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Suppression of *Clostridium difficile* in the Gastrointestinal Tracts of Germfree Mice Inoculated with a Murine Isolate from the Family *Lachnospiraceae*

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The indigenous microbial community of the gastrointestinal (GI) tract determines susceptibility to *Clostridium difficile* colonization and disease. Previous studies have demonstrated that antibiotic-treated mice challenged with *C. difficile* either developed rapidly lethal *C. difficile* infection or were stably colonized with mild disease. The GI microbial community of animals with mild disease was dominated by members of the bacterial family *Lachnospiraceae*, while the gut community in moribund animals had a predominance of *Escherichia coli*. We investigated the roles of murine *Lachnospiraceae* and *E. coli* strains in colonization resistance against *C. difficile* in germfree mice. Murine *Lachnospiraceae* and *E. coli* isolates were cultured from wild-type mice. The ability of each of these isolates to interfere with *C. difficile* colonization was tested by precolonizing germfree mice with these bacteria 4 days prior to experimental *C. difficile* challenge. Mice precolonized with a murine *Lachnospiraceae* isolate, but not those colonized with *E. coli*, had significantly decreased *C. difficile* colonization, lower intestinal cytotoxin levels and exhibited less severe clinical signs and colonic histopathology. Infection of germfree mice or mice precolonized with *E. coli* with *C. difficile* strain VPI 10463 was uniformly fatal by 48 h, but only 20% mortality was seen at 2 days in mice precolonized with the *Lachnospiraceae* isolate prior to challenge with VPI 10463. These findings confirm that a single component of the GI microbiota, a murine *Lachnospiraceae* isolate, could partially restore colonization resistance against *C. difficile*. Further study of the members within the *Lachnospiraceae* family could lead to a better understanding of mechanisms of colonization resistance against *C. difficile* and novel therapeutic approaches for the treatment and prevention of *C. difficile* infection.

Clostridium difficile is a Gram-positive toxin-producing bacterium first described as a commensal organism in the fecal microbiota of healthy newborn infants (18). Currently, *C. difficile* is the most common cause of health care-associated diarrhea and colitis. *C. difficile* infection (CDI) is responsible for significant morbidity and mortality and increased economic burden in hospitalized patients (9, 23). Risk for the development of CDI is associated with the use of broad-spectrum antibiotic therapy as well as increasing patient age and hospitalization (2).

The human gastrointestinal (GI) microbiota protects the host against colonization by exogenous pathogenic organisms, a function referred to as colonization resistance (17, 38, 40). Administration of broad-spectrum antibiotics is theorized to destroy this protective function of the indigenous microbiota, allowing *C. difficile* to proliferate and colonize the GI tract (32, 34, 39). In support of this hypothesis, mice or hamsters challenged with *C. difficile* are not readily susceptible to *C. difficile* colonization or disease (CDI) (5, 43, 45), while antibiotic administration will render animals susceptible to infection (3, 5, 12, 20, 30).

In previous studies, it was demonstrated that wild-type mice treated with a cocktail of five antibiotics and clindamycin prior to *C. difficile* challenge would follow one of two clinical courses. At the appropriate challenge dose, mice either would develop rapidly lethal CDI or were stably colonized, with the development of only mild disease (5, 30). We reported that members of the bacterial family *Lachnospiraceae* dominated the gut communities of animals with mild disease. Members of the *Lachnospiraceae* were also the primary component of the GI community in untreated mice from our colony. On the other hand, the GI community of moribund animals had a predominance of *Escherichia coli* (30). Based on this observation, we hypothesize that members of the *Lachnospiraceae* family (but not *E. coli*) were responsible for at least a portion of the natural colonization resistance against *C. difficile* in the murine GI tract.

In order to directly examine this hypothesis, we isolated *Lachnospiraceae* and *E. coli* from the ceca of mice and tested their ability to suppress *C. difficile* colonization, toxin production, and disease in germfree mice. Our results indicate that *Lachnospiraceae* can play an important role in limiting *C. difficile* colonization. Further study could lead to new modes of *C. difficile* suppression and greater insight into the function of these organisms in health and disease.

MATERIALS AND METHODS

Animals and housing. Wild-type C57BL/6 mice from a breeding colony established using animals purchased from Jackson Laboratories were housed with autoclaved food, bedding, and water under specific-pathogen-free conditions. Cage changes were performed in a laminar flow hood. Infection studies were performed with 6- to 8-week-old germfree Swiss Webster mice from a breeding colony established at the University of Michigan germfree core facility. Mice were housed in sterile soft-sided

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plastic isolators with autoclaved food, bedding, and water for the duration of the experiments. Each experimental group was housed in a separate isolator. All animal protocols used during the conduct of these experiments were reviewed and approved by the University Committee on Use and Care of Animals of the University of Michigan, Ann Arbor. The protocol was reviewed following guidelines for the care and use of laboratory animals set by the Office of Laboratory Animal Welfare, U.S. Department of Health and Human Services.

Development of *Lachnospiraceae* 16S rRNA-encoding gene primers. Partial 16S rRNA gene sequences for *Lachnospiraceae* from our previous study (30) were used to generate CLUSTALW multiple-sequence alignments. Regions of conserved homology from the most common *Lachnospiraceae* operational taxonomic units (OTUs) were identified and used for PCR primer design. These conserved regions were compared against 16S rRNA gene sequences of other, non-*Lachnospiraceae* bacteria to ensure primer specificity. Primer specificity was confirmed by performing PCR amplification on representative 16S rRNA sequences of non-*Lachnospiraceae* clones, including *E. coli*, *Pseudomonas*, *Porphyrmonodaceae*, *Bacteroides*, *Verrucomicrobia*, *Lactobacillus*, *Clostridiaceae*, *Staphylococcus*, *Ruminococcaceae*, and *Peptococcaceae* strains. The final *Lachnospiraceae*-specific forward primer (LachnoF, 5′-ACC GCA TAA GCG CAC AGC-3′) was used with the broad-range reverse bacterial primer 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′) for PCR with the following cycling conditions; initial denaturation at 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 45 s, and extension at 72°C for 90 s. A final extension at 72°C for 10 min was performed. PCR was performed with 20 pmol of each primer (LachnoF and 1492R), 8 mM deoxynucleoside triphosphate (dNTP) master mix (Promega, Madison, WI), 1 unit GoTaq DNA polymerase (Promega), PCR buffer (Promega), and water in a total of 25 μl per reaction mixture.

