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Bacteria and Bacterial rRNA Genes Associated with the Development of Colitis in IL-10^{-/-} Mice

Jingxiao Ye, MS[#], Jimmy W. Lee, PhD^{#†}, Laura L. Presley, BS^{*}, Elizabeth Bent, PhD^{*}, Bo Wei, MD, PhD[‡], Jonathan Braun, MD, PhD[‡], Neal L. Schiller, PhD[†], Daniel S. Straus, PhD[†], and James Borneman, PhD^{*}

^{*}Department of Plant Pathology and Microbiology, University of California, Riverside, California

[†]Division of Biomedical Sciences, University of California, Riverside, California

[‡]Department of Pathology and Laboratory Medicine, University of California, Los Angeles, California.

[#] These authors contributed equally to this work.

Abstract

Background—Microorganisms appear to play important yet ill-defined roles in the etiology of inflammatory bowel disease (IBD). This study utilized a novel population-based approach to identify bacteria and bacterial rRNA genes associated with the development of colitis in IL-10^{-/-} mice.

Methods—Mice were housed in 2 environments: a community mouse facility where the mice were fed nonsterile chow (Room 3) and a limited access facility where the mice were fed sterile chow (Room 4). Every month the disease activity levels were assessed and fecal bacterial compositions were analyzed. At the end of the experiments histological and bacterial analyses were performed on intestinal tissue.

Results—Although disease activity increased over time in both environments, it progressed at a faster rate in Room 3 than Room 4. Culture and culture-independent bacterial analyses identified several isolates and phylotypes associated with colitis. Two phylotypes (GpC2 and Gp66) were distinguished by their negative associations with disease activity in fecal and tissue samples. Notably, rRNA genes from these phylotypes had high sequence identity (99%) to an rRNA gene from a previously described flagellated *Clostridium* (Lachnospiraceae bacterium A4).

Conclusions—The negative associations of these 2 phylotypes (GpC2 and Gp66) suggest that these bacteria were being immunologically targeted, consistent with prior findings that the Lachnospiraceae bacterium A4 bears a prevalent flagellar antigen for disease-associated immunity in murine immune colitis and human Crohn's disease. Identification of these associations suggests that the experimental approach used in this study will have considerable utility in elucidating the host–microbe interactions underlying IBD.

Keywords

IL-10^{-/-} mice; rRNA genes; Crohn's disease; IBD; bacteria

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Reprints: Daniel S. Straus, Division of Biomedical Sciences, University of California, Riverside, CA 92521 (daniel.straus@ucr.edu) or James Borneman, Department of Plant Pathology and Microbiology, University of California, Riverside, CA 92521 (borneman@ucr.edu).

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Microorganisms appear to play important yet ill-defined roles in the etiologies of Crohn's disease (CD) and ulcerative colitis (UC).¹⁻³ CD and UC occur in regions of the intestine where enteric bacteria are found in the highest concentrations.⁴ Contact with intestinal contents triggers mucosal inflammation in CD,⁵ while diversion of the fecal stream promotes intestinal healing.⁶ The most direct evidence for the importance of microorganisms in disease etiology comes from investigations with animals. In numerous rodent models, colitis is absent when the animals are kept in a "germ-free" state, but it rapidly develops when standard intestinal microorganisms are introduced.⁷

Evidence also indicates that disease etiology involves specific bacteria, and/or an aberrant immunological response to specific intestinal microorganisms. In animal studies, monoassociation experiments have shown that the type of colitis is dependent on the bacterial species.^{8,9} In addition, various antibiotics, possessing different taxonomic targets, have been shown to exhibit varying abilities to prevent and treat colitis in HLA-B27 rats¹⁰ and IL-10^{-/-} mice.¹¹ In human studies, evidence for the involvement of specific bacteria includes the differing abilities of various antibiotic therapies to induce disease remission.¹²⁻¹⁴ Evidence that the immunological responses to these bacteria are involved in disease etiology include the ability of CBir1-selective T cells to cause colitis when transferred to immunodeficient mice¹⁵ and the ability to utilize seroreactivity to specific microbial antigens for disease stratification.¹⁶

Prior investigations in our laboratories have demonstrated the utility of population-based approaches for identifying microorganisms involved in specific in situ processes such as plant-pathogen suppression in soil.^{17,18} The first step in this approach is to create or identify a series of samples/subjects with various levels of a specific functional parameter/phenotype. Extensive microbial community analyses are then performed on these samples or subjects. Finally, analyses are performed to identify associations between the abundance of specific taxa and the levels of the functional parameter/phenotype.

In this study we used this experimental approach to identify bacteria and bacterial rRNA genes associated with the development of colitis in IL-10^{-/-} mice. Various levels of colitis were examined by monitoring bacterial populations in 2 different environments, where the disease progression rates were different, and over a 6-month period, throughout the development of colitis. Bacteria and bacterial rRNA genes with both positive and negative associations with colitis were identified. Although such trends can be interpreted in several ways, we suggest that negative associations may represent particularly important trends in inflammatory bowel disease (IBD), because they may facilitate the identification of resident microbiota that are being targeted by aberrant immunological responses.

