Antibiotics Potentiate Adherent-Invasive E. coli Infection and Expansion

Alexander M. Oberc,*† Aline A. Fiebig-Comyn, MSc,*† Caressa N. Tsai,*† Wael Elhenawy, PhD,*† and Brian K. Coombes, PhD*†‡

Background: Crohn’s disease (CD) is an inflammatory bowel disease with a complex etiology. Paradoxically, CD is associated with the use of antibiotics and with an increased abundance of an unusual phenotypic group of Escherichia coli known as adherent-invasive E. coli (AIEC). However, the impact of antibiotics on AIEC infection has not been well studied in controlled models of infection.

Methods: We infected mice with AIEC before or after treatment with a variety of different classes of antibiotics. We assessed levels of AIEC in the feces and tissues, AIEC localization by immunofluorescence microscopy, and tissue pathology.

Results: We found that a wide range of antibiotic classes strongly potentiated initial AIEC infection and expanded AIEC in chronically infected mice. We found that the ability of antibiotics to potentiate AIEC infection did not correlate with a stereotyped shift in the gut bacterial community but was correlated with a decrease in overall diversity and a divergence from the pre-antibiotic state. We found that antibiotic-induced inflammation provided a fitness advantage for AIEC expansion through their use of oxidized metabolites in the postantibiotic period.

Conclusions: Our results show that antibiotics can render hosts more susceptible to initial AIEC infection and can worsen infection in previously colonized hosts. AIEC appears to exploit host inflammatory responses that arise in the postantibiotic period, highlighting a previously unknown interaction between CD risk factors.

Key Words: adherent-invasive E. coli, Crohn’s disease, antibiotics

INTRODUCTION

Crohn’s disease (CD) is an inflammatory bowel disease characterized by transmural intestinal inflammation and a range of debilitating symptoms. The etiology of CD is manifold and includes genetic, microbial, and environmental factors. Although the genetic component of CD has been explored in many studies, most risk loci impart a relatively small risk toward developing CD.1 Furthermore, the rapid rise in CD incidence in industrializing countries such as China suggests that non-genetic factors are an important driver in CD pathogenesis.2 Among the many changes that occur in countries with rising economic status is better access to medicines including antibiotics.3 Antibiotic use has been correlated with an increased risk of CD in several epidemiological studies of high-income countries.4 The relative risk of CD onset after antibiotic use appears to be strongest within a few months after antibiotic exposure, and this relationship is generally not found for ulcerative colitis, a related inflammatory bowel disease. The classes of antibiotics associated with CD risk vary between studies; however, this may be due to differences in patient populations and prescribing practices.4 Certain antibiotics are also used in the treatment of CD, although their effectiveness appears to be limited to certain manifestations of the disease.5

Although epidemiological studies correlating antibiotic use to CD do not prove causality, antibiotics are known to cause changes within the microbiome that can lead to mild inflammation and altered nutrient availability within the intestine.6,7 Furthermore, a study found that CD patients who were treated with antibiotics displayed microbial dysbiosis with increased levels of several Proteobacteria families including Enterobacteriaceae.8 The Enterobacteriaceae family includes Escherichia coli, which has been found to be elevated in the intestinal microbiome of CD patients.9 In particular, many of the E. coli found in CD patients have been phenotypically classified as adherent-invasive E. coli (AIEC).10 AIEC have the ability to adhere to intestinal epithelial cells, survive within macrophages, and stimulate the secretion of pro-inflammatory cytokines. AIEC also lack well-defined virulence factors that discriminate other enteric E. coli pathogens such as enteroinvasive,
enteropathogenic, and enterohemorrhagic \textit{E. coli}. AIEC are found in over half of CD patients and are typically found within the mucosa of patients with ileal involvement. The presence of AIEC in CD patients is an independent risk factor for both a penetrating disease phenotype and severe postoperative recurrence.\textsuperscript{11} Despite the lack of a conserved genetic marker for AIEC, most strains are able to adhere to inflamed intestinal epithelia, are able to evade inflammation-associated antimicrobial peptides,\textsuperscript{12} and are resistant to multiple classes of antibiotics.\textsuperscript{13}

Antibiotic use and AIEC infection are both CD-associated factors; however, the interaction between these 2 features has not been investigated. Here we show that multiple classes of antibiotics can promote initial AIEC infection in mice with remarkably low infectious doses. We found that the ability of different classes of antibiotics to promote AIEC infection was not dependent on a characteristic shift in the intestinal microbiome, and we show that vancomycin treatment of mice with existing AIEC infection permits profound bacterial expansion. We additionally demonstrate that AIEC derive a fitness advantage from using alternative carbon sources and electron acceptors that become available after antibiotic treatment.\textsuperscript{7} These data provide new insights into how xenobiotics might influence expansion of CD-associated pathogens, either through de novo acquisition due to a susceptible host state that antibiotics engender or through expansion of resident microbes that derive advantage from a postantibiotic state.