Bacterial isolation from specific-pathogen-free mice. The plate wash PCR technique (35) was adapted for the isolation of murine *Lachnospiraceae* strains. Ceca from wild-type C57BL/6 mice were removed in a sterile manner and immediately transferred to an anaerobic chamber (Coy Industries, Grass Lake, MI). Anaerobically equilibrated 1× phosphate-buffered saline (PBS) was added to the cecum, and the organ was opened with a sterile scalpel to release the cecal contents. Serial dilutions of this cecal suspension were plated in triplicate on various culture media, including Trypticase peptone (BD, San Jose, CA) with 5% blood (blood agar), chocolate agar (consisting of 5% lysed blood) with Trypticase peptone base, reinforced clostridial agar (BD), modified peptone yeast glucose agar (ATCC medium 1237), routine growth media (8), and brain heart infusion agar (BD) with 0.1% cysteine (Sigma-Aldrich, St. Louis, MO) added (BHIS) in combination with aztreonam (Sigma-Aldrich) and gentamicin (Sigma-Aldrich) to determine which media would provide the greatest enrichment of *Lachnospiraceae*. The surface of one agar plate was scraped to remove all bacterial colonies, and bacterial DNA was extracted using an automated system (Roche MagNA Pure). Enrichment for *Lachnospiraceae* was determined by performance of the *Lachnospiraceae*-specific 16S rRNA gene PCR and the amplification of the expected 1,320-bp band. The greatest enrichment for *Lachnospiraceae* was obtained using BHIS plates supplemented with 2 mg/liter gentamicin and 1 mg/liter aztreonam (BHIS gen/az). Subsequently, individual colonies from the duplicate BHIS gen/az plates were inoculated into 250 μl of BHIS gen/az broth in a sterile 96-well plate and then incubated anaerobically at 37°C, and growth was monitored. PCR using 1 μl of bacterial culture was used to identify putative *Lachnospiraceae*. Genomic bacterial DNA was extracted using an automated system (Roche MagNA Pure) from cultures of putative *Lachnospiraceae* isolates. Sanger sequencing of full-length 16S rRNA gene amplicons was obtained using the following primers: 8F (5′-AGA GTT TGA TCC TGG CTC AG-3′), 515F (5′-GTG CCA GCM GCC GCG GTA-3′), E939R (5′-CTT GTG CGG GCC CCC GTC AAT TC-3′), and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′). DNA sequencing was performed by the DNA Sequencing Core facilities at the University of Michigan.

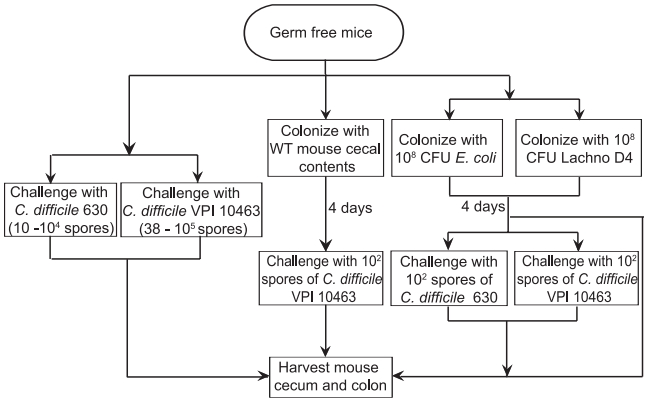

FIG 1 Schematic for examining the effects of *Lachnospiraceae* D4 and *E. coli* on *C. difficile* colonization in germfree mice. Mice were infected with titrating doses of *C. difficile* 630 spores (10, 10^2, 10^3, and 10^4; $n = 3$ per dose) or *C. difficile* VPI 10463 spores (38, 3×10^2, 3.3×10^3, and 1×10^5; $n = 4$ per dose) and monitored for colonization. Animals were colonized with cecal contents from a wild-type (WT) mouse for 4 days prior to *C. difficile* VPI 10463 challenge ($n = 5$). Additionally, mice were either precolonized with *E. coli* and then challenged with *C. difficile* 630 ($n = 9$) or *C. difficile* VPI 10463 ($n = 7$) spores or precolonized with *Lachnospiraceae* D4 and then challenged with *C. difficile* 630 ($n = 8$) or *C. difficile* VPI 10463 ($n = 14$) spores. Mice monocolonized with *C. difficile* 630 ($n = 11$) or *C. difficile* VPI 10463 ($n = 15$) were used as controls. Other groups of mice were colonized with either *E. coli* ($n = 4$) or *Lachnospiraceae* D4 ($n = 4$) for 4 days and harvested. The cecum and colon from each animal were harvested for bacterial and cytotoxin quantification.

Isolation of *Escherichia coli* was performed by selectively plating murine cecal contents on MacConkey agar anaerobically at 37°C. The identity of putative *E. coli* isolates was confirmed by sequencing the 16S rRNA-encoding gene amplicon. 16S rRNA gene sequence analysis, and taxonomic classification was performed using the RDP Bayesian classifier implemented in mothur (33).

***Clostridium difficile* strains and growth conditions.** *C. difficile* spores were prepared from strains VPI 10463 (ATCC 43255) and 630 (ATCC BAA-1382) as follows. *C. difficile* was cultured overnight anaerobically at 37°C in brain heart infusion broth with 0.1% cysteine (BHIS). On the following day, 100 μl of these cultures was spread onto BHIS plates (four plates per strain) and the plates incubated for 7 days anaerobically at 37°C. The plates were removed from the anaerobic chamber and exposed to ambient oxygen for 24 h at room temperature to kill vegetative cells. Plates were flooded with 15 ml cold water, and bacteria were removed by scraping with a sterile loop. Bacterial suspensions were centrifuged and washed in cold water at least three times. Spore stocks were stored at 4°C in sterile water. As an additional measure to remove vegetative cells, the *C. difficile* spore preparation was heat treated for 20 min at 65°C prior to experimental animal infection (37). The presence of spores was confirmed using phase-contrast microscopy, and spores were enumerated by plating for viable CFU on taurocholate-cycloserine-cefoxitin-fructose agar (TCCFA).

