GUT MICROBIOTA

A role for bacterial urease in gut dysbiosis and Crohn's disease

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Gut dysbiosis during inflammatory bowel disease involves alterations in the gut microbiota associated with inflammation of the host gut. We used a combination of shotgun metagenomic sequencing and metabolomics to analyze fecal samples from pediatric patients with Crohn's disease and found an association between disease severity, gut dysbiosis, and bacterial production of free amino acids. Nitrogen flux studies using ^{15}N in mice showed that activity of bacterial urease, an enzyme that releases ammonia by hydrolysis of host urea, led to the transfer of murine host-derived nitrogen to the gut microbiota where it was used for amino acid synthesis. Inoculation of a conventional murine host (pretreated with antibiotics and polyethylene glycol) with commensal *Escherichia coli* engineered to express urease led to dysbiosis of the gut microbiota, resulting in a predominance of Proteobacteria species. This was associated with a worsening of immune-mediated colitis in these animals. A potential role for altered urease expression and nitrogen flux in the development of gut dysbiosis suggests that bacterial urease may be a potential therapeutic target for inflammatory bowel diseases.

INTRODUCTION

The gut hosts a dense microbial community that responds to environmental stressors such as diet (*1*, *2*), antibiotic use (*3*), inflammation of the intestinal tract (*4*–*6*), and infection of the host with enteric pathogens. The term "dysbiosis" refers to the alteration of microbiota composition when it is associated with a host disease state. When characterized in patients with inflammatory bowel diseases (IBDs), such as ulcerative colitis or Crohn's disease, the dysbiotic microbiota often shows a decrease in richness and an increase in taxa belonging to the Proteobacteria phylum. This is typically associated with an increase in facultative anaerobes such as Enterobacteriaceae and a decrease in Firmicutes, particularly of the *Clostridium* clade (*7*). By studying the gut microbiota in pediatric patients with Crohn's disease, we have recently shown that multiple factors such as diet, antibiotic use, and intestinal inflammation each independently contribute to the development of gut dysbiosis (*5*, *6*, *8*, *9*).

Although intestinal inflammation may contribute to dysbiosis, chronic alteration of the gut microbiota may also play a role in perpetuating inflammation (*8*–*10*). The Proteobacteria phylum is enriched in intestinal pathogens, and several have been shown to cause intestinal inflammation in animal models of IBD (*11*–*13*). Reduced abundance of Firmicutes, principally Clostridia, may reduce the production of short-chain fatty acids (SCFAs) that are known to augment regulatory T cells that support immune tolerance in the intestinal mucosa (*14*, *15*). These observations have led to a growing interest in reversing the dysbiotic microbiota and augmenting the production of microbial metabolites such as SCFAs as a therapeutic modality. Potentially supporting this idea, a randomized controlled trial demonstrated the potential of fecal microbiota transplantation in patients with ulcerative colitis (*16*).

The biological mechanisms responsible for the development of dysbiosis remain incompletely characterized. The outgrowth of facultative anaerobic organisms has led some to hypothesize that dysbiosis results from an alteration in redox potential in the gut associated with intestinal inflammation (*17*). An alternative hypothesis is that inflammation may result in host production of small molecules such as nitrate, which serve as electron acceptors that facilitate anaerobic respiration (*18*). Evidence that nitrogen metabolism may play a role in the development of dysbiosis in IBD comes from the Pediatric Longitudinal Study of Elemental Diet and Stool Microbiome Composition (PLEASE) study (*5*). Here, gene pathway analysis of shotgun metagenomic sequencing data revealed an association of Crohn's disease with sulfur relay systems and nitrogen metabolism. Both of these pathways are involved in nitrogen utilization by bacteria, particularly in Proteobacteria (*19*), a prominent phylogenetic signature of a dysbiotic gut microbiota (*4*).

To investigate the possible role of nitrogen metabolism in the development of dysbiosis, we performed an untargeted metabolomic analysis of fecal samples obtained from participants in the PLEASE study. The results showed that fecal amino acids were associated with Crohn's disease and positively correlated with increasing disease activity. Fecal amino acids and their derivatives were also positively associated with taxa in the Proteobacteria phylum, such as *Escherichia*, *Klebsiella*, *Haemophilus*, and *Proteus*, all of which have been associated with gut microbiota

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dysbiosis in patients with IBD (7, 20, 21). These bacterial lineages also induce intestinal inflammation in animal models of IBD (11, 12, 22).

Ammonia, a product of urea hydrolysis produced by bacterial urease in the mammalian intestinal tract, is a major source of nitrogen for bacteria. To track nitrogen metabolism by the gut microbiota, we used ^{15}N-labeled urea to trace nitrogen incorporation into amino acids by bacteria in a mouse model and then focused on the role of urease enzymatic activity. Using our recently described method for altering the gut microbiota in previously colonized mouse hosts (23), we show that inoculation of a single aerotolerant bacterial species, *Escherichia coli*, into conventional mice pretreated with antibiotics and polyethylene glycol (PEG) altered the gut microbiota in a manner dependent on the presence or absence of a bacterial urease gene. The presence of urease led to a dysbiotic microbiota with enhanced abundance of Proteobacteria taxa and a reciprocal decrease in Firmicutes (namely, Clostridia); in contrast, inoculation of mice with urease-negative (Ure[−]) *E. coli* did not lead to dysbiosis. Finally, we demonstrated the functional consequences of the dysbiotic microbiota engineered through inoculation of a urease-positive (Ure[+]) *E. coli* strain by showing exacerbation of disease in a T cell adoptive transfer mouse model of colitis (24). The ability to engineer the mouse gut microbiota into either a dysbiotic or a more normal configuration by the inoculation of a single aerotolerant taxon with or without urease enzymatic activity provides a proof of concept that a single defined bacterial strain can reconfigure the gut microbiota.

RESULTS

The presence of amino acids in fecal microbiota from patients with Crohn's disease positively correlates with disease activity

Using shotgun metagenomic sequencing, we previously reported the characterization of the fecal microbiome of 90 pediatric patients with Crohn's disease receiving either anti–tumor necrosis factor (TNF) or dietary therapy and compared these data to those from 26 healthy control children (5). An analysis of gene pathways using Random Forest showed that sulfur relay systems, nitrogen metabolism, and several biosynthetic pathways for amino acids were enriched in the Crohn's disease fecal samples (5). Here, we performed an untargeted fecal metabolomic analysis on these samples using liquid chromatography–mass spectrometry (LC-MS) and identified 341 known small molecules. Nearly all of the molecules that fell into the category "Amino acids and their derivatives" were statistically significantly associated with Crohn's disease [false discovery rate (FDR)–adjusted $P < 0.05$] (Fig. 1A). This was confirmed using quantitative high-performance LC (HPLC) (fig. S1A). Dietary intake of protein was explored as a possible explanation for the association between fecal amino acids and Crohn's disease, but there was no significant difference based on 24-hour dietary recalls performed at study entry ($P = 0.79$; fig. S1B).

