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Intestinal Bacteria Associated with Colitis and Inflammatory Bowel Disease

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

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in

Microbiology

by

Jingxiao Ye

December 2009

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Finally, I would like to acknowledge my family and friends who have been always supporting and encouraging me, with special thanks to my husband Liande Li and my daughter Yejia Li.

Dedicated to

My family and friends

ABSTRACT OF THE DISSERTATION

Intestinal Bacteria Associated with Colitis and
Inflammatory Bowel Disease

by

Jingxiao ye

Doctor of Philosophy, Graduate Program in Microbiology
University of California, Riverside, December 2009
James Borneman, Ph.D., Chairperson

Inflammatory bowel disease (IBD) etiology likely involves several factors including gut bacteria, immunological responses and genetic characteristics. Results from investigations with animal models and human subjects suggest that resident microbiota play an important role in the IBD. To better understand IBD etiology, in this dissertation, I attempted to identify associations between bacterial rRNA gene populations and disease activity and disease type.

Associations between several bacteria including *Lachnospiraceae* and *Clostridium ramosum* and colitis disease activity were identified in IL-10$^{-/-}$ mice. These associations, along with other results from the literature, led to the development of a model of IBD etiology that involved intracellular infections as a key element. Subsequent investigations of IL-10$^{-/-}$ mice with different genotypes provided several results supporting this new model. Investigations of human samples endeavored to identify bacteria associated with IBD disease type. A *Ruminococcus* phylotype, which is a member of Lachnospiraceae, was significantly reduced in IBD subjects. A *Faecalibacterium* phylotype was found to

be reduced in Crohn's disease but not in ulcerative colitis. This study also identified a new type of bacteria that exhibits differential associations with IBD, which is the mucin-degrading bacteria *Akkermansia muciniphila.*

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Chapter One: Introduction

Disorders of the immune system in the GI tract can lead to chronic inflammatory diseases such as inflammatory bowel disease (IBD). IBD is a group of chronic relapsing inflammatory disorders of the intestinal mucosa that includes Crohn's disease (CD) and ulcerative colitis (UC) (Podolsky 2002). These two primary forms of IBD can be characterized by a variety of parameters including the region of the intestine that is affected, the type of tissue damage as well as the type of immune response. UC occurs primarily in the colon. CD can occur anywhere in the GI tract, but often occurs in the ileum. Histologically, intestinal damage in CD is more transmural and ulceration and crypt abscesses are less obvious, while the acute phase of UC is characterized by crypt abscesses and ulceration that can move into the lamina propria. Precise categorization can be a challenge because of the considerable overlap in the presentation of IBD subtypes.

The prevalence of IBD has increased in Europe and North America in recent years. Incidence of IBD in North America and Europe is approximately 1.4 million and 2.2 million people, respectively, which is higher than that in Asia and Africa (Loftus 2004). Common symptoms of Crohn's disease and UC include abdominal pain and diarrhea. CD patients more commonly loose weight while UC

patients more commonly exhibit symptoms of arthropathy (Beattie, Croft et al. 2006).

Crohn's disease is an immune system disease that is mediated by the Th1 immune pathway. Proinflammatory molecules such as IL-1β, TNF, IL-6, and IL-8 are increased in active Crohn's disease (Sartor 2006). Crohn's disease is also often characterized by altered T-cell profiles and increased levels of IFN-r, IL-17 and IL-23 (Fujino, Kawasaki et al. 2003; Schmidt, Giese et al. 2005). On the other hand, ulcerative colitis is a Th2 mediated immune disease, characterized by increases in IL-1β, TNF, IL-5 and IL-13 (Fuss, Neurath et al. 1996). Although the cause of IBD is not yet known, the literature points toward a multifactor etiology involving genetics, microorganisms, and other environmental factors.

In twin studies, there is a 50% rate of coincidence in CD while only 10% in UC (Halfvarson, Bodin et al. 2003), suggesting a familial relationship in CD (Halfvarson, Bodin et al. 2003). Genes including *NOD2*, *IL23R*, *SLC22A4/5*, *DLG5*, and *MDR1* have been reported to be associated with CD (Hugot, Chamaillard et al. 2001; Duerr, Taylor et al. 2006; Sartor 2006). Of these genes, *NOD2* has received the greatest attention. *CARD15/NOD2* encodes a protein that binds the muramyl dipeptide of peptidoglycan (MDP) in bacterial cell walls. NOD2 mediates immune responses by regulating the activation of nuclear factor NF-κB. Mutations of this gene (leucine-rich region) have been studied and

possible mechanisms have been proposed (Ismail and Hooper 2005; Sartor 2006). Ileal CD patients that have a genetic defect in *CARD15* had lower levels of human defensin 5v(HD5) which is an antimicrobial molecule produced by Paneth cells (Wehkamp, Salzman et al. 2005). Reductions in the amounts of defensins could cause changes in bacterial composition and therefore influence IBD pathology. However, only 25-35% of Crohn's disease patients in Europe have a *NOD2* mutation (Newman and Siminovitch 2003), suggesting that other factors are involved in the disease. *SLC22A4* and *SLC22A5* encode organic cation transporters OCTN1 and OCTN2. Mutations in these two genes have been associated with Crohn's disease with the *CARD15* mutation (Peltekova, Wintle et al. 2004). Similar to OCTN1 and OCTN2, the *DLG5* gene, which encodes a protein related to epithelial integrity, was associated with CD with *CARD15* mutations (Stoll, Corneliussen et al. 2004). The *MDR1* gene, which encodes P-glycoprotein 170, has been associated with both UC and CD (Farrell, Murphy et al. 2000). *IL23R*, which encodes a subunit of the IL-23 cytokine receptor, was also recently identified as a potential IBD susceptibility gene. Although genetic factors likely play a role in CD pathogenesis, the disconcordance rate in the monozygotic twin studies suggest other factors may be involved, including food, lifestyle and intestinal microbes.

Environmental factors have been implicated in the pathogenesis of both Crohn's disease and ulcerative colitis. Smoking causes opposite effects in UC

and CD (Johnson, Cosnes et al. 2005). Smokers have almost twice the risk of developing CD, along with a more severe course of CD, than non-smokers. Recurrence is also more frequent in smokers with CD after surgery. However, smoking appears to be beneficial in UC patients, but the mechanism for this is not clear (Johnson, Cosnes et al. 2005). Food may also be a factor in CD, which may explain the incidence difference between western and eastern countries. High consumption of sugar loaded foods such as chocolate and soft drinks may be a risk factor for CD (Seibold 2005), and margarine may also be involved in CD (Sonnenberg 1988). Stress may also be another factor that plays a role in IBD (Meddings and Swain 2000). Finally, microorganisms appear to play an important role in disease etiology.

Microbes in the gastrointestinal tract (GI tract)

The human GI tract harbors a diverse array of microorganisms including members of the Archaea, Eukaryota and Bacteria. Of these, bacteria have been studied the most. It has been estimated that there are around 10^{14} bacteria in the human GI tract, which is about 10 to 20 times greater than the total number of the eukaryotic cells in the human body. In a recent high throughput study examining rRNA gene sequences, 395 different bacterial phylotypes were identified (Eckburg, Bik et al. 2005). Ninety three percent of those phylotypes were Firmicutes and Cytophaga-Flavobacterium-Bacteroides (CFB). The majority of Firmicutes fell into two main groups: Clostridium rRNA subcluster XIVa and

Clostridium rRNA subcluster IV. Clostridium subcluster XIVa contains a large number of uncultured bacterium species. Clostridium subcluster IV contains a high number of butyrate-producing fibrolytic bacteria such as *Faecalibacteria prausnitzii.* There is significant individual-to-individual variation in the gut microbiota composition. In addition, the prevalence and diversity of bacteria in different areas of the GI tract is also altered. For example, the microbiota in jejunum is very different from that in the distal ileum, ascending colon and rectum. Finally, the distal intestine contains the highest bacterial cell density (Zoetendal, von Wright et al. 2002; Wang, Heazlewood et al. 2003).

Functions of the intestinal microbiota

Bacteria colonizing the intestinal tract likely include commensal and pathogenic bacteria. Commensal bacteria likely live in a symbiotic state with the host and provide benefits to the host, such as preventing the entry of pathogens. Bacteria such as Firmicutes ferment polysaccharides into short-chain fatty acids, which is an energy source for intestinal host cells that also appears to contribute to colonic homeostasis through regulation of host genes (Hooper, Midtvedt et al. 2002; Daly, Cuff et al. 2005).

The intestinal epithelium provides a physical barrier preventing bacteria and bacterial products from entering host tissue. Specialized cells within the epithelium, such as goblet and paneth cells, which produce mucins and

antimicrobial peptides, function to trap and selectively kill pathogens. Interaction between intestinal epithelial cells and bacteria play a major role in the immunomodulation of the mucosal immune system. Specific cell receptors known as Toll-like receptors (TLRs) are important components of the host microbe recognition system. TLR2, TLR4 and TLR5 detect pathogen-associated molecular patterns (PAMPs) such as LPS, lipoteichoic acid and bacterial flagellin, respectively (Sartor 2006). The recognition of specific microbial components by TLRs triggers both innate and adaptive immune responses that eliminate pathogens and shape the intestinal microbiota. Among these responses is the production of proinflammatory cytokines and chemokines, which is also increased in IBD.

The role of gut bacteria in IBD is currently unclear. It is not known whether IBD etiology involves a specific pathogen, a normal immune response to disordered commensal bacteria, a disordered immune response to the normal commensal microbiota, or other.

Pathogens

Several bacteria have been implicated in the etiology of CD, including *Mycobacterium avium paratuberculosis* (MAP) and *Escherichia coli*. MAP was suggested to be the microorganism that causes CD after it was isolated from a sample of CD tissue (Martin and Rhodes 2000). MAP has been cultured from

blood of both CD and UC patients, but not from healthy controls (Naser, Ghobrial et al. 2004; Sechi, Scanu et al. 2005). However, other studies have not found MAP to be more abundant in CD patients than normal controls (Ellingson, Cheville et al. 2003; Baksh, Finkelstein et al. 2004). In addition, the immunosuppressive therapies which should exacerbate a mycobacterial infection, ameliorate symptoms in CD patients. It therefore may be more likely that the colonization of MAP is the result of the inflammation rather than its cause.

Escherichia coli has also been implicated as the pathogen of CD. Recovery of a virulent *E. coli* strain from mucosal biopsies of CD patients, exhibiting increased mucosal adherence compared to controls, have supported the concept of it being a pathogen causing CD (Darfeuille-Michaud, Boudeau et al. 2004; Sokol, Seksik et al. 2006). Increased abundance of *E. coli* was found in CD patients when compared to their healthy twins (Willing, Halfvarson et al. 2009). Adherent invasive *E. coli* was found to often colonize ileal lesions and its numbers are abnormally high in ileal CD, which indicates that it may be specifically associated with ileal CD (Darfeuille-Michaud, Boudeau et al. 2004). However, it is not clear whether the presence of *E. coli* is the result of the inflammation or the cause. Other bacteria such as *Helicobacter pylori* have also been reported to be associated with CD (Martin and Rhodes 2000).

Dysbiosis

Shifts in the normal composition of commensal bacteria have also been associated with IBD. Biopsy analyses showed that concentrations of mucosal bacteria in IBD patients were 100 times higher than those in healthy controls, with Bacteroides being a dominant taxon (Swidsinski, Weber et al. 2005). Another study showed that there were no differences in bacterial composition between ulcerated and nonulcerated areas (Seksik, Lepage et al. 2005). Similar results were obtained in UC patients, where there were no significant differences in the mucosa-associated bacteria between injured and healthy samples (Sokol, Lepage et al. 2007).

Reductions in the numbers of the mucosa-associated bacteria, including *Bacteroides*, *Eubacterium* and *Lactobacillus* were also found when comparing CD patients to normal controls (Ott, Musfeldt et al. 2004). Notably members of the phyla Firmicutes and Bacteroides were depleted in CD and UC patients (Frank, St Amand et al. 2007). A decrease in the abundance of a major member of the Firmicutes, *Faecalibacterium prausnitzii*, was also found associated with the recurrence of ileal CD (Sokol, Pigneur et al. 2008). In a study by Swidsinski and colleagues, *Faecalibacterium prausnitzii* numbers were lower in CD patients than UC and healthy controls (Swidsinski, Loening-Baucke et al. 2008). Finally, lower levels of this organism were observed in twin studies, which revealed that those with predominantly ileal CD had a dramatically lower abundance of

Faecalibacterium prausnitzii compared to healthy co-twins and those with CD localized in the colon (Willing, Halfvarson et al. 2009).

In clinical studies, antibiotics have been shown to have a beneficial effect in some instances of Crohn's disease. These antibiotics included metronidazole, ciprofloxacin or a combination of the two (Bamias, Marini et al. 2002). Besides antibiotics, probiotics might also be a potential treatment for IBD.

Mouse models of IBD

Several different kinds of mouse models have been used to examine IBD like diseases. One mouse model of Crohn's disease is the interleukin-10 knock-out (IL-$10^{-/-}$). IL-10 is an anti-inflammatory cytokine with diverse effects on most hemopoietic cell types. The principal function of IL-10 appears to be to limit and ultimately terminate inflammatory responses (Moore, O'Garra et al. 1993). It inhibits many steps of the pathway of antimicrobial immunity, including inhibiting activation and effector function of T cells, monocytes and macrophages. The inhibitory effects of IL-10 on IL-1 and TNF production are crucial to its anti-inflammatory activities. IL-$10^{-/-}$ mice develop colitis gradually after birth, which mimics Crohn's disease in humans. One of the similarities between colitis in this animal model and CD in humans is that both of them are Th1 mediated. Another similarity is that the intestinal damage in IL-10 KO mice is similar to Crohn's disease.

Experiments have been done to show that microorganisms play important roles in colitis development in this mouse model. IL-10$^{-/-}$ mice do not develop colitis when raised in "germ-free" conditions. Yet these mice develop enterocolitis when maintained in conventional conditions and they develop colitis when kept in specific-pathogen-free conditions (Sellon, Tonkonogy et al. 1998).

In addition, mice inoculated with different enteric bacteria develop variable phenotypes of colitis. *Enterococcus faecalis*-monoassociated IL-10$^{-/-}$ mice developed distal colitis at 10-12 weeks; colitis was also more severe and associated with duodenal inflammation and obstruction by 30 weeks, which did not occur in wild-type mice. *E. coli*-monoassociation induced mild cecal inflammation in IL-10$^{-/-}$ mice at 3 weeks, which was distinct from the phenotype caused by *E. faecalis* (Kim 2005). Klebsiella-mono-associated IL-10$^{-/-}$ mice induced moderate pancolitis and *Bifidobacterium animalis* caused distal colonic and duodenal inflammation. However, when IL-10$^{-/-}$ mice were mono-associated with other bacteria (*Bacteroides vulgatus*, *Clostridium sordellii*, or Viridans group *Streptococcus*), colitis was not observed (Sydora, Tavernini et al. 2005). In sum, different bacteria appear to have different effects on the development of colitis, and the exact mechanisms need to be further investigated.

HLA-B27 transgenic (TG) rats are another animal model of IBD. They develop colitis, gastritis, and systemic inflammation in a specific pathogen free (SPF) environment. *Bacteroides vulgatus* has been shown to induce colonic inflammation in HLA-B27 TG rats, while *E. faecalis* and *E. coli* did not.

Approaches to identify microorganism associated with IBD

Since many microorganisms are not readily cultured, molecular techniques such as 16S rRNA gene sequencing, qPCR, DGGE, TTGE and RFLP have been used to identify the composition of the microorganisms in the gut associated with IBD.

16S rRNA gene sequencing has been widely used to analyze bacteria in the gut. In the first high throughput molecular study, 13,000 sequences of the 16S rRNA gene were obtained by Relman's group (Eckburg, Bik et al. 2005). In IBD, over 15,000 16S rRNA genes were examined to compare the microbial composition between CD and UC patients and non-IBD controls; here, significant reductions of Firmicutes and Bacteroides were discovered in IBD patients (Frank, St Amand et al. 2007).

Denaturing gradient gel electrophoresis (DGGE) was used to study the bacterial community of feces and biopsy samples in different regions of the GI tract. This work found that the tissue-associated bacteria were uniformly

distributed along the colon and were different from the fecal bacteria (Zoetendal, von Wright et al. 2002). Temporal temperature gradient gel electrophoresis (TTGE) of 16S rRNA genes was used to evaluate the dominant species in the colon of IBD patients. No significant differences between the ulcerative and non-ulcerative areas of the patients were found (Seksik, Rigottier-Gois et al. 2003; Sokol, Lepage et al. 2007).

Metagenomics were recently used to analyze the microbiota of the GI tract and the microbes associated with IBD. Around 78 million base pairs of unique DNA sequence of human intestinal microbiota were analyzed from two healthy human fecal samples (Gill, Pop et al. 2006). Metagenomics provides a broader view of the microbiota and their potential activities. A metagenomics analysis of 6 healthy and CD patients also revealed reduced diversity of fecal microbiota in CD compared to healthy subjects (Manichanh, Rigottier-Gois et al. 2006).