Germfree mouse infection studies. Six- to 8-week-old germfree Swiss Webster mice were divided into groups of 3 to 9 animals. Each treatment group was housed in separate sterile isolators. Initially, mice were challenged via oral gavage with various spore doses of two *C. difficile* strains, VPI 10463 or strain 630, to determine the appropriate dose of spores to use for the remaining experiments. For *C. difficile* VPI 10463, groups of three to five mice were challenged with 3.8×10^1, 3×10^2, 3.3×10^3, and 1×10^5 spores, while for *C. difficile* strain 630, mice were challenged with 1×10^1, 1×10^2, 1×10^3, and 1×10^4 spores, administered in a volume of 100 μl (Fig. 1). Based on these experiments, 100 spores were used as the challenge dose for all experiments.

For experiments testing colonization resistance, mice were precolonized via oral gavage with 1×10^8 CFU of either *Escherichia coli* or *Lachnospiraceae* for 4 days. As a control, mice were also orally gavaged with a single dose of cecal content homogenate obtained from a wild-type mouse at 4 days prior to *C. difficile* challenge. The cecal content homogenate was prepared by adding 100 mg of cecal contents to 900 μl of prereduced 1× PBS. One hundred microliters of the homogenate was then orally gavaged into germfree mice. Each treatment group consisted of 5 to 9 animals. Mice were challenged by oral gavage with 100 spores of *C. difficile* (VPI 10463 or strain 630) and monitored daily for signs of disease such as diarrhea, hunched posture, and weight loss. Control groups consisted of animals challenged with only *C. difficile* ($n = 11$ and $n = 15$ for strains 630 and VPI 10463, respectively), *E. coli* alone ($n = 4$), *Lachnospiraceae* alone ($n = 4$), cecal content homogenate from wild-type mice ($n = 3$), or no bacteria ($n = 5$) (Fig. 1). Bacterial colonization was monitored daily by anaerobic culture of fecal pellets and cecal contents at 37°C for 24 h. *E. coli* colonization was specifically monitored by culture on MacConkey agar, while the levels of *Lachnospiraceae* were monitored by culture on BHIS gen/az. The colonization status of *C. difficile*-challenged animals was monitored by anaerobic culture on TCCFA.

Necropsy and histologic procedures. Mice were euthanized by CO_2 asphyxiation. Cecum and colon tissue were placed intact into histology tissue cassettes, stored in 10% buffered formalin for 24 h, and then transferred to 70% ethyl alcohol. Tissue cassettes were then processed and paraffin embedded, and 5-μm sections were prepared. Hematoxylin-and-eosin (H&E)-stained slides were prepared for histologic examination (McClinchey Histology Lab Inc. Stockbridge, MI.).

Histopathologic examination. Histologic changes were coded, randomized, and scored in a blinded manner by a board-certified veterinary pathologist. A scoring system was adapted from previously published methods (5, 22). Edema, neutrophilic inflammation, and epithelial damage in colon and cecum were scored from 0 to 4 according to the following defined criteria. Edema scores were graded as follow: 0, no edema; 1, mild edema with minimal (<2× normal width) multifocal submucosal expansion; 2, moderate edema with moderate (2× to 3× normal width) multifocal submucosal expansion; 3, severe edema with severe (>3× normal width) multifocal submucosal expansion; and 4, same as score 3 with diffuse submucosal expansion. Neutrophilic inflammation scores were graded as follows: 0, no inflammation; 1, minimal multifocal neutrophilic inflammation (marginating or perivascular neutrophils in submucosa, minimal intraepithelial and proprial neutrophils); 2, moderate multifocal neutrophilic inflammation (perivascular and interstitial neutrophils in submucosa, mild to moderate intraepithelial and proprial neutrophils); 3, severe multifocal to coalescing neutrophilic inflammation (perivascular and increased interstitial neutrophils in submucosa with or without extension to muscular wall, moderate intraepithelial neutrophils); and 4, same as score 3 with abscesses. Epithelial damage was scored as follows: 0, no epithelial changes; 1, minimal multifocal superficial epithelial damage (vacuolation, apoptotic figures, villus tip attenuation/necrosis); 2, moderate multifocal superficial epithelial damage (vacuolation, apoptotic figures, villus tip attenuation/necrosis); 3, severe multifocal epithelial damage (same as score 2 with or without pseudomembrane [intraluminal neutrophils, sloughed epithelium in a fibrinous matrix]); and 4, same as score 3 with significant pseudomembrane or epithelial ulceration (focal complete loss of epithelium).

***C. difficile* cytotoxin assay.** Quantification of *C. difficile* toxin present in luminal contents was performed using a method adapted from that described by Corthier et al. (7). Green African monkey kidney epithelial cells (Vero) (provided by M. Imperiale, University of Michigan) were grown to confluence in Dulbecco modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin solution (Invitrogen). The cells were trypsinized using 0.25% trypsin (Invitrogen) and washed with 1 volume of DMEM. Cells were diluted in DMEM, and approximately 1×10^5 cells were distributed per well in a 96-well flat bottom microtiter plate (Corning) and incubated at 37°C for 18 to 24 h. One hundred milligrams of luminal contents was weighed, and 500 μl of 1× PBS was added to make a suspension. Samples were mixed, particulate material was removed by centrifugation at 9,000 × *g* for 5 min, and then the supernatant was filtered through a 0.2-μm membrane. Each sample was titrated in 10-fold dilutions within the wells to a maximum dilution of 10^{-12}, and each well had a corresponding control to which both neutralizing *Clostridium sordelli* antitoxin (TechLabs, Blacksburg, VA) and sample were added. After an overnight incubation at 37°C, plates were fixed with 10% buffered formalin for 2 h and then stained with Giemsa stain (50 μl per well) for 15 min, followed by a wash with 1× PBS. Wells with approximately 100% round cells were easily recognized under a magnification of ×200. The cytotoxic titer was defined as the reciprocal of the highest dilution that rounds 100% of Vero cells per gram of sample.

