Metabolism of Bile Salts in Mice Influences Spore Germination in Clostridium difficile

Jennifer L. Giel1*, Joseph A. Sorg2, Abraham L. Sonenshein2, Jun Zhu1*

1 Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, United States of America, 2 Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, Massachusetts, United States of America

Abstract

Clostridium difficile, a spore-forming bacterium, causes antibiotic-associated diarrhea. In order to produce toxins and cause disease, C. difficile spores must germinate and grow out as vegetative cells in the host. Although a few compounds capable of germinating C. difficile spores in vitro have been identified, the in vivo signal(s) to which the spores respond were not previously known. Examination of intestinal and cecal extracts from untreated and antibiotic-treated mice revealed that extracts from the antibiotic-treated mice can stimulate colony formation from spores to greater levels. Treatment of these extracts with cholestyramine, a bile salt binding resin, severely decreased the ability of the extracts to stimulate colony formation from spores. This result, along with the facts that the germination factor is small, heat-stable, and water-soluble, support the idea that bile salts stimulate germination of C. difficile spores in vivo. All extracts able to stimulate high level of colony formation from spores had a higher proportion of primary to secondary bile salts than extracts that could not. In addition, cecal flora from antibiotic-treated mice was less able to modify the germinant taurocholate relative to flora from untreated mice, indicating that the population of bile salt modifying bacteria differed between the two groups. Taken together, these data suggest that an in vivo-produced compound, likely bile salts, stimulates colony formation from C. difficile spores and that levels of this compound are influenced by the commensal gastrointestinal flora.

Introduction

Clostridium difficile is a spore-forming, Gram-positive bacterium estimated to be responsible for about one-quarter of hospital-acquired infections [1]. C. difficile causes a watery diarrhea, and transmission of this pathogen likely occurs through ingestion of C. difficile spores. C. difficile-associated disease (CDAD) can progress to intestinal lesions, resulting in pseudomembranous colitis characterized by raised yellow plaques throughout the mucosa of the colon. Though progression to toxic megacolon, intestinal perforations, peritonitis, and death is uncommon, it does occur [1,2]. Although C. difficile is an obligate anaerobe when in its vegetative state, its spores have been estimated to persist on dry, inanimate surfaces for months [3], contributing to its role as a major nosocomial pathogen. In fact, C. difficile has been suggested to be the major infectious cause of diarrhea caused by antibiotic usage in human adults [2] and is especially a problem in elderly and immunocompromised patients. Although CDAD has a low mortality rate, C. difficile infection causes longer hospital stays, and treatment costs are estimated to be more than $3 billion per year in the U.S. [4]. The beginning of this century has been marked by a doubling of the rate of CDAD throughout the United States [1], and the recent emergence of hypervirulent C. difficile strains has resulted in higher rates of CDAD-associated morbidity, mortality, and health care costs [5,6].

Two events usually occur prior to development of CDAD: administration of antibiotics [7], leading to disruption of commensal bacteria in the host intestine [8], and infection with C. difficile, likely via the spore form. Since C. difficile infection manifests in patients undergoing antibiotic treatment, it is not surprising that C. difficile is naturally resistant to a number of antibiotics [9]. Treatment has mainly relied on the antibiotics metronidazole and vancomycin [1,10], which are unsatisfactory given that they prevent reestablishment of the commensal flora so relapses are common [1], and failure rates for metronidazole are on the rise [11,12].

Two important aspects of C. difficile infection are germination of the spores and how this process is regulated in the intestinal environment. Despite the fact that C. difficile is likely acquired via ingestion of spores (as the vegetative form will die in the presence of oxygen), only the vegetative form produces toxins. Thus, to more fully understand pathogenesis of C. difficile, a better understanding of spore germination is needed. In general, bacterial spores germinate in response to the binding of one or more small molecules, and the small molecules that can cause germination vary among different species and strains of bacteria [13]. The signals to which C. difficile responds have not been well-characterized, but the primary bile salts cholate, taurocholate, and glycocholate have been shown to stimulate germination in vitro [14,15,16]. In fact, this effect of bile salts is the basis for a standard method of titering C. difficile spores by colony formation [16]. However, signals to which C. difficile spores respond in vivo have not

