach up to 25%. Thus, standard treatments are rendered less effective, making new methods of prevention and treatment more critical. Previously, the bile salt analog CamSA (cholic acid substituted with *m*-aminosulfonic acid) was shown to inhibit spore germination *in vitro* and protect mice and hamsters from *C. difficile* strain 630. Here, we show that CamSA was less active in preventing spore germination by other *C. difficile* ribotypes, including the hypervirulent strain R20291. The strain-specific *in vitro* germination activity of CamSA correlated with its ability to prevent CDI in mice. Additional bile salt analogs were screened for *in vitro* germination inhibition activity against strain R20291, and the most active compounds were tested against other strains. An aniline-substituted bile salt analog, CaPA (cholic acid substituted with phenylamine), was found to be a better antigerminant than CamSA against eight different *C. difficile* strains. In addition, CaPA was capable of reducing, delaying, or preventing murine CDI signs with all strains tested. CaPA-treated mice showed no obvious toxicity and showed minor effects on their gut microbiome. CaPA's efficacy was further confirmed by its ability to prevent CDI in hamsters infected with strain 630. These data suggest that *C. difficile* spores respond to germination inhibitors in a strain-dependent manner. However, careful screening can identify antigerminants with broad CDI prophylaxis activity.

KEYWORDS antibiotic-associated diarrhea, bacterial spores, bile salts, *Clostridioides difficile*, prophylaxis, spore germination

Clostridioides difficile infection (CDI) is a major cause of antibiotic-associated diarrhea. The Centers for Disease Control and Prevention (CDC) reported that in 2017, over 223,900 people were diagnosed with CDI in the United States (1). In that same year, approximately 12,800 people died of CDI-related complications within 30 days of initial diagnosis (1). With an average of \$35,000 to treat a single inpatient case, the cost burden to the U.S. health care system can reach between \$3.2 billion and \$4.8 billion annually (2, 3). In some regions of the United States, CDI has surpassed methicillin-resistant *Staphylococcus aureus* (MRSA) as the most common cause of hospital-associated infections (4). Globally, the incidence of CDI is complicated by the appearance of highly

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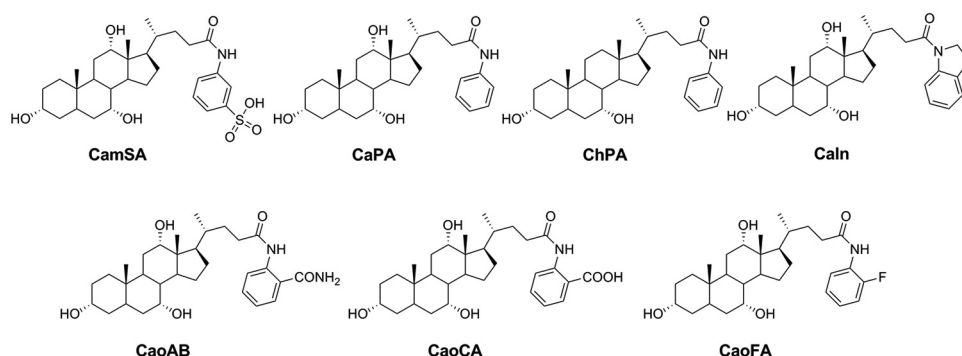


FIG 1 Structures of antigerminant compounds tested in animals. Seven compounds were found to have strong antigermination activity against spores from multiple *C. difficile* strains: cholic acid substituted with *m*-aminosulfonic acid (CamSA), cholic acid substituted with phenylamine (CaPA), chenodeoxycholic acid substituted with phenylamine (ChPA), cholic acid substituted with indoline (Caln), cholic acid substituted with *o*-aminobenzamide (CaoAB), cholic acid substituted with *o*-carboxyaniline (CaoCA), and cholic acid substituted with *o*-fluoroaniline (CaoFA).

resistant and hypervirulent BI/NAP1/027 strains that have an attributable mortality rate of an astonishing 22% (5, 6).

A key characteristic of *C. difficile* is its ability to form endospores (7, 8). These spores are transmitted through the fecal-oral route and can be ingested after contact with various media such as hard surfaces, food, animals, and unwashed hands (9). The spores' dormant nature allows them to survive in the gastrointestinal tract of susceptible patients. When the spores reach the nutrient-rich intestinal lumen, they can germinate into toxin-producing vegetative cells that cause symptomatic infection (10–12). Since spore germination is a necessary step for CDI establishment, methods that target this process could prevent infection (13, 14).

C. difficile spore germination is promoted by the bile salt taurocholate (10, 15). Another naturally occurring bile salt, chenodeoxycholate (CDCA), has been shown to compete with taurocholate to inhibit spore germination (11). Interestingly, a study by Heeg et al. showed that the spore response to germination activators and inhibitors was heterogeneous and strain specific (16). Furthermore, recent reports have shown that strains of *C. difficile* can use calcium as a cogerminant (17).

Previously, CamSA (cholic acid substituted with *m*-aminosulfonic acid) (Fig. 1) was found to be a more potent germination inhibitor than CDCA when tested against strain 630 (14). CamSA was also able to prevent CDI in mice and worked synergistically with subclinical doses of the antibiotic vancomycin to prevent CDI in hamsters (14, 18, 19).

In the present study, multiple bile salt analogs were screened for increased potency compared to CamSA. A cholic acid substituted with phenylamine (CaPA) was shown to be an effective antigerminant against eight *C. difficile* strains representing five different ribotypes, including hypervirulent BI/NAP1/027 strain R20291. Germination inhibition assays showed that CaPA inhibited spore germination with half-maximal inhibitory concentration (IC_{50}) values in the low-micromolar range in each strain tested. Furthermore, CaPA-treated mice challenged with each of the *C. difficile* strains had significantly reduced CDI signs and delayed sign onset or were completely protected from CDI. In mice, both CamSA and CaPA induced small but significant changes in the gut microbiome, potentially through inhibition of spore germination in other *Firmicutes*. Nevertheless, CaPA-treated mice showed no obvious acute or subchronic toxicity effects. CaPA, in combination with subclinical doses of vancomycin, was also able to protect the more susceptible hamster model from *C. difficile* strain 630.

RESULTS

Germination profiles of various *C. difficile* strains against cholic acid analogs. In this work, we utilized eight *C. difficile* strains representing diverse ribotypes. These

strains were selected for their differential responses to activators and inhibitors of germination (16). To verify their identity, we ribotyped all strains tested. We found that all strains were correctly identified, except for strain 9001966. Nevertheless, this *C. difficile* strain is pathogenic to mice and encodes both the TcdA and TcdB toxins. Hence, we renamed this strain putative strain 9001966 (p9001966) and proceeded to characterize its activity both *in vitro* and *in vivo*.

In our hands, six strains (630, R20291, p9001966, CDC38, DH1834, and 8085054) were able to germinate efficiently when incubated with taurocholate and glycine. On the other hand, strain 05-1223-046 and 7004578 spores failed to germinate even in the presence of saturating germinant concentrations. The addition of up to 40 mM calcium chloride to the germination mixture did not improve the germination efficiency of spores from strains 05-1223-046 and 7004578. Even though we could not obtain reliable antigermination data for these two strains *in vitro*, we used them in the murine model of CDI prevention.

Selection of antigerminants effective against multiple *C. difficile* strains. CamSA was previously reported to inhibit *C. difficile* strain 630 spore germination *in vitro* with an IC_{50} (half-maximal inhibitory concentration) of 58.3 μ M (13). However, CamSA did not appear to be biologically active against any of the other tested strains up to 100 μ M (Table 1).

To improve the efficacy of CamSA, we previously tested a series of related compounds for antigermination activity *in vitro* and found CaPA to be effective against hypervirulent *C. difficile* strain R20291 with IC_{50} values in the low-micromolar range (20). Based on this result, we continued testing CaPA analogs *in vitro* and found four new cholate derivatives (cholic acid substituted with indoline [Caln], cholic acid substituted with *o*-aminobenzamide [CaoAB], cholic acid substituted with *o*-carboxyaniline [CaoCA], and cholic acid substituted with *o*-fluoroaniline [CaoFA]) that were as effective as or better antigerminants than CaPA against multiple *C. difficile* strains and ribotypes. Furthermore, a chenodeoxy analog of CaPA (ChPA) was able to inhibit the spore germination of *C. difficile* R20291 effectively. Dimethyl sulfoxide (DMSO) alone did not have any effect on spore germination.

CDI prevention against *C. difficile* clinical isolates in a murine CDI model. *In vivo* screening of the most active antigerminants from the *in vitro* screens against strain R20291 revealed that CaPA was most effective at preventing CDI. Despite showing strong antigermination activity *in vitro*, ChPA, Caln, CaoAB, CaoCA, and CaoFA in DMSO were unable to prevent CDI when tested in the murine CDI model (data not shown). Therefore, only CaPA and CamSA were compared for the remaining *C. difficile* strains.

Mice challenged with *C. difficile* strain 630 spores mostly exhibited mild to moderate disease, with one animal developing severe CDI and needing to be culled by 48 h post-infection. Strain 630 CDI sign severity was heterogeneous, with maximum scores ranging from 3 to 8 for individual mice (Fig. 2A). Surviving mice quickly recovered by 72 h postinfection and remained asymptomatic throughout the remainder of the experiment. Mice challenged with strain 630 and treated with CamSA or CaPA did not develop any CDI signs.