SCFA measurement. *In vivo* measurement of short-chain fatty acids (SCFAs) was performed as follows. Groups of four 6- to 8-week-old Swiss Webster germfree mice were colonized with 10^8 CFU of either *E. coli* or *Lachnospiraceae* D4 for 4 days. Additionally, a third group remained germfree as a control. All mice were necropsied, and cecal contents were removed, weighed, flash frozen, and stored at −80°C. Samples were sent to the Michigan Metabolomics and Obesity Center at the University of Michigan for SCFA measurement by gas chromatography-mass spectrometry (GC-MS). GC (Agilent 6890) separation was performed using a ZB-Wax Plus column (0.25 μm by 0.25 mm by 30 m), and a quadruple mass spectrometer (Agilent, Santa Clara, CA) was used to identify and quantitate SCFAs using Agilent Chemstation software.

Statistical analysis. Statistical analyses were performed using Prism 5 for Mac OS X GraphPad software. The nonparametric Kruskal-Wallis test was used to determine significance for all treatment groups, while Student's *t* test was used for individual comparisons. Statistical significance was set at a P value of <0.05.

RESULTS

Isolation and characterization of murine *Lachnospiraceae* and *E. coli*. A strain of *E. coli* was isolated from the murine gut by selectively plating murine cecal contents on MacConkey agar. The identification of potential *E. coli* cultivars was confirmed by 16S rRNA gene sequence analysis. To isolate *Lachnospiraceae*, primers targeting 16S-targeted rRNA-encoding genes were designed based on the partial 16S rRNA gene sequences from our previous study (30) to guide cultivation efforts via the plate wash PCR technique (35). The greatest enrichment for *Lachnospiraceae* was achieved by selectively plating murine cecal contents on brain heart infusion agar supplemented with 0.1% cysteine, 2 mg/liter gentamicin, and 1 mg/liter aztreonam (BHIS gen/az) under anaerobic conditions. A total of 14 *Lachnospiraceae* isolates were confirmed by 16S rRNA-encoding gene sequence analysis (Fig. 2). We determined the phylogenetic relationship of these isolates to previously characterized members of the low-moles-percent G+C Gram-positive bacteria in the *Firmicutes* phylum (Fig. 2). All 14 *Lachnospiraceae* isolates were members of the clostridial cluster XIVa (10).

These *Lachnospiraceae* isolates were further characterized based on growth on BHIS gen/az plates and BHIS gen/az broth. Of the 14 *Lachnospiraceae* isolates, 13 took 3 to 4 days to grow on solid media and grew poorly in broth culture. The remaining isolate (referred to as D4) was most closely related to *Clostridium clostridioforme* based on 16S rRNA gene sequence (Fig. 2). *Lachnospiraceae* isolate D4 grew well on solid media and in broth culture, which allowed us to test its ability to inhibit *C. difficile* growth and toxin production in germfree mice.

Colonization of germfree mice with *Lachnospiraceae* D4 interferes with subsequent *C. difficile* colonization. We recently demonstrated that cefoperazone-treated mice were readily colo-

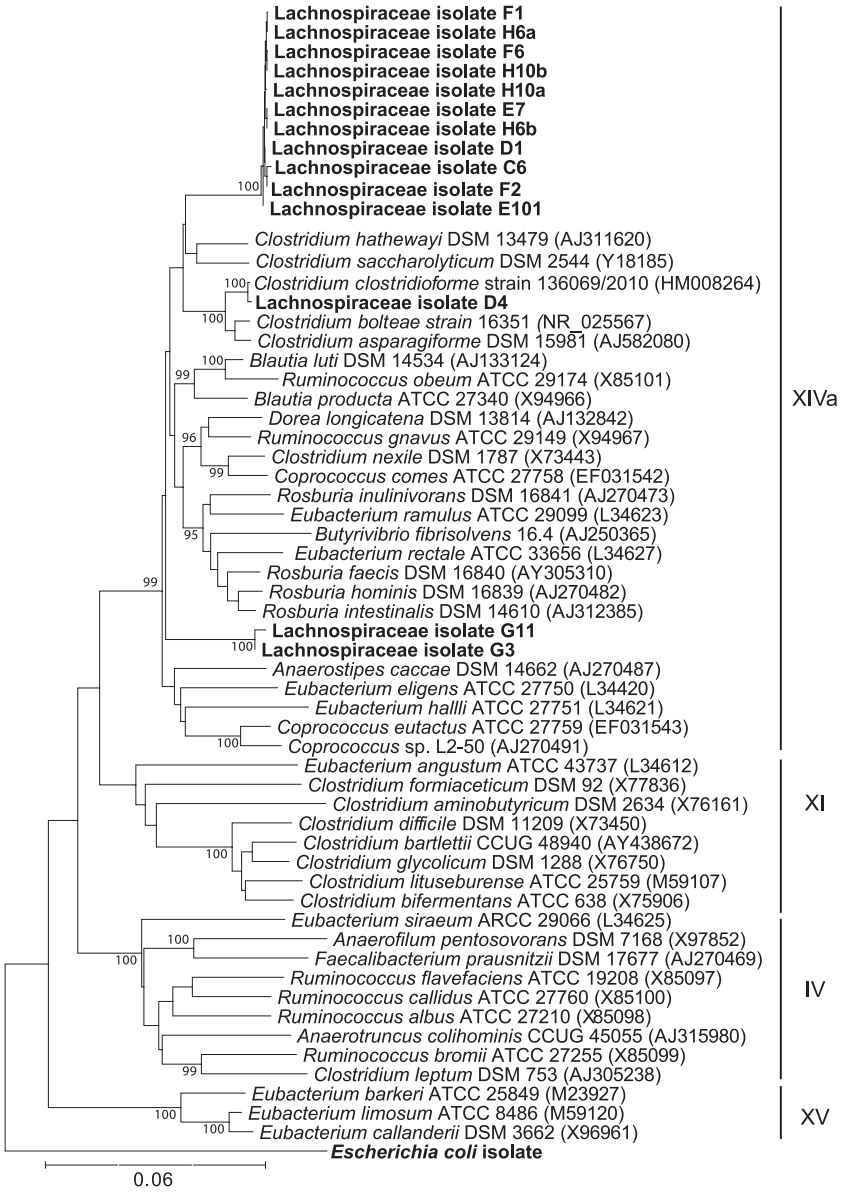

FIG 2 Phylogenetic tree showing clostridial clusters of low-moles-percent G+C Gram-positive bacteria based on 16S rRNA sequence. The tree was constructed using the neighbor-joining method, with the murine *E. coli* isolate used as the outgroup. Newly isolated murine *Lachnospiraceae* strains are shown in boldface. Accession numbers for sequences are given in parentheses. Bootstrap values greater than 95 (per 500 replicates) are shown at branch points. The scale bar represents genetic distance, and clostridial clusters are indicated by Roman numerals.