METHODS

Ethics Statement

Animal experiments were conducted according to guidelines from the Canadian Council on Animal Care. The Animal Review Ethics Board at McMaster University approved all procedures under Animal Use Protocol #17-03-10.

Bacterial Strains

Strains used in this study are listed in Supplementary Table 1. AIEC strain NRG857c (serotype O83:H1) was isolated from an ileal tissue biopsy from a CD patient in Charite Hospital (Berlin, Germany), and its genome sequence was determined previously.\textsuperscript{14} \textit{E. coli} HS was originally isolated from a healthy adult and has been shown to colonize the human intestine without causing symptoms.\textsuperscript{15} Primer sequences used for cloning and mutant generation are listed in Supplementary Table 2. Allelic exchange was used to delete the genes \textit{moaA}, \textit{gurD}, \textit{napA}, and \textit{catL}.\textsuperscript{16} Briefly, 2 pairs of primers and overlap-extension polymerase chain reaction (PCR) were used to make constructs containing ~500-bp regions flanking each gene. These constructs were ligated into a modified \textit{pRE112} plasmid conferring gentamicin resistance and transformed into \textit{E. coli} S17-1 \textit{λpir}. The construct was conjugated into NRG857c, and deletion mutants were screened by PCR after counterselection on sucrose. All other mutants were made using Lambda Red recombination.\textsuperscript{17} Due to the extensive antibiotic resistance profile of AIEC NRG857c, we first constructed a modified plasmid system for use with gentamicin selection. To generate the Lambda Red recombinase-expressing plasmid, a kanamycin resistance cassette was amplified from \textit{pKD4} using primers neoFwXmnI and neoRvXmnI. The amplified product was digested with XmnI and ligated into \textit{pKD46} to generate \textit{pKD46_km}. To generate a template for knockout constructs, primers GmFrtFwNeoI and GmFrtRvNeoI were used to amplify a gentamicin resistance cassette from \textit{pSAM_Gm}.\textsuperscript{18} The PCR product was digested with NcoI and ligated into \textit{pCDF-1b} to generate \textit{pCDF_GmFrt}. Knockout constructs were amplified from \textit{pCDF_GmFrt} using primers with extensions complementary to 48-bp in-frame regions at the 5' and 3' ends within each gene of interest. The knockout constructs were transformed into AIEC containing \textit{pKD46_km} in the appropriate genetic background. Transformants were selected on LB media with 15 \textmu{}g/mL of gentamicin and were confirmed by PCR. The Flp recombinase system was cloned by amplifying \textit{pCP20} using the primers cp20FwBamHI and cp20RvBamHI and by amplifying a kanamycin resistance cassette from \textit{pKD4} using the primers neoKD4FwBamHI and neoKD4RvBamHI. Both DNA products were digested with BamHI and then ligated together to generate \textit{pCP20_km}. When needed, the gentamicin resistance cassette was deleted by the transformation and expression of the FLP recombinase from \textit{pCP20_km}.

When appropriate, chloramphenicol (BioShop, Burlington, Canada; 34 \textmu{}g/mL), ampicillin (BioShop, Burlington, Canada; 100 \textmu{}g/mL), and gentamicin (Gibco, Grand Island, USA; 15 \textmu{}g/mL) were used in LB (BioShop, Burlington, Canada) broth or agar plates to provide selection. Before mouse infections or in vitro assays, AIEC strains were grown in LB-containing selective antibiotics for 16–18 hours at 37°C with shaking. For mouse infections, AIEC was resuspended in phosphate-buffered saline (PBS) at the appropriate density. For in vitro competitive assays, strains were subcultured into NCE media supplemented with mucin with or without nitrate, as previously described,\textsuperscript{19} or in NCE media containing 50 mM of glucose, glucarate, or galactarate (Sigma Aldrich, Oakville, Canada) and 0.1% yeast extract (BioShop, Burlington, Canada) and were grown for 24 hours at 37°C in anaerobic jars without shaking.