Among the pediatric patients with Crohn's disease, fecal amino acids and their derivatives positively correlated with activity of intestinal
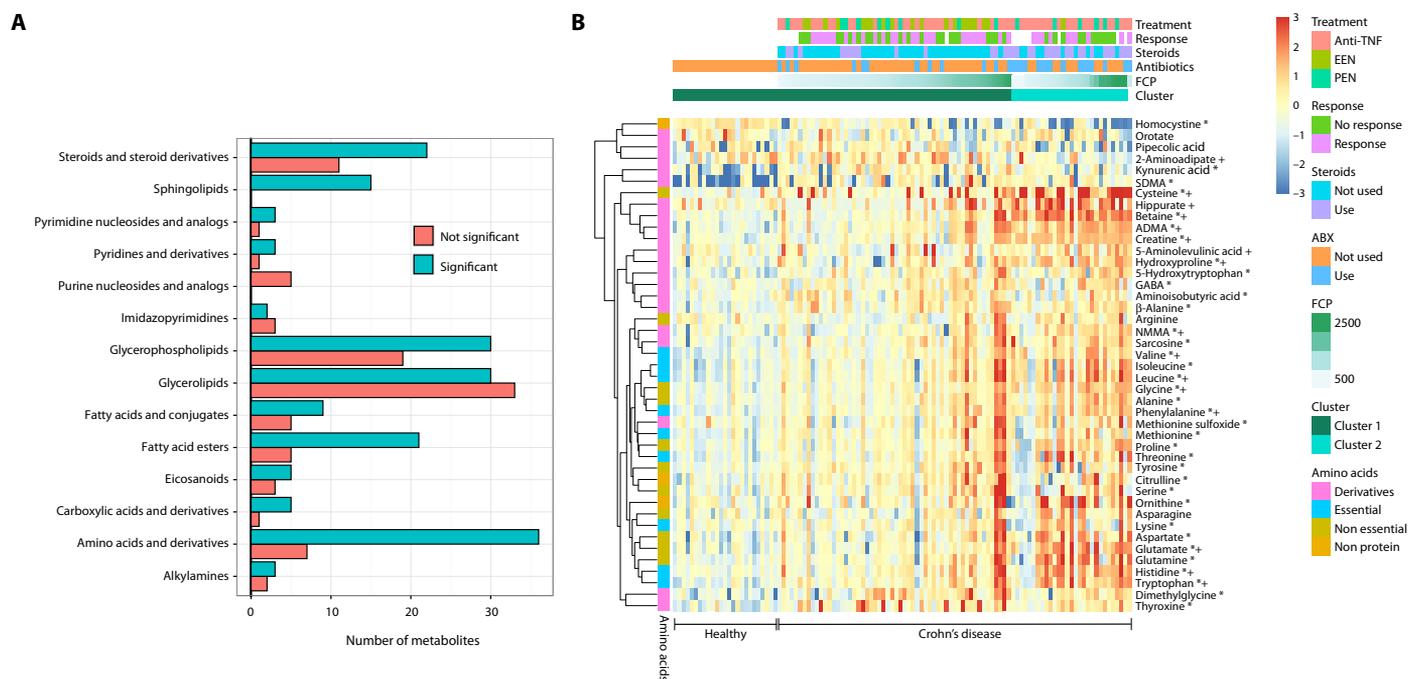

Fig. 1. Associations between fecal amino acids and their derivatives with disease in pediatric patients with Crohn's disease compared to healthy subjects. (**A**) Associations with metabolic pathways as determined by Wilcoxon rank-sum test, with FDR-adjusted $P < 0.05$ as the cutoff. Bars indicate the number of metabolites in each category, as defined by the Human Metabolome Database (56). (**B**) A heat map demonstrating relative abundance of fecal amino acids and their derivatives before therapy according to the presence of disease, cluster assignment, fecal calprotectin concentration, use of antibiotics and corticosteroids, response or no response to therapy, and treatment assignment. Metadata are indicated by the color code at the top of the figure. Metabolite categories are color-coded along the left side of the heat map. White cells indicate missing data. All metabolites that were statistically different (FDR-adjusted $P < 0.05$) in abundance between Crohn's disease samples and healthy controls are marked with an asterisk (*); metabolites differing significantly (FDR-adjusted $P < 0.05$) between cluster 1 and cluster 2 (previously defined clusters based on bacterial abundances in which cluster 1 resembled the healthy controls and cluster 2 was dysbiotic) are marked by a plus sign (+). The clusters were defined by partitioning around medoids (a centroid within a data set whose average dissimilarity to all the data points in the cluster is minimal) with estimation of number of clusters [PAMK (partitioning around medoids with estimation of number of clusters)], as previously described (5). EEN, exclusive enteral nutrition; PEN, partial enteral nutrition; FCP, fecal calprotectin; SDMA, symmetric dimethylarginine; ADMA, asymmetric dimethylarginine; GABA, γ-aminobutyric acid; NMMA, N^G-methyl-L-arginine.

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inflammation as measured by the concentration of fecal calprotectin (Fig. 1B and fig. S1C). It was not possible to determine the contributions of the host versus the gut microbiota to the quantities of fecal amino acids and their derivatives measured. However, the reduction in the positive correlation between fecal calprotectin and the quantities of fecal amino acids and their derivatives associated with antibiotic treatment of the pediatric patients (fig. S1, C and D) suggested that the presence of

increased fecal amino acids in pediatric Crohn's disease patients was, in part, due to the gut microbiota.

Fecal amino acids positively correlate with Proteobacteria in pediatric patients with Crohn's disease

In pediatric patients with Crohn's disease, quantities of fecal amino acids and their derivatives positively correlated with a dysbiotic composition of
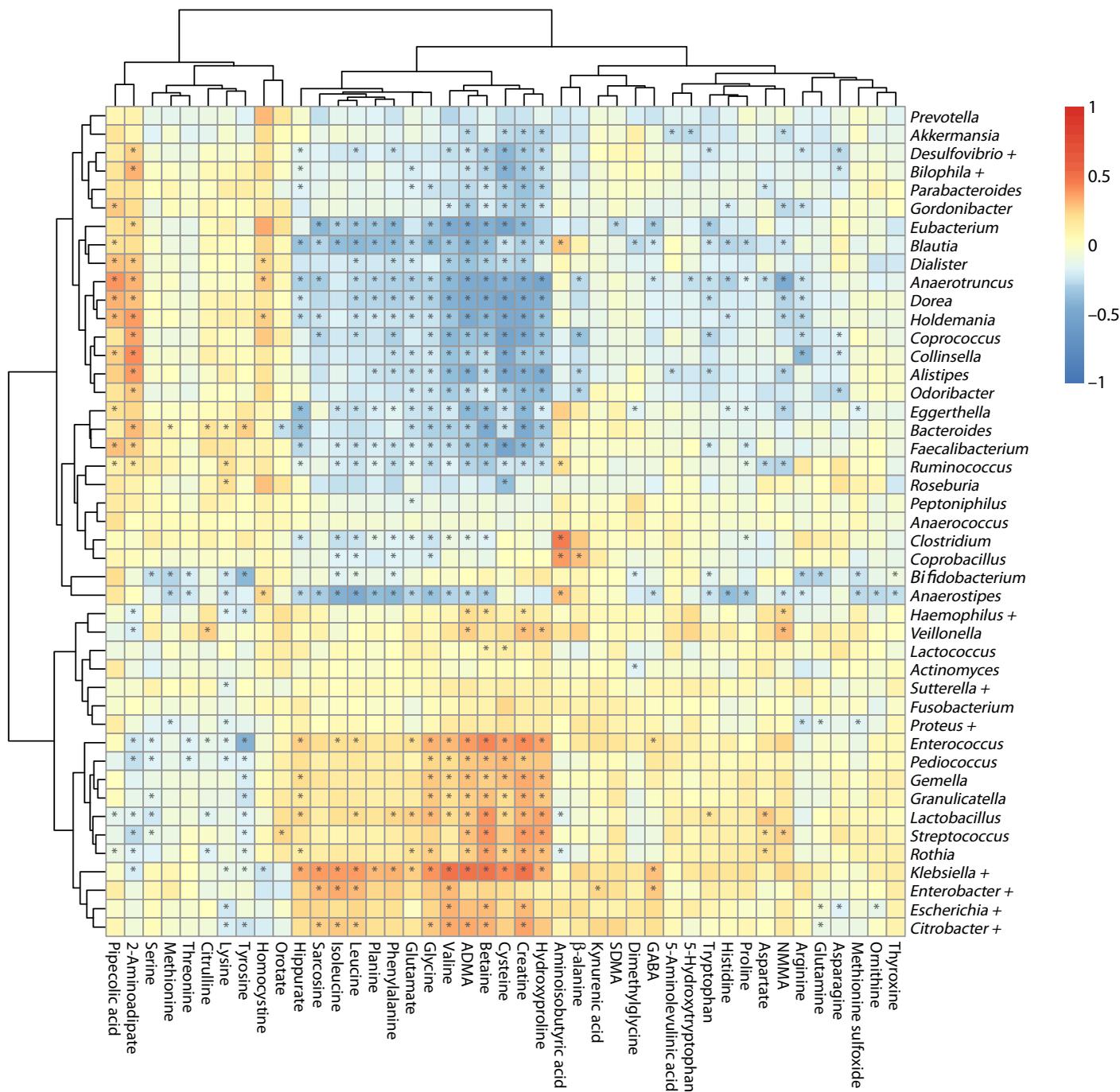
Fig. 2. Associations between bacterial taxa abundance ascertained by fecal shotgun metagenomic sequencing and the fecal metabolome in healthy pediatric subjects and those with Crohn's disease. Spearman rank correlation with the direction and strength of association indicated by the color index in the figure. Only metabolites classified as amino acids and their derivatives are shown. Statistically significant correlations after adjusting for healthy and disease status (FDR < 0.01 based on linear regression analyses) are indicated by an asterisk (*). Taxa belonging to the Proteobacteria phylum are indicated with a plus sign (+).