nized with *C. difficile* strain 630 and developed an acute, histologically apparent colitis but did not exhibit signs of clinically severe CDI such as weight loss, diarrhea, or hunched posture (37). Therefore, infection of cefoperazone-treated mice with *C. difficile* 630 permits the examination of the effect of *Lachnospiraceae* D4 and *E. coli* on *C. difficile* colonization and cytotoxin production in a nonlethal infection model that mimics many aspects of human infection with the pathogen. We initially challenged germfree mice with various doses of *C. difficile* 630 spores ranging from 10^1 to 10^4 (Fig. 1) to determine if the lack of lethality observed in antibiotic-treated animals would extend to monocolonization with this strain. *C. difficile* colonization levels were monitored daily by culture of fecal pellets and culture of cecal contents at necropsy. Levels of *C. difficile* cytotoxin were measured using a Vero cell assay. As with conventional mice, germfree mice challenged with *C. difficile* 630 did not exhibit clinical signs of CDI and survived the infection (Fig. 3A). Monocolonized animals had high levels of *C. difficile* colonization ($>10^9$ CFU/gram) (Fig. 3C). Similar results were seen regardless of the infectious dose administered. Subsequently, we employed an infectious dose of 100 *C. difficile* 630 spores for the following experiments.

Germfree mice received 10^8 CFU of either *Lachnospiraceae* D4 or *E. coli* via oral gavage. Mice challenged with either bacterium were readily colonized. Animals colonized with *E. coli* shed 10^{12} CFU/gram of feces by 4 days postchallenge, while animals that received *Lachnospiraceae* D4 shed 10^{10} CFU/gram of feces (data not shown). Four days after precolonization with either *Lachnospiraceae* D4 or *E. coli*, animals were challenged with 100 spores of

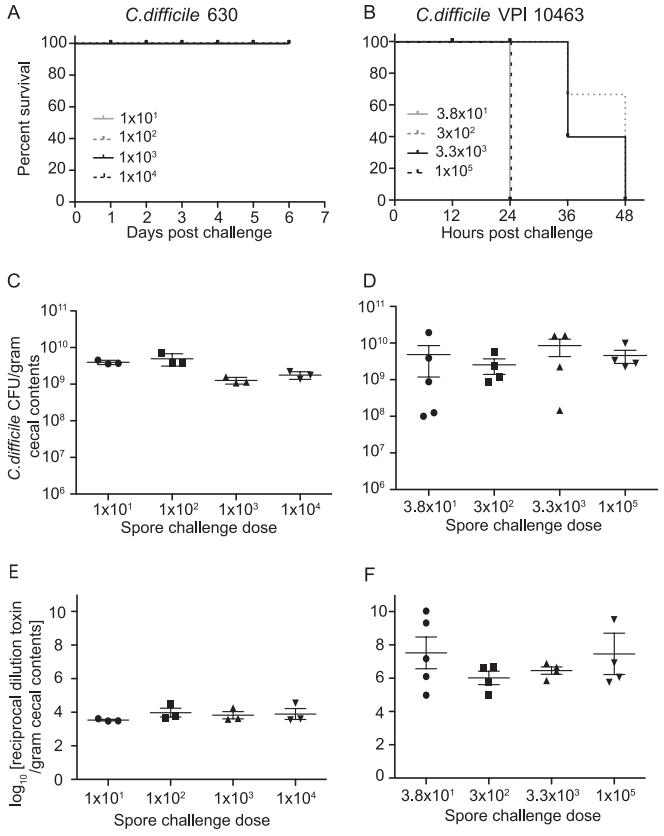

FIG 3 *C. difficile* infection in germfree mice. (A and B) Kaplan-Meier survival plots for mice infected with titrating doses of *C. difficile* 630 spores (1×10^1, 1×10^2, 1×10^3, and 1×10^4) (A) and *C. difficile* VPI 10463 spores (3.8×10^1, 3×10^2, 3.3×10^3, and 1×10^5) (B). (C and D) Quantification of *C. difficile* was determined by culturing cecal contents at the time of necropsy: at day 6 from mice infected with *C. difficile* 630 for each challenge dose ($n = 3$) (C) or at days 1 or 2 for mice infected with *C. difficile* VPI 10463 for each challenge dose ($n = 4$) (D). (E and F) Vero cell tissue culture was used to determine the $\log_{10}$ reciprocal cytotoxin dilution per gram of cecal contents from mice used for panels C and D infected with *C. difficile* 630 (E) or *C. difficile* VPI 10463 (F) for each challenge dose. Points on each graph represent individual animals. Error bars represent standard deviations.

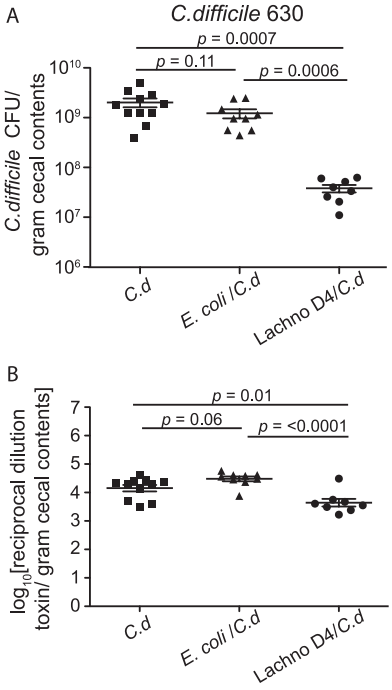

FIG 4 Decreased levels of *C. difficile* 630 and cytotoxin in *Lachnospiraceae* D4-precolonized mice. (A) Quantification of *C. difficile* was determined by culturing cecal contents at the time of necropsy (day 6) from mice infected with *C. difficile* only ($n = 11$) or precolonized with either *E. coli* ($n = 9$) or *Lachnospiraceae* D4 ($n = 8$) and then infected with *C. difficile*. Each point represents the *C. difficile* level from an individual animal. Mice precolonized with *Lachnospiraceae* D4 had significantly decreased levels of *C. difficile* compared to those in *C. difficile*-infected controls or *E. coli*-precolonized mice. Error bars represent standard deviations. Comparisons between groups were performed using the nonparametric Kruskal-Wallis test. (B) Vero cell tissue culture was used to determine the $\log_{10}$ reciprocal cytotoxin dilution per gram of cecal contents from mice used for panel A infected with *C. difficile* only or precolonized with either *E. coli* or *Lachnospiraceae* D4 and then infected with *C. difficile*. Error bars represent standard deviations. Comparisons between groups were performed using the nonparametric Kruskal-Wallis test. *C.d*, *C. difficile*; Lachno D4, *Lachnospiraceae* D4.