Animal Infections

Six- to eight-week-old female C57BL/6N or CD1 mice were purchased from Charles River Laboratories or acquired from in-house breeding colonies. For each experiment, groups of animals were from the same supplier and were subject to the same animal husbandry practices. Animals were housed in a specific pathogen-free barrier unit under level 2 conditions at the Central Animal Facility at McMaster University. When delivered as a pretreatment, the antibiotics streptomycin (BioShop, Burlington, Canada; 200 mg/mL), vancomycin (BioShop, Burlington, Canada; 100 mg/mL), and gentamicin
(12 mg/mL) were dissolved at the indicated doses in PBS, filter-sterilized, and given as a 100-μL oral gavage 24 hours before infection. Metronidazole (Sigma Aldrich, Oakville, Canada) was suspended in PBS with 1% methylcellulose and given in a 100-μL oral gavage. Vancomycin (50 mg/L) used for drinking water treatment was dissolved in water, filter-sterilized, and given to mice ad libitum. Mice were infected with the indicated doses of AIEC in a 100-μL suspension by oral gavage.

**Bacterial Enumeration**

Fecal pellets were weighed, homogenized in 1 mL of PBS, serially diluted, and plated onto LB agar plates containing selective antibiotics. Intestinal tissues were flushed with PBS to remove luminal contents, homogenized with a sterile metal bead, and plated in the same manner as feces. Plates were incubated overnight at 37°C, and colonies were counted to determine colony-forming units (cfu) per gram of tissue. Competitive assays were enumerated by replica-plating the total isolated AIEC onto selective plates to discriminate the 2 competing strains and by normalizing cfu counts to the input ratio.

**Histopathology and Immunofluorescence Staining**

At various times after infection, cecal sections were fixed in 10% neutral-buffered formalin overnight and were processed into paraffin blocks. Standard hematoxylin and eosin (H&E) staining was performed on 5-μm sections, and cecal pathology was scored from 9 representative images taken from 2–3 different sections for each cecum using the scoring system described previously. For immunofluorescence staining, paraffin-embedded blocks were processed as previously described. AIEC were stained using rabbit anti-O83 (1:200; Statens Serum Institute, Copenhagen, Denmark) followed by antirabbit Alexa 568 (1:200, Abcam ab175471, Location Cambridge, USA). Coverslips were mounted with ProLong Diamond Antifade (Invitrogen, Burlington, Canada) and imaged on an EVOS FL Auto 2 Cell Imaging System using a 40× objective. To count tissue-associated AIEC, regions of interest (ROI) were manually drawn around tissue sections on ImageJ. AIEC within each tissue ROI were counted by thresholding, watershed analysis, and particle analysis using Fiji (1.51n), as described in the ImageJ manual. Representative images were taken with a Zeiss Axio Imager 2 at 40× objective magnification.

**16S rRNA Gene Profiling**

DNA was isolated from feces or cecal contents as previously described, with the exception that the homogenates were not lyophilized but were instead diluted 1:2 into CTAB buffer. Extracted DNA was used to amplify the v3 region of the 16S rRNA gene by PCR. Fifty ng of DNA was used as a template with 1U of Taq, 1× buffer, 1.5 mM of MgCl₂, 0.4 mg/mL of BSA, 0.2 mM of dNTPs, and 5 pmoles of Illumina-adapted primers 341F, as previously described. The following PCR cycling conditions were used: 94°C for 5 minutes; 25 cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds; and a final extension of 72°C for 10 minutes. PCR products were visualized on a 1.5% agarose gel. DNA was normalized using the SequaPrep normalization kit (ThermoFisher, Carlsbad, USA) and sequenced on the Illumina MiSeq platform at the McMaster Genomics Facility. The resulting sequences were processed with the sl1p pipeline.
are not well understood. The intestinal niche is protected, in part, by microbe-mediated colonization resistance, which restricts the ability of orally ingested microbes to colonize the gut through both direct competition and host immune modulation.26 However, colonization resistance can be compromised by antibiotic administration, which has been shown to lower the infectious dose of many bacterial species such as Clostridium difficile.27 Experimentally, antibiotics have been incorporated into multiple animal models of infection to permit bacterial colonization, for example, in the Salmonella colitis model that uses streptomycin pretreatment.28 We have previously shown that streptomycin treatment allows chronic infection with AIEC strain NRG857c in several mouse lines,29 and other groups have used antibiotics to facilitate mouse infections with different AIEC strains.30 However, the degree to which antibiotics increase host susceptibility to de novo AIEC infection or to which they potentiate existing AIEC infections is not known.