Citation: Giel JL, Sorg JA, Sonenshein AL, Zhu J (2010) Metabolism of Bile Salts in Mice Influences Spore Germination in Clostridium difficile. PLoS ONE 5(1): e8740. doi:10.1371/journal.pone.0008740

Received: October 21, 2009; Accepted: December 23, 2009; Published: January 15, 2010

Copyright: © 2010 Giel et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by National Institutes of Health grant R01AI072479 to J.Z. A.L.S. acknowledges funding from the National Institutes of Health contract N01 AI60050 (S. Tzipori, principal investigator). J.A.S. acknowledges support through National Institutes of Health Federal Training in Education and Critical Research Skills (TEACRS) fellowship K12 GM074869. The funders had no role in study design, data collection and analysis, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: junzhu@mail.med.upenn.edu

Current address: UBC Envision Group, Philadelphia, Pennsylvania, United States of America
been identified. While it has been shown that commensal microflora may inhibit C. difficile growth and downregulate C. difficile virulence gene expression [17,18,19], it is not known what effects normal intestinal flora have on C. difficile spore germination. The identification of intestinal signals that affect C. difficile spore germination could lead to the discovery of compounds that inhibit germination and thus CDAD. Here, we examined colony formation from C. difficile spores in the presence of cecal and intestinal extracts from untreated and antibiotic-treated mice as well as the ability of flora from these mice to modify the primary bile salt taurocholate.

Materials and Methods

Strains and Growth Conditions

C. difficile CD196 [20] was grown in BHI liquid medium (Bacto brain-heart infusion [BD]) or BHIS plates (BHI supplemented with yeast extract to 5 mg/ml and L-cysteine to 0.1% [w/v]) at 37°C in a Don Whitley MiniMACS anaerobic chamber (80% N₂, 10% H₂, 10% CO₂). Taurocholic acid (TA) (Sigma) was added to the drinking water to a final concentration of 50 mg/ml to small intestinal or cecal extracts with the addition of 1 mM IPTG to induce expression.

Antibiotic Treatment and Preparation of Cecal and Intestinal Extracts

Mouse protocols were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. CD-1 mother mice were given clindamycin hydrochloride (Spectrum Chemical) or ampicillin (Research Products International) orogastrically in two doses each of 200 mg/kg of body weight in a 20 hour period and were sacrificed 24 hours after the initial antibiotic treatment. Streptomycin sulfate (Research Products International) was added to the drinking water to a final concentration of 5 mg/ml for 24 hours as previously described [22]. Mice were sacrificed by CO₂ asphyxiation and cervical dislocation, and the small intestine, cecum, and large intestine were harvested. Organs were weighed and homogenized in 1 ml H₂O per mg weight, pellet, and supernatant was filtered through a 0.2 m filter or boiled to sterilize.

Determination of Bile Salt Levels

To construct a strain overexpressing 7α-hydroxydehydrogenase (7α-HSDH), first the hdhA gene (Entrez GeneID 946151), which encodes 7α-HSDH, was PCR-amplified from the MG1655-based E. coli strain PK7743 [21] using primers 5'CTAGTCTGATATGTTCATATTGTAGCAACCTGTAAC-3' and 5'TCTCGAGTTAATTGAGCTCCTGTACCCCACC-3.' This DNA fragment was restriction digested and cloned into the NdeI and XhoI sites of pET-32a. The resulting plasmid, pJG32, was transformed into E. coli strain BL21 to give strain JG73.

Incubation of Taurocholate with Small Intestinal and Cecal Contents

The contents of small intestines and ceca from freshly euthanized mice treated with or without clindamycin were removed in the anaerobic chamber and washed five times with PBS to remove any bile salts present. Pellets were resuspended in 0.1% TA and incubated anaerobically for 24 hours at 37°C. Samples were pelleted, and the supernatant was boiled to sterilize for use in CFU recovery assays.