the gut microbiota, defined by gut bacterial composition and gene types, that we previously labeled as "cluster 2" (Fig. 1B) (5). Further evaluation demonstrated that concentrations of fecal amino acids and their derivatives from pediatric patients with Crohn's disease positively correlated with the abundance of Proteobacteria (Fig. 2). Bacteria of this phylum are associated with gut microbiota dysbiosis in IBD (7, 20, 21) and have been shown to exacerbate colitis in animal models of immunologically mediated intestinal inflammation (11, 12, 22). These findings are consistent with our previously reported observations of positive associations between dysbiosis in Crohn's disease and enrichment of bacterial genes for nitrogen metabolism and amino acid synthesis (5).

Bacterial urease promotes the flux of nitrogen into amino acids synthesized by the murine gut microbiota

The gut microbiota plays an essential role in nitrogen flux. Bacterial urease hydrolyzes host-derived urea in the colon into ammonia that is subsequently absorbed by the host or used by the microbiota as a nitrogen source. To investigate the role of bacterial urease in microbial amino acid synthesis, we administered isotopically labeled $[^{15}N_2]$urea via oral gavage to three groups of mice: (i) healthy control mice colonized with a conventional murine gut microbiota, (ii) mice treated with antibiotics (ABX) and PEG to deplete their endogenous gut microbiota (23), and

(iii) mice treated with ABX/PEG and then inoculated with altered Schaedler flora (ASF), a defined consortium of eight murine commensal bacteria with minimal urease gene content (23). 16S rRNA gene-tagged sequencing of fecal pellets from mice in group 3 confirmed effective engraftment, as previously described (Fig. 3A) (23). We then collected fecal pellets and used liquid/gas chromatography–MS to measure atom percent excess of ^{15}N in fecal amino acids. Given that bacterial urease is necessary for the hydrolysis of $[^{15}N_2]$urea into $^{15}NH_3$ and that lysine does not undergo transamination via host utilization of $^{15}NH_3$, the detection of $[^{15}N]$lysine in the mouse feces could be attributed solely to synthesis by bacteria (25). Relative to control mice colonized with conventional microbiota, ASF-inoculated mice showed significantly lower quantities of fecal $[^{15}N]$lysine ($P < 0.05$) (Fig. 3B). These quantities were comparable to the reduction observed in the feces of ABX/PEG-treated mice and thus demonstrate the importance of urease in the flux of nitrogen for amino acid synthesis by the gut microbiota.

Reduction of gut bacterial load promotes the establishment of a new steady state in colonized mice

We have previously shown that the combined use of antibiotics and PEG reduces the endogenous bacterial load in the murine gut (23) and that this reduction is required for engraftment of a defined bacterial consortium. To determine the effects of different host preparations on the gut microbiota, we administered PEG, antibiotics, or a combination of antibiotics and PEG (ABX/PEG) to three different groups of mice. Treatment with antibiotics in the presence or absence of PEG led to alterations in mouse gut microbiota composition, as measured by unweighted UniFrac (26) distances (Fig. 4A); PEG alone was not sufficient to induce these alterations.

For this reason, the difference in the composition of the mouse gut microbiota at day 30 after antibiotics versus ABX/PEG treatment was unlikely to be a result of PEG treatment. Rather, the reduction in microbiota biomass by antibiotic treatment could have allowed the development of a different community state that was dependent on cage effects. Cage effects, where mice housed in the same cage share a similar gut microbiota due to mixing by coprophagia, constitute a large source of variance in murine studies (17, 27, 28). To distinguish between these two mechanisms, we analyzed the composition of the mouse gut microbiota in three additional cages where mice were treated with ABX/PEG. Although the composition of the microbiota after 1 month was significantly different from the baseline in each cage, there were significant differences at 1 month between the three cages (fig. S2). These results suggest that our gut-cleansing protocol resulted in a permissive environment, leading to the development of a new community state determined by cage environment. This implies that inoculation of the mouse host with specific organisms after ABX/PEG treatment may provide a method for engineering a new community state in a consistent fashion.

Although antibiotics have been shown to alter the composition of the human gut microbiota, their effect on reducing bacterial load has not been fully quantified (3). Treatment of five adult human subjects with three antibiotics (rifaximin, trimethoprim/sulfamethoxazole, and metronidazole) over 3 days showed no effect on bacterial load as quantified by 16S rRNA gene copy number in stool samples (Fig. 4B). There was a surprisingly small effect on the composition of the human gut microbiota, with the dominant effect being intersubject variability (Fig. 4C). However, using a similar protocol in our human subjects as used in our mouse studies, that is, two antibiotics (vancomycin and neomycin) taken
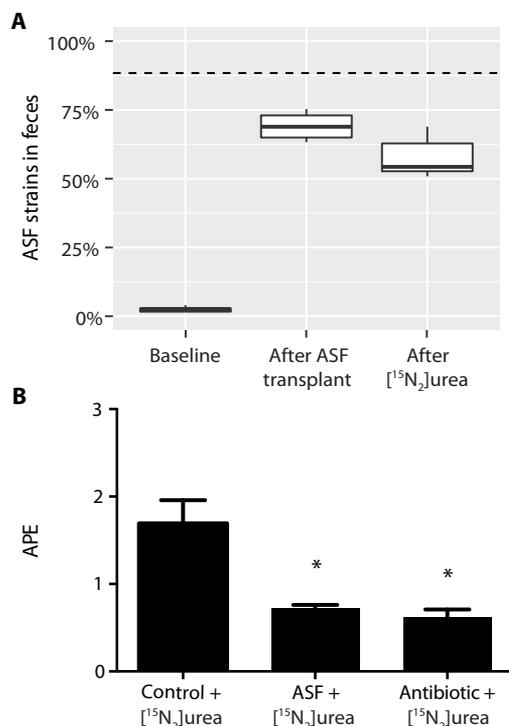
Fig. 3. In vivo heavy isotope assays using ^{15}N-labeled urea to determine the effect of bacterial urease on nitrogen flux in the murine gut microbiota. (**A**) 16S ribosomal RNA (rRNA) gene-tagged sequencing showing effective engraftment of the murine host with ASF after 3 days after completion of ASF inoculation (after ASF transplant) and 8 days after initiation of $[^{15}N_2]$urea administration (after $[^{15}N_2]$urea). (**B**) Atom percent excess (APE) of ^{15}N in fecal lysine for mice with a normal commensal microbiota (control), a minimal urease microbiota (ASF-transplanted), and a substantially reduced microbiota biomass due to antibiotic treatment. Mice were given ^{15}N-labeled urea via oral gavage. $n = 5$ per group, means ± SD; statistical analysis performed by two-tailed Student's t tests, *$P < 0.05$.

for 3 days, with PEG taken on the second day, we observed a marked reduction in both culturable bacteria and 16S rRNA gene copy number of about 4 to 5 logs after 72 hours ($P < 0.01$) (Fig. 4D) (23). The difference between a 5-log decrease in culturable bacteria and a 2-log decrease in 16S rRNA gene copy number at 48 hours likely represented residual DNA in the gut lumen from nonviable bacteria that were cleared from the gut with the PEG purge by day 4. These results suggest that a specific ABX/PEG protocol may be needed to prepare the human gut for inoculation and engineering of the gut microbiota.