C. difficile strain 630. All mice challenged with *C. difficile* were successfully colonized, and this colonization did not alter the level of fecal *Lachnospiraceae* or *E. coli* shedding. No clinical disease was apparent in any of the experimental groups.

In mice precolonized with *E. coli* prior to *C. difficile* challenge, there was no difference in the cecal levels of colonization by *C. difficile* compared to those in mice monocolonized with *C. difficile* strain 630 (Fig. 4A). On the other hand, mice precolonized with *Lachnospiraceae* D4 prior to *C. difficile* challenge had a significant decrease in the levels of *C. difficile* colonization (>1.5 log) (Fig. 4A) and a corresponding decrease in the amount of *C. difficile* cytotoxin in the cecal contents compared to animals monocolonized with *C. difficile* (Fig. 4B).

Colonization of germfree mice with *Lachnospiraceae* isolate D4 decreases subsequent disease severity after challenge with *C. difficile* strain VPI 10463. As opposed to *C. difficile* strain 630, *C. difficile* strain VPI 10463 causes a hyperacute, severe, and often lethal form of CDI in antibiotic-treated wild-type and germfree mice (21, 30, 37). Germfree mice challenged with various doses of *C. difficile* VPI 10463 spores (Fig. 3) all developed clinically severe CDI with diarrhea, hunched posture, and significant (>20% from baseline) weight loss. All mice, regardless of challenge dose, were either dead or moribund by 1 to 2 days postchallenge (Fig. 3B). High levels of *C. difficile* ($>10^9$ CFU/gram) (Fig. 3D) and cytotoxin (Fig. 3E and F) were detected in cecal contents at the time of necropsy compared to those in animals monocolonized with *C. difficile* 630. Similar to the case for animals monocolonized with *C. difficile* VPI 10463, 100% of mice precolonized with *E. coli* prior to *C. difficile* challenge lost >20% of baseline body weight by 2 days postinfection (Fig. 5) and had high levels of *C. difficile* and cytotoxin production at necropsy (Fig. 6). Conversely, mice precolonized with *Lachnospiraceae* D4 demonstrated significantly less clinically severe disease following challenge with *C. difficile* VPI 10463. Of a total of 14 mice, 3 (20%) were moribund and lost significant weight, while the remaining had minimal weight loss and were clinically well at 2 days postinfection (Fig. 5). The 11 mice that survived had lower levels of *C. difficile* colonization and measureable cytotoxin (Fig. 6) than the moribund mice and mice challenged with *C. difficile* alone or following prior colonization with *E. coli*.

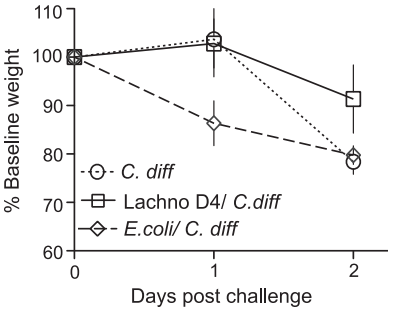

FIG 5 Weight loss in *C. difficile*-infected mice. Weight loss curves for *C. difficile*-infected mice ($n = 15$), mice precolonized with *Lachnospiraceae* D4 and infected with *C. difficile* ($n = 14$), and mice precolonized with *E. coli* and infected with *C. difficile* ($n = 7$) are shown. *Lachnospiraceae* D4-precolonized mice lost less weight than *C. difficile*-infected control or *E. coli*-precolonized mice. Weight loss percentage is based on the starting weight on day 0. Error bars represent the standard deviations of the weights for animals within each group. Lachno D4, *Lachnospiraceae* D4, *C. diff*, *C. difficile*.

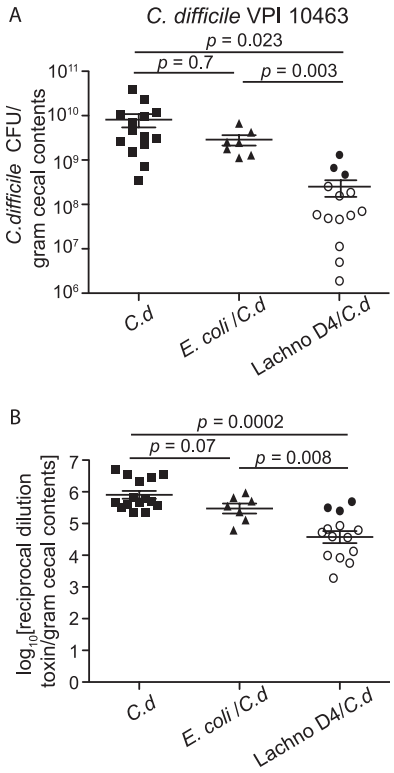

FIG 6 Decreased *C. difficile* VPI 10643 colonization and cytotoxin levels in *Lachnospiraceae* D4-precolonized mice. (A) Quantification of *C. difficile* was determined by culturing cecal contents at the time of necropsy (day 2) from mice infected with *C. difficile* only ($n = 15$) or precolonized with *E. coli* ($n = 7$) or *Lachnospiraceae* D4 ($n = 14$) and then infected with *C. difficile*. The levels of *C. difficile* colonization were decreased in *Lachnospiraceae* D4-precolonized mice compared to those in *C. difficile*-infected controls or *E. coli*-precolonized mice. Each point represents the *C. difficile* level from an individual animal. The open symbols represent animals that had improved CDI signs and did not lose significant weight, while the closed symbols represent animals that were moribund or dead at the time of necropsy. (B) Vero cell tissue culture was used to determine the $\log_{10}$ reciprocal cytotoxin dilution per gram of cecal contents from mice used for panel A infected with *C. difficile* only or precolonized with *E. coli* or *Lachnospiraceae* D4 and then infected with *C. difficile*. Error bars represent standard deviations. Comparisons between groups were performed using the nonparametric Kruskal-Wallis test. *C.d*, *C. difficile*; Lachno D4, *Lachnospiraceae* D4.