Mice challenged with strain R20291 spores exhibited higher CDI scores than those challenged with strain 630. Untreated mice developed moderate to severe CDI at 48 h postinfection (Fig. 2B). As expected, due to the lack of antigermination activity *in vitro*, CamSA-treated mice were not protected from CDI caused by strain R20291. On the other hand, CaPA-treated mice showed very mild CDI signs that were delayed by 24 h. None of the surviving mice showed CDI relapse after fully recovering from initial CDI.

C. difficile strain 8085054-infected mice displayed moderate CDI signs, similar to those of mice infected with strain 630 and strain R20291 (Fig. 2C). CamSA protected most mice from strain 8085054, with only one mouse developing moderate signs. Similarly, treatment with CaPA completely prevented CDI signs from this strain.

C. difficile strain p9001966-challenged mice developed moderate to severe CDI within 24 h of infection (Fig. 2D). By 72 h postinfection, all animals in the untreated group showed severe CDI signs. Interestingly, even though CamSA was unable to prevent strain p9001966 spore germination *in vitro*, it was able to reduce CDI signs

TABLE 1 IC₅₀ values for selected bile salt antigerminants against *C. difficile* strains^a

Strain	Ribotype	Mean IC ₅₀ (μM) (SD)						
		CamSA	CaPA	ChPA	Caln	CaoAB	CaoCA	CaoFA
630	012	58.3 (35) ^b	8.19 (0.89)	5.09 (1.01)	0.085 (0.006)	0.75 (0.09)	0.37 (0.30)	2.66 (2.97)
R20291	027	>100	1.92 (0.23)	5.51 (1.30)	0.098 (0.025)	1.54 (0.31)	0.32 (0.42)	1.17 (0.61)
8085054	014	>100	1.28 (0.43)	>100	0.54 (0.10)	2.19 (0.15)	8.07 (14.27)	16.22 (7.19)
CDC38	027	>100	4.62 (0.68)	5.93 (0.11)	0.067 (0.008)	0.33 (0.08)	0.12 (0.04)	1.36 (0.30)
DH1834	027	>100	4.15 (1.10)	5.24 (1.10)	0.038 (0.001)	0.42 (0.03)	0.090 (0.02)	1.29 (0.33)
p9001966	106 ^c	>100	7.60 (1.01)	>100	0.74 (0.40)	2.65 (0.41)	2.08 (0.38)	6.67 (0.61)
05-1223-046	027	ND	ND	ND	ND	ND	ND	ND
7004578	078	ND	ND	ND	ND	ND	ND	ND

^a*C. difficile* spores were germinated in the presence of 6 mM taurocholate, 12 mM glycine, and increasing concentrations of the bile salt analog. Germination inhibition data were used to calculate mean IC₅₀ values. Standard deviations are shown in parentheses. ND denotes that IC₅₀ values could not be determined since spore germination for those *C. difficile* strains could not be detected under the conditions tested.

^bValue obtained from reference 13.

^cPreviously reported as ribotype 002.

in mice. Although neither CamSA nor CaPA was able to completely prevent CDI signs in strain p9001966-challenged mice, both compounds reduced disease signs significantly (Fig. 2D). Indeed, both CamSA-treated and CaPA-treated animals developed delayed and moderate CDI compared to untreated animals.

Similar to *C. difficile* strain p9001966, untreated mice challenged with *C. difficile* strain CDC38 spores displayed a rapid onset of moderate to severe CDI signs at 24 h postinfection and remained symptomatic for at least 5 days (Fig. 2E). CamSA and CaPA were able to delay symptom onset in animals challenged with *C. difficile* strain CDC38 for 24 h. However, all treated animals eventually developed symptomatology similar to that of untreated animals.

C. difficile strain DH1834-challenged mice developed moderate to severe CDI signs and remained symptomatic for at least 4 days (Fig. 2F). CamSA did not prevent CDI in mice challenged with strain DH1834 spores. In contrast, mice treated with CaPA developed only mild CDI signs that resolved within 48 h postinfection.

Even though *C. difficile* strain 05-1223-046 spores did not germinate in the presence of taurocholate *in vitro* (Table 1), mice challenged with *C. difficile* strain 05-1223-046 developed mild to moderate CDI signs (Fig. 2G). Both CamSA and CaPA were able to completely prevent CDI signs in mice challenged with this strain.

Similar to strain 05-1223-046, *C. difficile* strain 7004578 spores did not show strong germination *in vitro* (Table 1). However, animals challenged with strain 7004578 developed CDI signs with intensities that varied significantly among animals (Fig. 2H). Two animals remained asymptomatic throughout the experiment, while two other animals developed severe CDI and reached the clinical endpoint. The last mouse developed mild CDI at between 24 h and 72 h postinfection and began to recover at 96 h. This heterogeneous response yielded large standard deviations for untreated animals compared to both treated groups. Because of the lack of normality in the data for infection in untreated animals, analysis of variance (ANOVA) and *ad hoc* analysis were not possible. Similarly, the loss of two animals from the untreated group due to severe CDI resulted in a sample size too small ($n < 5$) for nonparametric testing.

Nevertheless, a pairwise comparison of the daily CDI symptom data showed interesting trends. CamSA was not effective at preventing or reducing CDI signs in mice challenged with strain 7004578 spores. In contrast, whereas two of the untreated animals died of severe CDI, all animals treated with CaPA did not develop any CDI signs and were completely protected from strain 7004578.

Toxicity of bile salt analogs in mice. Mice that received neat DMSO, 300 mg/kg of body weight of CamSA, or 300 mg/kg of CaPA once daily for 30 days did not experience weight loss of >10% over the course of the trial (data not shown) and were statistically indistinguishable from untreated animals. Moreover, mice did not appear to

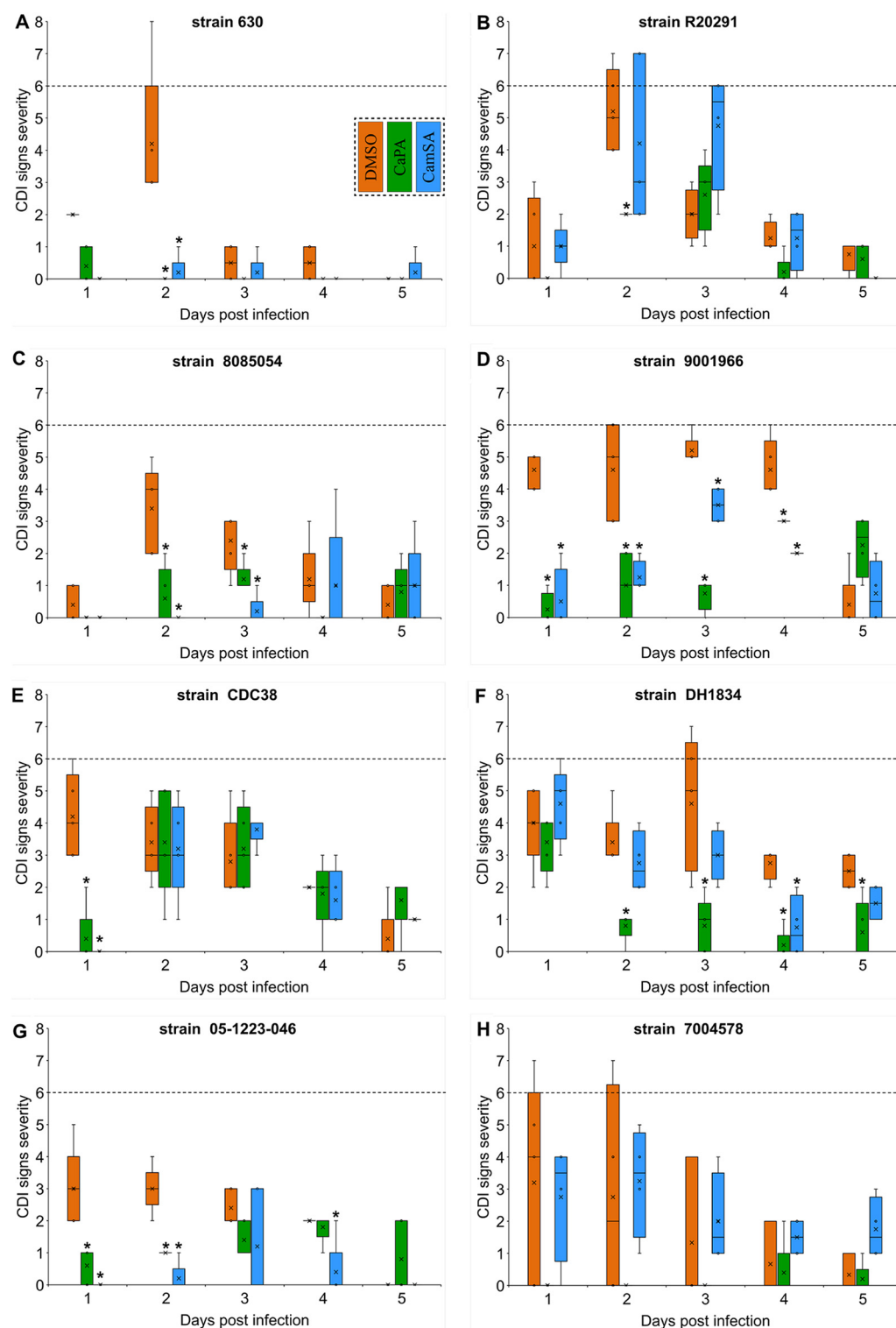


FIG 2 Murine CDI prophylaxis properties of CamSA and CaPA. Box-and-whisker plots of CDI sign severity in mice challenged with *C. difficile* spores from strain 630 (A), strain R20291 (B), strain 8085054 (C), strain p9001966 (D), strain CDC38 (E), strain DH1834 (F), strain 05-1223-046 (G), and strain 7004578 (H) are shown. The inset in panel A shows the color scheme used to represent DMSO vehicle-treated animals, CaPA-treated animals, and CamSA-treated animals. Signs of severity were determined based on the CDI scoring rubric reported previously (24). Animals with a score of <3 were indistinguishable from noninfected controls and were considered nondiseased. Animals with a score of 3 to 4 were considered to have mild CDI. Animals with a score of 5 to 6 were considered to have moderate CDI. Animals with a score of >6 (dashed lines) were considered to have severe CDI and were immediately culled. Horizontal bold lines represent median values, asterisks represent mean values, bars represent interquartile ranges, whiskers represent