To explore the impact of streptomycin on the short- and long-term infectivity of AIEC, we infected mice with various doses of AIEC 1 day after treatment with streptomycin or PBS control. We considered any mouse with culture-positive fecal AIEC 1 day postinfection to be infected. In PBS-treated mice, the dose of AIEC required to infect half of the animal cohort (ID_{50}) was $6.3 \times 10^2$ cfu. In this control group, mice infected with the lowest dose carried a lower fecal burden and cleared AIEC infection by day 7 (Fig. 1A). After a single dose of streptomycin, as few as 20 bacterial cells could establish robust infection of mice. In contrast to AIEC fecal burdens that were proportional to the infective dose in the control group, streptomycin treatment resulted in dose-independent AIEC expansion and persistent colonization in vivo. To determine if the ability of streptomycin to promote infection was specific to AIEC or common among other E. coli strains, we infected mice with various doses of the commensal E. coli strain HS, with or without streptomycin (Fig. 1B). Similar to AIEC, streptomycin significantly reduced the infectious dose of E. coli HS needed to colonize mice. However, a much higher dose of HS was needed to provide transient colonization in control mice, and streptomycin treatment did not promote the persistence of HS. Taken together, these results show that streptomycin allows for host infection with low doses of E. coli, demonstrating reduced colonization resistance that is not specific to AIEC. However, unlike E. coli HS, AIEC persists at relatively high levels in the postantibiotic period, suggesting additional adaptations that allow it to thrive after antibiotic treatment.
Multiple Classes of Antibiotics Potentiate AIEC Infection With Diverse Changes to the Microbiome

To determine if the potentiating effect of antibiotic treatment on AIEC infection was specific to an antibiotic class, we examined other antibiotics used in the clinical setting of Crohn’s therapy, including vancomycin, gentamicin, and metronidazole. These antibiotics differ in mechanism: vancomycin is a cell wall–targeting antibiotic used primarily in the treatment of Gram-positive bacteria and is administered orally to treat colitis,31 gentamicin is a broad-spectrum aminoglycoside antibiotic targeting the ribosome, and metronidazole is used to treat certain manifestations of Crohn’s disease and targets nucleic acid synthesis in anaerobic bacteria.5 We orally treated mice with a single dose of either vancomycin, gentamicin, metronidazole, or streptomycin or PBS as a control. The following day, mice were infected with a low dose of AIEC (2 × 10^3 cfu), which we previously showed results in transient infection of untreated mice (Fig. 1A). Compared with untreated controls, mice treated with streptomycin and vancomycin showed significantly increased fecal AIEC burden at day 1 postinfection that persisted out to day 14 (Fig. 2A). The effect of gentamicin was more variable, showing AIEC potentiation at day 1 that did not reach statistical significance. By day 14, mice treated with gentamicin had AIEC outputs that were indistinguishable from the untreated controls. Mice treated with a single oral dose of metronidazole showed no AIEC potentiation at either time point; however, in separate experiments, prolonged exposure to metronidazole in the drinking water did lead to AIEC expansion (Supplementary Fig. 1). We considered the possibility that the ability of some antibiotics to potentiate AIEC might be related to a reduction in total intestinal bacteria after antibiotic treatment or a characteristic shift in the microbial composition. We measured total fecal bacteria present before and after antibiotic treatment using 16S qPCR and found that only streptomycin was associated with a significant reduction in fecal bacteria (Fig. 2B). These data suggest that although multiple classes of antibiotics can potentiate AIEC, this does not strictly correlate with a reduction in total bacterial numbers.

