Human *Clostridium difficile* infection: inhibition of NHE3 and microbiota profile

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*Clostridium difficile* is a Gram-positive anaerobic bacterium from the phylum Firmicutes that is responsible for the majority of antibiotic-associated diarrhea (17). *C. difficile* infection (CDI) affects thousands of patients each year and treatment costs of over 1 billion dollars in the United States (11, 18, 49, 59). Furthermore, *C. difficile*-related deaths have been steadily rising since 1999 (54) and will likely remain a problem, especially in the face of current antibiotic regimens. CDI has been associated with a spectrum of symptoms ranging from mild to watery diarrhea and abdominal pain to life-threatening pseudomembranous colitis and toxic megacolon (8). Although most of the symptoms of CDI have been linked to *C. difficile* toxin production (31, 37, 41), the mechanism of *C. difficile* colonization is still unclear. Thus a better understanding of *C. difficile* pathogenesis is critical for developing new therapeutics.

*C. difficile* pathogenesis has been hypothesized to be a three-step process: 1) antibiotic disruption of the normal gut microbiota provides a potential niche for growth from its normal gut spore form; 2) the colonization phase, which includes bacterial-host interaction and adhesion; and 3) multiplication that maintains high numbers of vegetative *C. difficile* and toxin production, both of which exacerbate the infectious process (15, 34). Antibiotic use has been shown to decrease the dominant gut microbiota bacterial phyla Bacteroidetes and Firmicutes (40) and increase Proteobacteria (1, 14, 16, 30, 35, 43, 62), resulting in increased gut susceptibility to *C. difficile* infection (2, 5, 36, 52, 55, 67, 78). Once *C. difficile* binds to the gastrointestinal (GI) mucus layer (15, 69), the bacterium can deliver two exotoxins, toxin A (TcdA) and toxin B (TcdB) (17, 32, 74). The Tcd toxins bind to uncharacterized host receptors and are then internalized into the enterocyte cytoplasm, where they become enzymatically active and glycosylate the Rho family of GTPases (19, 29). Inhibition of such GTPases has been shown to have several effects including 1) disorganization of the host actin cytoskeleton, 2) loss of cellular tight junctions, 3) disruption of signaling cascades, and 4) arrest of cell cycle progression (3, 15, 19, 33). In addition, toxin B inhibition of Rho GTPase in cell lines leads to the internalization of the Na+/H+ exchanger isoform 3 (NHE3) (29), but this has yet to be established in vivo in animals or in humans. Inhibition of NHE3 in mice results in chronic diarrhea (25, 60), elevated Na+ and alkaline luminal fluid, and an altered microbiota composition with decreased members of Firmicutes and increased Bacteroidetes (20). It has been suggested that the diarrhea associated with CDI is a result of damage to the host epithelium or a response designed to “flush out” the pathogen. However, we hypothesize that *C. difficile* toxin production inhibits NHE3, creating an altered intestinal microenvironment and gut microbiota composition, which favor *C. difficile* proliferation and colonization of the mucosal lining. In this study, we demonstrate that biopsy specimens from patients with CDI have decreased NHE3 with increased Bacteroidetes and decreased Firmicutes phyla in their stool. In vitro, *C. difficile* growth depends on the high Na+ concentration ([Na+]i) and a
more alkaline environment, which can be caused by downregulation of NHE3. This study is the first to demonstrate downregulation of NHE3 and an altered luminal environment in patients with CDI.

METHODS

Patient information. All patients and healthy volunteers at the University of Cincinnati Medical Center Hospital, Cincinnati, OH, provided informed consent approved by the University of Cincinnati Institutional Review Board. Samples were evaluated from patients with recurrent CDI. Initial CDI cases, defined as only one C. difficile-positive laboratory test with no prior history of CDI, were not included in this study. Recurrent CDI was defined as onset of new diarrhea after a symptom-free period of >3 days, more than one C. difficile-positive laboratory test, and completion of at least one round of antibiotic treatment. C. difficile infection (CDI) was defined as a new onset of diarrhea (>3 loose stools/day for >24 h) and at least one positive C. difficile laboratory test. Diagnosis of CDI was determined by at least one ELISA-positive toxin test or a positive lysosome-associated membrane protein (LAMP) test. Over the course of fecal collections, two types of toxin tests were used. From November 2010 to August 2011, the enzyme immunosassay for toxins A and B was used. After August 2011, the Meridian Illumigene LAMP test was used. This shift in toxin testing represents a switch to in-house testing, lowering the cost, and an upgrade to a more sensitive method.