MATERIALS AND METHODS

Mouse Experiments

C3H/HeJBir.IL-10^{-/-} (C3H.IL-10^{-/-}) mice¹⁹ were bred in a room (Room 4) with limited access, isolated from other mice, and fed radiation-sterilized chow (Purina LabDiet 5053, St. Louis, MO).^{20,21} Beginning at around 5 weeks of age, mice were separated into 2 groups. One group was housed in a conventional community mouse facility where the animals were fed nonsterile chow (Purina LabDiet 5001) (Room 3). The other group was maintained on sterile chow in the aforementioned Room 4. Mice in the 2 groups were paired according to sex, age, weight, and initial disease activity index (DAI). DAI scores (0-6) are the sum of 4 individual parameters: fur appearance, length of prolapsed anus, stool consistency, and presence of occult bleeding.²¹ DAI measurements were performed monthly for all mice. Experiments were terminated after 6 months. At this time all surviving animals were

ethanized and tissue samples were collected and processed for histological and microbiological analyses. These experiments were performed twice and designated Experiments 1 and 2. Animal use protocols were approved by the Institutional Animal Care and Use Committee at the University of California, Riverside.

Histopathology

Segments of the proximal colon were fixed, embedded, sectioned, and stained as described previously.^{20,21} Coronal sections of the colon were stained with hematoxylin and eosin (H&E) plus Alcian Blue and photographed with a Zeiss Axioplan photomicroscope equipped with a digital camera.

Fecal and Tissue Sample Collection

Fecal samples were collected from each mouse every month. Mice were placed in separate plastic beakers. Fresh fecal samples were transferred to standard microcentrifuge tubes for the culture-based bacterial analysis or FastDNA Lysis Tubes (Qbiogene, Carlsbad, CA) with buffer for culture-independent analyses. The FastDNA tubes were stored at -70°C until the DNA was extracted. For each tube at least 2 fecal pellets were collected, which weighed between 0.1 and 0.4 g.

Tissue samples were collected at the end of each experiment. For each region of the intestine, 2 pieces of tissue (0.5 cm in length) were excised and placed in FastDNA Lysis Tubes with buffer and stored at 70°C ; these were the unwashed tissue samples. To obtain the washed tissue samples a similar procedure was performed, except before the samples were placed in the FastDNA tubes the intestinal pieces were opened longitudinally and washed in PBS buffer 3 times (tissue samples were placed in microcentrifuge tubes with 0.5 mL buffer and gently vortex mixed for 30 seconds; this procedure was repeated 2 more times using new tubes with fresh buffer). One 0.5-cm piece of the proximal colon was collected for histology.^{20,21}

DNA Extraction from Fecal and Tissue Samples

Extractions were performed using the FastDNA Spin Kit as described by the manufacturer, with a 30-second bead-beating step at a FastPrep Instrument setting of 5.5 (Qbiogene). DNA was further purified and size-fractionated by electrophoresis in 1% agarose gels. DNA larger than 3 kb was excised without exposure to UV or ethidium bromide, and recovered using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) following the manufacturer's instructions except that the gel pieces were not exposed to heat.

Oligonucleotide Fingerprinting of rRNA Genes (OFRG)

OFRG was performed as previously described²² with the following exceptions.

Library Construction—One bacterial rRNA gene clone library was produced from DNA from each of the fecal samples from Experiment 1. Polymerase chain reaction (PCR) amplifications were performed using the HPLC-purified bacterial rRNA gene primers BacOFRGpUSER, GGAGACAUAGRRTTTGATYHTGGYTCAG, and BacOFRGpUSER, GGGAAAGUGBTACCTTGTTACGACTT. Thermal cycling parameters were 94°C for 2 minutes; 30 cycles of 94°C for 20 seconds, 48°C for 30 seconds, and 72°C for 90 seconds; followed by 72°C for 10 minutes. Amplification reactions were performed using the PicoMaxx High Fidelity PCR System (Stratagene, La Jolla, CA). PCR products were cloned into pNEB 205 using the USER Friendly Cloning Kit (New England Biolabs, Beverly, MA).

OFRG Array Construction—Sixty 11 × 7 cm macroarrays on nylon membranes were produced, each containing ≈160 clones from each of 56 the fecal libraries and 384 control clones.