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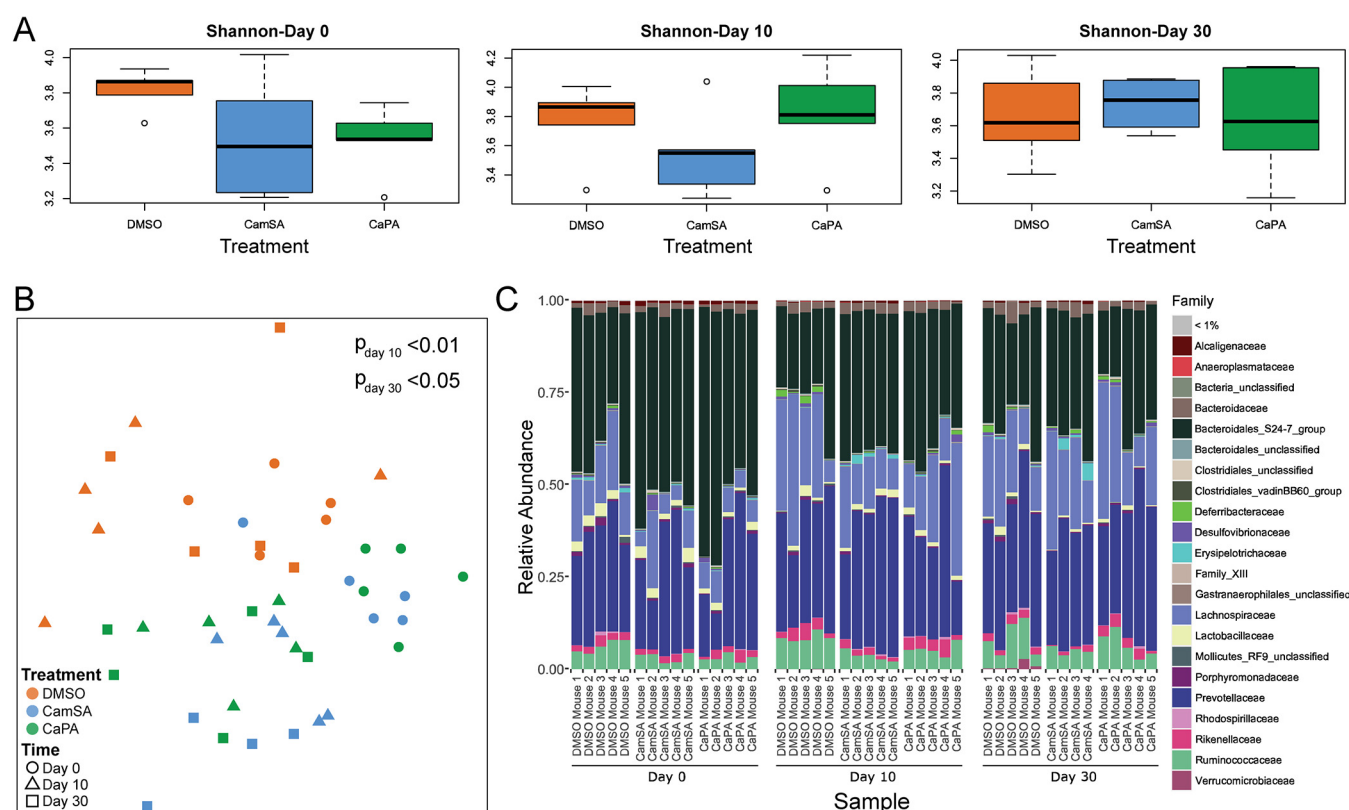


FIG 3 Effect of CamSA and CaPA on the gut microbiome. Mouse fecal microbiomes from three different cohorts of mice, DMSO vehicle-treated mice (orange), CamSA-treated mice (blue), and CaPA-treated mice (green), and for days 0, 10, and 30 were compared. (A) Comparison of Shannon diversity metrics. Horizontal bold lines represent median values, bars represent interquartile ranges, whiskers represent maximum and minimum values, and dots represent statistical outliers. No values were significantly different ($P > 0.05$ by one-tailed ANOVA and Tukey's HSD test). (B) Nonmetric multidimensional scaling (NMSD) analysis based on Bray-Curtis community dissimilarity. Microbiomes were significantly different according to ANOSIM at 10 days ($P < 0.01$) and 30 days ($P < 0.05$) posttreatment. (C) Taxonomic bar plots showing that the most abundant families are similar throughout the experiment, indicating that microbiome effects are mostly due to changes in less abundant taxa or lower taxonomic ranks.

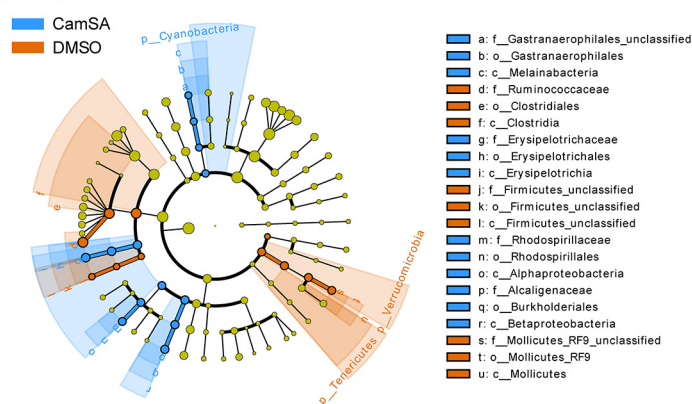
display signs of distress or a change in behavior or appearance that could be attributed to toxicity. Furthermore, necropsy of animals culled after 30 days of antigerminant treatment showed normal organs indistinguishable from those of untreated animals (data not shown).

Effects of bile salt analogs on the murine gut microbiome. CamSA and CaPA dissolved in DMSO were administered to uninfected control animals to assess potential disruption of the gut microbiome. Shannon diversity was not significantly different from that of the DMSO vehicle control after 10 days or 30 days of continuous administration of the germination inhibitors ($P > 0.05$ by one-tailed analysis of variance [ANOVA] and Tukey's honestly significant difference [HSD] test) (Fig. 3A). However, nonmetric multidimensional scaling (NMSD) clustering based on Bray-Curtis dissimilarity revealed small but significant differences in beta diversity among the three microbiomes at day 10 ($P < 0.01$ by analysis of similarity [ANOSIM]) and day 30 ($P < 0.05$ by ANOSIM) (Fig. 3B), although the proportions of the dominant families appeared similar throughout the experiment across the treatments (Fig. 3C). Pairwise linear discriminant analysis (LDA) effect size (LEfSe) analysis between the DMSO vehicle control and CamSA or CaPA treatments showed that both compounds induced similar microbiome changes, although fewer taxa changed due to CaPA. Few taxa were differentially abundant at

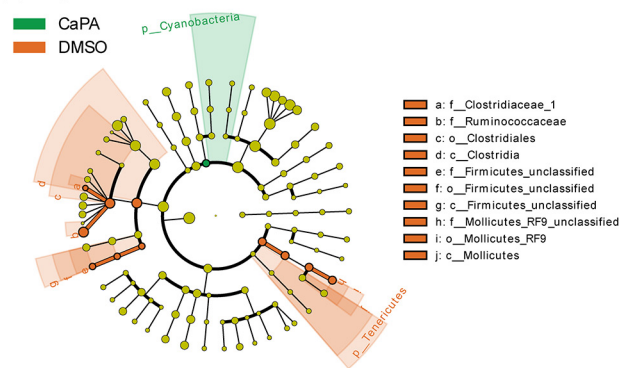
FIG 2 Legend (Continued)

maximum and minimum values, and dots represent inner point values. Single-factor ANOVA was performed at every time point to assess differences among the sign severity means of the untreated, CaPA-treated, and CamSA-treated groups. ANOVA results with P values of < 0.05 were analyzed *post hoc* using the Holm method for pairwise comparisons between individual treatments and untreated control groups. Statistical significance was determined as P values of < 0.05 between treated and untreated groups and is indicated with an asterisk.