Results

An Ex Vivo Factor Stimulates Colony Formation from C. difficile Spores

To examine whether compounds present in the mouse gastrointestinal tract could stimulate germination of C. difficile spores, separate extracts of small intestines, ceca, and large intestines from untreated adult mice were prepared and used in CFU recovery assays. The density of the extracts precluded the use of a spectrophotometric assay for germination [14]. Dormant spores have a very low efficiency of colony formation on BHIS medium unless the medium contains an appropriate bile salt; in contrast, germinated spores form colonies with high efficiency on BHIS medium [14]. Incubation of spores with cecal or large intestinal extracts led to low levels of colony formation, whereas incubation of spores with small intestinal extracts resulted in about 15% CFU recovery (Fig. 1A). These results indicate that there is a factor present in mouse small intestines that can stimulate germination of C. difficile spores.

Disruption of the Commensal Flora with Antibiotics Leads to Higher Levels of Colony Formation from C. difficile Spores

Treatment with clindamycin and ampicillin often precipitate CDAD in humans [24], and both have been used in animal...
models to disrupt normal flora and induce CDAD [25,26], so cecal extracts from mice treated with clindamycin, ampicillin, and streptomycin were also tested for their ability to stimulate colony formation from \textit{C. difficile} spores. Cecal extracts from all of the antibiotic-treated mice showed roughly equal abilities to stimulate colony formation from \textit{C. difficile} spores, which was about 50 to 65-fold higher than cecal extracts from the untreated mice (Fig. 1A and B). The small intestinal extracts from the clindamycin-treated mice also stimulated high levels of colony formation, but the large intestinal extract did not.

Since the CFU recovery assay measures not only spore germination but also outgrowth of the vegetative cells, germination efficiency was also measured by the loss of heat resistance. Briefly, spores were incubated with the cecal extracts from untreated or clindamycin-treated mice for 30 min. at 37°C, heat shocked for 20 min. at 60°C, then diluted and plated on BHIS plates. Spores incubated with cecal extract from untreated mice showed roughly equal abilities to stimulate colony formation from \textit{C. difficile} spores, which was about 50 to 65-fold higher than cecal extracts from the untreated mice (Fig. 1A and B). The small intestinal extracts from the clindamycin-treated mice also stimulated high levels of colony formation, but the large intestinal extract did not.

Characterization of the Germination Factor

Several approaches were used to characterize the physical properties of the factor causing germination of \textit{C. difficile} spores. First, to ensure that antibiotics alone did not stimulate spore germination, spores were incubated with streptomycin at a final concentration of 200 \( \mu \text{g/ml} \). The CFU recovery from streptomycin-treated spores was comparable to the water control, indicating that streptomycin alone does not stimulate spore germination (data not shown). To test the heat stability of the germination factor, cecal extracts from clindamycin-treated mice were boiled for 10 min.; these extracts showed no difference in CFU recovery compared to extracts sterilized by passage through a 0.2 \( \mu \text{m} \) filter, indicating that the germination factor is heat-stable and can pass through a 0.2 \( \mu \text{m} \) filter (Fig. 2A). The germination factor appears to be small, since dialysis of cecal extract from the clindamycin-treated mice in a membrane with a 1 kDa cutoff abolished its ability to stimulate CFU recovery from spores (Fig. 2B). Testing the organic and aqueous fractions from ethyl acetate extraction indicated that the germination factor goes to the aqueous, not the

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{An in vivo-produced factor can stimulate CFU recovery from spores of \textit{C. difficile}. A) CD196 spores were incubated for 30 min. with water, 0.1% taurocholate (TA) (gray bar), or extracts from mice that were untreated (black bars) or treated with clindamycin (striped bars), streptomycin (white bar), or ampicillin (cross-hatched bar), then diluted and spread to BHIS plates. The CFU/ml were compared to the CFU/ml on BHIS plates containing taurocholate, which was considered to reflect 100% germination. Data are the mean of at least three experiments, with error bars representing the standard error of the mean. B) Plates from a CFU recovery experiment in which spores were incubated with water, TA, or cecal extract from untreated or clindamycin-treated mice as in (A), then serially diluted ten-fold and spotted onto BHIS or BHIS + TA plates. A representative experiment is shown.}
\end{figure}
organic, phase (Fig. 2C). Taken together, these data indicate that the germination factor is a compound present at higher levels in vivo in response to antibiotic treatment (but is not itself the added streptomycin), and is small, heat-stable, and water-soluble.