To profile the shifts in microbiome composition after antibiotic treatment, we performed 16S profiling on fecal samples from all treatment groups before and after antibiotic treatment. We observed a significant decrease in alpha diversity in the streptomycin, vancomycin, and gentamicin treatment groups, consistent with a loss of bacterial taxa (Fig. 2C). We measured how microbial composition changed after treatment using Bray-Curtis analysis and found that Bray-Curtis dissimilarities between before and after antibiotic treatment significantly increased in streptomycin-, vancomycin-, and gentamicin-treated mice. These data suggest a significant change in bacterial diversity (Fig. 2D) and that the fecal microbiome of antibiotic-treated mice was primarily composed of a select few taxa (Fig. 2E). Principle coordinate analysis on the operational taxonomic units (OTU) revealed that the AIEC-potentiating antibiotics clustered separately, suggesting that, despite being able to potentiate AIEC infection, these treatments resulted in unique changes to bacterial composition (Fig. 2F).

We did not find evidence for a common change in the proportions of bacterial groups or taxa for AIEC-potentiating antibiotics, which may reflect the diverse spectrum of activity of these antibiotics. The ratio of Firmicutes:Bacteroidetes, which is commonly used as a measure of dysbiosis and tends to be lower in CD patients,32 was significantly decreased only in gentamicin-treated mice (Fig. 2G). Gentamicin resulted in a relative expansion of taxa within Bacteroides (Fig. 2H), whereas streptomycin resulted in a significant increase in Ruminococcaceae (Fig. 2I). Interestingly, vancomycin was significantly enriched for members of the genus Escherichia (Fig. 2J). Overall, we found that antibiotics that potentiate AIEC infection cause an overall loss in bacterial diversity and produce nonsimilar shifts in microbial composition.

Vancomycin Leads to AIEC Expansion in Chronically Infected Mice

Crohn’s disease is often treated with immunosuppressants, which have been shown to predispose patients to bacterial infection and thus increase the likelihood of antibiotic exposure.33 We tested the effect of antibiotic exposure in mice that were already infected with AIEC. We used vancomycin in these experiments because it is used clinically to treat CD patients with Clostridium difficile infections,31 and our profiling data suggested that vancomycin supports the expansion of E. coli. We established AIEC infections using streptomycin pretreatment as previously described,29 and after 8 days, we treated half of the mice with drinking water containing vancomycin while maintaining regular drinking water for the control cohort. Vancomycin exposure caused an almost immediate expansion of AIEC that reached statistical significance within 48 hours as measured in AIEC fecal burden (Fig. 3A). The expansion of AIEC after vancomycin treatment was also observed in the tissue-associated AIEC population in the colon, cecum, and ileum on days 13 and 28 (Fig. 3B). Vancomycin-treated mice exhibited mild cecal pathology at day 13 that resolved by day 28 in the uninfected group. Both groups infected with AIEC showed increased cecal pathology by day 28, suggesting that AIEC induced modest inflammation without additional antibiotics (Fig. 3C). Inflammation is known to increase luminal nitrate through Nos2 expression.4,19 Accordingly, we found that total cecal nitrate was significantly elevated in both vancomycin treatment groups at day 13 (Fig. 3D). In line with these observations, Nos2 expression was significantly increased in AIEC-infected mice treated with vancomycin at day 13 (Fig. 3E). Both vancomycin treatment groups displayed elevated Il1b, an established marker of intestinal inflammation (Fig. 3F).
Diverse classes of antibiotics promote AIEC infection and cause unique changes to the fecal microbiome. CD1 mice (n = 8) were treated with PBS, 20 mg of streptomycin, 10 mg of vancomycin, 1.2 mg of gentamicin, or 4.8 mg of metronidazole by oral gavage and were infected with 2 × 10³ AIEC. A, Fecal AIEC at days 1 and 14 postinfection. B, Total fecal bacteria measured by 16S rDNA qPCR 24 hours after antibiotic treatment (1-way ANOVA with Kruskal-Wallis test). C, Shannon index of fecal 16S profiles from mice 1 day before or 1 day after antibiotic treatment (2-way ANOVA with Holm-Sidak test). D, Bray-Curtis dissimilarities of fecal samples before or after antibiotic treatment (Mann-Whitney test). E, Stacked bar chart showing relative proportions of bacterial phyla from mice before or after treatment. The category “Taxa <1%” is representative of all phyla with less than 1% abundance, whereas the category “Others” refers to reads for which our pipeline was unable to predict taxonomic assignment with sufficient confidence. F, Unweighted Bray-Curtis principal coordinate analysis plot of fecal 16S profiling data. G, Ratio of the proportion of Firmicutes to Bacteroidetes phyla from mice treated with various antibiotics (2-way ANOVA with Holm-Sidak test). H–J, Relative abundances of Bacteroides, Ruminococcaceae, and Escherichia, respectively, from mice treated with various antibiotics (2-way ANOVA with Holm-Sidak test). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.
Using immunofluorescence microscopy, we observed significantly increased numbers of tissue-associated AIEC in vancomycin-treated mice compared with controls (Fig. 4A and B). Taken together, these data are consistent with vancomycin inducing mild cecal inflammation and increased nitrate availability, which coincides with AIEC expansion in both the luminal and tissue-associated populations.