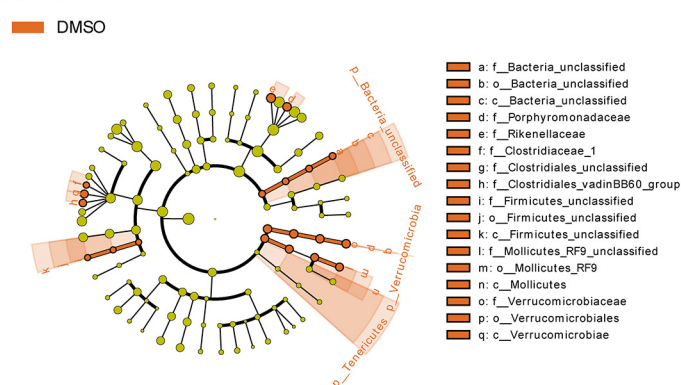
(A) Day 10: CamSA vs. DMSO



(B) Day 10: CaPA vs. DMSO



(C) Day 30: CamSA vs. DMSO



(D) Day 30: CaPA vs. DMSO

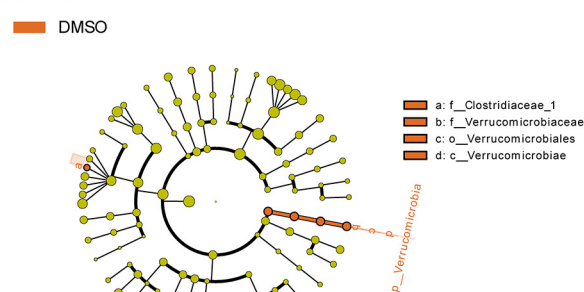


FIG 4 Microbial taxa most affected by CamSA and CaPA. Linear discriminant analysis (LDA) effect size (LEfSe) cladograms show taxa whose relative abundance was significantly ($P < 0.05$) affected by CamSA (A and C) or CaPA (B and D) treatments. Cladograms and taxon labels show taxa that were significantly more abundant in the DMSO vehicle controls in pairwise comparisons or in response to CamSA or CaPA at day 10 (A and B) or day 30 (C and D) posttreatment.

30 days, compared to 10 days, in both treatments, suggesting microbiome restoration (Fig. 4). Both CamSA and CaPA treatments depleted several lineages of *Firmicutes* within 10 days of administration, including the families *Ruminococcaceae* and *Clostridiaceae*, and multiple unclassified groups. Unclassified *Mollicutes* (phylum *Tenericutes*) were also depleted in both microbiomes, whereas *Verrucomicrobia* were depleted only in the CamSA-treated microbiome at day 10. Both treatments increased the relative abundance of *Cyanobacteria* (order *Gastranaerophilales*) within 10 days, whereas the firmicute *Erysipelotrichia* and multiple lineages of *Alpha*- and *Betaproteobacteria* expanded only in the CamSA-treated microbiome. Some of these same microbiome changes were also evident at 30 days posttreatment; however, fewer taxa differed between experimental treatments and controls than at the 10-day time point.

Establishing the delayed CDI hamster model. In our hands, treatment of hamsters with 30 mg/kg clindamycin alone induced colitis and death by day 4 posttreatment, even in the absence of *C. difficile* spore challenge (data not shown). Necropsy revealed loops of gas-filled colon consistent with colitis. However, plating of chyme and feces from deceased hamsters onto selective CDCA medium and PCR analysis failed to detect *C. difficile* contamination in these animals. This suggests that the animals were colonized *a priori* with a nonclostridial opportunistic pathogen. Clindamycin-induced colitis in hamsters is a common veterinary problem that has been addressed with vancomycin treatments (21).

To ensure that disease would be caused only by *C. difficile* infection, we tested sub-clinical doses of vancomycin coadministered with clindamycin before spore challenge. Based on previously reported protocols (19), we treated hamsters with 5 mg/kg

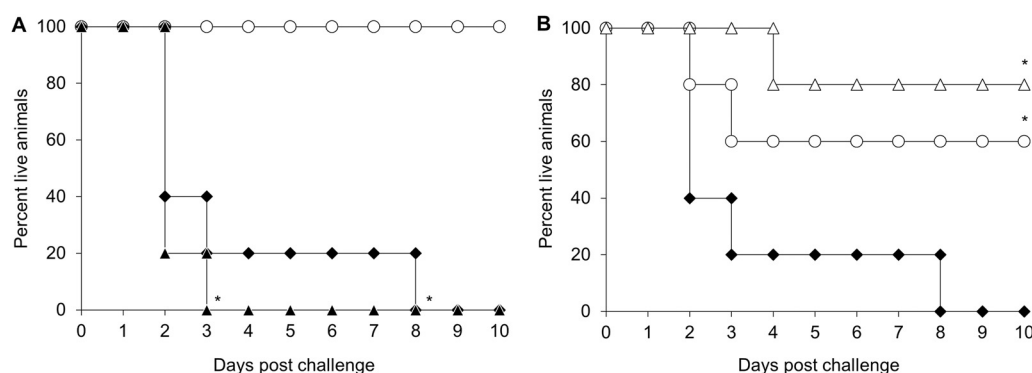


FIG 5 Protection of hamsters from CDI by CaPA. (A) Kaplan-Meier survival plot of hamsters treated with 30 mg/kg clindamycin and 1 mg/kg vancomycin. Animals were challenged with an inoculum of 0 (white circles), 50 (black diamonds), or 100 (black triangles) *C. difficile* strain 630 spores. Statistical survival comparisons of challenge groups and the unchallenged control group were performed via a log rank test (* represents a *P* value of <0.05 compared to untreated animals). (B) Kaplan-Meier survival plot of hamsters challenged with *C. difficile* strain 630 spores and subclinical doses of vancomycin. Animals were treated with DMSO (black diamonds), one daily dose of 300 mg/kg of CaPA (white circles), or two daily doses of 150 mg/kg of CaPA (white triangles) for 10 days following spore challenge. Statistical survival comparisons of CaPA-treated groups and the untreated control group were performed via a log rank test (* represents a *P* value of <0.05 compared to untreated animals).

vancomycin for 5 days starting 2 days before challenge with an inoculum of 100 *C. difficile* strain 630 spores. This regime prevented clindamycin-induced colitis, but it also prevented *C. difficile* infection from being established. The results were similar for animals given 5 mg/kg vancomycin for 3 days starting on the day of infection (data not shown).

In contrast, treatment with subclinical doses of 1 mg/kg vancomycin for 3 days postinfection protected hamsters from clindamycin-induced colitis. When challenged with 100 *C. difficile* strain 630 spores, animals receiving the 1-mg/kg vancomycin regimen succumbed to CDI by 3 days postinfection (*P* = 0.002). Using the same vancomycin regimen but reducing the inocula to 50 spores resulted in animals becoming moribund by 8 days postinfection (*P* = 0.002). Thus, preconditioning with 30 mg/kg clindamycin and 1 mg/kg vancomycin followed by challenge with 50 *C. difficile* strain 630 spores was used for all subsequent hamster CDI experiments (Fig. 5A).

CDI prevention against *C. difficile* clinical isolates in the hamster CDI model.

Hamsters treated with 1-mg/kg subclinical doses of vancomycin and challenged with *C. difficile* strain 630 spores started to become symptomatic and moribund 2 days after challenge. No untreated animals survived by day 8 postinfection (Fig. 5B).

In contrast, challenged animals treated with one daily 300-mg/kg dose of CaPA were partially protected from CDI. Indeed, 40% of animals died between days 2 and 3 postinfection, but the rest of the treated hamsters never developed CDI and remained asymptomatic for at least 30 days (*P* = 0.06 compared to untreated animals). When the CaPA treatment was divided into two 150-mg/kg daily dosages, only 20% of the animals were lost to CDI, while the rest were asymptomatic for the remainder of the experiment (*P* = 0.006 compared to untreated animals). As expected, unchallenged hamsters treated with clindamycin and vancomycin did not show any disease or toxicity signs and survived through the experimental timeline (data not shown).

DISCUSSION

C. difficile has a large and dynamic pangenome that allows rapid adaptation (22). Previous work has shown that spore germination is heterogeneous among strains (16). Since our approach is to use antigerminants as prophylactics for both primary CDI and CDI relapse, we screened bile salt analog compounds as inhibitors of *C. difficile* spore germination (20) against both epidemic-type strain 630 (ribotype 012) and hypervirulent strain R20291 (ribotype 027). The most potent antigerminants (Fig. 1) were then tested against six additional strains (9001966, CDC38, DH1834, 8085054, 05-1223-046, and 7004578) that have been shown to have different germination profiles (16).

Although most strains demonstrated typical germination behavior toward the known *C. difficile* germinants taurocholate and glycine, spores from strains 05-1223-046 and 7004578 failed to germinate with these molecules, even when supplemented with calcium. In contrast, Heeg et al. showed 74% germination for strain 05-1223-046 and 46% for 7004578 when incubated with 0.1% taurocholate in brain heart infusion (BHI) medium (16). It is possible that glycine is not a cogerminant for these strains and that other amino acid combinations present in complex media are required to activate the germination pathway. Indeed, we have previously shown that in addition to glycine, *C. difficile* strain 630 can use aromatic and basic amino acids as cogerminants with taurocholate (13). This possibility is further supported by the fact that both strain 05-1223-046 and strain 7004578 must germinate to cause CDI in the complex environment of mouse intestines.