Treatment of Extracts with Cholestyramine Decreases Their Ability to Stimulate Colony Formation from *C. difficile* Spores

These physical characteristics and the presence of the factor in untreated and antibiotic-treated mice (albeit at different locations along the gastrointestinal tract) are consistent with bile salts as an in vivo germination factor. Previous in vitro work has shown that purified primary bile salts such as taurocholate, glycocholate, and cholate stimulate spore germination [14,16]. As a test of whether bile salts are an in vivo germination factor, the bile acid sequestrant cholestyramine was used. Treatment of 0.1% taurocholate with cholestyramine resin decreased its ability to germinate spores about 200-fold (Fig. 3). Similarly, cholestyramine treatment of the cecal and small intestinal extracts from the clindamycin-treated mice and the small intestinal extracts from the untreated mice severely decreased their abilities to stimulate colony formation from *C. difficile* spores (Fig. 3). Spores that had been incubated with cholestyramine-treated samples were still able to germinate on plates containing TA, indicating that the resin itself did not interfere with germination. Importantly, bile salt levels in the cholestyramine-treated samples were below the limits of detection in hydroxysteroid dehydrogenase (HSDH) assays (data not shown). These results support the idea that the germination factor may be a bile salt that is present in the small intestines of untreated mice and in the cecal and small intestinal extracts of antibiotic-treated mice.

Murine Small Intestinal and Cecal Extracts That Can Stimulate Colony Formation from Spores Contain Higher Proportions of Primary Bile Salts

To examine whether bile salts levels differ among small intestinal and cecal extracts from untreated and clindamycin-treated mice, HSDH assays were carried out as previously described [23]. Total bile salt levels in extracts from small intestines were about 10-fold higher than those from cecal extracts (Fig. 4), also consistent with the greater ability of small intestinal extracts to stimulate colony formation from spores. Surprisingly, the total levels of bile salts in cecal extracts from untreated and clindamycin-treated mice were similar despite their differing abilities to stimulate CFU recovery from spores. Because some bile salts can inhibit spore germination and/or outgrowth [14,27], additional HSDH assays were performed to quantify primary and secondary bile salts (Fig. 4). Indeed, extracts that stimulated colony formation from spores contained higher levels and proportions of primary bile salts than did an extract that stimulated colony formation much less (i.e., cecal homogenate from untreated mice).

Treatment of Taurocholate with Small Intestinal and Cecal Flora Reduces Its Ability to Stimulate Colony Formation from *C. difficile* Spores

Several species of commensal bacteria have been shown to modify primary bile salts to secondary bile salts via 7α-dehydroxylation in vitro [28]. Secondary bile salts such as deoxycholate, formed by the deconjugation and dehydroxylation of primary bile salts by normal anaerobic intestinal bacteria, inhibit the growth of *C. difficile* [14,15]. Since antibiotic treatment disrupts the commensal gastrointestinal flora, there may be differences in the bile salt modifying ability of bacteria from the...
small intestines and ceca of organisms treated with antibiotics versus those untreated. To test this point, cecal contents were anaerobically isolated from untreated and clindamycin-treated mice, washed with PBS to remove endogenous bile salts, and incubated with 0.1% taurocholate under anaerobic conditions for 24 hours at 37°C. The bile salt levels in the supernatant from this incubation were quantified (Fig. 5A), and the TA that had been incubated with cecal contents from clindamycin-treated mice had not been converted to secondary bile salts. In contrast, over 80% of the bile salts in the supernatant from incubation of TA with cecal contents from untreated mice were in the secondary form. Thus, it appears that the populations of bile salt modifying bacteria differ between untreated and antibiotic-treated mice.