In this dissertation, toward a better understanding of IBD, I attempted to identify bacteria associated with colitis and disease activity and IBD subtypes.

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Chapter Two: Bacteria and bacterial rRNA genes associated with the development of colitis in IL-10$^{-/-}$ mice

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 two environments: a community mouse facility where the mice were fed non-sterile chow (Room 3) and a limited access facility where the mice were fed sterile chow (Room 4). Every month, 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 negative associations with disease activity in fecal and tissue samples. Notably, there was high sequence identity (99%) of their small-subunit rRNA genes with a previously described flagellated Clostridium (Lachnospiraceae bacterium A4). **Conclusions**: The negative associations of these two 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.

Introduction:

Microorganisms appear to play important yet ill-defined roles in the etiologies of Crohn's disease (CD) and ulcerative colitis (UC) (Sartor 2006; Eckburg and Relman 2007; Xavier and Podolsky 2007). CD and UC occur in regions of the intestine where enteric bacteria are found in the highest concentrations (Rath 2003). Contact with intestinal contents triggers mucosal inflammation in Crohn's disease while diversion of the fecal stream promotes intestinal healing (Rutgeerts, Goboes et al. 1991; D'Haens, Geboes et al. 1998). 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 (Sartor 2004).

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 upon the bacterial species (Kim 2005; Kim, Tonkonogy et al. 2007).

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In addition, various antibiotics, possessing different taxonomic targets, have been shown to exhibit varying abilities to prevent and treat colitis in HLA-B27 rats and IL-10$^{-/-}$ mice (Rath 2002; Hoentjen, Harmsen et al. 2003). In human studies, evidence for the involvement of specific bacteria includes the differing abilities of various antibiotic therapies to induce disease remission (Sutherland, Singleton et al. 1991; Arnold, Beaves et al. 2002; Sartor 2004). 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 mice and the ability to utilize seroreactivity to specific microbial antigens for disease stratification (Lodes, Cong et al. 2004; Braun and Targan 2006) .

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 (Borneman, Becker et al. 2007; Borneman and Becker 2007). 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 two 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$^{-/-}$) mice (Bristol, Farmer et al. 2000) were bred in a room (Room 4) with limited access, isolated from other mice and fed radiation-sterilized chow (Purina LabDiet 5053) (Lytle, Tod et al. 2005; Lee, Bajwa et al. 2007). Beginning at around 5 weeks of age, mice were separated into two groups. One group was housed in a conventional community mouse facility where the animals were fed non-sterile chow (Purina LabDiet 5001) (Room 3). The other group was maintained on sterile chow in the aforementioned Room 4. Mice in the two groups were paired according to sex, age, weight, and initial disease activity index (DAI). DAI scores (0-6) are the sum of four individual parameters: fur appearance, length of prolapsed anus, stool consistency and

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presence of occult bleeding (Lytle, Tod et al. 2005). DAI measurements were performed monthly for all mice. Experiments were terminated after 6 months. At this time, all surviving animals were euthanized 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 (Lytle, Tod et al. 2005; Lee, Bajwa et al. 2007). 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 two 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, two 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 three times (tissue samples were placed in microcentrifuge tubes with 0.5 ml buffer and gently vortex mixed for 30 seconds; this procedure was repeated two more times using new tubes with fresh buffer). One 0.5 cm piece of the proximal colon was collected for histology (Lytle, Tod et al. 2005; Lee, Bajwa et al. 2007).

DNA Extraction from Fecal and Tissue Samples

Extractions were performed using the FastDNA Spin Kit as described by the manufacturer, with a 30 s 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 3kb 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 described (Bent, Yin et al. 2006) 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. PCR amplifications were performed using the HPLC-purified bacterial rRNA gene primers BacOFRGpUSER, GGAGACAUAGRRTTTGATYHTGGYTCAG, and BacrOFRGpUSER, GGGAAAGUGBTACCTTGTTACGACTT. Thermal cycling parameters were 94°C for 2 min; 30 cycles of 94°C for 20s, 48°C for 30s, and 72°C for 90s; followed by 72°C for 10 min. Amplification reactions were performed using the PicoMaxx High Fidelity PCR System (Stratagene, LaJolla, CA). PCR products were cloned into pNEB 205 using the USER Friendly Cloning Kit (New England Biolabs, Beverly, MA).

OFRG Array Construction

Sixty 11 x 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, CATTCAGTTG, GTCTCAGTTC, AGGCTAGAGT, CAACCCTTGT, GCGTGAGTGA, GGTGCAAGCG, AGCTAGTTGG, AATACCGGAT, TGTGGGAGGG, CCCGCACAAG, CGGTACAGAG, ACCAAGGCAA,TTGCCAGCGG, ACCGCGAGGT, GCCTTCGGGT, TTGCCAGCAT, GCTAACGCGT, ACGGTACCTG, GCCACACTGG, GGCCGCAAGG and GCAAGTCGAG. The reference probe was GCTGCTGGCA (Valinsky, Della Vedova et al. 2002). Arrays were washed twice in 1X SSC for 30 min at 11°C.

Data analysis

A UPGMA dendogram of the OFRG fingerprints was constructed using GCPAT (Figueroa, Borneman et al. 2004). 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 Inc., Hercules, CA) using iCycler iQ PCR Plates with Optical Flat 8-Cap Strips (Bio-Rad Laboratories Inc). Twenty-five μl reaction mixtures contained the following reagents: 50 mM Tris (pH 8.3), 500 μg/ml BSA, 2.5 mM MgCl2, 250 μM of each dNTP, 400 nM of each primer, 1 μl of template DNA, 2 μl of 10X 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, GTCCGCCACTAACTCATAC, 51 bp); Gp76, (IL10Gp76F11, CAGTTACCAGCAAGTCAA, IL10Gp76R11, GCCGCATTGCTTCTCT, 147 bp); Gp156, (IL10Gp156F5, ATGAATTACGGTGAAAGCCG, IL10Gp156R9, ATCTTACGATGGCAGTCTTGT, 169 bp); Gp244, (GP244F1, TAAAGAATTTCGGTATGGGA, GP244R1, TTACCCCGCCAACTAA, 55 bp); Gp254, (IL10GP254F3, TGCTTGCACTAAATGAAACT, IL10GP254R2, GTTACTCACCCGTCCG, 52 bp); Gp572, (IL10GP572F5,

GTGCTCGAGTGGCG, IL10GP572R5, CCAATAGTTATCCCCCTGT, 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 (i) 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 (ii) using the

recently developed PRISE software (Fu, Ruegger et al. 2008). The thermal

cycling conditions were 94°C for 5 min; 40 cycles of 94°C for 20 s, X^{o}C for 30 s,

and 72°C for 30 s; followed by 72°C for 10 min; 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 two clones were determined; these

experiments confirmed that the target sequences were being amplified (data not

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shown). The qPCR analysis 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 µl) 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 and Company, Sparks, MD) plates containing 5% sheep red blood cells and incubated at 37°C in humidified incubators with 5% CO_2, or grown at 37°C in anaerobic jars, using AnaeroGen (Oxoid Ltd, Basingstoke, UK). Samples were also plated on MacConkey agar (Becton, Dickinson and Company) plates and incubated at 37°C. After 24, 48 or 72 h 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 two means was evaluated using two-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 v3.1 Cycle Sequencing Kit and an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). Sequence identities were determined using BLAST (NCBI) (Altschul, Madden et al. 1997).

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 two different environments: a conventional community mouse facility where the animals were fed non-sterile chow (Room 3) and a limited access facility where the animals were fed sterile chow (Room 4). Although disease activity index (DAI) increased over time in both environments, DAI progressed at a faster rate in Room 3 than Room 4 (Figure 2.1A). Consistent with the disease progression rates, Room 3 mice gained less weight (Figure 2.1B) and had more severe histopathologically assessed damage in the colon (Figures 2.1C-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 (Figures 2.1C-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 9,216 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 2.1).

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 (Figure 2.2; Tables 2.2, 2.3, 2.4). 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 (*Lachospiraceae* 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 large intestine. 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) large intestine 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

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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 one phylotype (GpC1) exhibited a positive association with time (Figure 2.2). 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 large intestine (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 2.5). β-hemolytic gram-positive cocci, *Clostridium beijerinckii/butyricum*, and Pseudomonas were found more often in Room 3. *Clostridium paraputrificum* and *Porphyromonas asaccharolytica* were more abundant in Room 4. *Actinomyces viscosus* and *Clostridium barati* were isolated from four and five 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 2.6). β-hemolytic gram-positive cocci and *Citrobacter amalonaticus* were more frequently found in

mice with high DAI. *Prevotella melaninogenica/oralis* was isolated in six of the mice with high DAI, but was not found in mice with low DAI. *E. coli* was only found in two 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 (Fredericks and Relman 1996; Inglis 2007). 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 towards resident microbiota, negative associations could also represent bacteria that are being immunologically targeted. Towards 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 (Duck, Walter et al. 2007). In addition, A4 flagella are related to the CBir1 flagellum, which is an antigen exhibiting considerable utility in Crohn's disease serotyping (Lodes, Cong et al. 2004; Targan, Landers et al. 2005; Papadakis, Yang et al. 2007). 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 (Kuhn, Lohler et al. 1993; Berg, Davidson et al. 1996). 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 towards this bacterium, it was not altering lumenal populations. These results are consistent with investigations that showed ulcerated and non-ulcerated biopsies from UC patients had different *Lactobacillus* communities and that UC subjects with active disease had lower levels of *Lactobacillus* in their colonic mucosa than those with inactive disease (Fabia, Ar'Rajab et al. 1993; Zhang, Liu et al. 2007).

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 (Matsuda, Fujiyama et al. 2000). In addition, as *Ruminococcus schinkii* is a member of the Lachnospiracaea, a taxon that contains the flagellated bacteria to which many Crohn's patients develop a serological response (Lodes, Cong et al. 2004; Duck, Walter et al. 2007), 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 towards 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 large intestine and, to a lesser extent, washed distal large intestine. These findings are concordant with a study that showed mucosal *Bacteroides* biofilms were common features of IBD and that tissue-associated *B. vulgatus* levels were higher in CD and UC subjects than in the controls (Fujita, Eishi et al. 2002; Swidsinski, Weber et al. 2005). It is also interesting that a UC study found serum reactivities to *B. vulgatus* (Matsuda, Fujiyama et al. 2000), 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 towards 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 (Swidsinski, Weber et al. 2005). For *E. coli*, higher levels of *E. coli* have been found in Crohn's disease patients and seroreactivity to an *E. coli* outer membrane protein (OmpC) is a useful marker for CD (Braun and Targan 2006;

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Sasaki, Sitaraman et al. 2007). Another member of the *Enterobacteriaceae* (*Citrobacter amaonaticus*), 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 (Figure 2.2).

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 (Scanlan, Shanahan et al. 2006). Decreases in bacterial species richness and diversity have been observed in both feces and tissue (Ott, Musfeldt et al. 2004; Scanlan, Shanahan et al. 2006; Sokol, Lepage et al. 2006). Reductions in specific taxa have also been observed, including tissue-associated *Clostridia* in CD (Gophna, Sommerfeld et al. 2006), mucosa-associated *Bacteroidetes* and *Enterobacteriaceae* in CD and UC (Ott, Musfeldt et al. 2004), and fecal lactic acid producing bacteria in CD (Scanlan, Shanahan et al. 2006). Finally, decreases in bacteria related to flagellated *Clostridia* associated with Crohn's disease have been shown in several reports (Manichanh, Rigottier-Gois et al. 2006; Sokol, Seksik et al. 2006; Baumgart, Dogan et al. 2007; Duck, Walter et al. 2007; Frank, St Amand et al. 2007). 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.

Data sets such as the one collected in this study could potentially (i) facilitate a greater understanding of disease etiology, (ii) lead to the identification of biomarkers for disease stratification and (iii) 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 two 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 (Arnott, Landers et al. 2004; Targan, Landers et al. 2005; Braun and Targan 2006; Papadakis, Yang et al. 2007). 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 large intestine, 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 rationale 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 response and was less likely to inhibit TNF secretion from macrophages (Ibnou-Zekri, Blum et al. 2003; Pena, Li et al. 2004). In addition, in probiotic experiments, *L. johnsonii* was shown to be ineffective in

inhibiting the recurrence of Crohn's disease after ileocaecal resection 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 (O'Mahony, Feeney et al. 2001; McCarthy, O'Mahony et al. 2003; Pena, Rogers et al. 2005; Van Gossum, Dewit et al. 2007).

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 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 (Marshall and Warren 1984; Suzuki, Hibi et al. 2007). This example and others point towards 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.
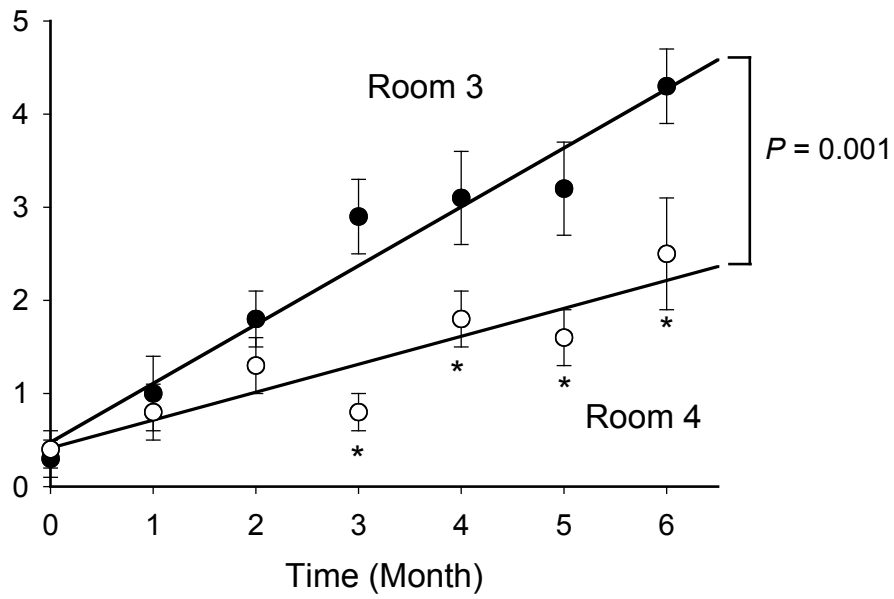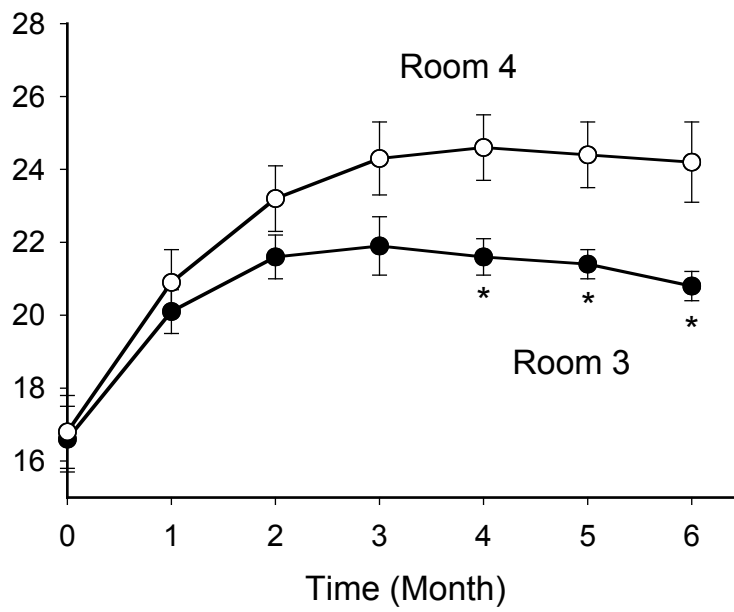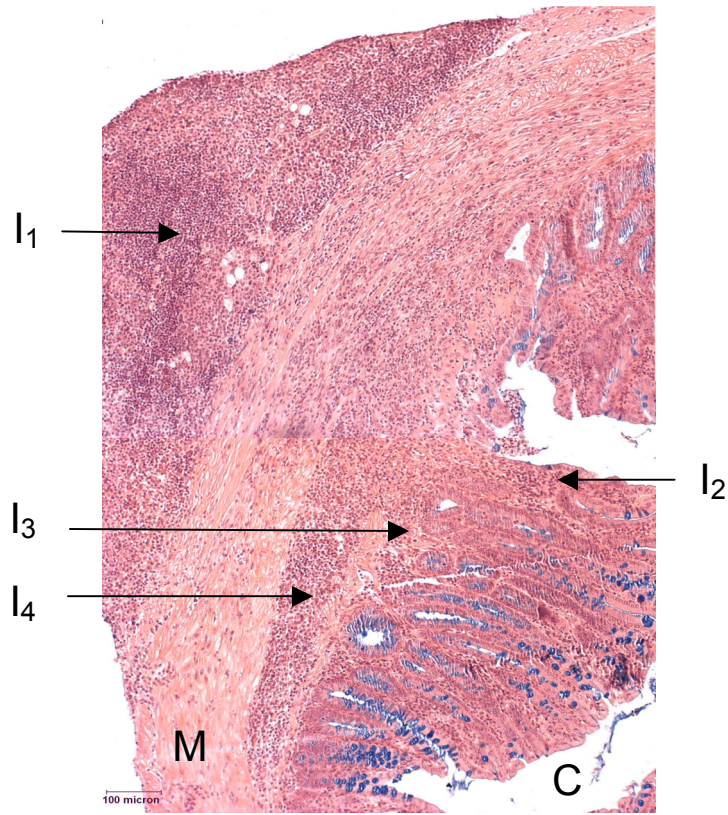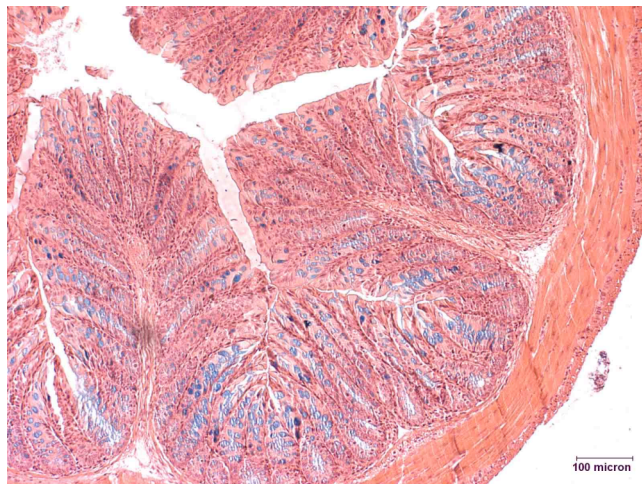
Figure 2.1A