Bacterial urease alters the composition of an engineered gut microbiota in colonized mice

We hypothesized that the bacterial enzyme urease may influence the gut microbiota after ABX/PEG treatment. As noted above, the dysbiotic gut microbiome in pediatric patients with Crohn's disease was associated with expression of genes involved in bacterial nitrogen metabolism (5). In addition, we observed a positive correlation between the severity of Crohn's disease in these patients, fecal amino acid concentrations, and bacterial taxa associated with dysbiosis

(Figs. 1 and 2, and fig. S1). This led us to hypothesize a role for urease enzymatic activity in shaping the dysbiotic microbiota, given its importance in bacterial nitrogen flux, ammonia production, and the subsequent incorporation of ammonia into bacterial amino acids (Fig. 3).

We have previously shown that aerotolerant organisms such as Enterobacteriaceae are the first to colonize the gut after cleansing (28), a possible consequence of oxygen diffusion into the intestinal luminal environment (17). We hypothesized that inoculation of the mouse gut microbiota, after gut cleansing, with *E. coli* engineered to express urease could facilitate alterations in the gut microbiota composition. To test this hypothesis, we engineered a derivative of the murine commensal *E. coli* MP1 (29) with the urease operon from *Proteus mirabilis* inserted in the chromosome. We used an inducible urease gene cluster that had previously been shown to be functional in *E. coli* (30) and verified urease activity with Christensen's urea agar plates (Fig. 5A). Both the wild-type MP1 murine *E. coli* strain, which was Ure⁻, and the engineered Ure⁺ strain were consecutively inoculated into mice for 5 days after the depletion of the native gut
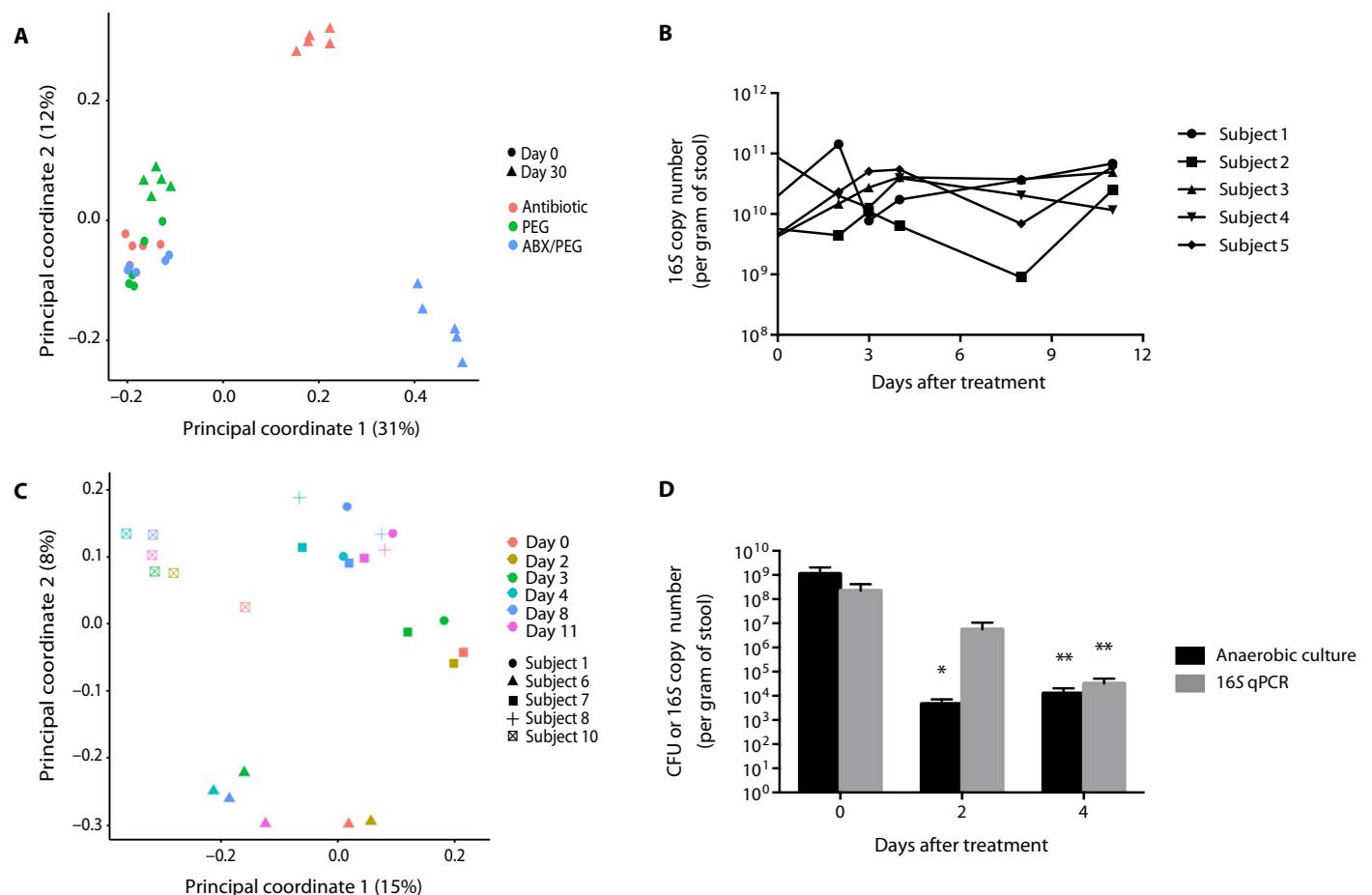

Fig. 4. Specific antibiotics reduce fecal biomass in mice and humans. (**A**) Unweighted UniFrac principal coordinates analysis of the murine fecal microbiota at baseline (day 0) and 30 days after treatment of mice orally with two nonabsorbed antibiotics (vancomycin and neomycin), PEG, or a combination of both (ABX/PEG). (**B**) Fecal bacterial load quantified by 16S rRNA gene copy number polymerase chain reaction (PCR) in five healthy human subjects at baseline and days 2, 3, 4, 8, and 11 during and after a 3-day treatment with three oral antibiotics (rifaximin, trimethoprim/sulfamethoxazole, and metronidazole). (**C**) Unweighted UniFrac principal coordinates analysis of fecal microbiota composition in the samples collected from five healthy human subjects as described in (B). (**D**) Fecal bacterial load quantified by 16S rRNA gene copy number PCR (per gram of stool) and anaerobic culture [colony-forming units (CFU) per gram of stool] in healthy human subjects, who received a bowel-cleansing protocol of oral antibiotics (neomycin and vancomycin) for 72 hours and a PEG purge initiated at 36 hours. $n = 5$, means ± SD; statistical analysis performed using two-tailed paired Student's t tests of log-transformed data, *$P < 0.05$, **$P < 0.01$. qPCR, quatitative PCR.

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microbiota with ABX/PEG. This gut-cleansing protocol resulted in successful colonization of the inoculated mice with both Ure⁻ and Ure⁺ *E. coli* MP1 strains for at least 3 months at numbers about 1000-fold greater than those achieved with a different colonization protocol previously reported in the literature (Fig. 5, B and C) (*29*).