To test its ability to stimulate colony formation from spores, sterilized supernatant from this incubation was used in CFU recovery assays (Fig. 5B). Taurocholate that had been incubated with cecal contents from the clindamycin-treated mice exhibited 2- to 5-fold higher levels of CFU recovery, respectively, than taurocholate incubated with contents from untreated mice. Thus, it appears that flora present in the ceca of non-antibiotic-treated mice can modify taurocholate and decrease its ability to stimulate colony formation from *C. difficile* spores.

**Discussion**

Here, we have shown that colony formation from *C. difficile* spores is stimulated by an in vivo-produced factor present in the gastrointestinal tract, and that this factor appears to be present at higher levels in mice treated with antibiotics. These results are consistent with those from a recent paper in which *C. difficile* spores germinated in the presence of small intestinal and cecal contents from mice treated with a proton pump inhibitor [29]. Intriguingly, a recent study showed that clindamycin treatment of *C. difficile*-infected mice appeared to induce a highly contagious “super-shedder” state, which indicates that antibiotic treatment also promotes transmission of the spores [30]. This mouse model appears to be relevant to the human disease since infection of germ-free mice with *C. difficile* resulted in large intestinal inflammation [31]. In addition, the histopathology of antibiotic-treated mice infected with *C. difficile* was similar to that of humans with CDAD, including the presence of pseudomembranous colitis [32].
The in vivo-produced germination factor may be bile salts, based on its physical properties as well as the result that treatment of extracts with cholestyramine, a bile salt sequestrant, eliminated their abilities to stimulate colony formation from *C. difficile* spores. Bile salts have been shown to have different effects in vitro: some (taurocholate, cholate, deoxycholate) stimulate germination, some inhibit germination (chenodeoxycholate), and some inhibit growth of vegetative cells (deoxycholate, chenodeoxycholate) [14,27]. For this reason, the ratio of primary to secondary bile salts was determined, and those extracts that could stimulate colony formation from *C. difficile* spores contained higher levels of primary bile salts than those that could not. It is important to note, however, that chenodeoxycholate is a primary bile salt and we were unable to perform the 12α-HSDH assay that would allow for comparison of the cholate and chenodeoxycholate levels. We hypothesize that the extracts able to stimulate CFU recovery have low levels of chenodeoxycholate (that is, that the majority of the primary bile salts were cholate or its derivatives) because chenodeoxycholate can inhibit spore germination even in the presence of cholate and taurocholate [27]. In addition, the HSDH assays do not detect sulfated bile salts, which are present in the murine gastrointestinal tract [33], and detect muricholate, whose effect on germination is unknown, so these reported bile salt levels may be approximate.

Based on the results presented here, cholestryamine may appear to be a good candidate for treatment of CDAD. In fact, cholestryamine (Questran) has been used to treat CDAD in humans [34] and in the hamster model [25] with mixed results [35,36]. Its mode of action was initially thought to be via toxin binding, which has been shown in vitro [37]. Perhaps the limited efficacy of cholestryamine in treating CDAD is due to the fact that bile acid synthesis increases 4–10-fold upon its ingestion and/or because ileal absorption of bile salts may outcompete the ability of cholestryamine to bind them [38]. In addition, cholestryamine can bind to vancomycin, one of the antibiotics used to treat CDAD [37].

We also investigated the ability of cecal flora from untreated and clindamycin-treated mice to modify the bile salt taurocholate. The results suggest that populations of bile salt modifying bacteria differ between untreated and antibiotic-treated mice, consistent with recent reports showing that antibiotic treatment of mice greatly reduces the diversity of their gastrointestinal flora [30,39]. In addition, taurocholate that had been incubated with cecal contents from clindamycin-treated mice harvested higher levels of CFU recovery than taurocholate incubated with contents from untreated mice. It is worth noting that the taurocholate that had been incubated with cecal contents was less able to stimulate colony formation from *C. difficile* spores compared to pure 0.1% taurocholate. Though 7α-dehydroxylation of taurocholate to deoxycholate may have occurred, the taurocholate also could have been dehydroxylated at C12 by Bacteroides species [40], resulting in conversion to chenodeoxycholate, which inhibits spore germination even when cholate or its derivatives are present, as mentioned above [27].