Fecal samples were collected from 12 recurrent CDI patients with an average age of 56, age range of 32–76. This group included eight females and four males. Selected patients did not have history of inflammatory bowel disease, small bowel obstruction, diverticulosis, colostomy, or cancer. Fecal samples were also collected from 12 healthy volunteers with an average age of 41, age range of 28–61. This group included seven females and five males. To address antibiotic use and stool composition, fecal samples were collected from eight patients with diarrhea, no antibiotics (age range: 34–67; mean age: 49; female: 3; male: 5), five patients with normal stool and antibiotics (vancomycin/clindamycin) but without CDI infection (age range: 29–56; mean age: 39; female: 4; male: 1), and seven patients with diarrhea and antibiotics (vancomycin/clindamycin) but without CDI infection (age range: 38–70; mean age: 52; female: 4; male: 3). Healthy volunteers and patients with diarrhea and/or antibiotics were without previous or current GI symptoms and history of chronic disease or cancer. All stool samples were processed for total DNA, ion concentration, and pH and stored at −20°C.

Colon biopsy specimens collected from five healthy volunteers were obtained by consent and fixed in neutral buffered formalin and paraffin embedded. Healthy subjects had an average age of 52 and patient age range of 45–63 and included three females and two males. Healthy volunteers were without previous or current GI symptoms and history of chronic disease or cancer. Paraffin sections of biopsies and colon resections were obtained from five de-identified patients with a current CDI diagnosis (C. difficile-positive toxin test) and no other known morbidity/disorder. The average patient age 44 and patient age range of 28–65 and included two females and three males. Selected patients did not have history of inflammatory bowel disease small bowel obstruction, diverticulosis, colostomy, or cancer. Confirmation of C. difficile infection was performed by tissue staining with C. difficile-specific antibody as described below.

Histology. Healthy and CDI biopsy and surgical resections were obtained from the transverse colon and fixed overnight at 4°C in neutral-buffered formalin and embedded in paraffin. Serial 6- to 7-μm-thick sections were applied to glass slides, and intestinal architecture was examined by hematoxylin and eosin staining. Expression of NHE3 was examined with rabbit anti-human NHE3 antibody (dilution 1:100, NBPI-82574; Novus Biologicals, Littleton, CO), and C. difficile binding was examined with rabbit anti-C. difficile cell surface protein antibody (dilution 1:100, ab93728; Abcam, Cambridge, MA). Briefly, sections were removed of paraffin and incubated for 40 min at 97°C with Tris-EDTA-SDS buffer as previously described (68). Sections were then blocked with PBS containing 10% serum and stained with primary antibody overnight at 4°C. Sections were then washed three times in PBS, incubated with goat-anti-rabbit IgG Alexa Fluor secondary antibody (dilution 1:100; Life Technologies, Grand Island, NY) for 1 h at room temperature, and counterstained with Hoechst (0.1 μg/ml; Fisher Scientific). Sections were analyzed by confocal laser scanning microscopy (Zeiss LSM Confo-cal 710; Carl Zeiss). Digital images of slides were evaluated by tabulating mean pixel intensity of the respective color channel on each image using ImageJ software (National Institutes of Health) and reported as relative fluorescence. Five regions of interest per image, four images per slide, and n = 5 healthy and CDI patients were used for semiquantification of stain intensity normalized to healthy subjects and referred to as relative fluorescence.

Human intestinal organoids and microinjection. Organoids resembling human proximal colon, hereafter referred to as human intestinal organoids (HIOs), were generated by the Cincinnati Children’s Hospital Medical Center (CCHMC) Pluripotent Stem Cell Facility through directed differentiation of human pluripotent stem cells (hPSC). Differentiation of hPSCs from a single subject was obtained by culturing hPSCs for 3 days in ActivinA, followed by fibroblast growth factor 4 (FGF4) and Wnt3a. HIOs achieved 3-day dimensional growth in Matrigel with epidermal growth factor (EGF), R-spondin, and Noggin as previously described (77). HIOs were obtained in Matrigel from the CCHMC Pluripotent Stem Cell core. These organoids have been previously shown to contain all major intestinal epithelial cell types: enterocytes (villin), goblet cells (mucin), paneth cells (lysozyme), and enteroendocrine cells (chromogranin A) (77). The luminal compartment of HIOs was microinjected with bacteria and stool supernatant to analyze host-microbe interactions as previously described (20). Injection needles were pulled on a horizontal bed puller (Sutter Instruments), and the tip was cut to a tip diameter of ~10–15 μm. HIOs were injected with C. difficile ATCC 1870 or stool from healthy or CDI patients. C. difficile ATCC 1870 was grown in tryptone yeast TY broth as previously described (22). For stool, 0.5 g of healthy or CDI stool were added to 4.5 ml tryptic soy broth (TSB; Fisher Scientific) in an anaerobic hood. Samples were vortexed and centrifuged at 150,000 g for 10 min to pellet solid materials. stool cultures or TSB broth was grown by 10.220.33.6 on June 25, 2017 http://ajpgi.physiology.org/ Downloaded from http://ajpgi.physiology.org/ by 10.20.33.6 on June 25, 2017
Supply (Burlington, NC), S. aureus, M. luteus, L. acidophilus, B. theta-tota-tomycin, E. coli, B. cepacia, and R. leguminosarum were used to generate quantitative (q)PCR standard curves as previously described (20). E. coli, S. aureus, M. luteus, B. cepacia, L. acidophilus, and R. leguminosarum were grown in Luria-Burtani (LB; Thermo Fisher Scientific) broth at 37°C in a shaking incubator. B. theta-tota-tomycin was grown in TSB (Fishier Scientific), and C. difficile was grown in tryptone-yeast extract-glucose broth (TYG; Thermo Fisher Scientific) at 37°C in a Coy Systems, dual-port anaerobic chamber (Coy Laboratory Products, Grass Lake, MI).