Array Hybridization—The bacterial probes were: GGGCGAAAGC, GAGACAGGTG, CCAGACTCCT, CGTGGGGAGC, ACGTAATGGT, TCCAGAGATG, CCTTCGGGAG, GATGAACGCT, GTGGGGTAAA, GGTAATGGCC, CCGTGAGGTG, AGTCGAACGG, TTGGTGAGGT, GCCGTAAACG, GTAACGGCTC, CCGCAAGGAG, GAACGCTGGC, GCTAACGCAT, CATTCAAGTTG, GTCTCAGTTC, AGGCTAGAGT, CAACCCTTGT, GCGTGAGTGA, GGTGCAAGCG, AGCTAGTTGG, AATACCGGAT, TGTGGGAGGG, CCCGCACAAG, CGGTACAGAG, ACCAAGGCAA, TTGCCAGCGG, ACCGCGAGGT, GCCTTCGGGT, TTGCCAGCAT, GCTAACGCGT, ACGGTACCTG, GCCCACTGG, GGCCGCAAGG, and GCAAGTCGAG. The reference probe was GCTGCTGGCA.²³ Arrays were washed twice in 1× SSC for 30 minutes at 11°C.

Data Analysis—A UPGMA dendrogram of the OFRG fingerprints was constructed using GCPAT.²⁴ To focus the subsequent analyses on the most abundant bacterial taxa, only clusters containing 5 or more clones were analyzed further. For each cluster, correlation analyses were performed between the number of clones and disease activity. For those clusters exhibiting a correlation at $P \leq 0.20$, nucleotide sequence analyses were performed on representative clones, and clusters containing clones with 99% or greater sequence identity were combined. At this point, each cluster (combined or not) was called an operational taxonomic unit (OTU) or phylotype.

Quantitative PCR

Sequence-selective quantitative PCR (qPCR) experiments were performed to quantify rRNA genes from selected bacteria or phylotypes. Real-time qPCR assays were performed in a Bio-Rad iCycler MyiQ Real-Time Detection System (Bio-Rad Laboratories, Hercules, CA) using iCycler iQ PCR Plates with Optical Flat 8-Cap Strips (Bio-Rad). Twenty-five μL reaction mixtures contained the following reagents: 50 mM Tris (pH 8.3), 500 $\mu\text{g}/\text{mL}$ BSA, 2.5 mM MgCl_2 , 250 μM of each dNTP, 400 nM of each primer, 1 μL of template DNA, 2 μL of 10× SYBR Green I (Invitrogen) and 1.25 U *Taq* DNA polymerase. The primers and amplicon sizes were: Gp000, (CitroSSUF1, CGGAGCTAACGCGTTAAA, CitroSSUR3, GCATCTCTGCAAAATTCTG, 163 bp); Gp66, (IL10GP66F4, AAGTCGAACGGACTCATAT, IL10GP66R4, GTCCGCCACT AACTCATAC, 51 bp); Gp76, (IL10Gp76F11, CAGTTACCAGCAAGTCAA, IL10Gp76R11, GCCGCATTGCTTCTCT, 147 bp); Gp156, (IL10Gp156F5, ATGAATTACGGTGAAAGCCG, IL10Gp156R9, ATCTTACGATGGCAGTCTTGT, 169 bp); Gp244, (GP244F1, TAAAGAATTCGGTATGGGA, GP244R1, TTACCCCGCCAACTAA, 55 bp); Gp254, (IL10GP254F3, TGCTTGCACTAAATGAAACT, IL10GP2-54R2, GTTACTCACCCGTCCG, 52 bp); Gp572, (IL10GP-572F5, GTGCTCGAGTGGCG, IL10GP572R5, GTGCTCGAGTGGCG, 66 bp); GpC1, (GpC1F2 GCTCAGGATGAACGCTG, GpC1R2, TCAACCGAAGTCTCTGTCA, 76 bp); GpC2, (IL10GPC2F3, GCAAGTCGAACGGACTCAT, IL10GPC2R1, CCGTCCGCCACTAACTC, 56 bp); GpC3, (GpC3F4, CATGCGACTCTTCGGAG, GpC3R4, CAGTCTCGCCAGAGTCC, 142 bp). Sequence-selective primers were designed either by 1) locating DNA sequences that were conserved among the rRNA gene sequences within each phylotype and which had few, if any, identical matches to rRNA gene sequences from unrelated taxonomic groups, or by 2) using the recently developed PRISE software.²⁵ The thermal cycling conditions were 94°C for 5 minutes; 40 cycles of 94°C for 20 seconds, X°C for 30 seconds, and 72°C for 30 seconds; followed by 72°C for 10 minutes; where X = 62.6 for Gp76 and Gp156, 63 for

GpC2, 64 for GpC3, 62.3 for GpC1, 64.9 for Gp66, 62.3 for Gp254, 63.4 for Gp572, 63.7 for Gp244, and 58.8 for Gp000. At each cycle, accumulation of PCR product was measured by monitoring the increase in fluorescence of the double-stranded DNA-binding SYBR Green dye. rRNA gene levels in the fecal sample DNA were quantified by interpolation from a standard curve comprised of a dilution series of cloned rRNA genes, respectively. To increase the likelihood that the real-time signals were produced by amplification of the target sequences, PCR fragments from fecal DNA were cloned into pGEM-T (Promega, Madison, WI), and the nucleotide sequences of 2 clones were determined; these experiments confirmed that the target sequences were being amplified (data not shown). The qPCR analyses were performed on DNA from individual fecal and tissue samples from both Experiments 1 and 2.