Figure 2.1B

Figure 2.1 C

Figure 2.1 D

Figure 2.1. Development of colitis in IL-10$^{-/-}$ mice housed in two 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 two rooms were different (multiple regression, P = 0.001). DAI was significantly lower in Room 4 than Room 3 at the indicated time points (*) (two-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 time points (*) (two-tailed Student's t-tests, P < 0.05). **C.** and **D.** Histopathology of the proximal colon from IL-10$^{-/-}$ mice. Sections were stained with hematoxylin and eosin (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 (these experiments were performed by Jimmy Lee and Dr. Daniel Straus).
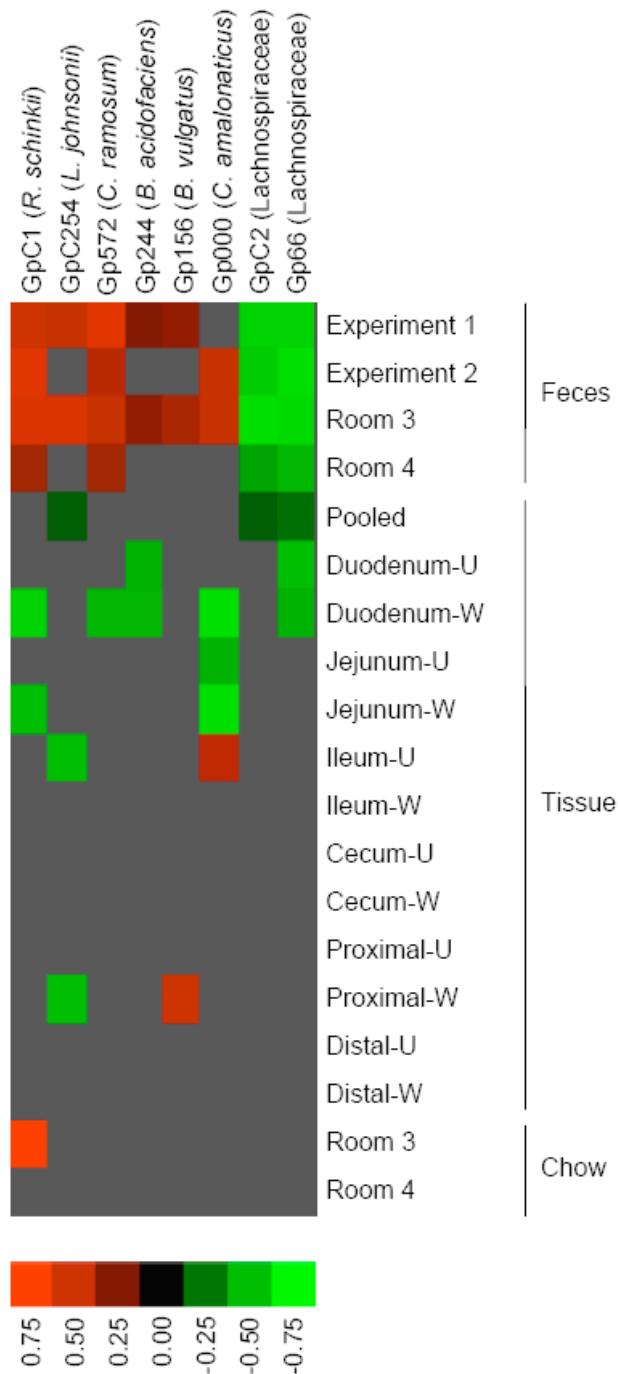
Figure 2.2. Associations between bacterial rRNA genes and disease activity in IL-10^{-/-} mice. Mice were raised in two different environments: a conventional mouse facility (Room 3) and a limited access facility (Room 4). Two replicate experiments were performed (Experiments 1 and 2). rRNA gene levels 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-U), 20 (Proximal-W), 20 (Distal-U), 20 (Distal-W), 7 (Room 3, chow), and 7 (Room 4, chow). The heat map was created using Java Tree View, version 1.1.1(Saldanha 2004).

Table 2.1. 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	
			r [a]	P [b]
GpC1 (EU402471)	*Ruminococcus schinkii* (X94964) (95%)	EF403301 (99%)	0.699	0.002
Gp254 (EU402472)	*Lactobacillus johnsonii* (AB295648) (99%)	AM183093 (99%)	0.358	0.158
Gp572 (EU402473)	*Clostridium ramosum* (AY699288) (99%)	DQ804171 (99%)	0.487	0.048
Gp244 (EU402468)	*Bacteroides acidofaciens* (AB021158) (99%)	EF602859 (99%)	0.406	0.106
Gp156 (EU402469)	*Bacteroides vulgatus* (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)	*Akkermansia muciniphila* (AY271254) (99%)	EF405092 (99%)	-0.592	0.012
Gp76 (EU402476)	*Barnesiella viscericola* (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 levels 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 2.2. Associations between bacterial rRNA genes in feces with disease activity or bacterial rRNA genes with time in IL-10$^{-/-}$ mice.

	Feces[a]								Chow[b]			
	Experiment 1		Experiment 2		Room 3		Room 4		Room 3		Room 4	
Phylotype	r	P	r	P	r	P	r	P	r	P	r	P
GpC1 (*R. schinkii*)	0.493	0.000	0.590	0.000	0.570	0.000	0.357	0.005	0.783	0.037	0.479	0.277
Gp254 (*L. johnsonii*)	0.479	0.000	0.209	0.082	0.563	0.000	0.000	0.997	0.217	0.640	0.687	0.088
Gp572 (*C. ramosum*)	0.585	0.000	0.438	0.000	0.474	0.000	0.364	0.004	0.349	0.443	-0.256	0.579
Gp244 (*B. acidofaciens*)	0.258	0.000	0.052	0.672	0.302	0.015	0.094	0.472	0.235	0.612	0.311	0.497
Gp156 (*B. vulgatus*)	0.302	0.029	0.122	0.361	0.375	0.005	-0.004	0.978	-0.612	0.144	0.612	0.144
Gp000 (*C. amalonaticus*)	0.060	0.661	0.476	0.000	0.478	0.000	0.101	0.436	-0.076	0.871	ND	ND
GpC2 (Lachnospiraceae)	-0.593	0.000	-0.543	0.000	-0.642	0.000	-0.387	0.004	-0.268	0.561	-0.233	0.614
Gp66 (Lachnospiraceae)	-0.583	0.000	-0.628	0.000	-0.624	0.000	-0.471	0.000	-0.322	0.482	0.470	0.287

Mice were raised in two different environments: a conventional mouse facility (Room 3) and a limited access facility (Room 4). Two replicate experiments were performed (Experiment 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 (see Table 1 for more details on the phylotypes). Disease activity was monitored every month for 6 months.
[a]Values are from correlation analyses between levels of bacterial rRNA genes in feces (collected every month) and DAI (r is the Pearson correlation coefficient, P is the probability value).
[b]Values are from correlation analyses between levels of bacterial rRNA genes in chow (collected every month) and time (r is the Pearson correlation coefficient, P is the probability value).
ND = not detected.
n for fecal analyses = 56 (Experiment 1), 70 (Experiment 2), 64 (Room 3) and 62 (Room 4).
n for chow analyses = 7 (Room 3) and 7 (Room 4).

Table 2.3 Associations between bacterial rRNA genes in different regions of the small intestine and disease activity in IL-10^{-/-} mice.

Phylotype	Pooled		Small intestine[a]											
			Duodenum-U		Duodenum-W		Jejunum-U		Jejunum-W		Ileum-U		Ileum-W	
	r	P	r	P	r	P	r	P	r	P	r	P	r	P
GpC1	0.047	0.491	-0.303	0.195	-0.592	0.006	-0.160	0.502	-0.493	0.027	-0.202	0.393	0.267	0.257
Gp254	-0.172	0.011	-0.295	0.206	-0.331	0.154	-0.174	0.462	-0.236	0.316	-0.498	0.025	-0.023	0.915
Gp572	-0.095	0.163	-0.210	0.373	-0.470	0.037	-0.291	0.213	-0.404	0.078	-0.185	0.434	0.109	0.648
Gp244	-0.052	0.443	-0.445	0.049	-0.461	0.041	-0.242	0.302	-0.228	0.334	-0.140	0.555	0.090	0.707
Gp156	0.033	0.620	-0.219	0.353	-0.329	0.157	-0.178	0.452	0.085	0.721	-0.126	0.597	0.165	0.486
Gp000	0.053	0.436	ND	ND	-0.644	0.002	-0.445	0.049	-0.640	0.002	0.461	0.041	-0.191	0.420
GpC2	-0.182	0.007	-0.227	0.335	-0.366	0.112	-0.199	0.399	-0.303	0.193	-0.381	0.097	-0.325	0.163
Gp66	-0.221	0.001	-0.488	0.029	-0.433	0.057	-0.227	0.336	-0.215	0.362	-0.198	0.403	-0.031	0.898

Mice were raised in two different environments: a conventional mouse facility (Room 3) and a limited access facility (Room 4). Two replicate experiments were performed (Experiment 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 (see Table 1 for more details on the phylotypes). Disease activity was monitored every month for 6 months.

[a]Values are from correlation analyses between levels of bacterial rRNA genes in tissue sample (collected at the end of the experiments) and DAI (r is the Pearson correlation coefficient, P is the probability value).

ND = not detected.

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 = 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-U), 20 (Proximal-W), 20 (Distal-U), and 20 (Distal-W).

Table 2.4. Associations between bacterial rRNA genes in different regions of the large intestine and disease activity in IL-10$^{-/-}$ mice.

Phylotype	Proximal-U r	*P*	Proximal-W r	*P*	Distal-U r	*P*	Distal-W r	*P*	Cecum-U r	*P*	Cecum-W r	*P*
GpC1	0.007	0.977	0.108	0.649	0.164	0.490	0.423	0.063	0.387	0.269	0.350	0.322
Gp254	-0.275	0.241	-0.502	0.024	-0.218	0.355	0.009	0.969	0.303	0.397	0.057	0.875
Gp572	-0.053	0.824	-0.298	0.201	0.218	0.357	0.345	0.137	0.088	0.809	-0.157	0.665
Gp244	0.118	0.622	0.132	0.579	0.084	0.724	0.197	0.405	-0.307	0.389	-0.261	0.467
Gp156	0.291	0.214	0.504	0.023	0.080	0.738	0.390	0.089	0.189	0.600	-0.356	0.312
Gp000	-0.051	0.832	ND	ND	0.067	0.778	-0.374	0.104	0.400	0.253	ND	ND
GpC2	-0.282	0.229	-0.218	0.356	-0.070	0.769	-0.079	0.740	-0.092	0.800	-0.002	0.996
Gp66	-0.242	0.304	-0.366	0.113	-0.262	0.265	-0.224	0.343	-0.127	0.726	-0.073	0.842

Mice were raised in two different environments: a conventional mouse facility (Room 3) and a limited access facility (Room 4). Two replicate experiments were performed (Experiment 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 (see Table 1 for more details on the phylotypes). Disease activity was monitored every month for 6 months. Values are from correlation analyses between levels of bacterial rRNA genes in tissue sample (collected at the end of the experiments) and DAI (r is the Pearson correlation coefficient, *P* is the probability value). ND = not detected. Suffixes indicate whether the tissue samples were washed in buffer before being analyzed: -U (unwashed), -W (washed). n = 20 (Proximal-U), 20 (Proximal-W), 20 (Distal-U), and 20 (Distal-W) and 10 (Cecum-U), 10 (Cecum-W).

Table 2.5. Analysis of Cultured Bacteria: Conventional (Room 3) vs. Limited Access (Room 4) Housing

Species	Room 3	Room 4	*P*
β-hem G$^+$ cocci	25 (9)	11 (7)	0.027
Clostridium beijerinckii/butyricum	17 (9)	7 (5)	0.067
Pseudomonas	17 (4)	3 (3)	0.003
Actinomyces viscosus	0 (0)	4 (4)	0.046
Clostridium barati	0 (0)	5 (5)	0.021
Clostridium paraputrificum	1 (1)	11 (8)	0.001
Porphyromonis asaccharolytica	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 that species were isolated from.

Table 2.6. Analysis of Cultured Bacteria: High vs. Low Disease Activity Index (DAI)

Species	High DAI	Low DAI	P
β-hem G$^+$ cocci	23 (8)	13 (8)	0.018
Citrobacter amalonaticus	22 (5)	8 (3)	0.001
Escherichia coli	4 (2)	0 (0)	0.046
Prevotella melaninogenica/oralis	6 (6)	0 (0)	0.009
Bifidobacterium spp.	24 (8)	40 (9)	0.028
Pantoea spp.	4 (2)	13 (3)	0.040
Proteus mirabilis	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 that species were isolated from.

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Chapter Three: Shifts in bacterial community composition associated with genotype and colitis disease activity in IL-10$^{-/-}$ mice

Abstract
Introduction: Chapter 2 showed that specific bacterial phylotypes were associated with disease activity in IL-10$^{-/-}$ mice. In this study, bacterial populations were analyzed in fecal and tissue samples from IL-10$^{-/-}$ mice with two different genotypes raised under the same environmental conditions. **Methods:** The mice were raised in very clean conditions for 10 months. They were then transferred to less clean conditions for 3 months. C3H is the abbreviation for C3H/HeJBir IL-10$^{-/-}$ mice, which are more prone to develop colitis than B6 (C57BL/6J IL-10$^{-/-}$) mice. Fecal samples were collected every month, and tissue samples were collected when the mice were sacrificed. A variety of bacterial analyses were performed on DNA extracted from these samples. **Results:** In the fecal samples, higher populations of *Clostridium ramosum* were found in the colitis prone C3H mouse than in the B6 control mice throughout the duration of the experiment. Lachnospiraceae populations in the B6 mice increased when they were moved to the less clean environment. Lachnospiraceae populations remained stable in the C3H after they were moved to the less clean environment, and then they decreased by more than an order of magnitude when the mouse developed colitis. Using the RISA method, a variety of bacterial phylotypes were found in B6 tissue and blood samples, but only *C. ramosum* and *Bacteroides vulgatus* were found in the C3H mouse (in the colon). **Conclusion:** Genotype and colitis disease activity were associated with several differences in bacterial

composition in both fecal and tissue samples. These results should enable new hypotheses to be developed addressing the bacterial role in this mouse model and Crohn's disease.

Introduction

There is considerable evidence that the etiology of IBD involves genetic alterations associated with innate immunity. The *NOD2* gene has been the most often studied. NOD2 is a component of an intracellular pattern recognition system. Variants of the *NOD2* gene have been linked Crohn's disease susceptibility (Hampe, Cuthbert et al. 2001; Hugot, Chamaillard et al. 2001; Ogura, Bonen et al. 2001). Although the exact roles of NOD2 are not well defined, it is clear that one key function likely involves managing intracellular infections.