All eight selected strains were tested for their ability to cause CDI in mice. Just as these strains showed a range of germination behaviors *in vitro*, they also showed variability in sign severity, disease progression, and sign duration in the mouse model of CDI. Both strains R20291 and p9001966 presented the highest CDI sign severity. This was expected for hypervirulent strain R20291. In contrast, strains 630, CDC38, and DH1834 all caused mild to moderate CDI signs. However, the onset of maximum signs varied among these three strains. Strains 05-1223-046, 7004578, and 8085054 resulted in mostly mild CDI signs, although sign heterogeneity was observed, with some mice becoming more symptomatic than others. Strains R20291, CDC38, and 05-1223-046 all belong to ribotype 027, yet they showed very divergent abilities to establish murine CDI. These variations in virulence between ribotype 027 strains are not unusual in mice (23). The cause of these differences is under investigation.

Since CamSA was the leading antigermination compound from the first group of bile salt analogs studied, its activity against spores was analyzed in all tested strains. However, CamSA was effective only against strain 630 *in vitro* and showed no activity against other ribotypes. Hence, CamSA was not expected to prevent CDI caused by other *C. difficile* strains based on *in vitro* data. Indeed, CamSA was not effective at protecting mice against strains R20291, CDC38, DH1834, and 7004578. Interestingly, CamSA was still able to protect mice challenged with strains 8085054 and 05-1223-046. Furthermore, decreased sign severity was observed in strain p9001966-challenged, CamSA-treated mice. The accumulated *in vivo* data suggest that it might be necessary to increase the levels of CamSA to detect the inhibition of spore germination *in vitro*. However, bile salt analogs may precipitate at high concentrations, making them difficult to test under these conditions.

To find a more universal antigerminant and CDI prophylactic, we tested over 200 CamSA analogs (20) and found 5 cholate analogs with aromatic side chains (CaPA, Caln, CaoAB, CaoCA, and CaoFA) and a chenodeoxycholate analog (ChPA) that inhibited the *in vitro* spore germination of strains R20291, p9001966, CDC38, DH1834, and 8085054 at concentrations of as low as 38 nM, even though the taurocholate germinant was at saturating millimolar concentrations (Table 1). Due to their powerful antigermination activity, these compounds were tested as potential CDI prophylactics.

To screen for CDI prophylactic efficacy *in vivo*, all seven compounds were tested as prophylactics against the *C. difficile* hypervirulent strain R20291 in the murine CDI model. Although compound Caln was the most potent germination inhibitor *in vitro* against all tested strains, it failed to prevent CDI in strain R20291 spore-challenged mice. Similarly, compounds CaoAB, CaoCA, and CaoFA were unsuccessful at preventing CDI in mice challenged with strain R20291. More intriguingly, ChPA, a chenodeoxycholate analog of CaPA, also failed to protect mice from hypervirulent strain R20291, even though these two compounds differ by only a single hydroxy group at position 12. These compounds might well be effective CDI prophylactics against other strains, but as they do not provide protection against the more clinically relevant hypervirulent strain, they were not pursued further.

CaPA significantly prevented, delayed, or reduced CDI signs in mice challenged with all strains tested. CaPA was also nontoxic, even after 30 days of dosing with

saturating concentrations, and did not cause a large-scale disruption of the gut microbiome compared to DMSO treatment, as evidenced by no loss in Shannon diversity (Fig. 3A) or large-scale changes in the most abundant families (Fig. 3C), consistent with our previous results with CamSA (19). Although we were able to detect significant differences in microbial community compositions (Fig. 3B), these changes were much less profound and lasting than changes incurred by conventional antibiotics, which can promote dysbiosis and subsequent CDI relapse (24–26). This is an important advantage for spore germination inhibitors over conventional antibiotic treatment for CDI.

Bile salt analogs have been shown to affect the germination of other *Clostridia*. Indeed, sterane compounds differentially affect the germination of *Clostridium sordellii* spores (27), while taurocholate is a cogerminant for *Clostridium perfringens* spores (M. Liggins, M. Pucci, and E. Abel-Santos, unpublished data). Endospore-forming *Firmicutes* were prominent among taxa that were less abundant during CamSA and CaPA treatments, suggesting some lack of specificity of the antigerminants. However, the microbiome of antigerminant-treated animals reached nearly complete recovery by day 30 postinfection. CamSA led to significant increases in respiratory *Proteobacteria*, a well-known sign of dysbiosis and inflammation (28); however, these taxa were present in very low abundances (Fig. 3C), unlike conventional antibiotic treatments, and the *Proteobacteria* that were enriched were not typical of dysbiosis induced by antibiotic treatments (e.g., absence of *Enterobacteriaceae*) (28). In all, the absence of detectable toxicity and the very minor microbiome disruptions induced by CaPA make this antigerminant a promising alternative to conventional antibiotics.

CaPA showed the lowest anti-CDI efficacy against the hypervirulent strain CDC38. In contrast, mice challenged with strains 630, 05-1223-046, 7004578, and 8085054 were completely protected from CDI when treated with CaPA. Although CaPA did not completely eliminate CDI in mice challenged with the hypervirulent strain R20291, sign severity was markedly reduced. Similarly, CDI signs were reduced and delayed for mice infected with strains p9001966 and DH1834.

The mouse CDI model has demonstrated the powerful potential of bile salt analogs in the prophylactic treatment of CDI. Mice, however, are quite resistant to CDI and its relapse. In contrast, studies have proposed the coadministration of vancomycin and clindamycin to hamsters as a surrogate for CDI relapse (19, 21). Indeed, subclinical levels of vancomycin were administered in conjunction with CamSA treatment to synergistically protect hamsters from CDI (19).

To further examine the potential of the leading aniline-substituted compound, CaPA's effectiveness was also investigated in the highly susceptible delayed hamster CDI model. In hamsters, CamSA was effective only when given concurrently with subclinical levels of vancomycin (19). In contrast, CaPA continued to be protective when administered after vancomycin treatment was stopped. Thus, CaPA appears to be an even more potent CDI prophylactic than CamSA.

The differences between *in vitro* and *in vivo* activities could be due to the influences that may be present only in the complex system of the gut, such as different spore germination rates *in vivo* due to additional biochemical cues not accounted for *in vitro*, the absorption of compounds decreasing the concentration in the gut lumen, microbial hydrolysis of the bile salt amide bond to render inactive compounds, or potential modification of antigerminants by resident microbiota *in situ*. This may explain why compounds such as ChPA, Caln, CaoAB, CaoCA, and CaoFA inhibited spore germination *in vitro* but not *in vivo*. Therefore, finding inhibitors that are stable and not systemically available (29) might be necessary to increase CDI prophylactic efficacy.

Bile salts are potential carcinogens (30) and can cause chronic diarrhea (31). On the other hand, bile salts have very high 50% lethal doses (LD₅₀s) in both mice and rats (32). The bile acid pool is approximately 2 to 3 g in fasting adult humans and increases to 6 to 10 g after a meal (33). In mice, CaPA prevented CDI at 50 mg/kg, which would correspond, based on body surface area, to a daily dose of 250 mg for a 60-kg human

(34). This amount of CaPA would increase the total bile salt pool to at most 15% during fasting and <5% after eating.

Similar to other bile salts, CaPA could be susceptible to degradation by microbial commensals (35). On the other hand, CamSA was shown to be stable in the microbiota of antibiotic-treated mice (29). Aniline, a hydrolysis product of CaPA, is potentially toxic (36). However, the LD₅₀ of aniline in mice is 8- to 10-fold lower than the maximum concentration released if the complete therapeutic dose of CaPA was degraded (36).

Aniline is even less toxic to humans than to rodents. Oral doses of aniline were well tolerated at up to 65 mg/kg by human volunteers (37). In mice, CaPA prevented CDI at 50 mg/kg, which would correspond to a daily dose of 250 mg for a 60-kg human. If this amount of CaPA were completely degraded in a patient's intestines, approximately 50 mg of aniline would be released. This corresponds to an aniline dose of 0.83 mg/kg for a 60-kg human, well below the tolerated range.

In conclusion, CaPA, an aniline-substituted cholate, was a more potent CDI prophylactic than its predecessor CamSA. Although CamSA can reduce or prevent CDI signs in mice for a few tested strains and can prevent CDI in mice when administered in combination with suboptimal vancomycin, CaPA is able to reduce, prevent, or delay CDI signs in mice with all but one strain tested. Furthermore, CaPA is more effective than CamSA in preventing hamster CDI. By modifying treatment while discovering new bile salt analogs, we may be able to find a potent prophylactic treatment option to be used on CDI-inflicting organisms.