Successful engraftment of *E. coli* MP1 resulted in changes in mouse gut microbiota composition that were dependent on the presence of urease but independent of caging. We carried out five interventions: ABX/PEG, ABX/PEG followed by inoculation with the feces of conventionally housed mice, ABX/PEG followed by inoculation with Ure⁻ MP1, ABX/PEG followed by inoculation with Ure⁺ MP1, and ABX/PEG followed by inoculation with a 1:1 mixture of Ure⁻ and Ure⁺ MP1. Although all of the groups had similar gut microbiota compositions at baseline, 16*S* rRNA gene sequencing revealed a pronounced difference among the five groups at day 29 after inoculation (Fig. 5D). All groups diverged from the ABX/PEG-treated group. Mice inoculated with conventional murine feces showed a similar gut composition to the donor, whereas mice that received either Ure⁺ MP1 or a mixture of Ure⁺ and Ure⁻ MP1 showed a gut composition different from those animals that received Ure⁻ MP1 (Fig. 5D). Using a Mann-Whitney test, the greatest distance among all pairwise comparisons before

(day 0) and after (day 29) gavage was between cages that had undergone different treatments (Fig. 5E), indicating that these effects were independent of caging. Testing for differences between groups using the permutational multivariate analysis of variance test (fig. S3) revealed that a number of groups were different at baseline (fig. S3), although with a small effect size ($R^2 < 0.15$). However, the differences between groups increased markedly on day 29, all of which were statistically significant ($P < 0.05$). The Ure⁺ MP1– and Ure⁺/Ure⁻ MP1–inoculated mice were most similar among the groups and had the smallest effect size between them. They were very different from the mice inoculated with conventional murine feces ($R^2 > 0.4$), the ABX/PEG group ($R^2 > 0.3$), and the Ure⁻ MP1 group ($R^2 > 0.25$), all with large effect sizes. These data suggest that *E. coli* can function as a keystone species that allows for the establishment of a stable microbiome (*31*) when inoculated into a properly prepared host and that the presence of the urease gene influences the composition of the new gut microbiota.

Bacterial urease influences dysbiosis and exacerbates colitis in a murine model
The difference in the composition of the gut microbiota induced by the inoculation of mice with Ure⁻ versus Ure⁺ MP1 is shown in Fig. 5F
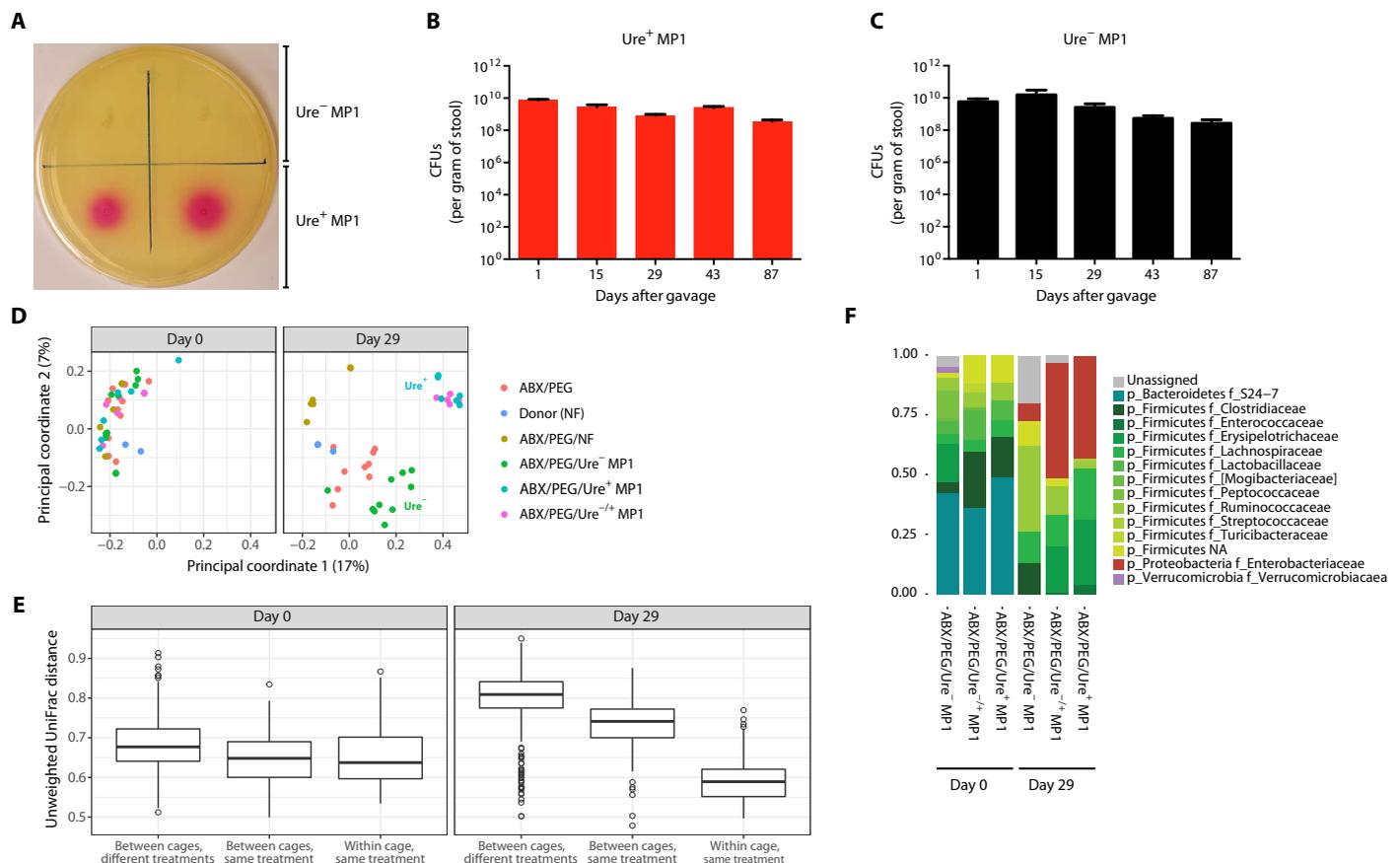

Fig. 5. Effect on gut microbiota composition of inoculating a murine host with *E. coli* MP1 strain engineered with a urease operon. (**A**) Christensen's urea agar plate shows urease activity, indicated by phenol red as a pH indicator, of Ure⁺ *E. coli* MP1 colonies and Ure⁻ *E. coli* MP1 colonies 6 hours after inoculation of murine hosts. (**B** and **C**) Bacterial load in mouse feces after inoculation of murine hosts with Ure⁺ *E. coli* MP1 (B) or Ure⁻ *E. coli* MP1 (C) at the indicated time points after oral gavage. (**D**) Unweighted UniFrac principal coordinates analysis of day 0 and day 29 fecal microbiota composition using 16*S* rRNA gene-tagged sequencing in five groups of mice: ABX/PEG, ABX/PEG then gavaged with normal feces (NF), ABX/PEG then gavaged with Ure⁺ *E. coli* MP1, ABX/PEG then gavaged with Ure⁻ *E. coli* MP1, and ABX/PEG then gavaged with a mixture of Ure⁺/Ure⁻ *E. coli* MP1. (**E**) Box plot of unweighted UniFrac distances between samples within and between cages and within and between study groups. (**F**) Taxonomic abundance of bacteria 29 days after inoculation of murine hosts with Ure⁻ *E. coli* MP1, Ure⁺ *E. coli* MP1, or a mixture of both.