To our knowledge, in all studies that have challenged mice (intragastrically) or cells with living intracellular pathogens, *NOD2* variants/knockouts have exhibited a diminished ability to kill or clear the microorganisms. In mice, a *NOD2* knockout exhibited higher levels of *Listeria monocytogenes* in the liver and spleen when the animals were inoculated intragastrically (Kobayashi, Chamaillard et al. 2005). In analyses of human epithelial cells, *NOD2* expression reduced intracellular survival of *Salmonella typhimurium* while a *NOD2* variant associated with Crohn's disease did not (Hisamatsu, Suzuki et al. 2003). DMBT1, a large glycoprotein involved in pathogen defense, is activated by NOD2. Crohn's disease patients with a *NOD2* mutation display a deficit in *DMBT1* induction and DMBT1 has been

shown to inhibit intracellular invasion by *Salmonella enterica* in human epithelial cells (Rosenstiel, Sina et al. 2007). In U937 cells, expression of the *NOD2* Blau variant caused decreased uptake of *S. typhimurium*, but also slower clearance of intracellular *S. typhimurium* (Kim, Payne et al. 2007). Finally, in human-subject-derived dendritic cells, *NOD2* variants were associated with gene expression profiles characterized by deficits in pathogen elimination (Zelinkova, van Beelen et al. 2008).

Additional data supporting the idea that intracellular defenses are involved in Crohn's disease have come from genetic analyses. These studies discovered Crohn's associated polymorphisms in both *ATG16L1* and *IRGM*, which are genes involved in autophagy (Hampe, Franke et al. 2007; Parkes, Barrett et al. 2007; Rioux, Xavier et al. 2007; Mathew 2008). Given that autophagy is a mechanism by which host cells eliminate intracellular pathogens (Schmid, Dengjel et al. 2006; Singh, Davis et al. 2006), these associations along with the aforementioned *NOD2* variants point toward persistent intracellular infections as a key element of IBD etiology.

In this study, to investigate the relationships between bacteria and genotype in the development of colitis, we examined two mouse models: C3H/HeJBir IL-10$^{-/-}$ (C3H) and C57BL/6J IL-10$^{-/-}$ (B6). The C3H genotype has been shown to possess deficits in innate immunity that are not present in B6 mice (Beckwith, Cong et al. 2005), and which are believed to promote chronic T-cell and B-cell responses to enteric flora that, in part, are cuasing the colitis (Brandwein, McCabe et al. 1997; Cong, Brandwein et al. 1998).

Methods:

Mice

Two male mice of each C3Bir.129P2(B6)-Il10[tmlcgn/Lt] (004326) and B6.129P2-Il10[tmlCgn/J] (002251) strains were obtained from the Jackson laboratory and were raised in the Boyce Hall Vivarium (Room 7, a clean room with filter cage tops) from the age of 6 weeks until 10 months. They were then transferred to Room 11, which was relatively dirty room, without cage filter tops. One of the C3H mice died at age 43 weeks, and was not further studied.

Disease activity index

Disease Activity Index scores (0-6) are the sum of four individual parameters (Lytle, Tod et al. 2005). This score includes 1 point each for occult blood in stools (determined with Hemoccult slides; Beckman Coulter, Fullerton, Calif.), rectal prolapse of < 1 mm, soft stool, and ruffled fur. An extra point was added for diarrhea or severe rectal prolapse (> 1 mm).

Fecal sample collection

Fecal samples from both mouse types were collected every month for the first 10 months and twice per week after being transferred to Room 11. Samples were put in lysis tubes with 1 ml CLS-Y buffer from a FastDNA spin kit (Qbiogene, Carlsbad, CA). DNA was extracted using the FastDNA Spin Kit (Qbiogene, Carlsbad, CA) as described by the manufacturer, with a 30-second bead-beading step at a FastPrep instrument setting of 5.5. DNA was gel purified without exposure to UV or ethidium bromide in a 1% agarose gel

and recovered using QIAquick Gel Extraction Kit (Qiagen, Valencia, Ca) as instructed by the manufacturer, except that the heating step was excluded.

Tissue sample collection

Mice were sacrificed at the age of 15 months (August, 2009) using the CO_2 method. Organ samples were collected and put in lysis tubes with 1 ml CLS-Y buffer from a FastDNA spin kit, and DNA was extracted as described above. Intestinal epithelial lymphocytes (IEL) (which is a mucosal associated fraction) and lamina propria (LP) cells from small intestine and colon were prepared as previously described (Van der Heijden and Stok 1987); DNA from these fractions was extracted as described above.

Blood sample collection

Blood samples were collected from the heart of sacrificed mice. 50 ul samples were incubated at 37℃ in 2 ml of Brucella Broth under three environmental conditions: standard atmospheric conditions (air), standard anaerobic conditions, and partial anaerobic (with one anaerobic envelope used instead of three). The mixtures were incubated for a week. Blood cells were collected by centrifugation and DNA was extracted as previously described.

PCR analyses

rRNA gene analyses of the samples were performed using universal small-subunit primers and ribosomal intergenic spacer analysis. Unibac PCR

primer, Userbacpuri27F (GGAGACAUAGRRTTTGATYHTGGYTCAG) and Userbacpuri1492R (GGGAAAGUGBTACCTTGTTACGACTT) were used to amplify 16S rRNA gene from the samples. Thermal cycling parameters were 94℃ for 2 min; 30 cycles of 94℃ for 20 s, 48℃ for 30 s, and 72℃ for 90 s; followed by 72℃ for 10 min.

Ribosomal intergenic spacer analysis (RISA) PCR primer were performed with 50 ul PCR reaction mixtures contained the following reagents: 50 mM Tris (pH 8.3), 2.5 mM MgCl$_2$, 500 ug/ml BSA, 250 uM of each dNTP, 40 nM of 1406FpUserpuri (GGAGACAUTGYACACACCGCCCGT) and 400 nM of 23RpUserpuri (GGGAAAGUGGGTTBCCCCATTCRG) and 2.5 U *Taq* DNA polymerase in a glass tube and PCR were performed in Idaho PCR machine. The thermal cycling conditions were 94℃ for 5 min, 40 cycles of 94℃ for 20 sec, 52℃ for 30 sec, and 72℃ for 1 min, followed by 72℃ for 5 mins.

Sequence-selective quantitative PCR (qPCR) experiments were performed to measure the rRNA gene population of *C. ramosum* (using primers IL10Gp575F5 and IL10Gp575R5) and Lachnospiraceae bacterium (using primers IL10GP66F4 and IL10GP66R4). Methods were same as described in chapter 2.

PCR amplification of Lachnospiraceae flagella was performed using the primers   CBirFla-XHIS

(CAATTACATATGCATCACCATCACCATCACGTAGTACAGCACAATC) and

CBir1TERMX (ATAGACTAAGCTTACTGTAAGAGCTGAAGTACACCCTG)

(Lodes, Cong et al. 2004). Thermal cycling parameters were 94℃ for 5 min;

40 cycles of 94℃ for 20 s, 52℃ for 30 s, and 72℃ for 60 s; followed by 72℃

for 10 minutes.

DNA was extracted from HT-29 cells (HTB-38) using the same protocol

describe above. Sequence-selective PCR was used to detect *C. ramosum*

rRNA genes using primers and conditions described in Chapter 2.

Results

Development of colitis

Two different strains of IL-10$^{-/-}$ mice were raised under the same

environmental conditions. During the course of the experiments, the B6 mice

did not develop any signs of colitis (Disease Activity Index score was 0). The

C3H mouse Disease Activity Index score was 1 until 57 days after being

moved to the less clean environment, when it changed to 3.

Bacteria in feces

Higher populations of *C. ramosum* were measured in fecal samples from

the C3H mouse than the B6 mice (Figure 3.1). In addition, the rate of increase

in the population densities over time was greater in the C3H mouse than in

the B6 mice.

During most of the experiment, Lachnospiraceae population densities increased in both mouse types, with the rate of increase being greater in the B6 mice (Figure 3.2). After moving the mice to the less clean room (see solid arrow), the rate of increase in the B6 mice was enhanced. When the Disease Activity Index score in the C3H mouse increased from 1 to 3 (see dashed arrow), the population densities of Lachnospiraceae appeared to decrease.

Bacterial analysis using RISA

RISA was performed on both purified and unpurified DNA from fecal samples from both types of mice (Figure 3.3, Table 3.1). From both mouse types, unpurified and purified DNA from fecal samples produced several amplicons of different sizes. There was not much difference in the banding patterns between the unpurified and purified DNA. However, the banding pattern from the C3H mouse was different from that of the B6. The predominant bacterial phylotypes in the C3H fecal samples were *Lactobacillus* and *Clostridium* spp*.* while *Eubacterium rectal*, *B. vulgatus* and *Robinsoniella peoriensis* were the predominant ones in the B6 samples.

RISA was also performed on both purified and unpurified DNA from tissue samples from both types of mice (Figure 3.4 A-D, Table 3.2). While there were numerous types of bacteria from various tissues in the B6 mouse (Figure 3.4 A, B), bacterial amplicons were only found in one location in the C3H mouse (mucosal compartment of colon), the phylotype of which was *C. ramosum* (Figure 3.4 C, D). Concerning the B6 mouse, *B. vulgatus* was found to be a common inhabitant of the intestinal tissue. *Enterococcus faecalis* was

a predominant phylotype in the lamina propria compartment of both the small intestine and colon. In most of the blood samples, *Lactobacillus* spp. were the only bacteria that were detected, although *Enterococcus saccharolyticus* was also found in the fresh blood.

Bacterial analysis using PCR with universal bacterial primers

PCR using universal bacterial rRNA gene primers was used to examine the fecal and tissue samples from both types of mice (Figure 3.5, Table 3.3). Similar to the RISA results, fewer bacteria were present in C3H mouse than in the B6 mouse. Commonly identified bacteria in the B6 mouse were *Lactobacillus* and *Enterococcus* spp.*,* which was similar to the RISA results. Lachnospiraceae bacterium A4 was commonly found in the colon samples from B6, which is consistent with the qPCR results of the fecal samples. In C3H mouse only one phylotype (*B. vulgatus*) was identified, and this occurred in the colon.

Lachnospiraceae flagella

The gene for the Lachnospiraceae flagella was PCR amplified from B6 mice. This gene was only amplifiable in the B6 feces after the mice were moved to the dirtier room (data not shown). This gene has been cloned and will be used in subsequent expression studies to assess seroreactivity.

Examination of *C. ramosum* in HT-29 cells

HT-29 cells were examined using a *C. ramosum* sequence-selective PCR assay. This analysis produced a PCR product of the correct size and

sequence, which was not found in control reactions (Figure 3.6).

Discussion

Based in part on the results from Chapter 2, we developed the following etiology model for IBD.

IBD etiology involves a sequential cascade of events comprised of genetic, immunological, and microbiological components.

(i) Epigenetic and genetic factors enable intracellular infection by *C. ramosum* and/or related Erysipelotrichi,

(ii) Infection of epithelial cells and mucosal leucocytes leads to barrier dysfunction and activation of innate immunity, both of which are exacerbated by deficits in pathogen clearance/killing and the ability of the pathogen to induce apoptosis resistance,

(iii) Persistent innate activation and barrier dysfunction, coupled with genetic impairment of immune regulation, leads to persistent adaptive immune activation (targeting in part resident bacteria such as Lachnospiraceae) and phenotypic colitis.

Various combinations of these variables produce the myriad of disease subtypes.

The new element of this model is the persistent infection by *Clostridium ramosum* and/or related Erysipelotrichi*.* This component provides a link connecting the genetic defects in innate immunity (NOD2 and autophagy) to barrier dysfunction, apoptosis resistance and chronic immune activation. In this study, we attempted to test a few components of this model.

If *C. ramosum* is involved in disease causation, one would expect its population densities to be positively associated with disease. In Chapter 2, such associations were observed in fecal samples. In this study, we observed greater population densities of *C. ramosum* in fecal samples in the more colitis prone C3H mice. In addition, in the RISA analyses of the tissue samples from the C3H mice, the only bacterial phylotype (there may be other phylotypes, but they were not detected because of methodological limitations) identified was *C. ramosum*; this phylotype was also only detected from one compartment – the mucosal fraction of the colon. This is intriguing as the colon is the primary site of disease in this mouse model.

Concerning the model and the ability of *C. ramosum* to infect cells, we would anticipate that deficits in innate immunity in C3H (Beckwith, Cong et al. 2005) would lead to higher levels of *C. ramosum* infection. The in vitro culture studies with HT-29 cells suggested that *C. ramosum* has the ability to infect this cell line. Subsequent follow-on investigations utilizing microscopic techniques will endeavor to verify that this phylotype is able to infect host cells

in C3H mice, and, more specifically, which type of host cells.

In the universal bacterial PCR analysis of the C3H tissue samples, PCR products were also only obtained from one region – the colon. Here, the only phylotype (there may be other phylotypes, but they were not detected because of methodological limitations) that was identified was *B. vulgatus*. Why the tissue analyses of the C3H mouse produced only two types of bacteria (*B. vulgatus* here and *C. ramosum* above) is unclear at this time. However, they are consistent with persistent immune activation that is reducing the population densities of most bacteria in these tissues.

The model would also predict that population densities of Lachnospiraceae would decrease when colitis disease activity increased, which is the result that was observed in this study. Subsequent investigations will endeavor to determine if these changes are caused by immune responses. Toward this goal, I have cloned the flagellin gene from Lachnospiraceae from these mice. Subsequent steps will involve in vitro expression this gene to obtain purified protein for seroreactivity analyses.