Associations Between rRNA Genes and Disease Activity

Correlation analyses between the log of the copy number of the bacterial rRNA genes and disease activity index values were performed using Minitab 15 (State College, PA).

Culture-Based Bacterial Analyses

Fecal samples, taken once per month for each mouse, were suspended in 0.01 M phosphate-buffered saline, pH 7.4 (PBS) and serially diluted in PBS. Aliquots (10 μ L) were plated on various media to determine the number of colony-forming units (CFU) per mL. Various dilutions of fecal samples were plated on Brucella agar (Becton Dickinson, Sparks, MD) plates containing 5% sheep red blood cells and incubated at 37°C in humidified incubators with 5% CO₂, or grown at 37°C in anaerobic jars using AnaeroGen (Oxoid, Basingstoke, UK). Samples were also plated on MacConkey agar (Becton Dickinson) plates and incubated at 37°C. After 24, 48, or 72 hours incubation colonies on each plate were counted and isolates of each colony type were subcloned and identified. Bacterial identification was done using Gram stain, catalase test, oxidase test, growth in 6.5% NaCl, and various standardized identification kits (such as the API 20E and API 20A kits, bioMerieux, Marcy l'Etoile, France). For each bacterial species identified by this systematic analysis the number of “incidences” was recorded. An incidence is the appearance of a species in the feces of a mouse at a given month.

Data Analysis of Cultured Bacteria

The significance of the difference between 2 means was evaluated using 2-tailed Student's *t*-tests. Simple and multiple regression analysis were performed using the StatCrunch program (www.statcrunch.com). Fisher's exact test was performed using the eXactoid program (www.exactoid.com/fisher).

Nucleotide Sequence Analysis of rRNA Gene Clones

Nucleotide sequences of rRNA gene fragments were determined using the ABI BigDye Terminator v. 3.1 Cycle Sequencing Kit and an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). Sequence identities were determined using BLAST (NCBI).²⁶

Nucleotide Sequence Data

The nucleotide sequences of the small-subunit rRNA genes of selected phylotypes were deposited in GenBank (NCBI): Gp66, EU402475; Gp76, EU402476; Gp156, EU402469; Gp244, EU402468; Gp254, EU402472; Gp572, EU402473; GpC1, EU402471; GpC2, EU402474; GpC3, EU402470.

RESULTS

Disease Activity in Conventional and Limited Access Facilities

IL-10^{-/-} mice were raised in 2 different environments: a conventional community mouse facility where the animals were fed nonsterile chow (Room 3) and a limited access facility where the animals were fed sterile chow (Room 4). Although the disease activity index (DAI) increased over time in both environments, DAI progressed at a faster rate in Room 3 than Room 4 (Fig. 1A). Consistent with the disease progression rates, Room 3 mice gained less weight (Fig. 1B) and had more severe histopathologically assessed damage in the colon (Fig. 1C,D). Examples of age-matched colons showed that mice in Room 3 had severe transmural inflammation and elongated crypts while mice in Room 4 did not exhibit these disease features (Fig. 1C,D). The mouse experiments were repeated once and designated Experiments 1 and 2.

Associations Between Bacterial rRNA Genes and Disease Activity

A bacterial OFRG analysis was performed on fecal samples collected throughout the development of colitis (Experiment 1 only). From the 9216 rRNA genes analyzed, 9 OTUs (or phylotypes) had both relatively large numbers of clones per phylotype (>64) and correlations ($P < 0.2$) with DAI (Table 1).

To further assess these associations, sequence-selective PCR analyses were performed on fecal samples collected throughout the experiments as well as on buffer washed and unwashed tissue samples, which were collected at the end of the experiments. Correlation analyses between the numbers of rRNA genes and DAI showed that all of the phylotypes had associations in one or more of the experiments or rooms (Fig. 2; Suppl. Tables 1–3). Two phylotypes (GpC1 *Ruminococcus schinkii* and Gp572 *Clostridium ramosum*) had positive associations between fecal rRNA gene levels and DAI in both experiments and rooms, and negative associations in a few intestinal compartments. Two phylotypes (*Lachnospiraceae* GpC2 and Gp66) exhibited negative associations between both fecal and tissue rRNA gene levels and DAI in both experiments and rooms. The *Lactobacillus* phylotype (Gp254) had positive associations in both experiments (P in Experiment 2 was 0.082) and Room 3 in the fecal analyses and negative associations in the pooled tissue and several specific tissue regions including the washed proximal colon. The two *Bacteroides* phylotypes (Gp244 and Gp156) had positive associations in Experiment 1 and Room 3 in the fecal analyses. Gp244 also exhibited negative associations in both buffer washed and unwashed duodenum samples while Gp156 had positive associations in the buffer washed proximal and distal ($r = 0.390$, $P = 0.089$) samples. Finally, the *Citrobacter* phylotype (Gp000) (identified by the culture-based analyses described below) had positive associations with the fecal samples in Experiment 2 and Room 3 and both positive and negative associations in different regions of the small intestine. Since there were only a few associations with the GpC3 and Gp76 phylotypes they were not included in the heat map figure. GpC3 had a negative association ($r = -0.501$, $P = 0.0245$) in the washed duodenum while Gp76 had a negative association in the fecal analysis ($r = -0.4652$, $P = 0.022$; from Room 4, Experiment 1) and a positive association in the unwashed jejunum samples ($r = -0.72583$, $P = 0.000$).