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and fig. S4. The Ure⁻ MP1–inoculated gut microbial community had higher proportions of taxa belonging to the Firmicutes phylum, including order Clostridiales, genus *Ruminococcus*, genus *Dorea*, genus *Oscillospira*, genus *Peptostreptococcus*, and family Clostridiaceae (fig. S4). Taxa within each of these clades have been associated with health of the host. In contrast, the Ure⁺ MP1–inoculated microbial community had higher proportions of Proteobacteria that were associated with dysbiosis, including family Enterobacteriaceae and family Sphingomonadaceae (fig. S4). Thus, inserting a single operon encoding bacterial urease in *E. coli* MP1 appeared to be sufficient to cause a shift in the inoculated mouse gut microbiota composition to a dysbiotic state.

To investigate the functional effects of the dysbiotic gut microbiota engineered by bacterial urease, we examined the effect of inoculation with either Ure⁻ or Ure⁺ MP1 on the development of colitis in a T cell adoptive transfer model of murine colitis (*24*). Thirty days after Rag⁻/⁻ mice were inoculated with the two different MP1 strains, adoptive transfer of CD45Rb^high naïve T cells was performed. Initial CFUs for both strains approximated numbers observed in wild-type mice (Fig. 6A) but then dropped transiently with a recovery to baseline by day 44. About 1 month after T cell transfer, mice that were inoculated with Ure⁺ MP1 began to lose weight. By day 44 after transfer, these mice exhibited a 20% loss in weight, triggering euthanasia (Fig. 6B). Consistent with a greater loss of weight in the Ure⁺ MP1–inoculated mice, colonic and cecal weights were increased, and colonic length was decreased relative to the colons of mice inoculated with Ure⁻ MP1 (Fig. 6C). Together with a higher clinical colitis disease activity index (Fig. 6C) (*32, 33*), these results indicate that inoculation of mice with Ure⁺ MP1 led to an exacerbation of colitis. This was confirmed by histological evidence of more pronounced chronic inflammation in the colons of mice inoculated with Ure⁺ MP1 compared to Ure⁻ MP1 (Fig. 6D). The Ure⁺ MP1–inoculated mice also exhibited an increase in Enterobacteriaceae and a decrease in Clostridiaceae (fig. S5, A and B). Consistent with our finding that fecal amino acid quantities were higher in Crohn's disease patients with a dysbiotic microbiota (Fig. 1B), the quantity of fecal amino acids was higher in mice inoculated with Ure⁺ MP1 with colitis compared to those receiving Ure⁻ MP1 (fig. S5C). These results implicate the dysbiotic microbiota established by inoculation with a commensal *E. coli* engineered to express a bacterial urease in the pathogenesis of colitis in a chronic immunologically mediated murine model of IBD.
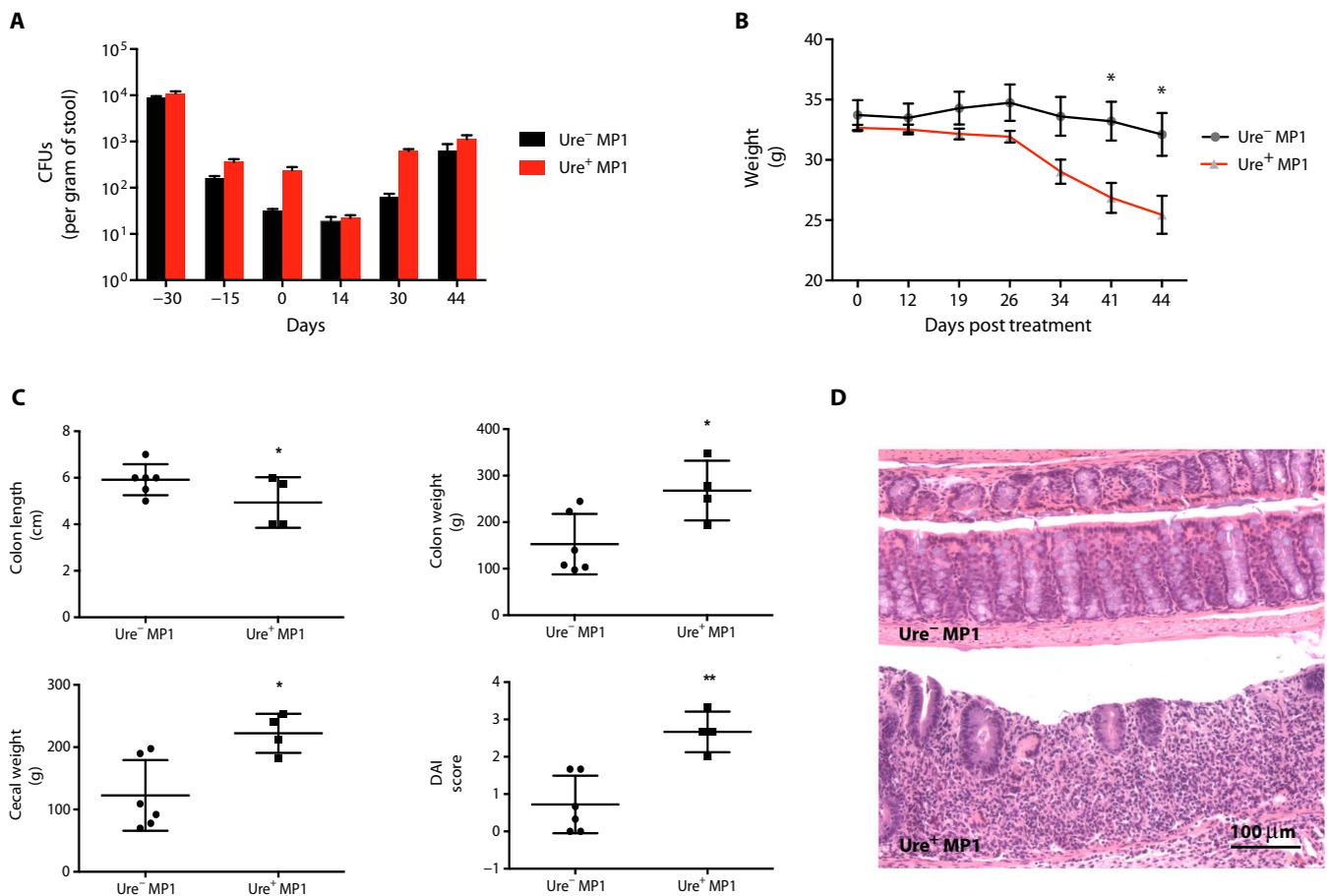

Fig. 6. Effect of *E. coli* urease on colitis in a T cell adoptive transfer mouse model of colitis. (**A**) Bacterial load represented as CFUs per gram of stool in the feces of mice after inoculation with Ure⁻ (black) or Ure⁺ (red) *E. coli* MP1 strains. Day 0 is the time point of T cell adoptive transfer. (**B**) Weight of T cell adoptively transferred Rag⁻/⁻ mice after inoculation with either Ure⁻ (black) or Ure⁺ (red) *E. coli* MP1 strains. Statistical analysis was performed using two-tailed unpaired Student's *t* test, *$P < 0.05$. (**C**) Mouse colonic and cecal weights, colonic length, and disease activity index (DAI) in T cell adoptively transferred Rag⁻/⁻ mice 44 days after inoculation with either Ure⁻ or Ure⁺ *E. coli* MP1 strains. Statistical analysis was performed using two-tailed unpaired Student's *t* test, *$P \leq 0.02$, **$P = 0.002$. (**D**) Photomicrographs of hematoxylin and eosin–stained mouse colon tissue from T cell adoptively transferred Rag⁻/⁻ mice 44 days after inoculation with either Ure⁻ or Ure⁺ *E. coli* MP1 strains.

DISCUSSION

If gut microbiota dysbiosis is in the causal pathway of a disease, then correction of the dysbiosis is a potential therapeutic strategy. Here, we provide evidence that urease-mediated nitrogen flux into the gut microbiota may play an important role in the development of the dysbiotic microbiota in IBD. We also show that inoculating a properly prepared murine host with a single aerotolerant commensal bacterial strain expressing urease caused worsening colitis in an immune-mediated mouse model.