One of many other future areas of research will be to repeat these experiments with a larger number of mice. I would also like to perform similar types of experiments with more defined knock-outs targeting genes such as *NOD2*.
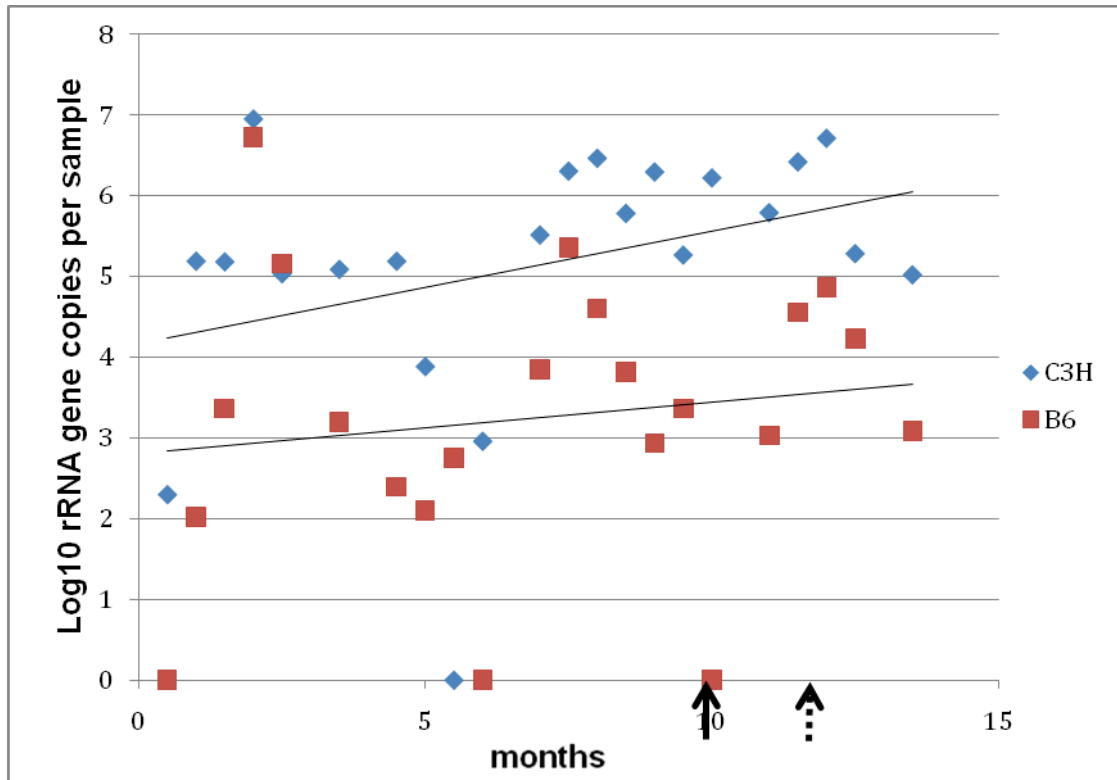
Figure 3.1A. Changes in the population densities of *C. ramosum* over time in C3H and B6 mice. Zero months indicates the time when the mice arrived at UCR, at which time the mice were 40 days. The arrow (solid line) shows the time when the mice were transferred from a clean environment to a less clean environment. The arrow (dashed line) shows the time when the Disease Activity Index of the C3H mouse changed from 1 to 3.
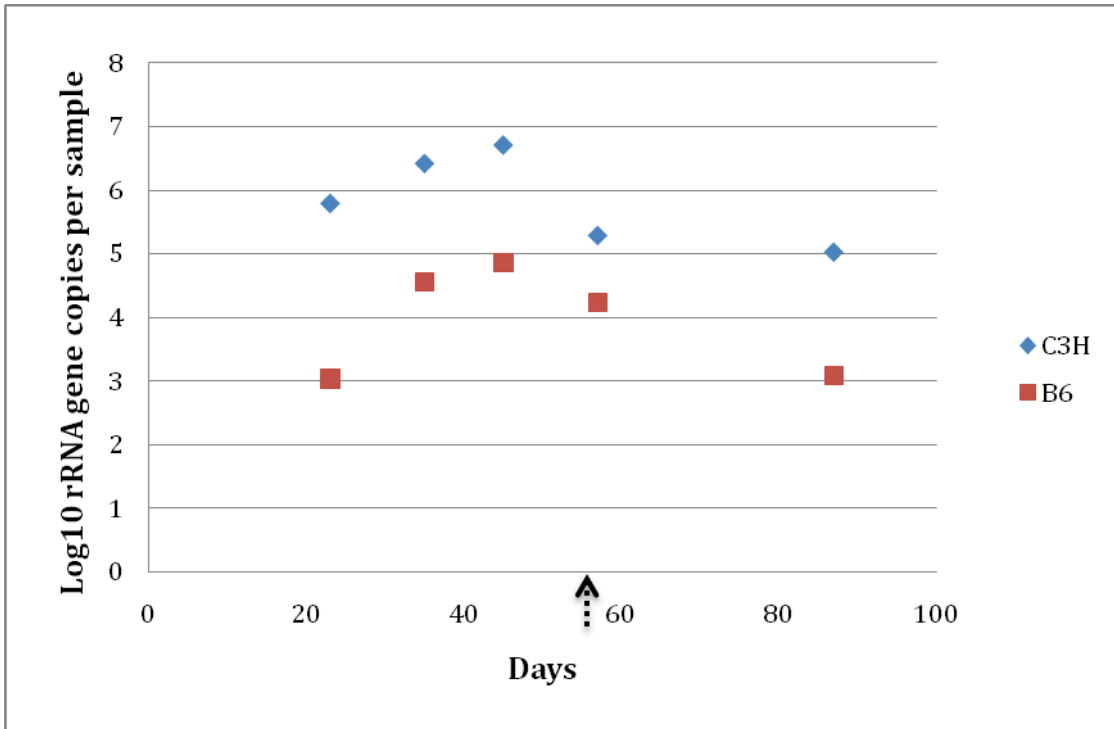
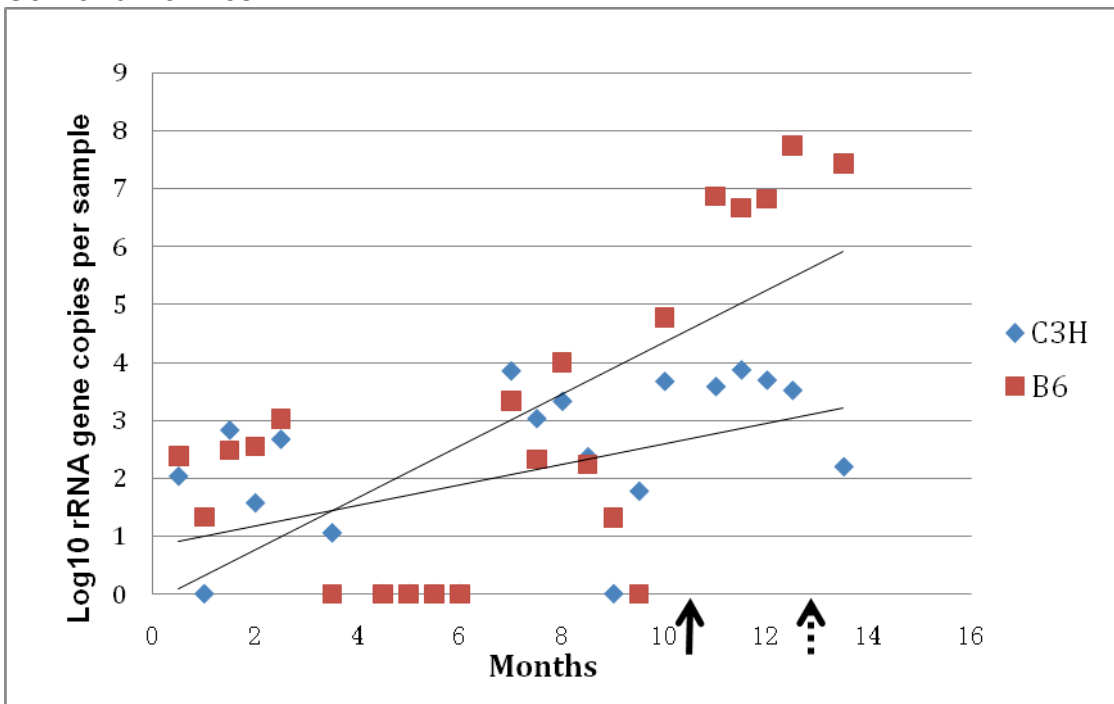Figure 3.1B. Changes in the population densities of *C. ramosum* over time in C3H and B6 mice after they were transferred to the less clean environment. Zero days indicates the time when the mice were transferred from the clean environment to a less clean environment. The arrow (dashed line) shows the time when the Disease Activity Index of the C3H mouse changed from 1 to 3.

Figure 3.1. Changes in the population densities of *C. ramosum* over time in C3H and B6 mice.

Figure 3.2A. Changes in the population densities of Lachnospiraceae over time in C3H and B6 mice. Zero months indicates the time when the mice arrived at UCR, at which time the mice were 40 days old. The arrow (solid line) shows the time when the mice were transferred from a clean environment to a less clean environment. The arrow (dashed line) shows the time when the Disease Activity Index of the C3H mouse changed from 1 to 3.
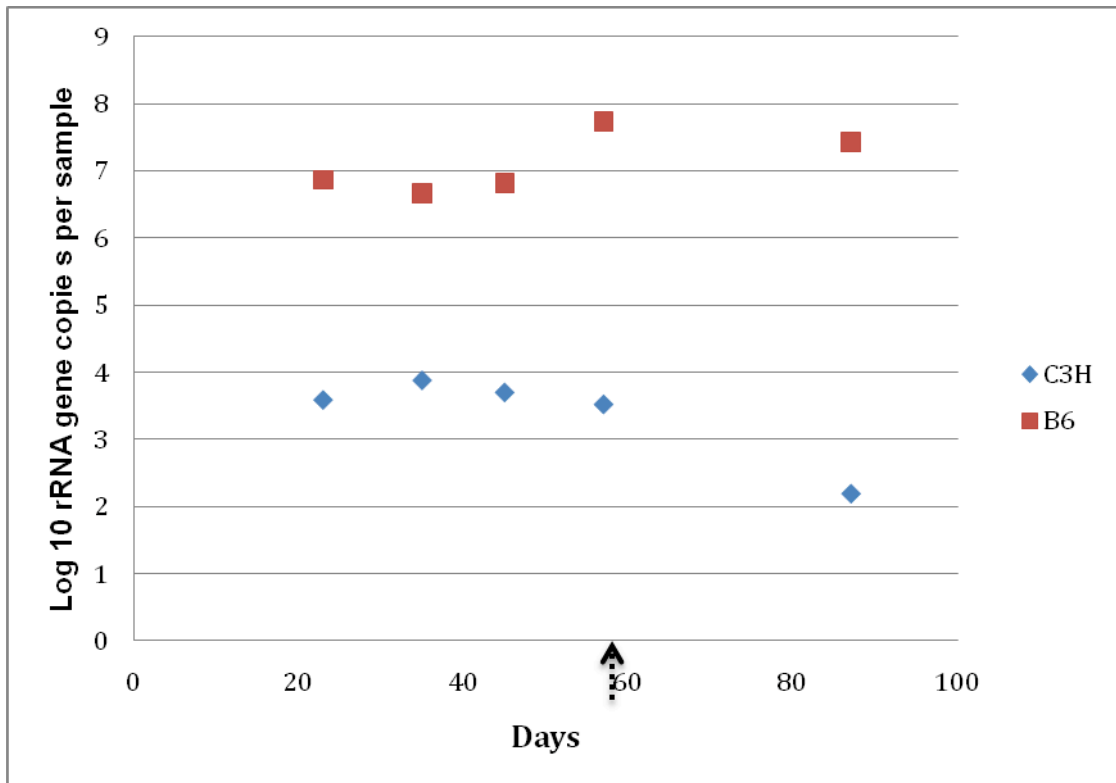

Figure 3.2B. Changes in the population densities of Lachnospiraceae over time in C3H and B6 mice after the mice were transferred to a less clean environment. Zero days indicates the time when the mice were transferred from a clean environment to a less clean environment. The arrow (dashed line) shows the time when the Disease Activity Index of the C3H mouse changed from 1 to 3.

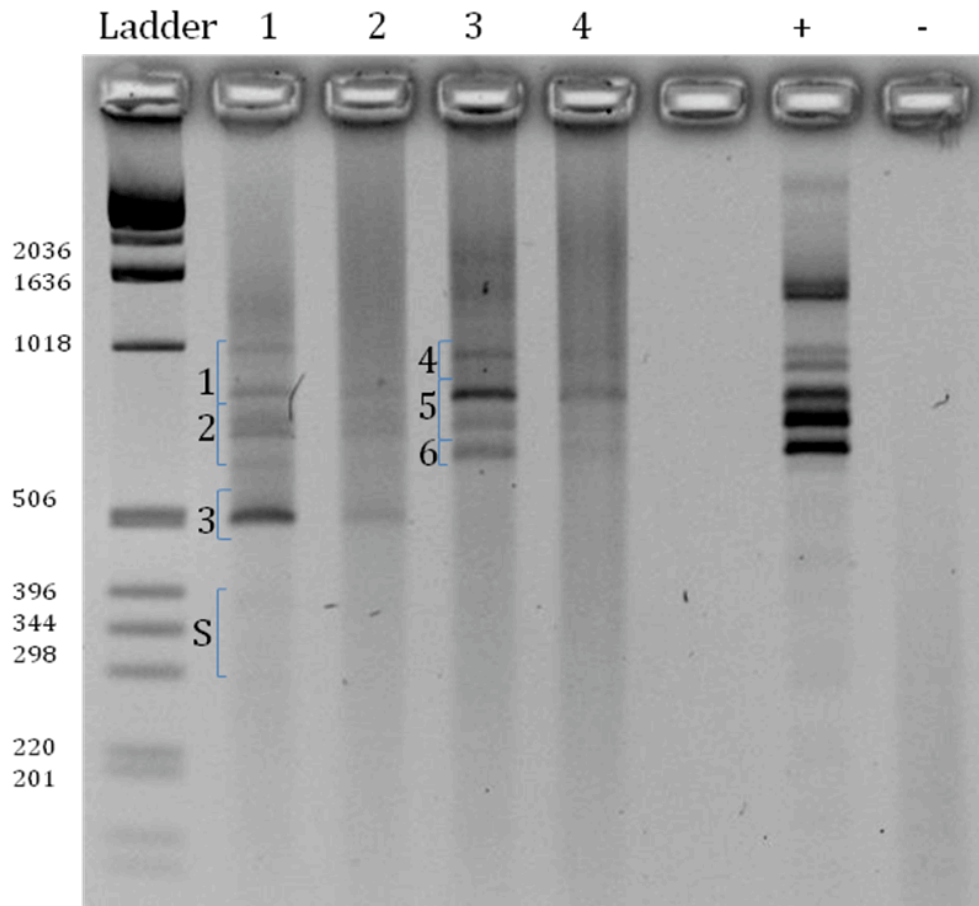Figure 3.2. Changes in the population densities of Lachnospiraceae over time in C3H and B6 mice.

Figure 3.3. RISA analysis of feces collected when the mice were 445 days old (the day before the mice were sacrificed). Sample 1, unpurified DNA from C3H feces; 2, purified DNA of 1; 3, unpurified DNA from B6 feces; 4, purified DNA of 3; +, E. coli DNA; -, negative control.

Table 3.1. Bacteria identified from the RISA analysis of unpurified DNA from feces from C3H and B6 mice. The fragment numbers refer to the band numbers in Figure 3.3.

|	Fragment	Nearest cultured related (accession, % identity)			
C3H	1	*Lactobacillus johnsonii*	AE017198	100	734
		Lactobacillus murinus	AB158764	100	668
		Clostridium piliforme	DQ352810	96	596
	2	*Clostridium piliforme*	DQ352810	96	595
		Lactobacillus murinus	AB158764	98	585
		Lactobacillus johnsonii	AE017198	100	762
		Lactobacillus reuteri	AP007281	99	488
	3	*Lactobacillus acidophilus*	AB092634	99	514
		Lactobacillus johnsonii	EU547283	99	504
		Lactobacillus animalis	AY526615	98	486
		Coprobacillus cateniformis	NR_024733	99	544
	S	*Clostridium phytofermentans*	CP000885	97	833
		Lactobacillus murinus	AB158764	98	585
		Lactobacillus johnsonii	AE017198	99	504
B6	4	*Eubacterium rectale*	CP001107	100	807
		Robinsoniella peoriensis	DQ681227	99	986
		Prevotella sp.	GQ422744	100	633
	5	*Robinsoniella peoriensis*	DQ681227	97	810
		Prevotella sp.	GQ422744	99	634
		Bacteroides vulgatus	FJ410382	96	714
	6	*Eubacterium rectale*	CP001107	89	837
		Prevotella sp.	GQ422744	99	714
		Bacteroides vulgatus	FJ410382	97	633
		Symbiobacterium thermophilum	AP006840	90	624
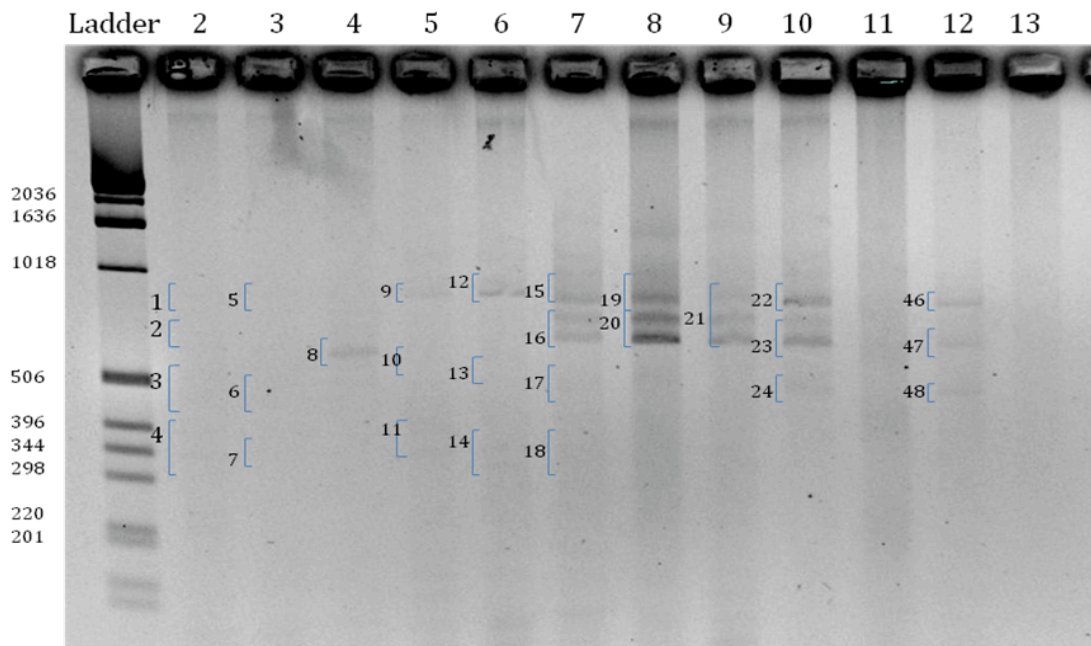
Figure 3.4A. RISA analysis of unpurified DNA from B6 mouse 1.
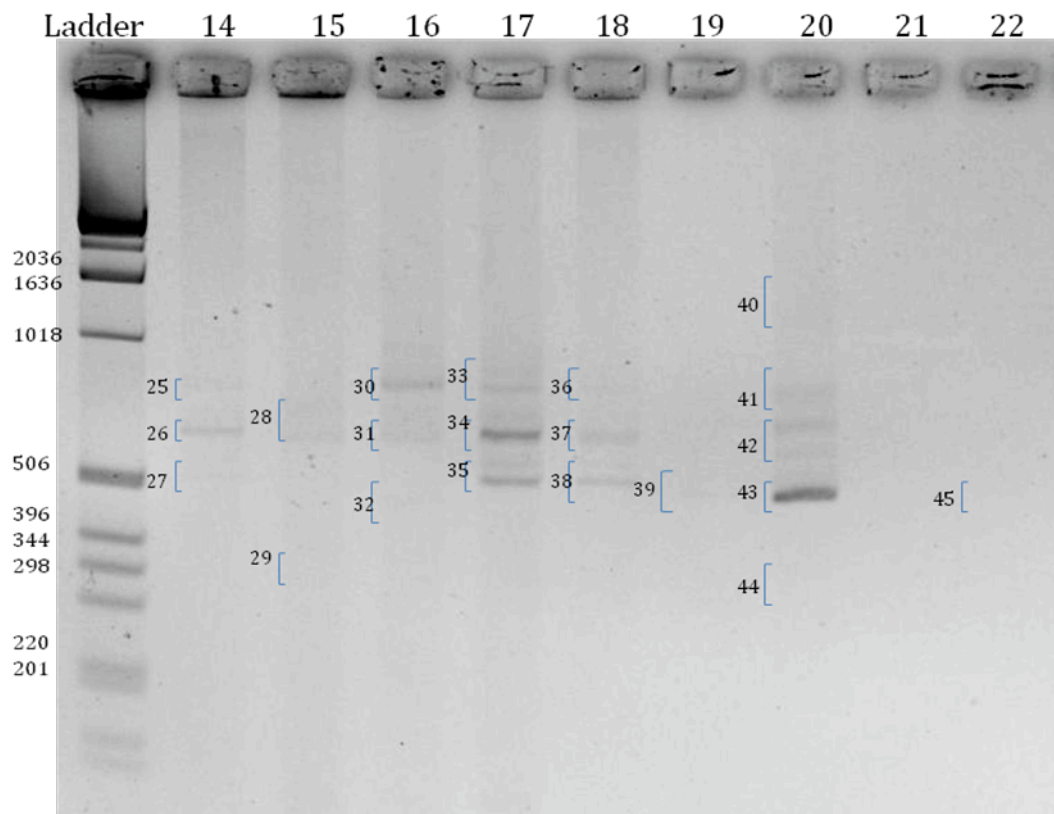