Representative histopathology in the colons of animals challenged with *C. difficile* VPI 10463 is shown in Fig. 7. Histopathologic changes in animals that developed colitis consisted predominantly of edema within the submucosa and the lamina propria. There was also neutrophilic inflammation perivascularly and interstitially within the submucosa and multifocally within the mucosa. Epithelial damage was not prominent but consisted of vacuolar degeneration and increased loss of apical tip enterocytes. Germfree mice precolonized with *Lachnospiraceae* D4 prior to challenge with *C. difficile* VPI 10463 had significantly less colonic inflammation and submucosal edema than either *C. difficile*-infected controls or animals precolonized with *E. coli* before *C. difficile* challenge (Fig. 7E and F). Mice that were maintained germfree and mice monoassociated with either *Lachnospiraceae* D4 or *E. coli* had no histologic alterations (data not shown). In addition to the differences in the colon, there was also significantly less mucosal epithelial damage in the ceca of mice precolonized with *Lachnospiraceae* D4 than in *E. coli*-precolonized and *C. difficile*-infected control mice (data not shown).

Prevention of *C. difficile* colonization in the GI tract of germfree mice inoculated with cecal contents from wild-type mice. The results described above indicate that *Lachnospiraceae* strain D4 partially restores colonization resistance against *C. difficile* in germfree mice. As a control, we tested whether the full complement of cecal microbiota from wild-type mice could completely restore colonization resistance in germfree mice. Germfree mice received cecal contents obtained from a wild-type mouse via oral gavage. Four days later, these mice were challenged with 100 *C. difficile* VPI 10463 spores (Fig. 1). Unlike *C. difficile*-monoassociated control mice, mice that were colonized with the cecal content homogenate prior to *C. difficile* challenge did not exhibit signs of clinical CDI such as weight loss, diarrhea, and hunched posture. No detectable *C. difficile* was present in feces at 1 day postchallenge or in the cecal contents at 2 days postchallenge or at the time of necropsy (data not shown).

DISCUSSION

The indigenous GI microbiota plays a fundamental role in colonization resistance against *C. difficile* (41). However, the specific components of the gut microbiota that are important in mediating colonization resistance are not well defined. In this study, we demonstrated that a single component of the murine gut microbiota, a member of the family *Lachnospiraceae*, is able to partially restore colonization resistance against *C. difficile* in germfree mice.

Until now, no study has examined the ability of *Lachnospiraceae* organisms to contribute to colonization resistance against *C. difficile* or other pathogens. We recently reported that antibiotic-treated mice with clinically severe CDI had a predominance of *E. coli* in their cecal microbiota, while mice with mild CDI had a predominance of *Lachnospiraceae* species. Likewise, other studies have associated the microbial gut community of patients with inflammatory bowel disease (IBD) with an increased prevalence of *E. coli* and decreased prevalence of *Lachnospiraceae* (15, 24, 25). Furthermore, patients with IBD are at a higher risk for developing CDI (14, 28). These findings prompted us to isolate and investigate the relative roles of *Lachnospiraceae* and *E. coli* in mediating *C. difficile* colonization resistance.

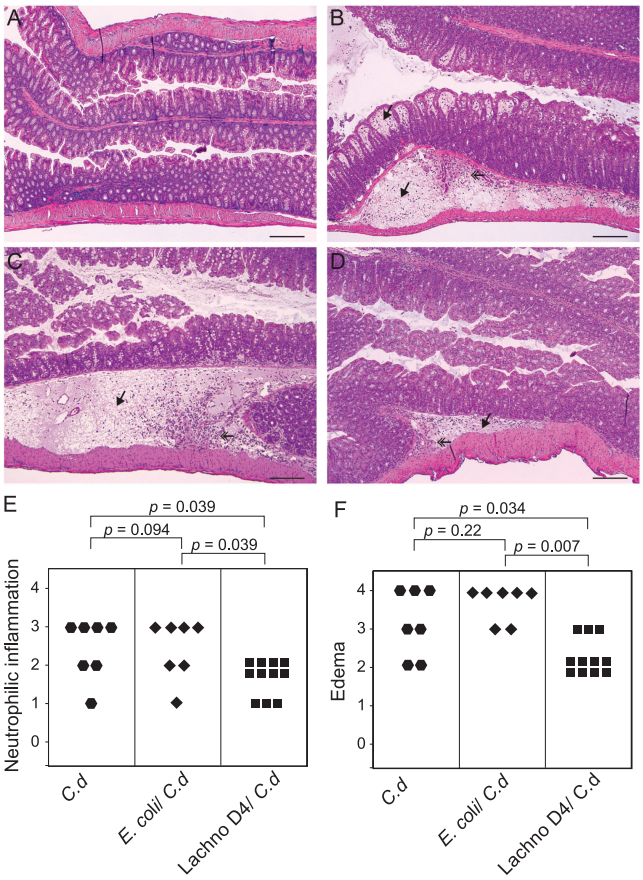

FIG 7 Germfree animals precolonized with *Lachnospiraceae* D4 have decreased colonic histopathology after challenge with *C. difficile* strain VPI 10463. (A) Colon of an uninfected germfree mouse. (B) Colon of a *C. difficile*-infected mouse showing severe edema in the submucosa and mucosal lamina propria (arrow) accompanied by neutrophilic inflammation. (C) Colon from a mouse precolonized with *E. coli* and infected with *C. difficile* showing submucosal edema (arrow) and neutrophilic inflammation similar to those for a *C. difficile*-infected mouse. (D) Colon of a mouse precolonized with *Lachnospiraceae* D4 and infected with *C. difficile* showing moderate neutrophilic mucosal inflammation and minimal submucosal edema (arrow). All micrographs are of H&E-stained tissue at an original magnification of ×100. Bars represent 100 μm. (E and F) Categorical scores of neutrophilic inflammation (E) and edema (F) in *C. difficile*-infected controls, *C. difficile*-infected, *E. coli*-precolonized mice, and *C. difficile*-infected, *Lachnospiraceae* D4-precolonized mice. Comparisons between groups were performed using the nonparametric Kruskal-Wallis test. *C.d*, *C. difficile*; Lachno D4, *Lachnospiraceae* D4.