qPCR analyses performed on chow showed that 1 phylotype (GpC1) exhibited a positive association with time (Fig. 2). Although this result should be taken into consideration when evaluating the fecal and tissue associations for this phylotype, the relatively strong positive association in the washed distal colon ($r = 0.423$, $P = 0.063$) suggests that the GpC1 PCR signal is derived, at least in part, from bacteria inhabiting the intestine.

Associations Between Cultured Bacteria and Room Type or Disease Activity

Of the 55 bacterial species cultured and identified from the fecal samples, only a few were differentially isolated in Rooms 3 and 4 (Table 2). -Hemolytic Gram-positive cocci and *Pseudomonas* were found more often in Room 3. *Clostridium paraprutificum* and *Porphyromonas asaccharolytica* were more abundant in Room 4. *Actinomyces viscosus* and *Clostridium barati* were isolated from 4 and 5 separate animals from Room 4, respectively, and both were not isolated from Room 3.

When the numbers of bacteria isolated from mice with high (4–6) and low (0–3) DAI were compared, 7 taxa were significantly different (Table 3). -Hemolytic Gram-positive cocci and *Citrobacter amalonaticus* were more frequently found in mice with high DAI. *Prevotella melaninogenica/oralis* was isolated in 6 of the mice with high DAI, but was not found in mice with low DAI. *E. coli* was only found in 2 mice with high DAI. *Bifidobacterium* spp, *Pantoea* spp, and *Proteus mirabilis* were all more prevalent in mice with low DAI.

DISCUSSION

Changing technologies and concepts, particularly in the context of resident microbiota, open new approaches for proving disease causation.^{27,28} In IBD, identifying microbial population trends associated with intestinal damage and immunological parameters should lead to a greater understanding of the host–microbe interactions underlying the disease process. For example, if a specific bacterium is positively associated with colitis, this organism could represent either a pathogen that is causing the disease or one that simply thrives in the environment created by the disease. Conversely, if a bacterium is negatively associated with colitis, this organism could represent either a bacterium that inhibits disease activity or one that does not thrive in the diseased environment. Alternatively, given that IBD appears to involve aberrant immunological responses toward resident microbiota, negative associations could also represent bacteria that are being immunologically targeted. Toward the goal of better understanding the role of microorganisms in IBD, in this study we monitored the population densities of bacteria throughout the development of colitis in IL-10^{-/-} mice.

Two of the bacterial phylotypes (GpC2 and Gp66) identified in this study had both negative associations with disease activity in fecal and tissue samples and small-subunit rRNA genes with high sequence identity (99%) to an rRNA gene from a flagellated *Clostridium* (Lachnospiraceae bacterium A4, accession DQ789118). These negative associations with disease activity suggest that these bacteria were being immunologically targeted, which is consistent with prior studies that have shown that sera from CD patients and colitic mice react with the A4 flagella.²⁹ In addition, A4 flagella are related to the CBir1 flagellum, which is an antigen exhibiting considerable utility in CD serotyping.^{15,30,31} The fact that our study identified these bacteria provides strong evidence for the general utility of the experimental approach, and, more specifically, for its ability to identify bacteria involved in immunological responses.

Another interesting association was observed with the *Lactobacillus johnsonii* phylotype (Gp254). In IL-10^{-/-} mice, colitis is most pronounced in the proximal colon.^{32,33} In tissue samples from this region the strongest negative association detected by our experiments came from the *L. johnsonii* phylotype, which, again, suggests an immunological targeting. Because this phylotype had a weaker association in unwashed ($r = -0.275$, $P = 0.241$) than washed ($r = -0.502$, $P = 0.024$) tissue, this suggests that the targeting was occurring in or on the tissue or mucus layer. In addition, although negative associations were identified from the tissue samples, there was also a strong positive association from the fecal samples, indicating that if there was an immunological response toward this bacterium, it was not altering luminal populations. These results are consistent with investigations that showed

ulcerated and nonulcerated biopsies from UC patients had different *Lactobacillus* communities³⁴ and that UC subjects with active disease had lower levels of *Lactobacillus* in their colonic mucosa than those with inactive disease.³⁵