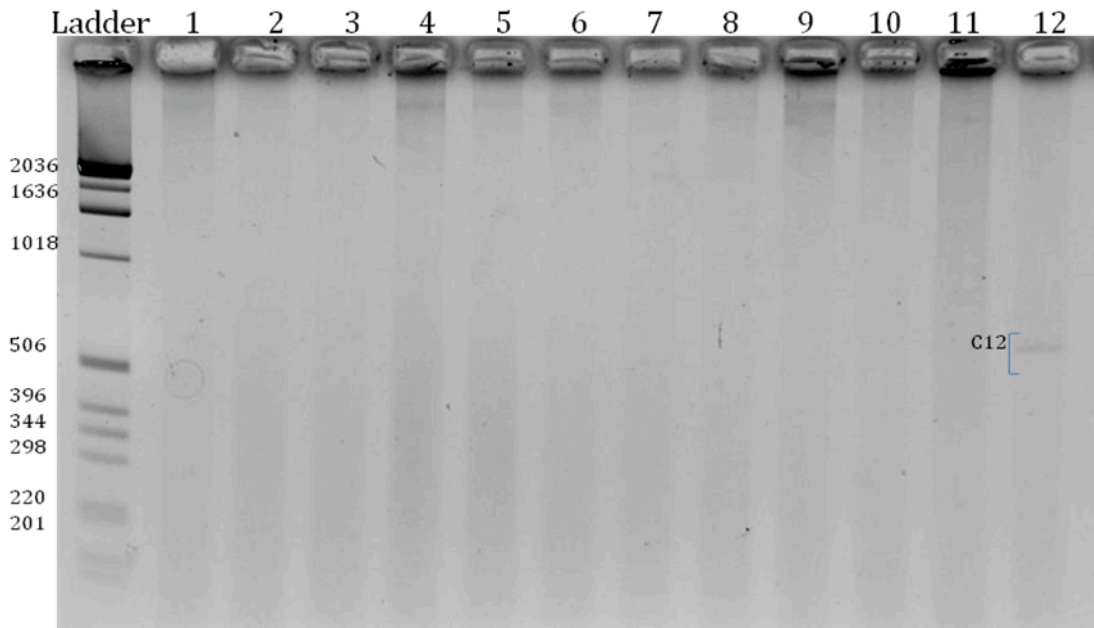Figure 3.4B. RISA analysis of unpurified DNA from B6 mouse 2.

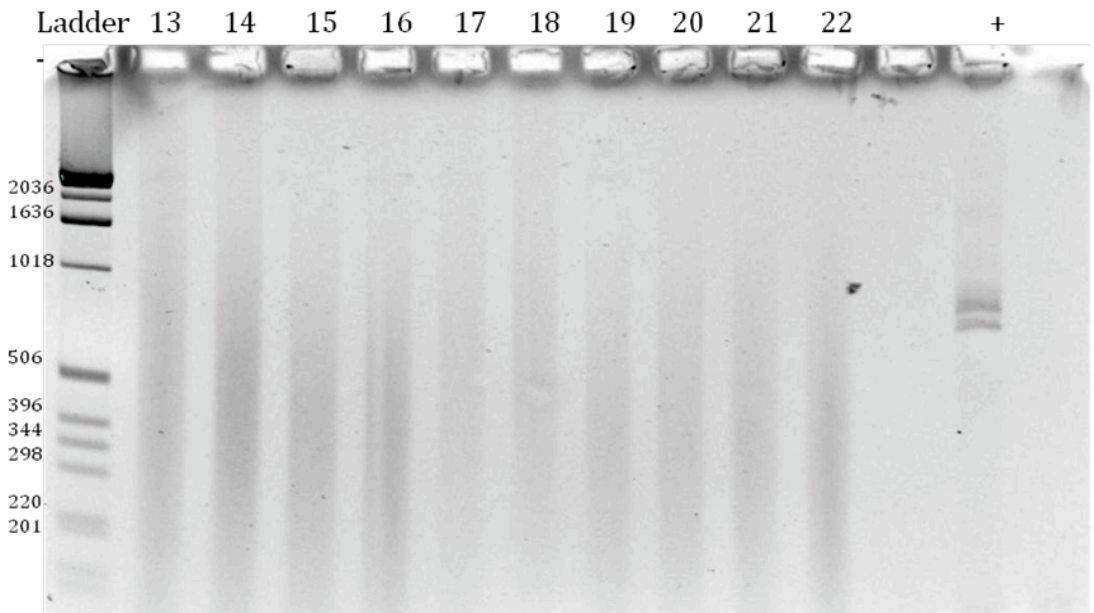Figure 3.4C. RISA analysis of unpurified DNA from C3H mouse 1.

Figure 3.4D. RISA analysis of unpurified DNA from C3H mouse 2.

Figure 3. RISA analysis of unpurified DNA from C3H and B6 mice tissue samples collected when the mice were 455 days old (when the mice were sacrificed). Samples: 1, spleen (C3H only); 2, liver; 3, pancreas; 4, testicular fat; 5, mesenteric lymph node; 6, pancreatic mesentery; 7, muscle; 8, bone and muscle; 9, small intestine; 10, colon; 11, IEL from small intestine; 12, IEL from colon; 13, LP from small intestine; 14, LP from colon; 15, IEL from small intestine with percoll; 16, IEL from colon with percoll; 17, LP from small intestine with percoll; 18, LP from colon with percoll; 19, fresh blood; 20, blood

incubated in Brucella Broth under air; 21, blood incubated in Brucella Broth under partial anaerobic conditions; 22, blood incubated in Brucella Broth under anaerobic conditions. No PCR products were obtained from purified DNA (possibility because a considerable amount of DNA was lost during the process).

Table 3.2. Bacteria identified from the RISA analysis of tissue samples from C3H and B6 mice. The fragment numbers refer to the band numbers in Figure 4.4.

Samples	PCR fragment*	Nearest cultured relative , accession, % identity, amplicon size			
Liver	RB3	*Streptococcus pneumoniae*	CP000919	98	529
		Clostridium ramosum	AY699288	98	573
T-fat	RB8	*Staphylococcus saprophyticus*	AP008934	99	572
MLN	RB9	*Leptothrix cholodnii*	CP001013	100	929
	RB11	G+ bacterium MOL 361	AF349724	99	981
Pancreas and mesentery	RB12	*Robinsoniella peoriensis strain*	DQ681227	97	810
	RB13	*Staphylococcus epidermidis*	CP000029	96	578
		Clostridium sp.	FJ625862	96	812
	RB15	*Clostridium celerecrescens*	NR_026100	94	836
Muscle	RB16	*Prevotella* sp	AY349398	89	646
	RB17	*Lactobacillus animalis*	AY526615	98	486
	RB19	*Clostridium* sp.	FJ625862	87	819
Bone and muscle		*Prevotella* sp.	GQ422744	95	639
	RB20	*Symbiobacterium thermophilum*	AP006840	90	623
Small intestine	RB21	*Bacteroides vulgatus*	FJ410382	96	764
		Robinsoniella peoriensis	DQ681227	97	810
Colon	RB22	*Bacteroides vulgatus*	FJ410382	96	718
	RB23	*Bacteroides vulgatus*	FJ410382	97	633
		Enterobacter sakazakii	CP000783	96	835
	RB25	*Bacteroides vulgatus*	CP000139	91	695
LP from colon	RB26	*Enterococcus faecalis*	AY351323	100	510
	RB27	*Enterococcus faecalis*	AE016830	97	518
IEL small w/percoll	RB28	*Bacteroides vulgatus*	FJ410382	97	633
		Eubacterium rectale	CP001107	100	806
	RB30	*Robinsoniella peoriensis*	DQ681227	96	847
		Lactococcus garvieae	AF225968	100	630
IEL colon w/percoll	RB31	*Bacteroides vulgatus*	FJ410382	97	634
	RB32	*Clostridium* sp.	AF157052	97	469

LP small w/ percoll			AF148208		
	RB33	*Pseudomonas fluorescens*		97	835
		Enterococcus faecalis	AE016830	100	611
	RB34	*Enterobacter sakazakii*	CP000783	95	552
	RB35	*Enterococcus faecalis*	AY351323	100	509
	RB36	*Enterococcus faecalis*	AE016830	100	724
		Enterococcus faecalis	AE016830	100	509
LP colon w/percoll	RB37	*Pseudomonas putida*	EU014559	93	548
	RB38	*Enterococcus faecalis*	AY351323	99	509
Blood	RB39	*Lactobacillus animalis*	AY526615	98	487
	RB40	*Lactobacillus animalis*	AY526615	98	488
	RB41	*Lactobacillus johnsonii*	AE017198	99	503
Blood in air	RB42	*Lactobacillus animalis*	AY526614	98	683
	RB43	*Lactobacillus animalis*	AY526615	98	487
		Lactobacillus animalis	AY526615	98	487
Blood in anerobic	RB45	*Lactobacillus reuteri*	AP007281	99	486
	RB46	*Robinsoniella peoriensis*	DQ681227	97	812
	RB47	*Bacteroides vulgatus*	FJ410382	97	633
IEL colon	RB48	*Rumen bacterium*	AF544207	95	590
IEL colon	RC12	*Clostridium ramosum*	AY699288	98	573

*RB and RC refer to the RISA analysis from the B6 and C3H mice, respectively.
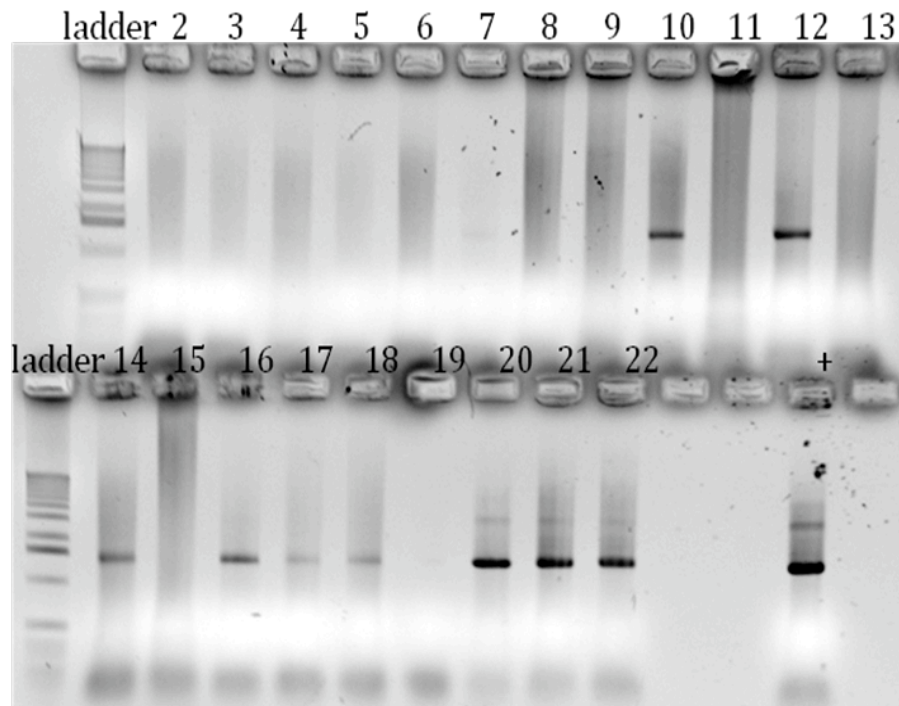
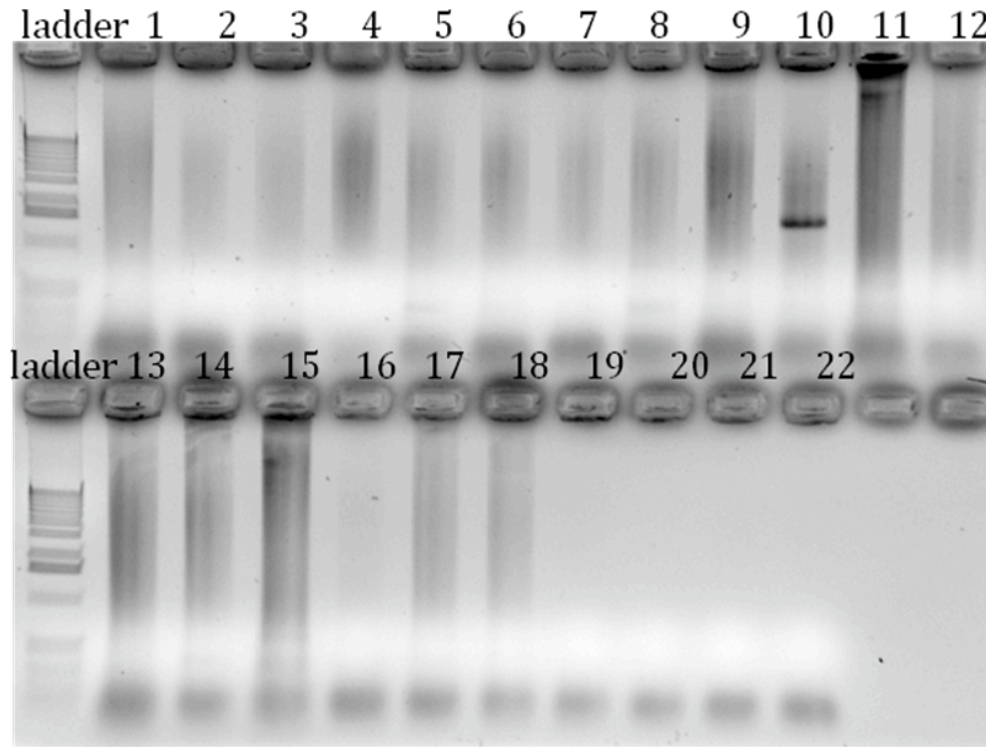Figure 3.5A: Universal bacteria analysis of unpurified DNA from B6 mouse.

Figure 3.5B: Universal bacteria analysis of unpurified DNA from C3H mouse.

Figure 3.5: Universal bacterial analysis of unpurified DNA from C3H and B6 mice tissue samples collected when the mice were 455 days old. Samples: 1, spleen (C3H only); 2, liver; 3, pancreas; 4, testicular fat; 5, mesenteric lymph node; 6, pancreatic mesentery; 7, muscle; 8, bone and muscle; 9, small intestine; 10, colon; 11, IEL from small intestine; 12, IEL from colon; 13, LP from small intestine; 14, LP from colon; 15, IEL from small intestine with percoll; 16, IEL from colon with percoll; 17, LP from small intestine with percoll; 18, LP from colon with percoll; 19, fresh blood; 20, blood incubated in Brucella Broth under air; 21, blood incubated in Brucella Broth under partial anaerobic conditions; 22, blood incubated in Brucella Broth under anaerobic conditions.

Table 3.3 Bacteria identified from the universal bacteria analysis of tissue samples from C3H and B6 mice. The fragment numbers refer to the sample numbers in Figure 4.5.