Lachnospiraceae are Gram-positive obligate anaerobes that are mostly non-spore forming (8, 19). Our murine *Lachnospiraceae* isolates were members of the clostridial cluster XIVa (Fig. 1). Clostridial cluster XIVa represents one of the most abundant phylotypes of the low-moles-percent G+C Gram-positive bacteria (6, 8) and makes up approximately 25% of the total bacterial species found in human fecal specimens (10). *Lachnospiraceae* organisms found in both humans and mice can have highly similar 16S rRNA gene sequences. In our study, the murine *Lachnospiraceae* isolate D4 was phylogenetically similar to *C. clostridioforme*, a *Lachnospiraceae* organism found in humans (Fig. 1) (13). This organism is present as part of the indigenous gut microbiota in humans and has also been associated with opportunistic infections (13). However, we did not observe any disease or histopathology in our mice monocolonized with *Lachnospiraceae* D4.

Germfree mice have proven to be a useful tool for studying host-microbe and microbe-microbe interactions within the GI tract (11). Germfree mice have been used to examine how individual bacteria or bacterial communities influence colonization resistance against *C. difficile* (7, 21, 36, 42, 44). In many cases, the bacteria employed in an attempt to interfere with *C. difficile* were previously described as "probiotic" organisms or undifferentiated groups of bacteria derived from healthy animals. In the current study, we examined bacteria that were previously observed to be associated with normal or diminished colonization resistance to *C. difficile* (30). In this way, we used the results of culture-independent study of gut microbial ecology to inform and guide subsequent hypothesis-testing studies utilizing cultured bacterial isolates. We leveraged the plate wash PCR technique to fine-tune our cultivation efforts using 16S rRNA-encoding gene sequence data (35). We feel that this coupling of sequence-based microbial ecology studies with more traditional methods such as bacterial cultivation and experimental animal infection represents a powerful way study bacterial pathogenesis.

Multiple mechanisms that explain how the indigenous microbiota can mediate colonization resistance have been proposed (reviewed in reference 4). It is likely that several factors are involved in mediating colonization resistance, but the production of bacterial products that directly inhibit pathogens has received significant experimental attention. Several investigators have examined the ability of bacterial fermentation products, including short-chain fatty acids (SCFAs), to inhibit *C. difficile* growth. Some studies have shown that butyrate is capable of inhibiting *C. difficile in vitro* (26, 31), although contradicting reports exist (36). *Lachnospiraceae* organisms are notable in that many are capable of fermenting complex carbohydrates to SCFAs, which have an important role in maintaining intestinal homeostasis (1, 8, 29, 46). We investigated whether SCFAs were associated with less *C. difficile* colonization in *Lachnospiraceae*-precolonized mice and found that SCFA production was not correlated with lower *C. difficile* colonization levels (data not shown). Therefore, the decrease in *C. difficile* colonization levels due to *Lachnospiraceae* colonization is most likely attributable to the production of other metabolites or the use of other mechanisms.

It has been proposed that rather than specific inhibition by the production of metabolites such as SCFAs or antimicrobial compounds, including bacteriocins, the indigenous microbiota could simply be competing for limiting nutrients, the so-called nutrient niche hypothesis. Stated in brief, this hypothesis maintains that an organism can outcompete another if it utilizes a limiting nutrient more efficiently. It is possible that the diminished levels of *C. difficile* in the presence of *Lachnospiraceae* is due to less effective utilization of specific nutrients by the former (16). It should be noted, however, that our experiments do not suggest that there is a simple mass effect with regard to nutrient utilization. When the levels of *Lachnospiraceae* D4 and *E. coli* colonization were measured, *Lachnospiraceae* D4 reached colonization levels 100-fold lower than those of *E. coli*, suggesting that simply occupying more "space" in the gut, and presumably consuming proportionately more of the available resources, does not necessarily contribute to colonization resistance. Furthermore, competition for adherence to the mucosal epithelium was not apparent, as neither *C. difficile*, *E. coli*, nor *Lachnospiraceae* D4 was found to adhere to the mucosal layer as judged by 16S RNA-based fluorescent *in situ* hybridization analysis (data not shown).

Corthier and colleagues demonstrated that a neonatal *E. coli* strain significantly inhibited *C. difficile* cytotoxin (7). In our studies, an *E. coli* strain indigenous to wild-type mice had no such effect on *C. difficile* cytotoxin or colonization levels in germfree mice. Naaber and colleagues examined the effects of over 50 *Lactobacillus* strains on *C. difficile* growth inhibition and found only five strains that had antagonistic activity toward *C. difficile* (27). These studies suggest that although strains may belong to the same genus/species, variation in their individual genetic content results in functional differences. Similarly, it will be important to test the ability of other *Lachnospiraceae* isolates to inhibit *C. difficile in vivo*. It is possible that this is an ability that is shared by the *Lachnospiraceae* as a group or is associated with a function that is more restricted to certain members.

Monocolonization with *Lachnospiraceae* isolate D4 only partially restored *C. difficile* colonization resistance, while there was complete restoration following the transfer of cecal contents from a wild-type mouse to germfree mice. This implies that there are likely additive effects of specific microbiota in determining colonization resistance. Each member of the microbiota may partially contribute, but the entire community (or a specific subset) is required for complete colonization resistance. Others have observed only partial restoration of colonization resistance against *C. difficile* (7, 21). For instance, Itoh and colleagues colonized germfree mice with multiple strains of *Bacteroides* and lactobacilli and observed little antagonism toward *C. difficile*. Only when feces containing clostridia were administered to mice was *C. difficile* eliminated (21).

In summary, our results show that a single component of the gut microbiota, a murine *Lachnospiraceae* isolate, was able to partially restore colonization resistance against *C. difficile* and greatly improve clinical CDI outcome. Further investigation of the members within the *Lachnospiraceae* family potentially in combination with other taxonomically distinct members of the indigenous microbiota could lead to a greater understanding of mechanisms of *C. difficile* suppression and the role that these organisms play in protection against a variety of other pathogens and disease states.

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