MATERIALS AND METHODS

Materials. *C. difficile* strains R20291, 9001966, 05-1223-046, CDC38, DH1834, 7004578, and 8085054 were donated by Nigel Minton at the University of Nottingham, United Kingdom. *C. difficile* strain 630 was obtained from the American Type Culture Collection (ATCC). Synthetic bile salt analogs were prepared by Steven M. Firestone (Caln, CaoAB, CaoCA, and CaoFA) or were previously synthesized in the Abel-Santos laboratory (CamSA and CaPA). All compounds were >95% pure as determined by nuclear magnetic resonance (NMR) analysis. Laboratory rodent diet was provided by the University of Nevada, Las Vegas, animal care facility from LabDiet (St. Louis, MO, USA). All growth of *C. difficile* strains was done in a Coy Laboratories vinyl anaerobic chamber (5% H₂, 5% CO₂, and 95% N₂).

Animals. All procedures involving animals in this study were performed according to the *Guide for Care and Use of Laboratory Animals* by the National Institutes of Health (38). The protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Nevada, Las Vegas (permit number R0914-297). Weaned female mice (strain C57BL/6) and weaned male Golden Syrian hamsters were purchased from Charles River Laboratories (Wilmington, MA, USA). Mice were housed in groups of five per cage, and hamsters were housed individually at the University of Nevada, Las Vegas, animal care facility. Upon arrival at the facility, animals were allowed to acclimate for at least 1 week prior to the start of experimentation. All bedding, cages, food, and water were autoclaved prior to contact with animals. All postchallenge manipulations were performed within a biosafety level 2 laminar flow hood.

Ribotyping of *C. difficile* strains. *C. difficile* strains from frozen stocks were grown for 48 h on *C. difficile* selective agar (CDSA) composed of 20 g Bacto agar, 32 g Bacto proteose peptone, 6 g mannitol, 1 g KH₂PO₄, 5 g Na₂HPO₄, 2 g NaCl, 0.1 g MgSO₄, and 0.03 g neutral red per L. After autoclaving, the melted agar was supplemented with 0.25 g D-cycloserine and 0.016 g cefoxitin. Three colonies from each strain were then selected and cultured overnight in BHI medium.

DNA from *C. difficile* cultures grown overnight was used to amplify the rRNA region by PCR using the universal primers GM3 forward (5'-AGAGTTTGATCMTGGC-3') and GM4 reverse (5'-TACCTTGTTACGACTT-3'), according to previously reported procedures (39–41). PCR bands for each sample were separated by capillary gel electrophoresis at the University of Nevada, Reno, Genomics Center. Digital data files for the PCR banding patterns were submitted to the Public Health England *Clostridium difficile* ribotyping network (CDRN) for comparison to the ribotype library. Ribotypes confirmed by these results are reported in Table 1. Each strain was also tested to confirm the presence of *C. difficile* toxin A (*tcdA*) and *C. difficile* toxin B (*tcdB*).

***C. difficile* spore harvest and purification.** *C. difficile* cells from frozen stocks were streak plated in an anaerobic chamber onto BHI agar supplemented with 2% yeast extract, 0.1% L-cysteine-HCl, and 0.05% sodium taurocholate (BHIS) to yield colonies (15). After 48 h, a single colony was inoculated into BHI broth supplemented with 0.5% yeast extract and incubated for 48 h. The inoculated broth was then spread plated onto multiple BHI agar plates prepared as described above. Inoculated plates were incubated for 7 days at 37°C.

Inoculated plates were then flooded with ice-cold deionized (DI) water. Cells and spores were harvested by scraping bacterial colonies from the plates. The cell and spore mixtures were pelleted via centrifugation at 8,000 × *g* for 5 min, resuspended in DI water, and pelleted again. This washing step was repeated twice more. The cell-spore mixture was then centrifuged through a 20%-to-50% HistoDenz gradient at 18,200 × *g*

for 30 min with no brake (42). Under these conditions, spores pelleted at the bottom of the centrifuge tube, while cell debris remains above the 20% HistoDenz layer. Pelleted spores were transferred to a clean centrifuge tube and washed five times before storing them in DI water at 4°C. To determine spore purity, selected samples were stained using the Schaeffer-Fulton endospore staining method (43) or visualized via phase-contrast microscopy. Spore preparations used were >95% pure.

Preparation of the spore germination assay. Spores were washed three times with DI water and heat shocked at 68°C for 30 min. Spores were then washed three more times. Heat-shocked spores were resuspended in germination buffer (0.1 M sodium phosphate supplemented with 0.5% sodium bicarbonate and adjusted to pH 6.0) to reach an optical density at 580 nm (OD_{580}) of 1.0. Germination buffer for strains 05-1223-046 and 7004578 were also supplemented with 10 mM, 20 mM, or 40 mM calcium chloride.

Sodium taurocholate and other bile salt analog solutions were prepared in DMSO. Glycine solutions were prepared in H₂O. *In vitro* spore germination assays were performed in 96-well plates in triplicate. To test for germination inhibition, bile salt analogs were added at increasing concentrations together with the cogerminants sodium taurocholate and glycine. As negative germination controls, spores were treated with neat DMSO. As positive germination controls, spores were incubated with sodium taurocholate and glycine. Following the addition of analogs and germinants, 180 μ L of spores in germination buffer (OD_{580} = 1.0) was pipetted into each well for a final volume of 200 μ L. The final concentration of sodium taurocholate was 6 mM, and the final concentration of glycine was 12 mM. Final bile salt analog concentrations varied but were mostly in the micromolar-to-nanomolar range. The optical density over time was read by a Labsystems iEMS Reader MF plate reader using Ascent software or a Tecan Infinite M200 plate reader using Tecan i-control software.

Germination curves were used to determine percent germination by comparing the slope from the linear part of the early germination curves of each concentration of bile salt analog to the slope from the germination curve of the positive germination control. Percent germination rates were plotted against the inhibitor concentration.

The resulting sigmoidal curves were analyzed in SigmaPlot version 11, 13, or 14 by fitting with the four-parameter logistic function to obtain the IC_{50} values for antigerminant compounds. Adjusted R^2 values from the regression analyses were above 0.95 in every case. IC_{50} values represent the concentration of the compound required to reduce the spore germination rate to half the maximal value and are used to compare the inhibitory potencies of bile salt analogs.

Thirty-day compound toxicity regimen in mice. To test for acute and subchronic toxicity, animals were given a saturating dose of 300 mg/kg of the bile salt analog compound once per day for 30 days. Bile salt analogs were administered via oral gavage with a total volume of 50 μ L per dose. Neat DMSO was used as a control in one cage of mice. Another cage of mice was given CamSA dissolved in DMSO, and mice in a third cage were given CaPA dissolved in DMSO. Fresh fecal matter was collected from mice on days 0, 10, and 30 to examine the dynamics of the gut microbiome. Fecal pellets were homogenized in Qiagen ASL buffer, flash-frozen, and kept at –80°C for later DNA extraction. Weight changes were recorded on those days. Body weight loss in mice was calculated as the percent change from the weight on day 0. Significant weight loss was defined as a loss of >15% of the original body weight. Mice were observed for signs of distress daily. At the end of the 30-day trial, animals were sacrificed and necropsied to investigate potential anatomical abnormalities.

Murine CDI prevention model. The murine CDI model used in this study was adapted from a model described previously by Chen et al. (44). Mice were given three consecutive days of an antibiotic cocktail containing kanamycin (0.4 mg/mL), gentamicin (0.035 mg/mL), colistin (850 U/mL), metronidazole (0.215 mg/mL), and vancomycin (0.045 mg/mL) *ad libitum* (14). Mice were then given autoclaved DI water for the remainder of the experiment. On the day prior to *C. difficile* challenge (day –1), mice were given an intraperitoneal (i.p.) injection of 10 mg/kg clindamycin. None of the animals developed clinical symptoms during antibiotic treatment. On the day of infection (day 0), experimental groups were challenged with 10^8 *C. difficile* spores via oral gavage and given 50-mg/kg daily gavage doses of either CamSA or CaPA at 0, 24, and 48 h postchallenge. One group of five infected mice was given neat DMSO and served as a positive infection control group for CDI. One group of five antibiotic-treated mice was used as a negative uninfected control group.

Mice were observed for signs of CDI twice daily, and disease severity was scored according to a CDI sign rubric adapted from previous work (14, 18, 19, 24). According to the rubric, pink anogenital area, mild (approaching) wet tail, and weight loss of 8 to 15% were scored as 1, and red anogenital area, lethargy/distress, increased diarrhea/soiled bedding, hunched posture, and weight loss of >15% each were scored as 2. Animals with a total score of <3 were indistinguishable from noninfected controls and were considered nondiseased. Animals with a score of 3 to 4 were considered to have mild CDI. Animals with a score of 5 to 6 were considered to have moderate CDI. Animals with a score of >6 were considered to have severe CDI and were immediately culled.

Microbiome analysis. Freshly thawed fecal pellets were rehomogenized and DNA was extracted using the QIAamp Fast DNA Mini stool kit and quantified using a NanoDrop 1000 spectrophotometer. The V4 region of the 16S rRNA gene was amplified and paired-end sequenced from extracted DNA using the Illumina MiSeq platform at Micro-Seq Enterprises (Las Vegas, NV), as described previously by Kozich et al. (45), except that the PCR primers were optimized to increase the coverage of *Archaea*, as described previously (19).

Amplicons were imported into QIIME 2 (version 2018.6) (46), demultiplexed, denoised, and dereplicated to obtain an amplicon sequence variant (ASV) table using the “dada2-denoise-paired” plug-in. The ASV table was rarified to the lowest number of reads per sample, and ASVs were classified using QIIME’s feature-classifier plug-in and the Silva 132 99% operational taxonomic unit (OTU) full-length sequences.