Intriguingly, disruption of intestinal bacteria by antibiotic treatment appears to cause differences in bile salt levels in vivo, since levels of secondary bile salts in the feces of rats treated with β-lactam antibiotics decreased while primary bile salt levels increased [41]. A similar result was observed in humans despite the bile salt differences between these two species [38]. Stools from human volunteers treated with neomycin, an aminoglycoside like streptomycin, exhibited an increase in the primary bile salt cholic acid compared to the level prior to antibiotic treatment [42] and showed a decrease in the amount of 7α-dehydroxylation activity [43], supporting the idea that changes in bile salt levels were due to a decrease in the commensal flora that carry out 7α-dehydroxylation of bile salts.

**Relationships among Antibiotic Treatment, Commensal Flora, Bile Salt Metabolism, and Germination of Spores of *C. difficile***

A model emerges in which *C. difficile* spores can be germinated by primary bile salts such as cholate and its derivatives in the small intestine and cecum. In healthy humans, CDAD may be avoided because spore germination can be inhibited by the primary bile salt chenodeoxycholate [27]. For those *C. difficile* spores that manage to germinate in response to cholate and its conjugates, outgrowth of the vegetative cells would be inhibited by the secondary bile salt deoxycholate in the large intestine, where bile salts are present in millimolar ranges [28], concentrations at which they can inhibit growth in vitro [14]. In contrast, when the host is treated with antibiotics, *C. difficile* spores are able to exploit the accompanying changes in bile salt levels. Antibiotic treatment disrupts the normal flora that carry out the 7α-dehydroxylation of bile salts, leaving a larger pool of primary bile salts such as cholate and its derivatives that can germinate the spores. There is likely also an unknown mechanism by which levels of chenodeoxycholate, a primary bile salt that inhibits spore germination and outgrowth, are concomitantly lowered. Once *C. difficile* spores have germinated, the vegetative cells must be able grow out in order to produce toxins and lead to CDAD. In fact, the CFU/ml and toxin B levels from *C. difficile* strains grown in vitro in cecal contents from clindamycin- or ampicillin-treated mice were about 10,000-fold higher after 24 hours relative to those grown in cecal contents from non-antibiotic-treated mice [19,44]. This indicates that treatment of the host with antibiotics creates a favorable environment for growth of and toxin production by *C. difficile* vegetative cells, which would lead to CDAD. The model presented here suggests that perhaps non-hepatotoxic analogs of chenodeoxycholic acid could be administered as a preventative measure to hospital patients at risk for infection with *C. difficile* or that those with the infection could be treated with antibiotic-resistant probiotics exhibiting 7α- and/or 12α-dehydroxylation activity.

**Acknowledgments**

We thank Ansel Hsiao (Washington University in St. Louis) and Matthew Bunce (Children’s Hospital of Philadelphia) for helpful comments and suggestions.

**Author Contributions**

Conceived and designed the experiments: JLG ALS JZ. performed the experiments: JLG ALS JZ. Analyzed the data: JLG JAS ALS JZ. Contributed reagents/materials/analysis tools: JAS ALS. Wrote the paper: JLG JAS ALS JZ.

**References**

1. Blossom DB, McDonald LC (2007) The challenges posed by reemerging *Clostridium difficile* infection. Clin Infect Dis 45: 222–227.
2. Hurley BW, Nguyen CC (2002) The spectrum of pseudomembranous enterocolitis and antibiotic-associated diarrhea. Arch Intern Med 162: 2177–2184.
3. Kramer A, Schwebke I, Kampf G (2006) How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. BMC Infect Dis 6: 130.
4. O’Brien JA, Labue BJ, Caro JJ, Davidson DM (2007) The emerging infectious challenge of *Clostridium difficile*-associated disease in Massachusetts hospitals: clinical and economic consequences. Infect Control Hosp Epidemiol 28: 1219–1227.
5. O’Connor JR, Johnson S, Gerdling DN (2009) Clostridium difficile infection caused by the epidemic BI/NAP1/027 strain. Gastroenterology 136: 1913–1924.