To determine the optimal [Na+] for growth, C. difficile (Cluster XI), C. butyricum (Cluster I), Blautia producta ATCC 27340D (C. cocoides Cluster XIVA), and Faecalibacterium prausnitzii (C. leptum Cluster IV) were grown in media where sodium chloride (NaCl) was either removed or replaced with cesium chloride (CsCl) or potassium chloride (KCl) as previously described (9, 10, 20). Briefly, low Na+ media was mixed with normal media at various ratios to obtain varying concentrations of Na+ for bacterial growth measurements. Actual Na+ and K+ concentrations ([Na+] and [K+]) were confirmed by flame photometry (Single-Channel Digital Flame Photometer Model 02655–10; Cole-Parmer Instrument, Vernon Hills, IL). [Cl–] was measured by chloridometry (Digital Chloridometer Model 4425100; Labconco, Kansas City, MO). Bacteria were grown under anaerobic conditions at 37°C to early stationary phase in normal TYG media [12 h, optical density at 560 nm (OD560nm) ~1] and used to inoculate media containing varying [Na+]. Growth was measured as the optical density (OD560nm) at an Ambersom Biosciences Ultraspec 3100 Spectrophotometer (GE Healthcare Life Sciences, Pittsburgh, PA). Clostridial titers were determined by bacterial cell counts using a Petroff-Hauser chamber (Hauser Scientific; Horsham, PA) and also by colony-forming units (CFU) (9, 10). No differences in growth patterns were observed between 4 (early exponential phase)–12 (early stationary phase)–24,–or 48 (stationary phase)–h time points (data not shown). As a result, all data are represented as the OD560nm and CFU at the 24-h time point. To determine the optimal pH for growth, C. difficile, C. butyricum, Blautia producta ATCC 27340D (C. cocoides), and Faecalibacterium prausnitzii (C. leptum) were grown in TYG media containing either normal media or low Na+ media adjusted to pH values ranging from 5.5 to 7.0 as determined electrochemically using a pH meter (Orion Model 720A; Thermo Fisher Scientific).

Quantitative real time-PCR amplification of 16S genes. QIAamp DNA Stool kit (Qiagen, Valencia, CA) was used to isolate total DNA from stool of healthy subjects or patients with recurrent CDI. To improve bacterial cell lysis, the temperature was increased to 95°C and incubation with lysozyme (10 mg/ml; 37°C for 30 min) was used as previously described (12, 20, 24, 48, 56, 57). qPCR was used to access the abundance of total bacteria and specific intestinal bacterial phyla using a Step One Real Time PCR machine [Applied Biosystems (ABI) Life Technologies] with SYBR Green PCR master mix (ABI) and bacteria-specific primers (Table 1) in a 20-μl final volume. Cycle of threshold values (Ct) were correlated to the calculated bacteria number using standard curves from the pure bacterial cultures as previously described (4, 20, 51, 56). Total bacteria were calculated using a universal bacterial primer that recognizes all bacterial groups and represents the total stool microbiota.

Quantitative real time-PCR amplification of mRNA. To examine C. difficile mRNA level, total RNA was extracted from HIOs with Trizol reagent (Invitrogen, Life Technologies) according to the manufacturer’s instructions. Briefly, the Matrigel surrounding the HIOs was removed by the addition of ice-cold PBS and 400 μl of Trizol were added to the HIOs and homogenized. RNA was extracted by the addition of chloroform and reverse transcription was performed using 50 μg/ml oligo(dT) 20 primer and SuperScript reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Amplification reactions of C. difficile mRNA were performed using SYBR Green PCR master mix (ABI) on a Step One Real Time PCR Machine (ABI). The following gene specific qRT-PCR primers derived from previous literature were used: human NHE3 forward 5′-GAGCTGAACCTGAAGGATG-3′, C. difficile reverse 5′-AGCGATCGAATTTCAGAGAGA-3′, human GAPDH forward 5′-TGCACCAACCTGATCC-3′, and GAPDH reverse 5′-GCGATGCACGATGTCATGAG-3′ (53). Data were reported as the ΔACt using GAPDH as the standard. Differences in mRNA expression were determined by qRT-PCR and expressed as the ΔΔCt relative fold difference.