**Anaerobic Respiration and Alternative Sugar Metabolism Provide a Fitness Advantage for AIEC After Antibiotic Exposure**

Streptomycin causes moderate inflammation and Nos2 upregulation in the mouse gut. Streptomycin causes moderate inflammation and Nos2 upregulation in the mouse gut. Streptomycin causes moderate inflammation and Nos2 upregulation in the mouse gut. Streptomycin causes moderate inflammation and Nos2 upregulation in the mouse gut. Streptomycin causes moderate inflammation and Nos2 upregulation in the mouse gut.

Previous work has shown that commensal E. coli can expand its metabolic niche to use carbon and energy sources made available by streptomycin treatment. The previous experiment indicated that vancomycin caused a transient increase in cecal Nos2 expression and nitrate content (Fig. 3). Thus, we surmised that the metabolites derived from antibiotic-induced inflammation might drive AIEC expansion. We constructed mutants of AIEC that were deficient in glucarate and galactarate (ΔgarD/gudDXP), nitrate reduction (ΔnarG/narZ/napA), and the synthesis of molybdenum co-factor used for multiple anaerobic respiration pathways (ΔmoaA). Through competitive growth assays in vitro, we first confirmed that the loss of these metabolic pathways resulted in the expected growth phenotypes. The ΔgarD/gudDXP mutant had reduced fitness in media containing glucarate or galactarate, and the ΔnarG/narZ/napA and ΔmoaA mutants had reduced fitness in media containing glucarate and galactarate. Previous work has shown that commensal E. coli can expand its metabolic niche to use carbon and energy sources made available by streptomycin treatment.

**FIGURE 3.** AIEC expands in chronically infected mice after vancomycin treatment. C57BL/6N mice (n = 13–14) were pretreated with streptomycin followed by infection with 2 × 10⁹ cfu AIEC. At 8 days postinfection, mice in the indicated groups received vancomycin in drinking water. A, Fecal AIEC at various days postinfection (t tests with Holm-Sidak). B, Tissue-associated AIEC at 13 and 28 days postinfection (t tests with Holm-Sidak). C, Cecal histopathology scores of H&E-stained sections at 13 or 28 days postinfection (1-way ANOVA with Kruskal-Wallis test). D, Total nitrate in cecal contents at day 13 postinfection (1-way ANOVA with Kruskal-Wallis test). E, Cecal Nos2 expression relative to water treatment group at day 13 postinfection measured by qPCR (1-way ANOVA with Kruskal-Wallis test). F, Cecal IL1β expression relative to water treatment group at day 13 postinfection measured by qPCR (1-way ANOVA with Kruskal-Wallis test). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.
FIGURE 4. Tissue expansion of AIEC after vancomycin treatment. A, Representative immunofluorescence images (red = AIEC, blue = nuclei) of ceca at day 28 showing AIEC tissue infiltration after water or vancomycin treatment. Two representative micrographs are shown for vancomycin-treated mice and water controls. Left panels are immunofluorescent images; right panels include differential interference contrast image overlay. White arrows indicate AIEC. Luminal AIEC are not indicated by arrows in the vancomycin-treated mice due to the large number of AIEC foci. B, Enumeration of AIEC tissue invasion using automated ImageJ counting (Mann-Whitney test). Data are compiled from 5 individual animals per group. **P < 0.01. Abbreviation: L, intestinal lumen.
supplemented with nitrate (Supplementary Fig. 2). As expected, these mutants had similar in vitro growth rates in both aerobic and anaerobic conditions in rich media where use of these metabolites is dispensable (Supplementary Fig. 2). To determine if antibiotic-induced AIEC expansion required the metabolic capacity to consume oxidized products of inflammation, we first quantified the fitness of the ΔgarD/gudDXP mutant in competition with wild-type AIEC in mice treated with streptomycin or PBS. In the absence of antibiotic treatment, ΔgarD/gudDXP bacteria were as fit as wild-type, competing equally or slightly better than wild-type by day 8 of infection. In contrast, after streptomycin treatment, the ΔgarD/gudDXP mutant was significantly compromised for competitive infection beginning on day 3 after infection (Fig. 5A). We then tested the effect of vancomycin treatment on wild-type and AIEC mutants that were infected for 8 days before antibiotics were initiated, modeling the previous experiments investigating