ASVs assigned to mitochondria or chloroplasts or that were unidentified at the domain level were filtered out. Alpha diversity metrics were calculated using the QIIME2 plug-in q2-diversity and evaluated statistically using one-way ANOVA and Tukey's honestly significant difference (HSD) test. Nonmetric multidimensional scaling (NMDS) was performed based on Hellinger-transformed OTU data (relative abundance) with Bray-Curtis distances using the *vegan* (version 2.5.7) and *ggplot2* (version 3.3.3) packages in R software (v4.0.3). Analysis of similarity (ANOSIM) was performed using the *anosim* function from the R *vegan* package with the Bray-Curtis metric. Proportions of taxa were calculated using *phyloseq* (version 1.20.0) and visualized using *ggplot2* (version 2.2.1). Linear discriminant analysis (LDA) effect size (LEfSe) statistical analysis was conducted using the ImageGP online interface (<http://www.ehbio.com/ImageGP/>).

Establishment of CDI in hamsters. Hamsters were treated orogastrically with 30 mg/kg clindamycin 1 day prior to infection (day -1). Hamsters were given one of the following regimens of vancomycin: 5 mg/kg vancomycin for 5 days starting at day -2, 5 mg/kg vancomycin for 3 days starting at day 0, or 1 mg/kg vancomycin for 3 days starting at day 0. Following the selection of a suitable vancomycin regimen, animals were given either 0 (negative control), 50, or 100 *C. difficile* strain 630 spores. Animals were observed for signs of disease. Any animal that developed CDI signs was immediately culled.

Hamster CDI prevention model. The hamster CDI model used in this study was adapted from procedures reported previously by Howerton et al. (19). Hamsters were orogastrically dosed with 30 mg/kg clindamycin 1 day prior to infection (day -1). On day 0, animals were challenged with 50 *C. difficile* strain 630 spores. Hamsters were also given a suboptimal dose of 1 mg/kg vancomycin daily from day 0 to day 2 to prevent clindamycin-induced colitis. Beginning the day of infection, experimental groups of hamsters were treated with either a once-daily 300-mg/kg CaPA ($n = 5$) dose or twice-daily 150-mg/kg CaPA doses ($n = 5$) via oral gavage until day 10. One group of challenged hamsters ($n = 5$) was treated with neat DMSO and served as a positive infected control group. A group of nonchallenged hamsters ($n = 5$) was treated with neat DMSO and served as a negative uninfected control group. Twice-daily dosages were administered 12 h apart. Animals were observed for CDI signs. Symptomatic animals were immediately culled, as described above.

Statistical analysis. For the murine CDI prevention model, severity of signs was analyzed via box-and-whisker plots with a minimum of three independent values ($n \geq 3$). Single-factor ANOVA was performed in R at every time point to assess differences among sign severities of the untreated, CaPA-treated, and CamSA-treated groups. ANOVA results with P values of <0.05 were analyzed *post hoc* using the Holm method for pairwise comparison between individual treatments and untreated groups. Statistically significant differences between untreated and treated groups were determined as P values of <0.05 . For the hamster CDI prevention model, survival was analyzed using Kaplan-Meier plots. The significance of differences between survival patterns was calculated in R using a log rank test.

Data availability. Raw, unfiltered 16S rRNA gene sequence data are available from the NCBI under BioProject accession number [PRJNA749826](https://www.ncbi.nlm.nih.gov/bioproject/PRJNA749826), according to recommendations from the Genome Standards Consortium (47).

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REFERENCES

- Centers for Disease Control and Prevention. 2019. Antibiotic resistance threats in the United States, 2019. Centers for Disease Control and Prevention, Atlanta, GA.
- Dubberke ER, Olsen MA. 2012. Burden of *Clostridium difficile* on the healthcare system. *Clin Infect Dis* 55:88–92. <https://doi.org/10.1093/cid/cis335>.
- Lessa FC, Mu Y, Bamberg WM, Beldavs ZG, Dumyati GK, Dunn JR, Farley MM, Holzbauer SM, Meek JI, Phipps EC, Wilson LE, Winston LG, Cohen JA, Limbago BM, Fridkin SK, Gerding DN, McDonald LC. 2015. Burden of *Clostridium difficile* infection in the United States. *N Engl J Med* 372:825–834. <https://doi.org/10.1056/NEJMoa1408913>.
- Miller BA, Chen LF, Sexton DJ, Anderson DJ. 2011. Comparison of the burdens of hospital-onset, healthcare facility-associated *Clostridium difficile* infection and of healthcare-associated infection due to methicillin-resistant *Staphylococcus aureus* in community hospitals. *Infect Control Hosp Epidemiol* 32:387–390. <https://doi.org/10.1086/659156>.
- Giancola SE, Williams RJ, Gentry CA. 2018. Prevalence of the *Clostridium difficile* BI/NAP1/027 strain across the United States Veterans Health Administration. *Clin Microbiol Infect* 24:877–881. <https://doi.org/10.1016/j.cmi.2017.11.011>.
- Wendt JM, Cohen JA, Mu Y, Dumyati GK, Dunn JR, Holzbauer SM, Winston LG, Johnston HL, Meek JI, Farley MM, Wilson LE, Phipps EC, Beldavs ZG, Gerding DN, McDonald LC, Gould CV, Lessa FC. 2014. *Clostridium difficile* infection among children across diverse US geographic locations. *Pediatrics* 133:651–658. <https://doi.org/10.1542/peds.2013-3049>.
- Paredes-Sabja D, Shen A, Sorg JA. 2014. *Clostridium difficile* spore biology: sporulation, germination, and spore structural proteins. *Trends Microbiol* 22:406–416. <https://doi.org/10.1016/j.tim.2014.04.003>.
- Setlow P. 2007. I will survive: DNA protection in bacterial spores. *Trends Microbiol* 15:172–180. <https://doi.org/10.1016/j.tim.2007.02.004>.
- McDonald LC, Gerding DN, Johnson S, Bakken JS, Carroll KC, Coffin SE, Dubberke ER, Garey KW, Gould CV, Kelly C, Loo V, Shaklee Sammons J, Sandora TJ, Wilcox MH. 2018. Clinical practice guidelines for *Clostridium difficile* infection in adults and children: 2017 update by the Infectious Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA). *Clin Infect Dis* 66:e1–e48. <https://doi.org/10.1093/cid/cix1085>.
- Sorg JA, Sonenshein AL. 2008. Bile salts and glycine as cogerminants for *Clostridium difficile* spores. *J Bacteriol* 190:2505–2512. <https://doi.org/10.1128/JB.01765-07>.
- Sorg JA, Sonenshein AL. 2010. Inhibiting the initiation of *Clostridium difficile* spore germination using analogs of chenodeoxycholic acid, a bile acid. *J Bacteriol* 192:4983–4990. <https://doi.org/10.1128/JB.00610-10>.