Ion and pH measurements. Stool fluid ion composition was analysis by flame photometry and chloridometry. Briefly, 0.3 g of human stool/liquid were added to tubes and 300 μl of deionized water were added and vortexed thoroughly. The samples were centrifuged at 3,000 rpm for 10 min at 4°C to pellet solids, and the supernatant Na+ and K+ concentrations were determined using a digital Flame photometer (Single-Channel Digital Flame Photometer Model 02655–10; Cole-Parmer Instrument). CI– ion concentrations were determined by a digital Chloridometer (Model 4425100; Labconco). All values were normalized to weight. pH measurements were performed electrochemically via a pH meter (Orion Model 720A; Thermo Fisher Scientific).

Statistics. Data are presented as means ± SE. Comparisons between groups were made with either one- or two-way ANOVA, and the Holm-Sidak post hoc (parametric) test was used to determine significance pairwise comparisons using SigmaPlot (Systat Software, San Jose, CA). A P < 0.05 value was considered significant while n is the number of experiments performed.

RESULTS

NHE3 has been shown to be essential for intestinal absorption of Na+ and, therefore, water (25, 60). Work in cell lines (LLC-PK1: pig kidney; OK: opossum kidney; and BeWo: human placenta) has demonstrated that C. difficile toxin B inhibits NHE3 by dephosphorylation and redistribution of ezrin, which normally anchors NHE3 to the cytoskeleton, resulting in the loss of NHE3 from the apical membrane (29).

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Table 1. Quantitative PCR primer sequences for of total bacteria, bacterial phyla, and C. difficile

<table>
<thead>
<tr>
<th>Type</th>
<th>Bacteria</th>
<th>Forward</th>
<th>Reverse</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>Universal (total bacteria)</td>
<td>ACTCTCTACGGGAGGCGCACAG</td>
<td>ATTACGCGGCGCTGCTG</td>
<td>(4, 23, 26)</td>
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<tr>
<td>Phyla Bacteroidetes</td>
<td>GGCAGCGCGGACCCAG</td>
<td>GRCCTTCTCTCTAGAAAC</td>
<td>(26)</td>
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<tr>
<td>Phyla Firmicutes</td>
<td>GACAGATGTTGGTGAATTAATGGAAGCA</td>
<td>AGTCAGACCAACAGCTGAC</td>
<td>(23)</td>
<td></td>
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<tr>
<td>Phyla Actinobacteria</td>
<td>CGCCGCGATAGGGCGAGATGGA</td>
<td>TCTAGATATTCGACATGC</td>
<td>(23)</td>
<td></td>
</tr>
<tr>
<td>Phyla β-Proteobacteria</td>
<td>ACTCCGCGAGGAGGCA</td>
<td>CGAACATTAAGCGCTGCCA</td>
<td>(23)</td>
<td></td>
</tr>
<tr>
<td>Phyla y-Proteobacteria</td>
<td>GAGGTTTGTATCATGCTCA</td>
<td>GTATACCGCGGCGATGCTG</td>
<td>(39)</td>
<td></td>
</tr>
<tr>
<td>Class Clostridium cocoides cluster XIVα</td>
<td>ACTCCGCGGAGGCGACG</td>
<td>GTCCTTATGACTGACCGTCA</td>
<td>(57)</td>
<td></td>
</tr>
<tr>
<td>Class Clostridium leptum cluster IV</td>
<td>GTTGCACAAAACGGAGGAAGG</td>
<td>GAGGCGCGGCGTGATACAA</td>
<td>(57)</td>
<td></td>
</tr>
<tr>
<td>Species Clostridium difficile</td>
<td>TTGAGCGGATTTACTCTGGAGAAGA</td>
<td>CCAATTCGCTACTGCTCCACT</td>
<td>(65)</td>
<td></td>
</tr>
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</table>
To determine if NHE3 was inhibited in CDI patients, intestinal architecture was examined by hematoxylin and eosin staining (Fig. 1A) and NHE3 expression was examined by immunofluorescence (Fig. 1B). Colonic biopsy specimens demonstrate normal healthy crypts in healthy subjects (Fig. 1A). Consistent with CDI pathology, colon segments demonstrated pronounced thickening of the colonic wall (black arrows) and pseudomembranes (grey arrows) as previously described (73). To confirm the presence of C. difficile in patients with CDI, slides were stained with an anti-C. difficile antibody. As expected, healthy colonic tissue and adjacent areas did not contain any C. difficile. In patients with CDI, C. difficile was found primarily in the expelled mucus layer (89 ± 3%) and occasionally in the crypts close to the host epithelium (11 ± 2%; n = 5; Fig. 1A).