![Graph A: Competitive indices of ΔnarG/narZ/narA vs WT AIEC from feces of mice treated with PBS or streptomycin](image1)

![Graph B: Total AIEC counts in days postinfection](image2)

![Graph C: Competitive indices of mutant and WT AIEC from indicated tissues taken from mice treated with vancomycin or no vancomycin control](image3)

![Graph D: Competitive indices of mutant and WT AIEC from indicated tissues taken from mice treated with vancomycin or no vancomycin control](image4)

**FIGURE 5.** AIEC mutants lacking nitrate or glucarate/galactarate metabolism show reduced fitness after antibiotic treatments. A, Competitive indices of ΔnarG/narZ/narA:WT AIEC from feces of mice treated with PBS or streptomycin (1 sample t test against theoretical CI of 1.0). B, Total AIEC counts from mice were treated with 20 mg of streptomycin before infection with 2 × 10^9 AIEC with a 1:1 ratio of the indicated mutant:WT strain. At day 8, groups were treated with vancomycin in the drinking water. B, Total fecal AIEC (mutant + WT) in days postinfection. Data are from 4–10 mice per group. C, Competitive indices of mutant and WT AIEC from indicated tissues taken from mice treated with vancomycin or D, no vancomycin control (1-way ANOVA with Kruskal-Wallis test). *P < 0.05; **P < 0.01; ***P < 0.001.
the effects of antibiotics on existing AIEC infection. To do these competitive infections, we constructed a chloramphenicol-sensitive wild-type strain (wt<sup>cm-s</sup>) to differentiate it from the unmarked AIEC mutants and to ensure the absence of competitive infection bottlenecks. Total numbers of AIEC in the feces (which included both mutant and wild-type) were similar in all competitive infections before antibiotic treatment, and AIEC expanded after vancomycin treatment began on day 8 (Fig. 5B). In the presence of vancomycin, the Δ<sup>nmoA</sup>, Δ<sup>garD/gudDXP</sup>, and Δ<sup>narG/narZ/napA</sup> mutants were significantly attenuated for infection of gut tissue compared with wild-type in the colon, cecum, and ileum (Fig. 5C). Interestingly, the attenuation of the Δ<sup>nmoA</sup> and Δ<sup>garD/gudDXP</sup> mutants was normalized to near wild-type levels in the absence of vancomycin treatment, whereas the Δ<sup>narG/narZ/napA</sup> mutant remained highly attenuated in tissues (Fig. 5D). The wt<sup>cm-s</sup> was recovered to similar levels as wild-type AIEC, indicating that this strain was equally fit and there were no significant infection bottlenecks under the conditions of these experiments. These data suggest that AIEC expansion after antibiotics is mediated by the ability to use inflammation-derived metabolites.

**DISCUSSION**

Crohn’s disease is a multifactorial illness that arises from complex interactions between genetic and environmental factors. The interactions of AIEC with other CD factors have been investigated in some CD-associated genotypes and infectious gastroenteritis. However, the interaction between AIEC and different antibiotics in the setting of host colonization has not previously been explored. Here we show that antibiotics promote AIEC expansion through the production of inflammation-derived metabolites. We found that streptomycin, vancomycin, and gentamicin, but not metronidazole, promoted initial AIEC infection. In contrast to the potentiating antibiotics, a single dose of metronidazole had a minimal impact on AIEC infectivity and the intestinal bacterial community. Metronidazole has high intestinal absorption compared with the other antibiotics tested, which may have resulted in subtherapeutic levels of the drug within the intestinal lumen when given as a single dose. Indeed, recurrent exposure to metronidazole in drinking water did lead to AIEC expansion over several days. Our data suggest that antibiotic-promoted AIEC expansion is correlated with a loss of bacterial diversity and a disruption from the pre-antibiotic state. This is consistent with early studies in *Salmonella* models that first demonstrated robust intestinal colonization in the mouse after streptomycin or vancomycin treatment. However, like in the *Salmonella* model, we do not yet know whether there is a common microbial signature in the postantibiotic state that accounts for this reduced colonization resistance.