Gene pathway analysis supports the notion that the gut microbiota responds to gut environmental stressors principally through the modification of metabolism. Given that Proteobacteria are increased in Crohn's disease and that these bacteria are enriched for genes that promote utilization of nitrogen, we anticipated that the microbiome in patients with Crohn's disease would demonstrate increased abundance of gene pathways associated with nitrogen metabolism. This was confirmed in our gene pathway analysis in which there was increased representation of gene pathways associated with IBD and dysbiosis in pediatric Crohn's disease patients. These included sulfur relay systems, nitrogen metabolism, and biosynthetic pathways for several amino acids, all of which are involved in bacterial nitrogen utilization. For example, the sulfur relay system provides sulfur via L-cysteine desulfurase for biosynthesis of molybdenum cofactor, as well as other biomolecules such as thiamin, iron-sulfur clusters, and thionucleosides. Specifically, we show that various genes encoding enzymes involved in the synthesis of molybdenum cofactor, which is responsible for catalyzing redox reactions in the bacterial metabolism of nitrogen, sulfur, and carbon compounds (34), are associated with both Crohn's disease and taxa belonging to the Proteobacteria phylum. In addition, molybdenum cofactor may also play a role in various nitrate reduction pathways associated with nitrogen respiration in the setting of intestinal inflammation (18). Whereas the shifts in both bacterial community structure and gene expression may be linked to a variety of factors, the predominance of the sulfur relay system in the gut microbiome of patients with Crohn's disease emphasizes the possible importance of nitrogen metabolism in the development of the dysbiotic gut microbiota.

The notion that bacterial nitrogen metabolism plays a role in the development of dysbiosis is supported by the strong positive correlation between Proteobacteria taxa found in the dysbiotic microbiota of patients with IBD (5) and our fecal metabolomic profiling showing a strong association between dysbiosis and quantities of fecal amino acids. However, one potential limitation of this study is that the metagenomic data focused on bacteria and not the contribution of fungal microbiome members (which also encode ureases) to nitrogen cycling in dysbiosis associated with Crohn's disease. Nitrogen metabolism pathways have been extensively characterized in $E.\ coli$ (35) where the preferred nitrogen source is ammonia (36). To provide mechanistic evidence supporting the metagenomic and metabolomic associations between nitrogen metabolism and fecal amino acid quantities with the dysbiotic microbiota in Crohn's disease, we examined the role of urease in bacterial nitrogen flux and amino acid synthesis. Urease activity in the gut microbiota is particularly important for nitrogen flux between the host and its gut microbiota (37). The catabolism of dietary protein by the host results in the formation of urea, a nitrogenous waste product that is excreted through the urine or delivered into the colon, where hydrolysis by bacterial urease results in the production of carbon dioxide and ammonia. Using $[^{15}N_2]$urea as an isotopic tracer, we provide direct evidence for the importance of bacterial urease and its production of ammonia for bacterial amino acid synthesis. The quantification of ^{15}N

enrichment specifically in lysine, an amino acid that cannot undergo mammalian transamination, established that bacteria are responsible for nitrogen flux from urea into ammonia, resulting in the incorporation of ^{15}N into amino acids synthesized by bacteria. Having established the importance of urease in bacterial nitrogen flux and amino acid synthesis in vivo in a mouse model, we then sought to assess the effect of urease on the composition of the gut microbiota in a mouse host. We have recently shown that it is possible to engineer the gut microbiota of conventionally housed mice using ASF, a defined bacterial consortium of eight organisms (23). Required preparation included administration of two orally delivered nonabsorbable antibiotics, vancomycin and neomycin, together with a gut-purging agent, PEG. Here, we extend these findings by showing that antibiotics are critical for reshaping the composition of the murine gut microbiota because PEG had little effect on its own. Moreover, in humans, the choice of antibiotics is important because a combination of three antibiotics administered without PEG had little impact on bacterial load and microbiota composition, whereas use of vancomycin, neomycin, and PEG resulted in a 5-log reduction in bacterial load. Finally, in mice, the composition of the resultant gut microbiota after gut cleansing was variable and dependent on cage effects. We inoculated the $E.\ coli$ MP1 strain, engineered to express urease or not, into mice after gut cleansing with ABX/PEG. We found that long-term engraftment of the inoculated bacterial strain was possible, independent of urease activity. Inoculation with Ure$^+$ $E.\ coli$, but not Ure$^-$ $E.\ coli$, was sufficient to engineer the murine gut microbiota into a dysbiotic state characterized by increased proportions of Proteobacteria and reduced Firmicutes. A 1:1 mixture of Ure$^-$ and Ure$^+$ $E.\ coli$ also led to the development of a dysbiotic microbiota, showing dominance of the Ure$^+$ phenotype. These results demonstrated that the composition of the gut microbiota can be engineered by altering expression of a single enzyme in one bacterial lineage when inoculated into a properly prepared murine host, supporting the importance of nitrogen flux and metabolism in the development of the dysbiotic gut microbiota.

The worsening of disease in a chronic immune-mediated murine model of colitis by inoculation with Ure$^+$ MP1 relative to the Ure$^-$ strain provides functional evidence for the relevance of the dysbiosis induced by urease. Our results may explain why the transfer of specific Enterobacteriaceae, namely, $Klebsiella\ pneumoniae$ and $P.\ mirabilis$ (both known to express urease) (38), correlates with the development of colitis in another chronic T cell–mediated model of colitis (11). Fecal microbiota transplantation has been used with mixed results in Crohn's disease and ulcerative colitis, a related IBD with similar dysbiosis to that seen in patients with Crohn's disease (16, 39–44). Some studies have identified differential effectiveness of fecal microbiota transplantation depending on the donor. It is possible that the inconsistent effectiveness of fecal microbiota transplantation could be related to inconsistent reduction in urease activity because about 15 to 30% of urea produced by humans is hydrolyzed by microbial ureases (45, 46).

There are limitations to our study. Although there was a clear correlation between fecal amino acids and the dysbiotic gut microbiota in both IBD and a murine colitis model, it was not possible to determine the degree to which this was due to host versus microbial biology because it was not technically possible to distinguish between fecal amino acids derived from the host versus the gut microbiota. In addition, our mouse models did not provide information about the mechanism by which bacterial urease induced dysbiosis. However, our human and murine data do reveal a role for bacterial nitrogen flux via urease activity in the development of dysbiosis and the pathogenesis of IBD. These observations will inform gut microbiota–based

strategies for the treatment of human diseases. It appears to be important to reduce microbial load to enhance the engraftment of engineered bacterial consortia. Not all antibiotics influence the bacterial load and composition in the human gut, but vancomycin and neomycin together with PEG reduced both culturable bacteria and 16S rRNA gene copy number in humans and mice (23). In addition, human commensal organisms that are available as probiotics might have greater utility in the treatment of disease when used in combination with a gut-cleansing protocol. For example, E. coli Nissle, which has been shown to have modest beneficial effects in ulcerative colitis and lacks urease activity (47), might be more effective when inoculated into patients in which bacterial load has been sufficiently diminished. Such a strategy might enable currently used probiotics, which have minimal effects on the composition of the human gut microbiota, to confer more benefits on patients with disease.