Our study also identified several other bacterial phylotypes whose population densities were significantly associated with disease activity. The *Ruminococcus schinkii* (GpC1) and *Clostridium ramosum* (Gp572) phylotypes exhibited positive associations in fecal samples and negative associations in certain regions of the tissue. These findings suggest that these bacteria are being immunologically targeted, but that the response is only effective in the tissue compartment. These results are also consistent with a study that showed serum reactivities to *C. ramosum* were higher in UC than control subjects.³⁶ In addition, as *Ruminococcus schinkii* is a member of the Lachnospiraceae, a taxon that contains the flagellated bacteria to which many Crohn's patients develop a serological response,^{15,29} the negative association observed with this phylotype indicates that it may be immunologically targeted. It is interesting to note that the association patterns exhibited by the flagellated *Clostridium* phylotypes (GpC2 and Gp66) were different in that negative associations were also observed in the fecal samples. This distinction may point toward varying immunological responses or differing abilities of the bacteria to evade the responses. This study also identified positive associations with the *Bacteroides vulgatus* phylotype (Gp156) in feces, washed proximal colon, and, to a lesser extent, washed distal colon. These findings are concordant with a study that showed mucosal *Bacteroides* biofilms were common features of IBD³⁷ and that tissue-associated *B. vulgatus* levels were higher in CD and UC subjects than in the controls.³⁸ It is also notable that a UC study found serum reactivities to *B. vulgatus*,³⁶ but that we did not observe negative associations with this phylotype, suggesting that a biofilm could be protecting the bacteria from immunological attack. These positive associations could also point toward organisms involved in disease causation.

Several of the bacteria identified in our culture-based analyses have also been previously associated with colitis. We found that *P. melaninogenica/oralis* (which belongs to the *Bacteroidetes*), *E. coli*, and *C. amalonaticus* were more frequently isolated in mice with high disease activity. This is consistent with a report that showed that *Bacteroides* were more abundant in tissue samples from IBD subjects.³⁷ For *E. coli*, higher levels of *E. coli* have been found in CD patients³⁹ and seroreactivity to an *E. coli* outer membrane protein (OmpC) is a useful marker for CD.¹⁶ Another member of the *Enterobacteriaceae* (*Citrobacter amalonaticus*), which was more prevalent in the mice with high disease activity, was further assessed using a sequence-selective qPCR assay targeting this organism. These analyses showed that *C. amalonaticus* (Gp000) had a positive association with disease activity in feces (Experiment 2) and negative associations in several tissue regions (Fig. 2).

Much of the microbiological data in the IBD literature, as well as those presented in this report, show trends that are consistent with the idea that these organisms are being immunologically targeted. Less stability in fecal bacterial community composition was observed in Crohn's subjects than in the controls.⁴⁰ Decreases in bacterial species richness and diversity have been observed in both feces^{40,41} and tissue.⁴² Reductions in specific taxa have also been observed, including tissue-associated *Clostridia* in CD,⁴³ mucosa-associated *Bacteroidetes* and *Enterobacteriaceae* in CD and UC,⁴² and fecal lactic acid producing bacteria in CD.⁴⁰ Finally, decreases in bacteria related to flagellated *Clostridia* associated with CD²⁹ have been shown in several reports.^{44–47} All of these results are consistent with the idea that IBD is associated with an immunological response to intestinal bacteria, resulting in decreases in specific taxa, bacterial community stability, richness, and diversity.

Datasets such as the one collected in this study could potentially 1) facilitate a greater understanding of disease etiology, 2) lead to the identification of biomarkers for disease