It has been previously suggested that streptomycin reduced colonization resistance to Proteobacteria by selectively reducing the number of competing facultative anaerobes while sparing obligate anaerobes. We found that streptomycin increased the abundance of obligate anaerobes within the family *Ruminococcaceae*, in agreement with the findings of a previous study. Furthermore, our data also show that levels of endogenous facultative anaerobes, such as those belonging to the phylum Proteobacteria, were already extremely low in all mice before antibiotic treatment. Proteobacteria, specifically *Escherichia*, increased dramatically in vancomycin-treated mice, yet these mice were equally susceptible to subsequent AIEC infection. These results suggest that the presence of closely related bacteria might not impede colonization resistance to AIEC. AIEC, including the strain used in this study, are enriched for genes needed for propanediol metabolism, which was shown to provide a fitness advantage for *Salmonella* in the inflamed gut. In addition to possessing additional metabolic clusters, it is also possible that AIEC can colonize a niche that allows access to additional sources of nutrition that are not available to commensal *E. coli* by adhering to the mucosal surface, which we commonly observed after antibiotic treatment. Whether AIEC can use carbon sources more similarly to commensal or to pathogenic *E. coli* has not been studied; however, the ability of AIEC to persist in both inflamed and healthy hosts suggests that it may vary its lifestyle and adapt to diverse environments.

The ability of AIEC to benefit from inflammation-derived metabolites after antibiotic treatment is consistent with previous work in commensal *E. coli* and *Salmonella*, which showed that streptomycin causes mild inflammation and the upregulation of *Nos2*. Our data showed that vancomycin treatment also causes mild colonic inflammation and a transient upregulation of Nos2 expression, and that AIEC capable of using inflammation-derived metabolites outcompete mutants lacking these capacities after vancomycin treatment. This suggests that antibiotic-induced inflammation is not specific to streptomycin. Although Nos2-derived metabolites appear to contribute to AIEC expansion after antibiotic treatment, AIEC levels did not decline at later time points even though *Nos2* levels did normalize to pre-antibiotic levels. This suggests that there are additional uncharacterized mechanisms by which antibiotics maintain high levels of AIEC burden. Antibiotics are also known to promote bacterial translocation through the formation of goblet cell–associated cell passages. This effect is strongest after a single high-dose antibiotic treatment or for a continuous low-dose antibiotic treatment, similar to the conditions we used in our study. As such, the formation of goblet cell–associated cell passages may represent 1 mechanism by which AIEC are able to persist during vancomycin treatment and to gain access to tissues. Additional experiments will be required to address this possibility.

A complete understanding of the role that antibiotics play in CD disease progression is likely hampered by the heterogeneous nature of host and environmental factors driving the disease. A variety of antibiotics are commonly prescribed in the treatment of CD; however, only a few specific CD manifestations have good-quality evidence supporting their use.
For example, metronidazole has been shown to be effective at preventing postoperative disease reoccurrence in CD patients, whereas vancomycin has been shown to be ineffective at treating CD exacerbations.1 This has led to concerns of antibiotic resistance in AIEC from CD patients.2–4 Accordingly, the acquisition of antibiotic resistance genes diminishes the possibility of using antibiotics to eradicate AIEC in CD patients. Clinical data regarding the behavior of AIEC in healthy individuals or CD patients who are taking antibiotics are lacking. Similarly, the temporal dynamics of AIEC acquisition, and whether CD patients were carriers before diagnosis, are still unknown. Our work shows that AIEC and antibiotic use may act synergistically to promote gut inflammation and create a susceptible host state. It is also possible that genetic susceptibility factors might exacerbate CD-like inflammation, and future experiments using mice predisposed to developing ileitis or colitis may be informative. Deciphering the interaction between AIEC and antibiotics in the gut will increase our understanding of the interplay between environmental and microbial factors that lead to CD manifestation.

SUPPLEMENTARY DATA
Supplementary data are available at Inflammatory Bowel Diseases online.

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REFERENCES