12. Shen A. 2015. A gut odyssey: the impact of the microbiota on *Clostridium difficile* spore formation and germination. *PLoS Pathog* 11:e1005157. <https://doi.org/10.1371/journal.ppat.1005157>.
13. Howerton A, Ramirez N, Abel-Santos E. 2011. Mapping interactions between germinants and *Clostridium difficile* spores. *J Bacteriol* 193:274–282. <https://doi.org/10.1128/JB.00980-10>.
14. Howerton A, Patra M, Abel-Santos E. 2013. A new strategy for the prevention of *Clostridium difficile* infection. *J Infect Dis* 207:1498–1504. <https://doi.org/10.1093/infdis/jit068>.
15. Ramirez N, Liggins M, Abel-Santos E. 2010. Kinetic evidence for the presence of putative germination receptors in *Clostridium difficile* spores. *J Bacteriol* 192:4215–4222. <https://doi.org/10.1128/JB.00488-10>.
16. Heeg D, Burns DA, Cartman ST, Minton NP. 2012. Spores of *Clostridium difficile* clinical isolates display a diverse germination response to bile salts. *PLoS One* 7:e32381. <https://doi.org/10.1371/journal.pone.0032381>.
17. Rohlfing AE, Eckenroth BE, Forster ER, Kevorkian Y, Donnelly ML, de La Puebla HB, Doublé S, Shen A. 2019. The CspC pseudoprotease regulates germination of *Clostridioides difficile* spores in response to multiple environmental signals. *PLoS Genet* 15:e1008224. <https://doi.org/10.1371/journal.pgen.1008224>.
18. Howerton A, Patra M, Abel-Santos E. 2013. Fate of ingested *Clostridium difficile* spores in mice. *PLoS One* 8:e72620. <https://doi.org/10.1371/journal.pone.0072620>.
19. Howerton A, Seymour CO, Murugapiran SK, Liao Z, Phan JR, Estrada A, Wagner AJ, Mefferd CC, Hedlund BP, Abel-Santos E. 2018. Effect of the synthetic bile salt analog CamSA on the hamster model of *Clostridium difficile* infection. *Antimicrob Agents Chemother* 62:e02251-17. <https://doi.org/10.1128/AAC.02251-17>.
20. Sharma SK, Yip C, Esposito EX, Sharma PV, Simon MP, Abel-Santos E, Firestone SM. 2018. The design, synthesis, and characterizations of spore germination inhibitors effective against an epidemic strain of *Clostridium difficile*. *J Med Chem* 61:6759–6778. <https://doi.org/10.1021/acs.jmedchem.8b00632>.
21. Bartlett JG, Onderdonk AB, Cisneros RL. 1977. Clindamycin-associated colitis in hamsters: protection with vancomycin. *Gastroenterology* 73:772–776. [https://doi.org/10.1016/S0016-5085\(19\)31782-2](https://doi.org/10.1016/S0016-5085(19)31782-2).
22. Knight DR, Elliott B, Chang BJ, Perkins TT, Riley TV. 2015. Diversity and evolution in the genome of *Clostridium difficile*. *Clin Microbiol Rev* 28:721–741. <https://doi.org/10.1128/CMR.00127-14>.
23. Lewis BB, Carter RA, Ling L, Leiner I, Taur Y, Kamboj M, Dubberke ER, Xavier J, Pamer EG. 2017. Pathogenicity locus, core genome, and accessory gene contributions to *Clostridium difficile* virulence. *mBio* 8:e00885-17. <https://doi.org/10.1128/mBio.00885-17>.
24. Mefferd CC, Bhute S, Phan JR, Villarama JV, Do DM, Alarcia S, Abel-Santos E, Hedlund BP. 2020. A high-fat/high-protein, Atkins-type diet exacerbates *Clostridioides (Clostridium) difficile* infection in mice, whereas a high-carbohydrate diet protects. *mSystems* 5:e00765-19. <https://doi.org/10.1128/mSystems.00765-19>.
25. Dieterle MG, Rao K, Young VB. 2019. Novel therapies and preventative strategies for primary and recurrent *Clostridium difficile* infections. *Ann N Y Acad Sci* 1435:110–138. <https://doi.org/10.1111/nyas.13958>.
26. Theriot CM, Koenigsnecht MJ, Carlson PE, Jr, Hatton GE, Nelson AM, Li B, Hufnagle GB, Li J, Young VB. 2014. Antibiotic-induced shifts in the mouse gut microbiome and metabolome increase susceptibility to *Clostridium difficile* infection. *Nat Commun* 5:3114. <https://doi.org/10.1038/ncomms4114>.
27. Liggins M, Ramirez N, Magnuson N, Abel-Santos E. 2011. Progesterone analogs influence germination of *Clostridium sordellii* and *Clostridium difficile* spores in vitro. *J Bacteriol* 193:2776–2783. <https://doi.org/10.1128/JB.00058-11>.
28. Reese AT, Cho EH, Klitzman B, Nichols SP, Wisniewski NA, Villa MM, Durand HK, Jiang S, Midani FS, Nimmagadda SN, O'Connell TM, Wright JP, Deshusses MA, David LA. 2018. Antibiotic-induced changes in the microbiota disrupt redox dynamics in the gut. *Elife* 7:e35987. <https://doi.org/10.7554/eLife.35987>.
29. Yip C, Okada NC, Howerton A, Amei A, Abel-Santos E. 2021. Pharmacokinetics of CamSA, a potential prophylactic compound against *Clostridioides difficile* infections. *Biochem Pharmacol* 183:114314. <https://doi.org/10.1016/j.bcp.2020.114314>.
30. Bernstein H, Bernstein C, Payne CM, Dvorakova K, Garewal H. 2005. Bile acids as carcinogens in human gastrointestinal cancers. *Mutat Res* 589:47–65. <https://doi.org/10.1016/j.mrrev.2004.08.001>.
31. Walters JRF. 2010. Defining primary bile acid diarrhea: making the diagnosis and recognizing the disorder. *Expert Rev Gastroenterol Hepatol* 4:561–567. <https://doi.org/10.1586/egh.10.54>.
32. Klaassen CD. 1973. Comparison of the toxicity of chemicals in newborn rats to bile duct-ligated and sham-operated rats and mice. *Toxicol Appl Pharmacol* 24:37–44. [https://doi.org/10.1016/0041-008x\(73\)90179-8](https://doi.org/10.1016/0041-008x(73)90179-8).
33. Hofmann AF. 1999. The continuing importance of bile acids in liver and intestinal disease. *Arch Intern Med* 159:2647–2658. <https://doi.org/10.1001/archinte.159.22.2647>.
34. Nair AB, Jacob S. 2016. A simple practice guide for dose conversion between animals and human. *J Basic Clin Pharm* 7:27–31. <https://doi.org/10.4103/0976-0105.177703>.
35. Patel AK, Singhania RR, Pandey A, Chincholkar SB. 2010. Probiotic bile salt hydrolase: current developments and perspectives. *Appl Biochem Biotechnol* 162:166–180. <https://doi.org/10.1007/s12010-009-8738-1>.
36. National Research Council (US) Committee on Acute Exposure Guideline Levels. 2008. Acute exposure guideline levels for selected airborne chemicals, vol 6, p 115–118. National Academies Press, Washington, DC.
37. Lu G, Zhou L, Zhang X, Zhu B, Wu S, Song W, Gong X, Wang D, Tao Y. 2016. The expression of metastasis-associated in colon cancer-1 and KAI1 in gastric adenocarcinoma and their clinical significance. *World J Surg Oncol* 14:276. <https://doi.org/10.1186/s12957-016-1033-z>.
38. National Research Council. 2011. Guide for the care and use of laboratory animals, 8th ed. National Academies Press, Washington, DC.
39. Bélanger SD, Boissinot M, Clairoux N, Picard François J, Bergeron MG. 2003. Rapid detection of *Clostridium difficile* in feces by real-time PCR. *J Clin Microbiol* 41:730–734. <https://doi.org/10.1128/JCM.41.2.730-734.2003>.
40. Guilbault C, Labbé AC, Poirier L, Busque L, Béliveau C, Laverdière M. 2002. Development and evaluation of a PCR method for detection of the *Clostridium difficile* toxin B gene in stool specimens. *J Clin Microbiol* 40:2288–2290. <https://doi.org/10.1128/JCM.40.6.2288-2290.2002>.
41. Gillers S, Atkinson CD, Bartoo AC, Mahalanabis M, Boylan MO, Schwartz JH, Klapperich C, Singh SK. 2009. Microscale sample preparation for PCR of *C. difficile* infected stool. *J Microbiol Methods* 78:203–207. <https://doi.org/10.1016/j.mimet.2009.05.020>.
42. Dodatko T, Akoachere M, Jimenez N, Alvarez Z, Abel-Santos E. 2010. Dissecting interactions between nucleosides and germination receptors in *Bacillus cereus* 569 spores. *Microbiology (Reading)* 156:1244–1255. <https://doi.org/10.1099/mic.0.030270-0>.
43. Schaeffer AB, Fulton MD. 1933. A simplified method of staining endospores. *Science* 77:194. <https://doi.org/10.1126/science.77.1990.194>.
44. Chen X, Katchar K, Goldsmith JD, Nanthakumar N, Cheknis A, Gerding DN, Kelly CP. 2008. A mouse model of *Clostridium difficile*-associated disease. *Gastroenterology* 135:1984–1992. <https://doi.org/10.1053/j.gastro.2008.09.002>.
45. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl Environ Microbiol* 79:5112–5120. <https://doi.org/10.1128/AEM.01043-13>.
46. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F, Bai Y, Bisanz JE, Bittinger K, Brejnrod A, Brislawn CJ, Brown CT, Callahan BJ, Caraballo-Rodríguez AM, Chase J, Cope EK, Da Silva R, Diener C, Dorrestein PC, Douglas GM, Durall DM, Duvallet C, Edwardsen CF, Ernst M, Estaki M, Fouquier J, Gauglitz JM, Gibbons SM, Gibson DL, Gonzalez A, Gorlick K, Guo J, Hillmann B, Holmes S, Holste H, Huttenhower C, Huttley GA, Janssen S, Jarmusch AK, Jiang L, Kaehler BD, Kang KB, Keefe CR, Keim P, Kelley ST, Knights D, Koester I, Kosciolk T, et al. 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol* 37:852–857. <https://doi.org/10.1038/s41587-019-0209-9>.
47. Yilmaz P, Kottmann R, Field D, Knight R, Cole JR, Amaral-Zettler L, Gilbert JA, Karsch-Mizrachi I, Johnston A, Cochrane G, Vaughan R, Hunter C, Park J, Morrison N, Rocca-Serra P, Sterk P, Arumugam M, Bailey M, Baumgartner L, Birren BW, Blaser MJ, Bonazzi V, Booth T, Bork P, Bushman FD, Buttigieg PL, Chain PSG, Charlson E, Costello EK, Huot-Creasy H, Dawyndt P, DeSantis T, Fierer N, Fuhrman JA, Gallery RE, Gevers D, Gibbs RA, San Gil I, Gonzalez A, Gordon JI, Guralnick R, Hankeln W, Highlander S, Hugenholtz P, Jansson J, Kau AL, Kelley ST, Kennedy J, Knights D, Koren O, Kuczynski J, Kyrpides N, et al. 2011. Minimum information about a marker gene sequence (MIMARKS) and minimum information about any (x) sequence (MIXS) specifications. *Nat Biotechnol* 29:415–420. <https://doi.org/10.1038/nbt.1823>.