6. Razavi B, Apsiri-Moradfar A, Mundy LM (2007) Clostridium difficile: emergence of hypervirulence and fluoroquinolone resistance. Infection 35: 300–307.

7. Jarvis WR, Schlosser J, Jarvis AA, Chinn BY (2009) National point prevalence of Clostridium difficile in US healthcare facility inpatients, 2008. Am J Infect Control 37: 263–270.

8. Vollaard EJ, Clasener HA (1994) Colonization resistance. Antimicrob Agents Chemother 38: 409–414.

9. Sebahia M, Wren BW, Mullany P, Fairweather NF, Minton N, et al. (2006) The multirug-resistant human pathogen Clostridium difficile has a highly mobile, mosaic genome. Nat Genet 38: 779–786.

10. Bartlett JG (2000) Historical perspectives on studies of Clostridium difficile and C. difficile infection. Clin Infect Dis 46 Suppl 1: S4–11.

11. Musher DM, Aslam S, Logan N, Nallacheru S, Bhaila I, et al. (2005) Relatively poor outcome after treatment of Clostridium difficile colitis with metronidazole. Clin Infect Dis 40: 1591–1599.

12. Pepin J, Alary ME, Valiquette L, Ruel J, et al. (2005) Increasing risk of relapse after treatment of Clostridium difficile colitis in Quebec, Canada. Clin Infect Dis 40: 1586–1590.

13. Seldov P (2003) Spore germination. Curr Opin Microbiol 6: 550–556.

14. Sorg JA, Sonenshein AL (2008) Bile salts and glycine as co-germinants for Clostridium difficile spores. J Bacteriol 190: 2505–2512.

15. Wilson KH (1983) Efficiency of various bile salt preparations for stimulation of Clostridium difficile spore germination. J Clin Microbiol 18: 1017–1019.

16. Wilson KH, Kennedy MJ, Fekety FR (1982) Use of sodium taurocholate to enhance spore recovery on a medium selective for Clostridium difficile. J Clin Microbiol 15: 443–446.

17. Cothier G, Dubes F, Raia P (1985) Modulation of cytotoxin production by Clostridium difficile in the intestinal tracts of gnotobiotic mice inoculated with various human intestinal bacteria. Appl Environ Microbiol 49: 250–252.

18. Keel MK, Songer JG (2006) The comparative pathology of Clostridium difficile-associated disease. Vet Pathol 43: 245–249.

19. Pultz NJ, Donskey CJ (2003) Effect of antibiotic treatment on growth of and toxin production by Clostridium difficile in the cecal contents of mice. Antimicrob Agents Chemother 47: 3299–3302.

20. Popoff MR, Robin EJ, Gill DM, Boquet P (1988) Actin-specific ADP-ribosyltransferase produced by a Clostridium difficile strain. Infect Immun 56: 2299–2306.

21. Yii JH, Rodonov D, Liu M, Blatter FR, Kiley PJ (2006) ICR-dependent gene expression links iron-sulphur cluster assembly to the control of O2-regulated genes in Escherichia coli. Mol Microbiol 60: 1050–1075.

22. Wadloowska EA, Laux DC, Colman FS (1985) Colonization of the streptomycin-treated mouse large intestine by a human fecal Escherichia coli strain. role of growth in mucus. Infect Immun 56: 1030–1035.

23. Macdonald IA, Williams CN, Musial BC (1980) 3α-, 7α-, and 12α-OH group specific enzymic analysis of biliary bile acids: comparison with gas-liquid chromatography. J Lipid Res 21: 301–305.

24. Monaghan T, Boswell T, Mahida YR (2009) Recent advances in Clostridium difficile-associated disease. Postgrad Med J 85: 152–162.