stratification, and 3) provide crucial knowledge directing the development of effective probiotics. Elucidating the population dynamics of intestinal microbiota in relation to immunological parameters, disease activity, and intestinal geography throughout the disease process should provide fundamental knowledge concerning the host–microbe interplay underlying disease etiology. For example, although the rRNA genes from our 2 flagellated *Clostridium* phylotypes (GpC2 and Gp66) have 98% sequence identity, they did not exhibit identical association patterns. In the duodenal samples, Gp66 exhibited a stronger negative association with disease activity than GpC2. These results are concordant with the idea that the type of bacteria, and the associated immune responses, are determining factors in disease etiology. In another example, when comparing the *Lactobacillus* (Gp254) and flagellated *Clostridium* (GpC2 and Gp66) phylotypes, although both showed negative associations in the tissue samples, they had opposite trends in the fecal samples (Gp254 positive, GpC2 and Gp66 negative). These kinds of data could provide important information concerning the types of immunological responses that are being mounted, and/or the ability of the bacteria to evade these responses. Concerning biomarker discovery, given that two of the associations identified in this study were highly similar to the flagellated *Clostridium*, which produces one of the more valuable CD seromarkers, this suggests that our other bacterial correlates could be useful biomarkers as well. Several studies have demonstrated the utility of serological assays for disease stratification.^{16,30,31,48} Identifying additional markers should facilitate improved diagnoses as well as predictions of future disease activity and response to therapy. Finally, this kind of data could provide knowledge directing the development of more effective probiotics. Thorough knowledge of bacterial community composition throughout disease development in each of the intestinal compartments should provide information to select for bacteria that do not provoke a strong immunological response but that fill a niche similar to those that do. For example, because our *Lactobacillus* phylotype has a negative association with disease activity in the proximal colon, this suggests that it may be immunologically targeted and therefore involved in disease etiology. Identifying other *Lactobacillus* strains that inhabit this region but do not provoke a strong immunological response (have smaller negative associations) would represent a rational strategy for identifying effective probiotics. Evidence supporting this concept includes what is known about *L. johnsonii*, as compared to other *Lactobacillus* species, it produced a greater IgA response⁴⁹ and was less likely to inhibit TNF secretion from macrophages.⁵⁰ In addition, in probiotic experiments, *L. johnsonii* was shown to be ineffective in inhibiting the recurrence of CD after ileocecal resection,⁵¹ whereas other *Lactobacillus* species, which likely fill a similar niche as *L. johnsonii*, have been shown to decrease colitis and cancer in IL-10 mouse models.^{52–54}

In sum, this study demonstrated the utility of using a population-based approach for identifying bacteria associated with the development of colitis in IL-10^{-/-} mice. The general principle of this approach is straightforward: organisms whose abundance correlates with a functional parameter may be involved in that function. Of course, such associations do not prove causation, but they do lead to the development of specific hypotheses that can be tested. For example, the discovery that *Helicobacter pylori* was frequently associated with gastric ulcer biopsies led to a series of follow-up investigations that not only determined that this bacterium caused gastric ulcers, but that it also was associated with the development of gastric cancer.^{55,56} This example and others point toward a model for future studies, where large-scale investigations to identify associations among microorganisms (and or their genes and gene products) and human physiological or disease processes will lead to the development and examination of hypotheses that address causality as well as a myriad of specific host–microbe interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Development of colitis in $IL-10^{-/-}$ mice housed in 2 different environments: a conventional mouse facility (Room 3) and a limited access facility (Room 4). A: Disease activity index (DAI) was monitored on a monthly basis. Data were pooled from 2 separate experiments. In all there were 9 mice raised in Room 3 and 8 in Room 4. The trend lines in the 2 rooms were different (multiple regression, $P = 0.001$). DAI was significantly lower in Room 4 than Room 3 at the indicated timepoints (*) (2-tailed Student's t -test, $P < 0.05$). B: Average monthly weight of mice was significantly higher in Room 4 than Room 3 at the indicated timepoints (*) (2-tailed Student's t -test, $P < 0.05$). C,D: Histopathology of the proximal colon from $IL-10^{-/-}$ mice. Sections were stained with H&E plus Alcian Blue, which stains

the goblet cells blue. C: Histology of the proximal colon of a mouse from Room 3 showing increased inflammatory infiltrate, crypt elongation, and decreased goblet cells. In panel C, I_1 = inflammatory cells on the outside of the serosa, I_2 = inflammatory cells in lamina propria (mucosa), I_3 = inflammatory cells in region below crypts (mucosa), I_4 = inflammatory cells in submucosa, C = elongated crypt, M = thickened muscularis externa. D: Histology of the proximal colon of a mouse from Room 4.

FIGURE 2.

Associations between bacterial rRNA genes and disease activity in IL-10^{-/-} mice. Mice were raised in 2 different environments: a conventional mouse facility (Room 3) and a limited access facility (Room 4). Two replicate experiments were performed (Experiments 1 and 2). rRNA gene levels were measured in monthly fecal samples and in tissue samples collected at the end of the experiments using sequence-selective qPCR assays for 8 phylotypes (horizontal axis, see Table 1 for more details on the phylotypes). Disease activity was monitored every month for 6 months. Correlation analyses were performed between rRNA gene levels and disease activity index values for the fecal and tissue analyses and between rRNA gene levels and time in the chow analyses. Associations with $P < 0.05$ are

shown as colored blocks (positive are red, negative are green). The brightness of the colors indicates the Pearson correlation coefficient (r) values (see scale bar at bottom). Suffixes indicate whether the tissue samples were washed in buffer before being analyzed: U (unwashed), W (washed). For the tissue analyses, “Pooled” is a combined analysis of all intestinal regions. $n = 56$ (Experiment 1, feces), 70 (Experiment 2, feces), 64 (Room 3, feces), 62 (Room 4, feces), 220 (Pooled), 20 (Duodenum-U), 20 (Duodenum-W), 20 (Jejunum-U), 20 (Jejunum-W), 20 (Ileum-U), 20 (Ileum-W), 10 (Cecum-U), 10 (Cecum-W), 20 (Proximal colon-U), 20 (Proximal colon-W), 20 (Distal colon-U), 20 (Distal colon-W), 7 (Room 3, chow), and 7 (Room 4, chow). The heat map was created using Java Tree View, v. 1.1.1.⁵⁷