In healthy subjects, NHE3 is located along the apical membrane of absorptive enterocytes (Fig. 1B). In contrast, CDI patient biopsy specimens demonstrated varying levels of NHE3. In CDI biopsy and surgical resections, there are areas with intact NHE3 and areas with decreased or no NHE3; together CDI samples demonstrate a 48% decrease in NHE3 compared with healthy colon samples (Fig. 1C). ImageJ analysis of C. difficile and NHE3 immunofluorescence revealed decreased NHE3 expression correlated with C. difficile presence (P = 0.042). This is the first study that demonstrates that NHE3 is inhibited in patients infected with C. difficile.

Mice lacking NHE3 have increased intestinal [Na+] and an alkaline pH (20, 25, 60). We predicted that loss of NHE3 activity in patients would likewise result in increased [Na+] and an altered intestinal pH. To determine if CDI patients had an altered ion composition, stool from healthy subjects with or without diarrhea and antibiotics and CDI patients were examined by flame photometry and chlorideometry (Fig. 2, A–D). Patients with CDI had higher [Na+] (P = 0.005) (Fig. 2A), and [Cl−] (P < 0.001; Fig. 2C) with no change in [K+] (P = 0.106; Fig. 2B) (two-way ANOVA; Fig. 2). To account for differences in stool composition and antibiotic use, patients with normal stool and diarrhea with or without antibiotics were included for analysis. For [Na+], an interaction existed between the presence of diarrhea and antibiotics (P = 0.045; Fig. 2A). A significant difference was found between healthy and diarrhea patients without antibiotics (P < 0.001) and with antibiotics (P = 0.039). An interaction was also observed for [Cl−] (P = 0.002) between healthy and diarrhea patients without antibiotics (P = 0.025) and with antibiotics (P = 0.015; Fig. 2C). Interestingly a difference was observed in [K+] between patients with or without antibiotics (P = 0.027; Fig. 2B).

Non-Cl anion gap calculations ([Na+] + [K+] − [Cl−]) demonstrated an increase in bulk non-Cl anions (Fig. 2D) for between patients with or with antibiotics (P = 0.012), patients with and without diarrhea (P = 0.032), and patients with and without CDI (P < 0.001). This suggests increase in bicarbonate or short chain fatty acids. In addition, CDI patients had a more alkaline stool (pH 6.9 ± 0.3) compared with healthy subjects (pH 6.0 ± 0.1) (P = 0.003; Fig. 2E). Differences in pH were also found between patients with and without antibiotics (P = 0.026). These data indicate that changes in ion composition and pH occur with antibiotic use, but infection with C. difficile alters these parameters further.

The altered intestinal environment observed in recurrent CDI patients (increased [Na+] and more alkaline pH) is similar to our observations in NHE3 null mice and may promote the growth of a different subset of gut microbiota. Mice lacking NHE3 exhibit an altered gut microbiota with increased Bacteroidetes and decreased Firmicutes phyla (20). To address whether patients with recurrent CDI exhibit a similar profile, stool microbiota extracted from nine healthy and nine CDI patients were examined by qPCR. Total stool bacteria density remained unchanged between the groups (Fig. 3A). In CDI patients C. difficile represented < 2% of total bacteria (data not shown). In healthy subjects, the gut bacterial phylum Firmicutes constituted the most abundant group, followed by Bacteroidetes (Fig. 3B), consistent with other reports (13). In contrast, patients with CDI had increased Bacteroidetes and decreased Firmicutes (P < 0.001, two-way ANOVA; Fig. 3C). CDI patients also had increased αβγ-Proteobacteria (P = 0.02) that may result from antibiotic use, since antibiotics have been shown to increase Proteobacteria titers (1, 14, 16, 30, 35, 43, 62). To determine if resident Clostridial groups (Firmicutes phylum) were changed in CDI, C. coccoides Cluster XIVa and C. leptum Cluster IV titer were examined (Fig. 3D) and both were decreased compared with healthy subjects. In a healthy patient, C. coccoides and C. leptum account in total for ～50% of the total stool bacteria (28, 38, 63) and decreases in these groups represent a significant decrease in the Firmicutes phylum. These observations point to the proclivity of C. difficile in generating an altered intestinal environment.