MATERIALS AND METHODS
Study design
The results reported in this manuscript were generated from a two-part study where the primary aim was to determine the biological mechanism by which fecal amino acids are correlated with the dysbiotic gut microbiota in IBD. The first component involved the analysis of biospecimens collected in a human subject study named PLEASE, where correlations between fecal amino acids and the dysbiotic gut microbiota were demonstrated using a combination of metabolomics and shotgun metagenomic sequencing (5). Details of the PLEASE human subject prospective study have been previously reported (48). In brief, enrolled in the PLEASE study were children under the age of 22 with active disease defined as having a Pediatric Crohn's Disease Activity Index score of greater than 10, who were initiating therapy with PEN, EEN, or anti–TNF-α therapy. Patients had stool samples collected for determining fecal calprotectin concentration and microbiome characterization at baseline and 1, 4, and 8 weeks after initiation of the treatment. Metabolomic and gene pathway analyses were performed on baseline stool samples from subjects with Crohn's disease compared to healthy controls. In addition, two interventional human subject studies were performed to determine the effect of various antibiotics and a PEG gut purge on fecal bacterial biomass using anaerobic culture and 16S rRNA gene copy number PCR. All human subject studies were performed with Institutional Review Board (IRB)–approved protocols that included informed consent.

The second component of this study was designed to determine the mechanism by which fecal amino acids correlated with dysbiosis in IBD. Using a combination of heavy isotope nitrogen flux studies, bacterial culture experiments, and mouse models, we show that bacterial urease can lead to the development of dysbiosis that exacerbates the course of murine experimental colitis. We used in vivo heavy isotope assays using ^{15}N-labeled urea to determine the effect of bacterial urease on bacterial nitrogen flux, where ^{15}N was incorporated into bacterial amino acids in a urease-dependent fashion. We then engineered a strain of commensal mouse E. coli to express urease. When this Ure$^+$ E. coli strain was inoculated into mice that were pretreated with ABX/PEG, resulting in stable engraftment, it led to the development of a dysbiotic gut microbiota with an increase in Proteobacteria and a decrease in Clostridia spp. Last, when this Ure$^+$ E. coli strain was inoculated into Rag$^{-/-}$ mice in which colitis was induced via T cell transfer, we observed an exacerbation of disease with an increase in fecal amino acids, providing functional evidence for the relevance of the dysbiosis induced by bacterial nitrogen flux via a urease-dependent mechanism.

Animal studies
All animal experiments were performed according to Institutional Animal Care and Use Committee–approved protocols. Mice were prepared for inoculation by oral delivery of antibiotics in the drinking water (1 g of aspartame, 0.1 g vancomycin, and 0.2 g of neomycin in 200-ml sterile water) for 72 hours with the addition of PEG 10% (w/v) for the last 12 hours. For the MP1 experiments, the mice were then inoculated daily with the desired E. coli strains by oral gavage for 5 days. For the T cell adoptive transfer model of colitis, the mice were inoculated with the desired E. coli strains by oral gavage then underwent induction of colitis detailed below at day 30 after gavage. Freshly sorted CD4$^+$CD45RB^{high} T cells from 6- to 8-week-old C57BL/6 mice were transferred intraperitoneally into 8-week-old Rag1KO (Rag$^{-/-}$) mice on a C57BL/6 background on day 0 per established protocols (24). ^{15}N flux experiments were performed on conventionally housed mice that had received fecal microbial transplant using ASF (with low urease activity), as previously described (23). These mice were compared to mice that were continuously treated with antibiotics (vancomycin and neomycin) and to control mice. All groups were gavaged with 50 mg of [^{15}N$_2$]urea per mouse daily for 7 days. ^{15}N enrichment in amino acids was determined by liquid/gas chromatography–MS and HPLC (49, 50).

Human studies
All studies were performed using IRB-approved protocols. Fecal samples used for metabolomic studies were collected from pediatric patients with Crohn's disease as part of our PLEASE study (5) and from healthy controls. Fecal samples used to determine the effect of antibiotics on the bacterial load of the human gut microbiota were obtained from two independent studies. The AFTER (Impact of Antibiotics on Fecal Bacterial Concentration) study was a single-arm clinical trial examining the composition of the stool microbiome from healthy adult volunteers exposed to 3 days of oral antibiotics [rifaximin (550 mg, orally twice daily) plus metronidazole (500 mg, orally twice daily) plus trimethoprim/sulfamethoxazole (160 to 800 mg, orally twice daily)]. In the FARMM (Food and Resulting Microbial Metabolites) inpatient study, fecal samples were collected from healthy adult volunteers before, during, and immediately after a bowel-cleansing protocol consisting of vancomycin (500 mg, every 6 hours) and neomycin (1000 mg, every 6 hours) daily for 3 days with a PEG purge (4 liters) on day 2. Anaerobic culture conditions for CFU determination of bacterial load were performed as previously described (51).

DNA extraction, 16S rRNA amplification, sequencing, and analysis
Total DNA was extracted from mouse fecal pellet and bacterial gavage samples using the MO BIO PowerSoil-htp kit. The first and second hypervariable regions of the 16S rRNA gene (V1 and V2) were amplified using Golay-barcoded 27F and 338R primers (52–54). 16S rRNA amplicons were sequenced using 2 × 250–base pair paired-end reads on the Illumina MiSeq platform (53, 54). The sequenced 16S rRNA reads were analyzed using the QIIME software package (55) and the R programming language.

Metabolomic analyses of fecal samples
Stool samples were homogenized using a bead mill (TissueLyser II, QIAGEN), and the aqueous homogenates were aliquoted for metabolite profiling analyses. Four separate LC–tandem MS methods were used to

measure polar metabolites and lipids in each sample. Methods 1, 2, and 3 were conducted using two LC-MS systems composed of Nexera X2 UHPLC systems (Shimadzu Scientific Instruments) and Q Exactive Hybrid Quadrupole-Orbitrap MSs (Thermo Fisher Scientific), and method 4 was conducted using a Nexera X2 UHPLC (Shimadzu Scientific Instruments) coupled to an Exactive Plus Orbitrap MS (Thermo Fisher Scientific).

Statistical analysis

Wilcoxon rank-sum test was used for two group comparisons, and Spearman rank correlation was used to assess the association between two quantitative variables such as taxon abundance and metabolite. To account for sample heterogeneity between normal and disease samples, linear regression analyses were applied to assess the statistical significance, including a disease indicator as a covariate. Linear regression analysis was also applied to associate the amino acid abundance and fecal calprotectin measurements adjusting for antibiotic use. Log transformation was applied to both taxon and metabolite abundances. Finally, FDR was applied to control for multiple comparisons. Two-tailed Student's t tests were used for comparisons of fecal amino acids, atom percent excess, mouse weights, and clinical end points of colitis.

SUPPLEMENTARY MATERIALS

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Extended experimental procedures
Fig. S1. Fecal amino acids in healthy control subjects versus patients with pediatric Crohn's disease.
Fig. S2. Composition of the gut microbiota at baseline and 1 month after ABX/PEG bowel cleansing in three independent experiments.
Fig. S3. Test for difference in microbiota community composition based on unweighted UniFrac distance.
Fig. S4. Heat map and Kruskal-Wallis analysis of the murine gut microbiota before and after inoculation with Ure⁻ and Ure⁺ E. coli MP1.
Fig. S5. Changes in microbiota composition and fecal amino acids in mice inoculated with Ure⁺ versus Ure⁻ E. coli MP1 in a T cell adoptive transfer model of colitis.

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A role for bacterial urease in gut dysbiosis and Crohn's disease

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Nitrogen flux and gut dysbiosis

Ni *et al.* used shotgun metagenomic and metabolomic analysis of fecal samples from pediatric patients with Crohn's disease. They demonstrated an association between disease severity, gut dysbiosis, and free amino a cids. A heavy isotope–labeled nitrogen flux analysis showed that bacterial urease activity led to the transfer of host-derived nitrogen to the gut microbiota, boosting amino acid synthesis. Inoculation of a murine host with *Escherichia coli* engineered to express urease led to dysbiosis associated with worsened immune-mediated colitis and increased amino acid production. A potential role for nitrogen flux in the development of gut dysbiosis suggests that urease may be a potential target for developing treatments for inflammatory bowel diseases.

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