Samples	PCR fragment*	Nearest cultured relative, accession, %identity		
Muscle	UB7	Bad sequences		
Colon	UB10	Lachnospiraceae bacterium A4	DQ789118	97
IEL from colon	UB12	Lachnospiraceae bacterium A4	DQ789118	97
LP from colon	UB14	Bad sequences		
IEL from colon w/ percoll	UB16	Lachnospiraceae bacterium A4	DQ789118	96
LP small w/ percoll	UB17	*Pseudomonas* sp.	GQ903480	97
LP from colon w/percoll	UB18	*Clostridium histolyticum*	M59094	98
Blood in air	UB20	*Lactobacillus murinus*	EU161635	99
Blood in partial air	UB21	*Enterococcus gallinarum*	GQ337026	99
Blood in anerobic	UB22	*Lactobacillus murinus*	EU161635	99
		Lactobacillus reuteri	EU381121	99
Colon	UC10	*Bacteroides vulgatus*	EU728705	99

*UB and UC refer to universal bacterial PCR from B6 and C3H mice, respectively. The numbers following UB and UC refer to the lane numbers from Figure 3.5.
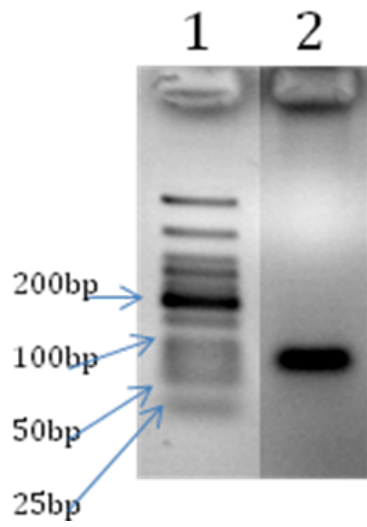
Figure 3.6. PCR of *C. ramosum* rRNA genes from HT-29 cells. 1, low molecular weight ladder; 2, PCR product from HT-29 cells.

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Chapter Four: Mucosal bacteria associated with ulcerative colitis and Crohn's disease

Abstract

Background: Microorganisms appear to play important roles in the etiology of inflammatory bowel disease (IBD) in both Crohn's disease (CD) and ulcerative colitis (UC). In this study, relationships between bacteria and disease type in intestinal mucosal samples were examined. **Methods:** Endoscopic lavage samples were collected by colonoscopy from various regions of the intestine including ascending colon (AI), sigmoid colon (SI) and cecum. Bacterial rRNA gene analysis was performed on these samples using qPCR targeting specific taxa. **Results:** Bacterial phylotypes Ruminococcus 312, Akkermansia 498, Eubacterium 2766, Ruminococcus 246 and Faecalibacterium 2994 were all found to have negative associations with IBD. This is the first report of an association of Akkermansia with IBD. **Conclusion:** This study could lead to a greater understanding of the bacterial roles in IBD etiology.

Introduction

Intestinal microbiota appear to play an important role in the etiology of Crohn's disease (CD) and ulcerative colitis (UC). However, it is not known whether IBD etiology involves a specific pathogen, a normal immune response to disordered bacterial communties, an inappropriate immune response to the normal microbiota, or other.

Several bacteria have been implicated in the etiology of CD, including *Mycobacterium avium paratuberculosis* (MAP) and *Escherichia coli*. MAP was suggested to be the microorganism that causes CD after it was isolated from a sample of CD tissue (Martin and Rhodes 2000). MAP was also cultured from blood of both CD and UC patients, but not from healthy controls (Naser, Ghobrial et al. 2004; Sechi, Scanu et al. 2005). However, other studies have not found MAP to be more abundant in CD patients than normal controls (Ellingson, Cheville et al. 2003; Baksh, Finkelstein et al. 2004). In addition, the immunosuppressive therapies which should exacerbate a mycobacterial infection, ameliorate symptoms in CD patients. It therefore may be more likely that the colonization of MAP is the result of the inflammation rather than its cause.

Escherichia coli has also been implicated as the pathogen of CD. Recovery of a virulent *E. coli* strain from mucosal biopsies of CD patients, as well as the increased *E. coli* mucosal adherence compared to controls, have supported the concept of it being a pathogen that is causing CD (Darfeuille-Michaud, Boudeau et al. 2004; Sokol, Seksik et al. 2006). Increased abundance of *E. coli* has been found in CD patients compared to their healthy twins (Willing, Halfvarson et al. 2009). Adherent invasive *E. coli* was found to often colonize ileal lesions and its numbers were abnormally high in ileal CD, which indicates that it may be specifically associated with ileal CD (Darfeuille-Michaud, Boudeau et al. 2004). However, it is not clear whether the presence of *E. coli* is the result of the

inflammation or the cause. Other bacteria such as *Helicobacter pylori* have also been reported to be associated with CD (Martin and Rhodes 2000).

It has been suggested that IBD may not be due to a specific pathogen but rather an imbalance in the microbiota, which may lead to intestinal inflammation. Shifts in the normal composition of intestinal bacteria have also been associated with IBD. Biopsy analyses have shown that concentrations of mucosal bacteria in IBD patients were 100 times higher than those in healthy controls, with Bacteroides being a dominant taxon (Swidsinski, Weber et al. 2005). A study using a metagenomics approach showed that there was reduced bacterial diversity in feces from Crohn's disease subjects compared to healthy controls (Manichanh, Rigottier-Gois et al. 2006). In this study, Bacteroides population densities were similar while Firmicutes were reduced in CD, with *Clostridium leptum* being one of the major Firmicutes phylotypes that was decreased. Comparisons between UC, CD and healthy subjects have also been made using tissue samples (Gophna, Sommerfeld et al. 2006). Here, Crohn's disease subjects showed decreases in Firmicutes (Clostridium) but increases in Bacteroides and Proteobacteria. Quantitative PCR analyses showed that there were 300-fold and 50-fold decreases in Lachnospiraceae and Bacteroides, respectively, in IBD compared to healthy controls (Frank, St Amand et al. 2007). In many studies, bacterial populations in UC subjects have been shown to be similar to those in healthy subjects (Sokol, Seksik et al. 2006).

In this study, rRNA gene clone libraries from mucosal samples obtained from a small cohort of UC, CD healthy control (HC) subjects were created and analyzed. For ten of the predominant phylotypes identified in this cohort, sequence-selective qPCR assays were developed. These assays were then used to measure the population densities of these phylotypes in a second cohort of subjects.

Materials and Methods

Patients

Two patient cohorts were examined in this study. The first was comprised of males and females with active and non-active UC (n = 6) and healthy individuals (n = 3). The second was comprised of males and females with 14 patients of CD and 16 patients of UC, and 12 healthy control subjects (samples were kindly provided by Jonathan Braun and colleagues at UCLA).

Mucosal sample collection

Mucosal samples were obtained using a saline rinse lavage procedure (Li, Borneman et al.). Samples were collected from various regions of the intestine (ascending colon, sigmoid colon, and cecum) during routine colonscopy procedures and frozen at -70°C within 20 minutes of procurement (samples were kindly provided by Jonathan Braun and colleagues at UCLA).

DNA extraction

Mucosal samples were centrifuged for 1 minute at 14,000 x g. Pellets were resuspended with 1 ml CLS-Y buffer and transferred to Lysis Tubes from a FastDNA Spin Kit (Qbiogene, Carlsbad, CA). DNA was extracted using the FastDNA Spin Kit (Qbiogene, Carlsbad, CA) as described by the manufacturer, with a 30-second bead-beading step at a FastPrep instrument setting of 5.5. DNA was gel purified without exposure to UV or ethidium bromide in a 1% agarose gel and recovered using QIquick Gel Extraction Kit (Qiagen, Valencia, Ca) as instructed by the manufacturer, except that the heating step was excluded.

Quantitative PCR

Sequence-selective quantitative PCR (qPCR) experiments were performed to measure the phylotypes in the mucosal samples from CD, UC and health control subjects. Specific primers were designed using the PRISE software. These primers were previously identified as having the potential to differentiate disease subtypes (Presley 2008). Real-time qPCR assays were performed in a Bio-Rad iCycler MyiQ™ Real-Time Detection System (Bio-Rad Laboratories Inc., Hercules, CA) using iCycler iQ PCR Plates with Optical Flat 8-Cap Strips (Bio-Rad Laboratories Inc). Twenty-five µl reaction mixtures contained the following reagents: 50 mM Tris (pH 8.3), 500 µg/ml BSA, 2.5 mM MgCl2, 250 µM of each dNTP, 400 nM of each primer, 1 µl of template DNA, 2 µl of 2.5X SYBR Green I

and 1.25 U Taq DNA polymerase. The primers and amplicon sizes were: Group603 (603F5, CTATGTAACTAGAGTGTCGG, 603R3, GAGCCTCAACGTCAGTG, 121 bp), Group501 (501F3, GATAACCTGCCTCACACT, 501R3 GCTAATCAGACGCGGA, 125 bp), Gp2766, (2766F5, ATTGCACAATGGGCGAAAG, 2766R3, CTCCGACACTCTAGTACG, 274bp), Gp2994, (2994F6, GAGTAACGCGTGAGG, 2994R6, GCGAGGCCATCTCAA, 128bp), Gp246, (246F2, CGCAACCCCTATCCTT, 246R6 , TATTTTTGAGATTTGCCCACC, 186bp), Gp312, (312F1, ACTACTAGGTGTCGGGC, 312R1, TTACTTGCCGGTCAGGGA, 195bp), Gp323, (323F1, ATCTTCAGTAGCCAGCG, 323R1, CGTTATTTTTGGGATTTGCC, 178bp), Gp575, (575F4, CGCCGCAAACGCAT, 575R6, GACACATTACTGTCCGGT, 167bp), Gp832 (Gp832F4, CGCGGTAATACGGAGGA, Gp832 R4, ACGGTTGAGCCGCAAACT, 101bp), Gp498 (GP498F2, GTGGGAGCAAATTAAAAAGATA, GP498R2, CTCGCAGTATCATGTGC, 187bp). For the qPCR of total Bacteria, (UnibacqPCRF3, ACGGGAGGCAGCAGT, UnibacqPCRR3, ACCAGGGTACTAATCCTGTT, 483 bp), master mix (New England) were used to decreased the possibility of contamination, 400 nM of each primer, 1 μl of template DNA, 2 μl of 2.5X SYBR Green I (Londaz) and 1.25 U *Taq* DNA polymerase. The thermal cycling conditions were 94℃ for 5 min; 36 cycles of 94℃ for 20 s, X℃ for 30 s, and 72℃for 30 s; followed by 72℃ for 10 min; where X = 61.5 for Gp603, 63.8 for Gp501, 65.5 for Gp2766, 59.5 for Gp2994, 61.8 for

Gp246, 61.8 for Gp312, 61.5 for Gp323, 65.5 for Gp575, 65.5 for Gp832, 63.8 for Gp498, and 58.8 for Total Bacteria. DNA templates for the PCR were 1 to 3 times diluted gel purified DNA samples.

Data analysis

Relationships between the bacterial phylotypes and disease type were examined using ANOVA and Turkey's method (Minitab 15, State College, PA). qPCR data (copies per sample) were either transformed using log10 or divided the copy numbers per sample obtained using the universal bacteria qPCR assay; the ratio values were normalized by first multiplying them by 1×10^{7} and then performing a log10 transformation. The heat map was created by performing an unsupervised clustering analysis of the qPCR values using Cluster (Eisen, Spellman et al. 1998) and displayed by using Java TreeView (Saldanha 2004).

Results

First cohort analyses

An rRNA gene sequence analysis of mucosal samples from the first cohort of subjects was performed. The first cohort was comprised of 9 individuals: 6 with UC and 3 healthy controls. Ten phylotypes from this analysis were identified as having the potential to differentiate IBD disease types (Presley 2008).

Second cohort analyses

To further examine these ten phylotypes, sequence-selective quantitative PCR (qPCR) assays were developed (Table 4.1). These assays were used to measure the population densities of the phylotypes in mucosal samples from a second cohort (Figure 4.1). The second cohort was comprised of 42 subjects: 14 with CD, 16 with UC, and 12 healthy controls. Bacteriodes 832 had the highest population densities, followed by Faecalibacterium 2994 and Clostridium 501. Akkermansia 498 had the lowest population densities.

Within the healthy control (HC) and UC subjects, there were no differences in the population densities of these phylotypes between the two most commonly sampled intestinal regions (sigmoid colon and cecum). Within CD, there were a few differences between these two intestinal regions: Akermansia 498 (Log 10 and ratio) and Clostridium 603 (ratio).

Concerning differences between disease types for each of the phylotypes (Figure 4.2), an ANOVA analysis identified differences between UC and HC samples with the Ruminococcus 312 assay in the cecum and sigmoid colon, but not between the two forms of IBD. Differences between IBD and HC, but not between the two forms of the IBD, were identified for the Akkermansia phylotype, both in the cecum and sigmoid colon. Differences between CD and UC and CD and HC were identified for the Faecalibacterium phylotype. Differences in the population densities of both the Ruminococcus 246 and Eubacterium 2766 phylotypes were found between CD and HC, but not between UC and HC nor between the two forms of IBD. In summary, most of the significant differences

were identified in sigmoid colon samples between CD and HC. There were not many differences between CD and UC except for the Faecalibacterium phylotype. Only Akkermansia showed differences between UC and HC in all types of samples and analyses.

An unsupervised clustering analysis of the bacterial population densities was also performed (Figure 4.3). This analysis used the phylotypes that exhibited differences from the ANOVA analysis (Figure 4.2). The heatmap shows that this analysis was able to sort many of the subjects by disease type.

Discussion

This project endeavored to test the hypothesis that IBD subtypes and healthy control subjects can be stratified by measuring the population densities of specific bacterial phylotypes in the mucosal compartment of the intestine. Indeed, five of the ten phylotypes examined in this study exhibited differences between the IBD disease types and healthy controls. To our knowledge, this high rate of success is unique, demonstrating the utility of examining the intestinal mucosal compartment, and, in general, paying attention to microgeography. We anticipate that host-microbe associations such as these should also provide new clues as to the pathophysiologies of IBD.

Our study and others have identified associations between the population densities of *Faecalibacterium prausnitzii* and CD. In recent work, *Faecalibacterium* was shown to be able to reduce colitis in a mouse model, and it

is believed to secrete metabolites that can block the activation of NF-kB and production of IL-8 (Sokol, Pigneur et al. 2008). In my study, I found that its population densities were the same in healthy control and UC subjects, but that they were reduced in Crohn's disease, which matches the results from Sokol's work (Sokol, Pigneur et al. 2008). Because of this relationship, this phylotype may be a useful biomarker for CD. It has also been considered to be a prospective probiotic for CD (Sokol, Pigneur et al. 2008).

Akkermansia 498 was another phylotype associated with IBD identified in this study. To our knowledge, this is the first report of such an association. This bacterium has been reported to be a mucin-degrading bacterium in the human intestine (Derrien, Vaughan et al. 2004). As determined by fluorescent in situ hybridization, *A. muciniphila* accounts for more than 1% of the total fecal bacteria (Derrien, Collado et al. 2008). In our study, the ratio of *Akkermansia* to total bacteria was around 0.5% in healthy controls, and its levels decreased in the IBD subjects. *Akkermansia* was also found be negatively associated with colitis disease activity in the IL-10$^{-/-}$ mouse model (see Chapter 2). One explanation for these data is that inflammation changes the environment to be less suitable for *Akkermansia*. Another possibility is that is being immunologically targeted.

We also attempted to determine if we could stratify individuals by disease type by analyzing the phylotypes that showed differences in population densities between IBD and healthy subjects. In this analysis (Figure 4.3), most of the healthy subjects were clustered together. However, there was less cohesion in

the CD and UC groups. It is certainly possible that this approach will never provide adequate clustering by disease type. Other factors that could influence the utility of this approach include the fact that we did not account for disease activity (active or not), medication type, genotype, and/or disease subtype such as ileal versus colonic CD.

Overall, in this study, lower levels of several bacterial phylotypes were found in IBD subjects than in the healthy controls. Why this is occurring is not yet known. It is very possible that in CD, lower levels of specific mucosal bacteria exist because there is an immune response toward bacteria. Increases in immune responses to bacterial flagellin from Lachnospiraceae have been shown in patients with CD (Lodes, Cong et al. 2004). In our study, population densities of Ruminococcus 312, which is a member of the Lachnospiracaea, were lower in the CD subjects. In UC, subjects have less mucous and thus this selective niche is changed, which could cause decreases in bacteria inhabiting the mucosa. This hypothesis is consistent with the fact that Akkermansia 498 populations were decreased in our UC subjects and that it has been shown to be a mucin-inhabiting organism.

Table 4.1: Bacterial phylotype designations and parameters used in the design of the phylotype-selective qPCR assays.