To address whether C. difficile prefers the environmental conditions mediated by inhibition of NHE3, C. difficile ATCC BAA-1870 was grown in vitro anaerobically in TYG media containing various [Na+] (8–106 mM Na+). Growth was examined by OD560nm and CFU enumeration at 4 (early exponential phase)-, 12 (early stationary phase)-, 24-, or 48 (stationary phase)-h time points as previously described (75). Growth patterns were found to be the same for all time points, and the data are represented as CFU at the 24-h time point. C. difficile was found to grow optimally at >16 mM [Na+] (media pH 6.0), conditions observed in stool of CDI patients (Fig. 4A). This experiment was repeated using KCi or CsCl replacement, and C. difficile again was noted to grow more efficiently at higher [Na+] (data not shown). C. difficile was also grown in vitro at [Na+] and pH values designed to mimic human stool (healthy: Na+ 8 mM, pH 6.0; CDI: Na+ 75 mM pH 7.0, refer to Fig. 2). As shown in Fig. 4B, C. difficile also grew better at pH 7.0 vs. pH 6.0 (P = 0.003) at both [Na+], indicating that C. difficile is also influenced by pH. The resident Clostridial members C. butyricum (Cluster I), Blautia producta (C. coccoides Cluster XIVa), and Faecalibacterium prausnitzii (C. leptum Cluster IV) were also grown in TYG media in high and low Na+ at pH 6.0 and 7.0 to determine if all Clostridial groups preferred the similar environmental conditions as C. difficile (Fig. 5). C. butyricum is a resident bacteria that has been used as a probiotic (58, 61) and has been shown to prevent experimental colitis via an IL-10-dependent mechanism (27). C. butyricum grew well at lower [Na+], but proliferation significantly dropped at high [Na+] (Fig. 5A). B. productus and F. prausnitzii had similar growth preferences to C. butyricum with decreased growth at high [Na+] (Fig. 5, B and C). These data demonstrate that C. difficile is distinct in its preference for a high [Na+], alkaline pH environment, adding credence to the hypothesis that C. difficile prefers an altered intestinal environment that may be caused by the loss of NHE3.
Fig. 1. Clostridium difficile infection (CDI) specimens exhibit altered intestinal structure and decreased apical Na\(^+\)/H\(^+\) exchanger 3 (NHE3) expression. A: hematoxylin and eosin stains of healthy and CDI patient biopsies or surgical resections demonstrate that these CDI patients have regions of pseudomembranes (composed of inflammatory cells, necrotic epithelium, and mucus) (grey arrows) and areas of thickened intestine wall (black arrows). Scale bar = 500 μM. The presence of C. difficile was confirmed with an anti-C. difficile antibody (purple). Healthy tissue contained no C. difficile stain, while CDI specimens contained C. difficile at the level of the mucus (89 ± 3%) and epithelium (11 ± 2%) (n = 5). B: confocal images from healthy and CDI patient biopsies or surgical resections depicting NHE3 (red) and nuclei (blue) stained with Hoechst. Scale bar = 100 μM. Representative micrographs of observations from n = 5 healthy and CDI patient specimens. NHE3 was found to varying degrees in CDI patients, representing a 48% decrease in NHE3 expression compared with healthy colon (arrows). C: semiquantitative analysis of surface NHE3 expression in healthy colon, CDI biopsy nondiseased adjacent tissue, and CDI biopsy diseased tissue. Data presented as relative fluorescence normalized healthy NHE3 expression. \( * P < 0.005 \), two-way ANOVA; n = 5.
function. Of note, at low [Na⁺], C. butyricum proliferated to much higher levels compared with C. difficile, even at the high [Na⁺]. This suggests that under healthy conditions, C. butyricum would be able to outcompete C. difficile for a given niche.

Although CDI patients demonstrate decreased NHE3 expression, it could be argued that a number of different bacterial groups could be responsible for changes in NHE3 levels. We have previously used intestinal organoids to address microbial-host interaction (20). To determine if C. difficile alone was sufficient to decrease NHE3, HIOs were used. HIOs have been shown to mimic native tissue: the cellular diversity and architecture are similar to tissue; HIOs contain all the intestine cell lineages; secretory and absorptive functions are present; and HIOs also contain a significant degree of epithelial and mesenchymal complexity and secrete mucus (77). To confirm that decreases in NHE3 were C. difficile specific, HIOs were injected with C. difficile, C butyricum, and stool supernatant from healthy and CDI patients. mRNA levels of NHE3 (Fig. 6a) demonstrate that C. butyricum and healthy stool does not inhibit NHE3 expression. However, injection of C. difficile and CDI stool supernatant resulted in a substantial decrease in NHE3 mRNA compared with broth-injected (control) organoids. This inhibition was also observed at the protein level (Fig. 6, B and C), demonstrating that C. difficile is capable of decreasing NHE3 expression in patients with CDI. Taken together, these data indicate that C. difficile is capable of decreasing NHE3 expression in vivo and in vitro. Stool from patients with CDI exhibit increased Na⁺ and Cl⁻ and are more alkaline in pH compared with healthy subjects and patients on antibiotics. Stool gut microbiota from patients with CDI are higher in Bacteroidetes and lower in Firmicutes compared with healthy subjects and patients on antibiotics. Stool microbiota bacterial phyla alterations in Firmicutes, Bacteroidetes, and Proteobacteria.