25. Fekety K, Silva J, Browne RA, Rilkan GD, Ebright JR (1979) Clindamycin-induced colitis. Am J Clin Nutr 32: 244–250.

26. Merrigan M, Sambo S, Johnson S, Gerdling DN (2003) Susceptibility of hamsters to human pathogenic Clostridium difficile strain BI following clindamycin, ampicillin or ceftriaxone administration. Anaerobe 9: 91–95.

27. Sorg JA, Sonenshein AL (2009) Chloroetheresylate is an inhibitor of Clostridium difficile spore germination. J Bacteriol 191: 1115–1117.

28. Ridlon JM, Kang DJ, Hylemon PB (2006) Bile salt biotransformations by human intestinal bacteria. J Lipid Res 47: 241–259.

29. Nerandzic MM, Pultz MJ, Donskey CJ (2009) Examination of potential mechanisms to explain the association between propan pump inhibitors and Clostridium difficile infection. Antimicrob Agents Chemother 53: 4133–4137.

30. Lawley TD, Clare S, Walker AW, Goulting D, Stabler RA, et al. (2009) Antibiotic treatment of Clostridium difficile carrier mice triggers a sparespreader state, spore-mediated transmission, and severe disease in immunocompromised hosts. Infect Immun 77: 3661–3669.

31. Onderdonk AB, Aiseners RL, Bartlett JG (1980) Clostridium difficile in gnotobiotic mice. Infect Immun 28: 277–282.

32. Chen X, Katchar K, Goldsmith JD, Nanthakumar N, Cheknas A, et al. (2008) A mouse model of Clostridium difficile-associated disease. Gastroenterology 135: 1894–1899.

33. Eyssen HJ, Parmentier GG, Mertens JA (1976) Sulfate bile acids in germ-free and conventional mice. Eur J Biochem 66: 507–514.

34. Ariano RE, Zhanel GG, Harding GK (1998) The role of anion-exchange resins in the treatment of antibiotic-associated pseudomembranous colitis. CMAJ 149: 1049–1051.

35. Laiocarous CA, Piccoli DA (1996) Whole-bowel irrigation as an adjunct to the treatment of chronic, relapsing Clostridium difficile colitis. J Clin Gastroenterol 22: 186–189.

36. Murphy C, Vernon M, Cullen M (2006) Intravenous immunoglobulin for resistant Clostridium difficile infection. Age Ageing 35: 85–86.

37. Taylor NS, Bartlett JG (1980) Binding of Clostridium difficile cytotoxin and vancomycin by anion-exchange resins. J Infect Dis 141: 92–97.

38. Hofmann AF, Hagey LR (2000) Bile acid chemistry, pathochemistry, biology, pathology, and therapeutics. Cell Mol Life Sci 65: 2461–2483.

39. Antonopoulos DA, Huse SM, Morrison HG, Schmidt TM, Sogin ML, et al. (2009) Reproducible community dynamics of the gastrointestinal microbiota following antibiotic perturbation. Infect Immun 77: 2367–2375.

40. Edenharter R (1984) Dehydroxylation of cholic acid at C12 and epimerization at C5 and C7 by Bacteroides species. J Steroid Biochem 21: 413–420.

41. Hashimoto S, Igimi H, Uchida K, Satoh T, Bento Y, et al. (1996) Effects of beta-lactam antibiotics on intestinal microflora and bile acid metabolism in rats. Lipids 31: 601–609.

42. Powell RC, Nunes WT, Harding RS, Vacca JB (1962) The influence of nonabsorbable antibiotics on serum lipids and the excretion of neutral sterols and bile acids. Am J Clin Nutr 11: 136–168.

43. Samuel P, Holtzman CM, Meilman E, Skowkowsi I (1973) Effect of neomycin and other antibiotics on serum cholesterol levels and on alpha-dehydroxylation of bile acids by the fecal bacterial flora in man. Circ Res 33: 393–402.

44. Stiefel U, Nerandzic MM, Koski P, Donskey CJ (2008) Oral administration of beta-lactamase enzymes represent a novel strategy to prevent colonization by Clostridium difficile. J Antimicrob Chemother 62: 1105–1108.