Phylotype designation (accession)	Nearest cultured relative (accession, % identity)[a]	qPCR primer design (number of target sequences[b] / % identity[c])	qPCR primer design (number of non-target sequences[b] / % identity[c])
Akkermansia 498 (accession)	*Akkermansia muciniphila* (CP001071, 99%)	632 / 100-97.8%	2110 / 95.9-70.3%
Bacteroides 832 (accession)	*Bacteroides fragilis* (DQ100447, 99%)	1366 / 100-91.5%	1134 / 88.1-70.3%
Clostridium 501 (accession)	*Clostridium bolteae* (NR_025567, 99%)	55 / 100-98.2%	1370 / 95.5-70.9%
Clostridium 603 (accession)	*Clostridium nexile* (X73443, 97%)	70 / 100-98.1%	1310 / 96.0-72.7%
Eubacterium 2766 (accession)	*Eubacterium rectale* (CP001107, 99%)	1517 / 100-98.0%	2325 / 93.0-72.0%
Faecalibacterium 2994 (accession)	*Faecalibacterium prausnitzii* (AJ413954, 97%)	2240 / 100-95%	739 / 93.0-72.3%
Roseburia 575 (accession)	*Roseburia inulinivorans* (AJ270473, 99%)	561 / 100-98.2%	1876 / 95.9-93.5%
Ruminococcus 246 (accession)	*Ruminococcus* sp. DJF_VR70k1 (EU728792, 97%)	77 / 100-98.0%	2569 / 95.5-73.0%
Ruminococcus 312 (accession)	*Ruminococcus* sp. Eg2 (FJ611794, 97%)	393 / 100-98.9%	1233 / 96.0-72.7%
Ruminococcus 323 (accession)	*Ruminococcus lactaris* (NR_027579, 98%)	70 / 100-98.2%	2598 / 96.0-72.8%

[a]Determined by analyses using BLAST (Altschul et al. 1997).
[b]Number of sequences from this study and GenBank used as target or non-target for the design of the phylotype-selective primers.
[c]Range of pairwise identities between the sequence used to represent the phylotype (see accessions in far left column) and the other sequences used as target or non-target for the design of the phylotype-selective primers.
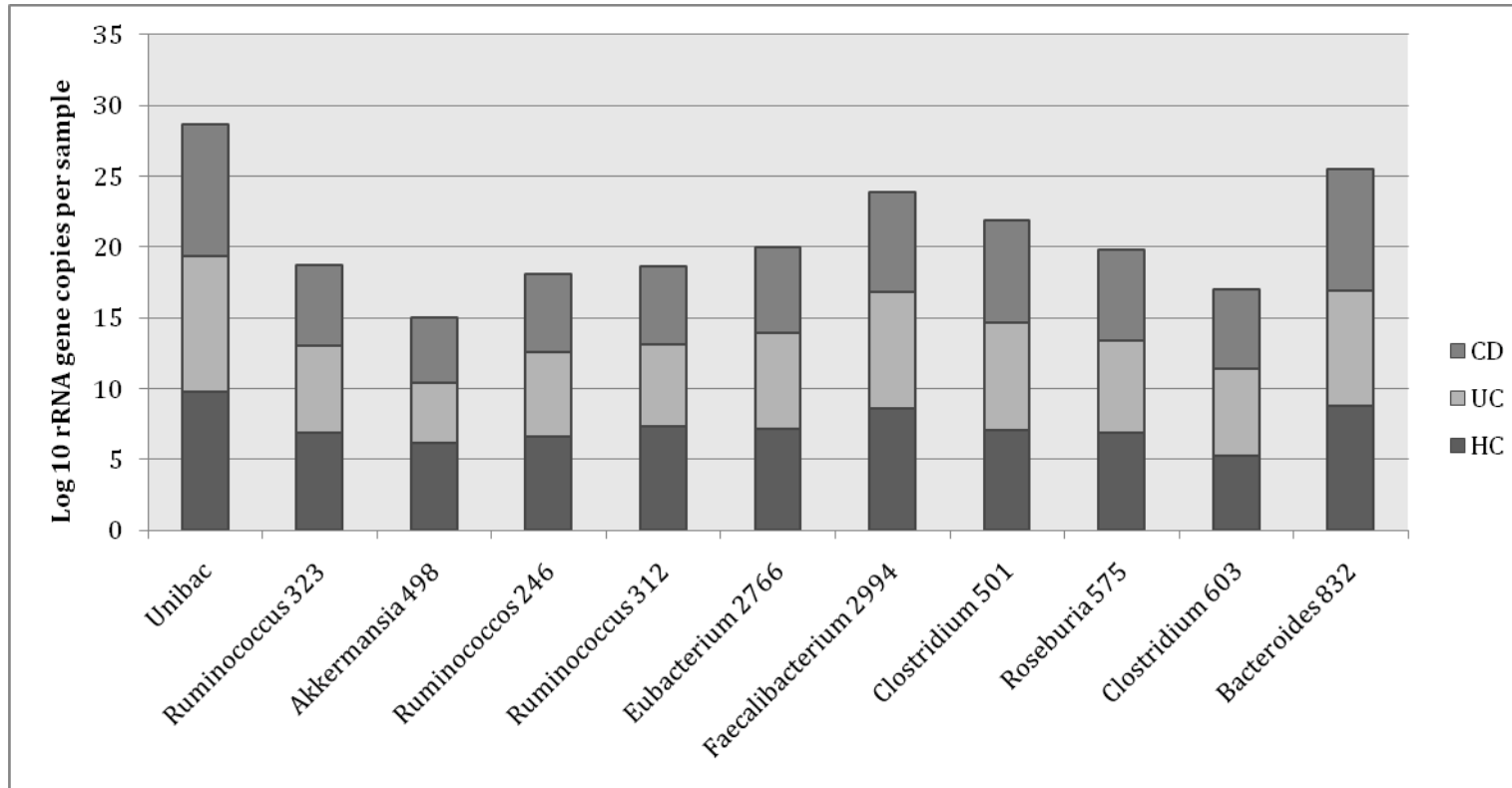
Figure 4.1. Bacterial phylotype population densities in intestinal mucosal samples from CD, UC and healthy control subjects. Phylotypes were enumerated by sequence-selective qPCR. Data were Log 10 transformed. Unibac data are from a qPCR assay targeting all bacterial 16S rRNA genes.
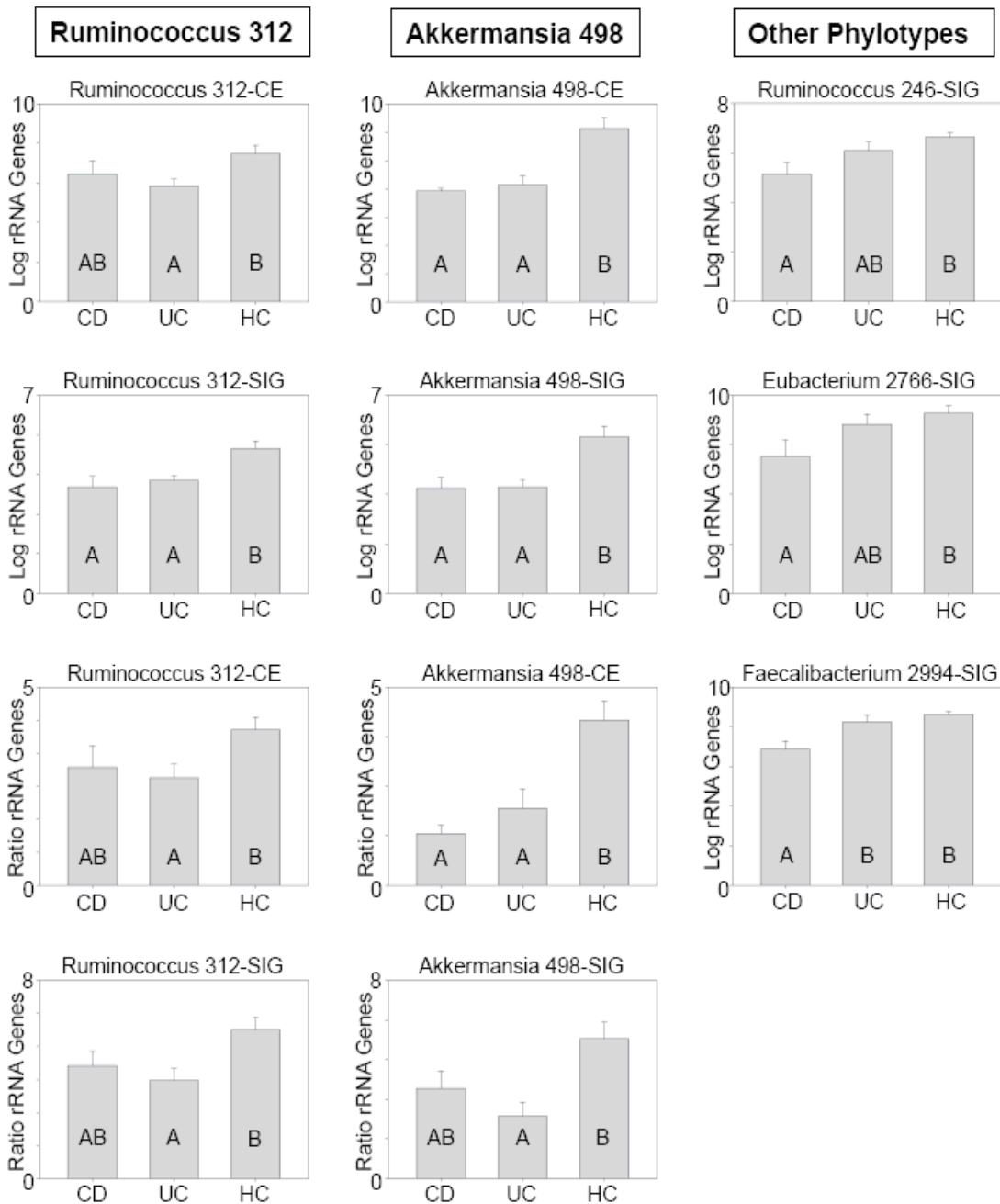
Figure 4.2. Differences in population densities of bacterial phylotypes and intestinal mucosal samples from CD, UC and HC subjects. "A" is smaller than "B" at $P < 0.05$. Log rRNA genes = Log 10 of rRNA gene copy number per samples. Ratio rRNA genes = rRNA gene copy number per sample for each phylotype divided by the total number of bacterial rRNA genes.
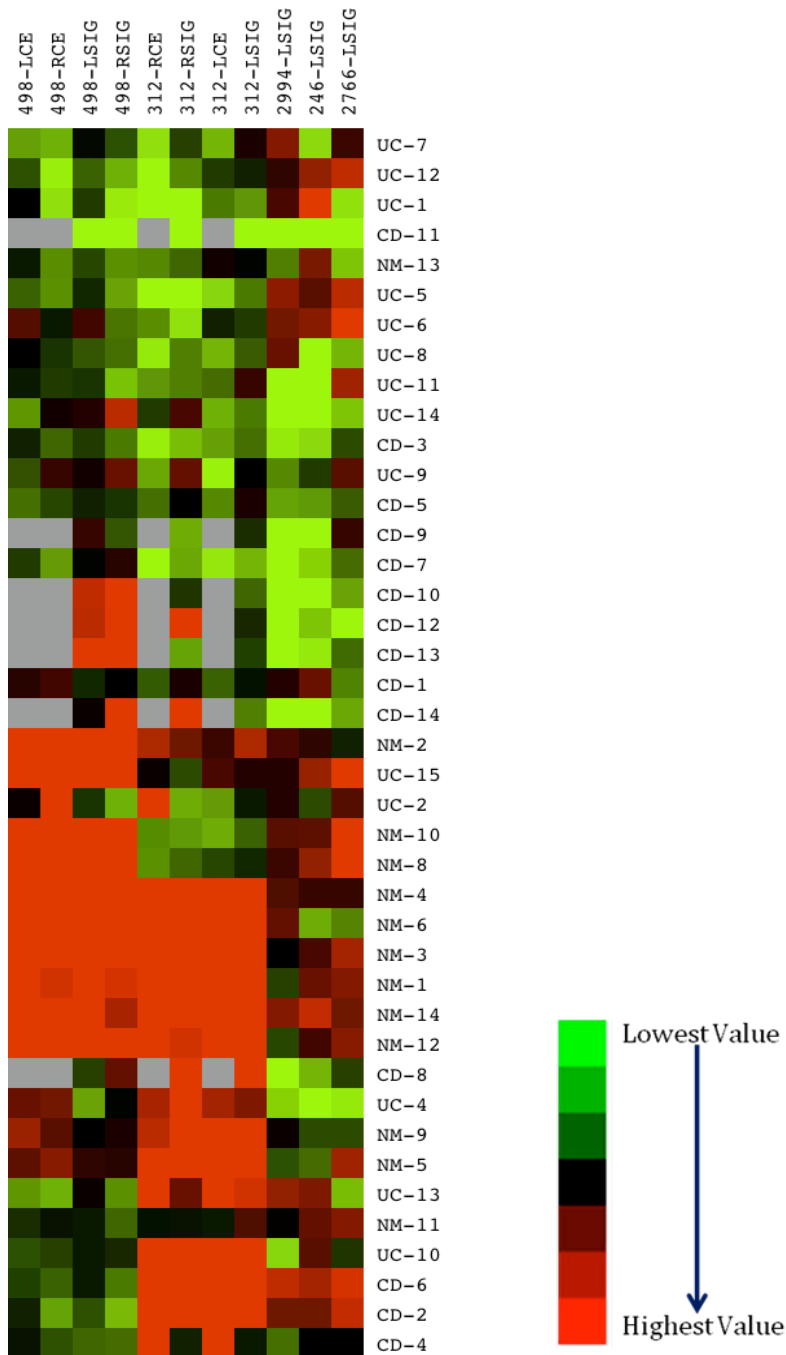
Figure 4.3. Associations of bacterial phylotypes with individual subjects (and their disease type). Horizontal axis: phylotypes with the intestine region designated. Vertical axis: individual subjects with disease type designated. LCE, Log transferred data from Cecum; Rce, Ratio of Cecum; LSIG, Log of sigmoid; RSIG, ratio of sigmoid. Color bar indicates abundance of the phylotypes.

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Chapter Five: Conclusion

The research described in this dissertation endeavored to provide a better understand of IBD etiology. The experimental strategy employed was to identify bacterial phylotypes associated with Colitis Disease Activity in IL-10$^{-/-}$ mice, genotype in IL-10$^{-/-}$ mice as well as IBD subtypes in humans.

Chapter 2 identified several bacterial phylotypes whose population densities exhibited associations with Colitis Disease Activity in IL-10$^{-/-}$ mice. Among other findings, this work identified a link between the population densities of specific bacteria and previously discovered immunologic responses to intestinal organisms. Namely, Lachnospiraceae populations exhibited a negative association with Colitis Disease Activity. This association suggests that these bacteria were being immunologically targeted, which is 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 this association suggests that the experimental approach used in this study will have considerable utility in elucidating the host-microbe interactions underlying IBD.

Based in part on the results from Chapter 2, the following etiology model for IBD was developed.

IBD etiology involves a sequential cascade of events comprised of genetic, immunological, and microbiological components.

(i) Epigenetic and genetic factors enable intracellular infection by *C. ramosum* and/or related Erysipelotrichi,

(ii) Infection of epithelial cells and mucosal leucocytes leads to barrier dysfunction and activation of innate immunity, both of which are exacerbated by deficits in pathogen clearance/killing and the ability of the pathogen to induce apoptosis resistance,

(iii) Persistent innate activation and barrier dysfunction, coupled with genetic impairment of immune regulation, leads to persistent adaptive immune activation (targeting in part resident bacteria such as Lachnospiraceae) and phenotypic colitis.

Various combinations of these variables produce the myriad of disease subtypes.

The new element of this model is the persistent infection by *Clostridium ramosum* and/or related Erysipelotrichi*.* This component provides a link connecting the genetic defects in innate immunity (NOD2 and autophagy) to barrier dysfunction, apoptosis resistance and chronic immune activation. In Chapter 3, we attempted to test a few components of this model.

If *C. ramosum* is involved in disease causation, one would expect its population densities to be positively associated with disease. In Chapter 2, such associations were observed in fecal samples in the IL-10$^{-/-}$ mice. In Chapter 3, greater population densities of *C. ramosum* in fecal samples were detected in the more colitis prone C3H mice than in the B6 control mice. In addition, in the RISA analyses of the tissue samples from the C3H mice, the only bacterial phylotype identified was *C. ramosum*; this phylotype was also only detected from one compartment – the mucosal fraction of the colon. This is intriguing as the colon is the primary site of disease in this mouse model.

Concerning the model and the ability of *C. ramosum* to infect cells, we would anticipate that deficits in innate immunity in C3H would lead to higher levels of *C. ramosum* infection. The in vitro culture studies with HT-29 cells suggested that *C. ramosum* has the ability to infect this cell line. Subsequent follow-on investigations utilizing microscopic techniques will endeavor to verify that this phylotype is able to infect host cells in C3H mice, and, more specifically, which type of host cells.

In Chapter 4, I examined bacteria in the mucosal compartment to determine if the population densities of such organisms could be used to stratify IBD types. In this study, five of the ten phylotypes examined exhibited differences between the IBD disease types and healthy controls. To our knowledge, this high rate of success is unique, demonstrating the utility of examining the intestinal mucosal compartment, and, in general, paying attention to microgeography. We anticipate that host-microbe associations such as these should also provide new clues as to the pathophysiologies of IBD.

Future studies. To verify our results that showed that the population densities of specific bacteria are associated with the disease type and host genotype, the

experiments described in Chapter 3 need to be repeated using larger numbers of mice. It would also be useful to repeat the experiments described in Chapter 3 using more defined genetic knockouts such as *NOD2*. Follow-on investigations should also be performed to determine if *C. ramosum* can infect host cells in mice, and, if they can, which cell types are infected. Finally, it would be useful to determine if the decreases in Lachnospiraceae population densities are caused by immune responses. Toward this end, I have cloned the flagellin gene from Lachnospiraceae. Subsequent steps will involve in vitro expression of this gene to obtain purified protein for seroreactivity analyses.