**DISCUSSION**

C. difficile represents an ever increasing public concern as the major cause of antibiotic-induced diarrhea and colitis. The
Fig. 4. In vitro growth of C. difficile ATCC BAA-1870 in varying Na+ and pH conditions. [Na+] ranges for healthy and CDI stool are displayed as bars along the x-axis. A: growth (CFU) of C. difficile in tryptone-yeast extract-glucose (TYG) broth at varying [Na+], which mimics those seen in vivo for healthy and CDI stool at 24 h. C. difficile grew optimally &gt;16 mM Na+ (pH 6.0), which is above the in vivo concentration of 3 mM Na+ for healthy patient stool. B: growth of C. difficile at varying pH, which mimics that seen in vivo for healthy and CDI stool. Growth was determined at 8 mM Na+ (●) mimicking healthy stool and 75 mM Na+ (○) mimicking CDI stool. Significant difference between 8 mM Na+ and 75 mM Na+ at pH 5.5 (P = 0.001), 6.0 (P &lt; 0.001), and 6.3 (P = 0.001). In addition, there is a significant difference between growth at pH 5.5 vs. 6.0–6.5 (P &lt; 0.001) and pH 6.0–6.5 and pH 7.0 (P &lt; 0.001) for both 8 mM Na+ and 75 mM Na+. *P &lt; 0.05, two-way ANOVA, Holm-Sidak.

C. difficile has increased proliferation at Na+ concentrations &gt;16 mM and at more alkaline pH levels in vitro, a pattern of proliferation that is not observed in resident C. butyricum, B. producta, or F. prausnitzii; and 3) C. difficile alone and in combination with a complex microbiota (CDI stool) are capable of decreasing NHE3 expression in HIOs. These new findings shed light on several novel aspects of the C. difficile colonization phase. This study represents the first in vivo analysis of NHE3 inhibition in response to C. difficile infection. Targeted disruption of the normal intestinal environment via regulation of ion transport may help explain both the diarrhea phenotype and how C. difficile maintains a competitive advantage.

Fig. 5. In vitro growth of Clostridial members in a range of Na+ and pH conditions. Growth (CFU) of Blautia producta (C. coccoides Cluster XIVa; A), Faecalibacterium prausnitzii (C. leptum Cluster IV; B), and C. butyricum (Cluster I; C) in TYG broth at varying [Na+], which mimics that seen in vivo for healthy (pH 6, ●) and CDI stool (pH 7, ○) at 24 h. All Clostridial groups grow optimally from 7–40 mM Na+ (pH 6.0 and 7.0) *P &lt; 0.05, two-way ANOVA, Holm-Sidak.
We propose that in healthy individuals the luminal and mucosa-associated gut microbiota compete for a *C. difficile* niche (see model in Fig. 7). In an antibiotic microbiota-depleted environment, *C. difficile* spores germinate and vegetative *C. difficile* likely use intestinal nutrients (such as cleaved mucus oligosaccharides) to enter a colonization phase. After a colonization phase, *C. difficile* enters a virulence phase and produces toxins that inhibit NHE3. *C. difficile* toxin inhibition

Fig. 6. Human intestinal organoids (HIOs) grown in 3-dimensional culture microinjected with bacterial or stool supernatant. A: NHE3 mRNA levels indicate decreased expression in HIOs injected with CDI stool and *C. difficile*. No changes were observed between healthy stool or *C. butyricum* culture. Left: organoid culture in Matrigel with injection needle. B: NHE3 protein expression as determined by immunofluorescence is decreased in HIOs injected with CDI stool and *C. difficile* compared with control, healthy, and *C. butyricum* infected HIOs (white asterisk designates lumen). Left: depicts widefield view of HIO injection. C: semiquantitative analysis of NHE3 florescence. *P < 0.05, two-Way ANOVA, Holm-Sidak; n = 6–9 organoids.
of NHE3 alters the intestinal environment producing a high [Na\(^+\)], and a more alkaline fluid, which enhances \textit{C. difficile} proliferation and inhibits competitive \textit{Clostridial} groups proliferation. This altered intestinal environment further shapes the reemerging gut microbiota (which is restructuring after antibiotic use), perhaps so that noninhibitory Bacteroidetes members proliferate. Altered gut microbiota may also play a role in further shaping the intestinal environment, making it more favorable for \textit{C. difficile} colonization. \textit{C. difficile} toxin B inhibition of NHE3 was demonstrated in cells lines (29), but this is the first study that has demonstrated that inhibition of NHE3 occurs in infected human patients. Loss of NHE3 in mice appears to mimic the effects of \textit{C. difficile} toxin production in humans as NHE3\(^{-/-}\) mice have higher [Na\(^+\)], alkaline intestinal fluid, and a distal colon microbiota that are higher in Bacteroidetes and lower in Firmicutes (20). It should be noted that NHE3\(^{-/-}\) mice have higher Bacteroidetes and lower Firmicutes in both the luminal and mucosa-associated bacterial populations, with the mucosa-associated bacterial population being more dramatic than the luminal population. If the mucosa-associated bacteria of CDI patients likewise reflects the luminal bacterial composition, this indicates that this altered composition represents a noncompetitive population to \textit{C. difficile}.