TABLE 1

Bacterial Phylotypes Associated with Disease Activity in IL-10^{-/-} Mice

Phylotype Designation (Accession)	Nearest Cultured Relative (Accession) (%ID)	Nearest Uncultured Relative Accession (% ID)	Correlation Values	
			<i>r</i> ^a	<i>P</i> ^b
GpC1 (EU402471)	<i>Ruminococcus schinkii</i> (X94964) (95%)	EF403301 (99%)	0.699	0.002
Gp254 (EU402472)	<i>Lactobacillus johnsonii</i> (AB295648) (99%)	AM183093 (99%)	0.358	0.158
Gp572 (EU402473)	<i>Clostridium ramosum</i> (AY699288) (99%)	DQ804171 (99%)	0.487	0.048
Gp244 (EU402468)	<i>Bacteroides acidofaciens</i> (AB021158) (99%)	EF602859 (99%)	0.406	0.106
Gp156 (EU402469)	<i>Bacteroides vulgatus</i> (CP000139) (99%)	EF404383 (99%)	0.489	0.046
GpC2 (EU402474)	Lachnospiraceae bacterium A4 (DQ789118) (99%)	EF604543 (99%)	-0.666	0.004
Gp66 (EU402475)	Lachnospiraceae bacterium A4 (DQ789118) (99%)	EF604543 (99%)	-0.751	0.000
GpC3 (EU402470)	<i>Akkermansia muciniphila</i> (AY271254) (99%)	EF405092 (99%)	-0.592	0.012
Gp76 (EU402476)	<i>Barnesiella viscericola</i> (AB267809) (86%)	EF406573 (99%)	-0.357	0.159

Feces were collected throughout the development of colitis in IL-10^{-/-} mice. An rRNA gene analysis (OFRG) was performed on 56 fecal samples (~160 clones per sample). Associations between rRNA gene abundance and disease activity were examined using correlation analyses. This analysis was performed on Experiment 1 only.

^a*r* is the Pearson correlation coefficient.

^b*P* is the probability value.

TABLE 2

Analysis of Cultured Bacteria: Conventional (Room 3) vs. Limited Access (Room 4) Housing

Species	Room 3	Room 4	P
-hem G ⁺ cocci	25 (9)	11 (7)	0.027
<i>Pseudomonas</i>	17 (4)	3 (3)	0.003
<i>Actinomyces viscosus</i>	0 (0)	4 (4)	0.046
<i>Clostridium barati</i>	0 (0)	5 (5)	0.021
<i>Clostridium paraputrificum</i>	1 (1)	11 (8)	0.001
<i>Porphyromonas asaccharolytica</i>	4 (4)	12 (8)	0.029

Mice were raised in two different environments: a conventional mouse facility (Room 3) and a limited access facility (Room 4). Fecal material was collected from each mouse every month and analyzed for bacterial content. Bacteria were grown on selective medium and identified using standard procedures. The number of incidences of each species was tabulated based on housing condition. Fisher's exact test was used to determine whether the differences between the two rooms were significant. 55 separate species were identified; only species showing significant differences ($P < 0.05$) between the rooms are shown. $n = 9$ mice in Room 3 and $n = 8$ mice in Room 4. Numbers in parentheses represent the numbers of mice from which that species was isolated.

TABLE 3

Analysis of Cultured Bacteria: High vs. Low Disease Activity Index (DAI)

Species	High DAI	Low DAI	P
-hem G ⁺ cocci	23 (8)	13 (8)	0.018
<i>Citrobacter amalonaticus</i>	22 (5)	8 (3)	0.001
<i>Escherichia coli</i>	4 (2)	0 (0)	0.046
<i>Prevotella melaninogenica/oralis</i>	6 (6)	0 (0)	0.009
<i>Bifidobacterium</i> spp.	24 (8)	40 (9)	0.028
<i>Pantoea</i> spp.	4 (2)	13 (3)	0.040
<i>Proteus mirabilis</i>	12 (5)	25 (5)	0.047

Mice were raised in two different environments: a conventional mouse facility (Room 3) and a limited access facility (Room 4). Fecal material was collected from each mouse every month and analyzed for bacterial content. Bacteria were grown on selective medium and identified using standard procedures. The number of incidences of each species was tabulated based on the level of disease activity (DAI). Fisher's exact test was used to determine whether the differences between the two DAI levels. 55 separate species were identified; only species showing significant differences ($P < 0.05$) between the rooms are shown. $n = 8$ mice with High DAI and $n = 9$ mice with Low DAI. Numbers in parentheses represent the numbers of mice from which that species was isolated.