NHE3\(^{-/-}\) mice also exhibited increased [Na\(^+\)], alkaline fluid, and Bacteroidetes in the small intestine (terminal ileum) in addition to changes in the colon. This suggests that the altered intestinal environment, due in part to the loss of NHE3, may occur upstream as well as in the colon. Although \textit{C. difficile} studies have focused on the colon, \textit{C. difficile} infection has also been reported in the small intestine (47, 70, 76) and can cause small bowel disease (66, 71, 72). These studies suggest that \textit{C. difficile} infection is not localized solely to the colon and may provide keys areas for the initial pathogenesis of \textit{C. difficile}. Knowledge of \textit{C. difficile} colonizing in the intestine (either small or large intestine) is critical for developing better therapies against CDI.

It has been speculated by some that the diarrhea observed in CDI patients is the direct result of epithelial integrity or secretory diarrhea by the host to remove the pathogen. Our work has demonstrated that patients with CDI have a dramatic increase in stool [Na\(^+\)] and a moderate increase in [Cl\(^-\)]. This suggests that loss of Na\(^+\) absorption, and concomitant water absorption, in combination with increased [Cl\(^-\)], may contribute to in the diarrhea observed in CDI patients. A caveat to such study is the fact that it is unfeasible to collect samples before patients become infected with \textit{C. difficile}, so it remains to be determined the extent of alterations in ion composition and pH in each patient before the acquisition of CDI. However, these data demonstrate that [Na\(^+\)] and [Cl\(^-\)] in CDI patients are significantly different from those of patients with or without diarrhea on antibiotics, indicating that the large alterations in Na\(^+\) and Cl\(^-\) content in CDI patients are likely due to the presence of \textit{C. difficile}.

Hayashi et al. (29) demonstrated in placental and renal cell lines that exposure to \textit{C. difficile} toxin B resulted in decreased expression of NHE3 at the apical membrane and translocation to a subapical endosome. This redistribution in NHE3 location was suggested to contribute to loss of NHE3 activity. However, our work with the HIOs has shown that injection with toxin-producing \textit{C. difficile} decreased NHE3 expression at the level of the protein and mRNA. This indicates that a dual mechanism of NHE3 inhibition may be occurring in patients with CDI. In renal cell lines, NHE3 activity has also been shown to be diminished at the level of apical protein and mRNA by parathyroid hormone (PTH) (6). PTH addition correlated with a significant decrease in promoter \(-152/+55\) activity. This promoter segment contains putative binding sites for Sp1, AP2,
and NF-Y, which seem to be essential for NHE3 gene transcription (42). *C. difficile* toxin, similar to PTH, may also lead to decreased NHE3 promoter activity and thus transcription, but this remains to be determined. *C. difficile* toxin production has been demonstrated to disrupt intestinal actin cytoskeleton, which is thought to lead to cell death (44). Loss of cell integrity could also contribute to decreased NHE3 in diseased segments of the intestine of CDI patients. However, decreased NHE3 mRNA may point to a selective inhibition of NHE3 to ensure a more favorable environment and reemerging bacterial composition for *C. difficile* growth.

In vitro *C. difficile* has optimal growth at higher sodium and more alkaline pH compared with healthy subjects. This might represent a biphasic event where *C. difficile* is capable of using [Na+] until a more alkaline pH is obtained. Patients on antibiotics (either with or without diarrhea) have elevated [Na+] compared with patients not on antibiotics (Fig. 2). Since patients who are on antibiotics and have diarrhea have a lower pH compared with CDI patients, *C. difficile* may use [Na+] until the pH becomes more alkaline. Once the pH is alkaline, in vitro data suggest that *C. difficile* is insensitive to [Na+]. Additional experiments are likely needed to identify the exact conditions that stimulate *C. difficile* growth in vivo.

Ion transport now represents a new route for combating CDI. For example, were NHE3 to be upregulated, this may provide a means to reestablish the normal intestinal environment and thus shift the microbiota toward one that is considered normal. The effects of normal commensal microbiota on NHE3 expression and function may prove valuable in this regard. *Lactobacillus* has been used as a probiotic treatment for CDI (7, 45, 50) and *Lactobacillus acidophilus* and NHE3 (64). In addition, *Clostridium* infection and function may prove valuable in this regard. Thus, shift the microbiota toward one that is considered normal. A novel therapeutic for CDI.

REFERENCES


