# Studying the enteric microbiome in inflammatory bowel diseases: getting through the growing pains and moving forward

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In this commentary, we will review some of the early efforts aimed at understanding the role of the enteric microbiota in the causality of inflammatory bowel diseases. By examining these studies and drawing on our own experiences bridging clinical gastroenterology and microbial ecology as part of the NIH-funded Human Microbiome Project (Turnbaugh et al., 2007), we hope to help define some of the "growing pains" that have hampered these initial efforts. It is our sincere hope that this discussion will help advance future efforts in this area by identifying current challenges and limitations and by suggesting strategies to overcome these obstacles.

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The notion that the indigenous enteric microbiota play an important role in the etiopathogenesis of inflammatory bowel diseases (IBD; Annese et al., 2005) is now well accepted. This realization has prompted a flurry of research activity that is aimed at determining the mechanisms by which changes in the gut microbiota contribute to the development and progression of IBD. Much of this research has been made possible by advances in culture-independent microbial ecology. Microbial ecologists who have been studying complex communities in natural environments such as soil and seawater have leveraged advanced molecular microbiological techniques to profile the structure and dynamics of these microbial consortia. However, the application of these techniques to human-associated microbial communities has not been particularly straightforward. Most clinicians and researchers who study clinically related problems are not familiar with the concepts and techniques employed by microbial ecology. Conversely, most microbial ecologists have not had a great deal of experience interpreting their data in a clinical context, therefore, both sets of investigators need to understand and develop new paradigms. By working together, clinical experts and microbial ecologists can design studies that will leverage their respective expertise and avoid potential pitfalls.

## CLINICAL CONSIDERATIONS IN APPROACHING THIS AREA
### IBD IS NOT A SINGLE DISEASE
Inflammatory bowel diseases is not a single disease, nor is it fully or accurately represented by the commonly used classifications of Crohn's disease and ulcerative colitis (UC). Crohn's disease includes a wide range of presentations and can affect any part of the gastrointestinal tract from the mouth to the rectum. Crohn's disease causes a transmural inflammation that can be associated with structuring, penetrating, and perianal disease. By definition, UC is limited to the colon and is characterized by confluent mucosal inflammation starting at the rectum and extending proximally to various lengths in the colon. The remaining 10–15% of patients are given the diagnosis of "indeterminate colitis," a classification based on having features of both Crohn's disease and UC, but also reflecting the ambiguities of clinical descriptors. None of the terms take into account the many types of genetic and pathophysiological processes that can lead to disease having similar clinical phenotypes. Thus, studies aimed at establishing a role of enteral microbial dysbiosis in the etiopathogenesis of "IBD" are not likely to be useful without recognition or consideration of the complexity and heterogeneity of IBD patient populations. The solution must involve designing studies where more homogeneous patient subsets can be defined. Unfortunately, this task remains a difficult as few molecular and genetic markers have emerged that identify a clear disease subset. Thus, the challenge remains in developing better metrics to identify IBD patients that share genetic and/or pathophysiological features. When this is achieved, we can truly begin to understand the relationship of enteric microbiomes to etiopathogenesis and clinical outcomes.

### MICROBIAL DYSBIOSIS IN IBD: CAUSE OR CONSEQUENCE?
Dysbiosis of the intestinal microbiota is commonly found in patients with IBD (Peterson et al., 2008), but in almost all cases, it

cannot be determined whether these changes are causal or merely consequences of the activated immune and inflammatory condition. Typically, large changes in 16S rRNA gene-based profiles are observed at the phylum level. These changes are characterized by blooms of Proteobacteria and decline in Firmicutes and Bacteroidetes (Frank et al., 2007), the latter typically associated with the microbiota of a normal, healthy colon (Eckburg et al., 2005). Similar patterns have been observed in other non-IBD inflammatory states (Lupp et al., 2007; Sekirov et al., 2008) and in experimental colitis (McKenna et al., 2008; Hoffmann et al., 2009; Nagalingam et al., 2011), suggesting that these changes are largely a consequence of the altered immune and inflammatory state. Similarly, studies of healthy and IBD-associated microbial functional metagenomes have shown significant differences (Manichanh et al., 2006). In the final analysis, however, these studies have resulted in the accumulation of large, descriptive datasets that have shed little light on fundamental mechanisms of IBD etiopathogenesis. What is needed are prospective studies, initiated at a time point before the onset of disease in order to capture information on functional and structural characteristics of the enteric microbiome that correlate with eventual development of disease or with maintained health. Unfortunately, these types of studies are difficult to do in IBD, because the means to identify subjects at risk do not yet exist. Moreover, long term, population-based studies that include microbiome analysis are impractical and cost-prohibitive. Nevertheless, a few opportunities exist for longitudinal studies of the enteric microbiome in IBD risk, particularly when the study questions and types of test subjects can be more precisely defined. As one example, the development of pouchitis in patients with UC is a condition where the incidence of disease is high and predictable (Hurst et al., 1996; Ferrante et al., 2008). Pouchitis is an inflammatory complication of the surgically created ileal pouch that serves as a pseudo-rectal vault in patients with UC who have undergone total colectomy. The condition is unique to UC, as it is rarely seen in patients with familial adenomatous polyposis (FAP) who undergo the same surgical procedure (Salemans et al., 1992). The study of pouchitis offers several advantages: (1) a clear "time-zero" can be defined when all patients are absent of disease and off medications, (2) each patient serves as his/her own control, (3) sampling of both luminal and mucosa-associated pouch microbiota is feasible, eliminating potential confounders such as the need for lavage preparation, and (4) the analysis of microbiome and host responses can be easily correlated with clinical outcomes.

### CONFOUNDING VARIABLES ASSOCIATED WITH CLINICAL RESEARCH

Very few studies to date that have examined the relationship between IBD and intestinal microbial dysbiosis have taken into account confounding variables such as age of onset, disease duration, patient age, gender, life style, smoking history, ethnic background, diet, environmental exposures, surgical history, and medications. Each of these factors may be important in IBD patients and can independently impact host biology and enteric microbiota, directly or indirectly. Conclusions drawn from data analysis in absence of careful multivariate analysis or patient stratification can be misleading and lead to both type I and type II errors. As one example, dietary modifications are common in the management or clinical course of IBD patients. Dietary components such as fat

and carbohydrates have also been shown to have dramatic effects on the enteric microbiota in animal models (Hildebrandt et al., 2009; Turnbaugh et al., 2009). Since the diets of patients in remission versus those that have active disease are likely to be quite different, this introduces a variable that confounds interpretation of data aimed at defining a causal relationship between changes in enteric microbiota and disease activity. Few studies have taken into consideration the types of medications patients may be taking. IBD patients take a number of medications which are aimed at altering the immune response and promoting intestinal healing. Although no data exists to indicate how the various classes of medications impact the intestinal microbiome in IBD, preliminary data from animal models suggest that medications may have a dramatic and sustained impact (Croswell et al., 2009; Hill et al., 2010; Manichanh et al., 2010; Robinson and Young, 2010). The nature of these confounding variables are perhaps more significant in complex immune-based disorders and challenge our ability to design studies that can yield unambiguous results. They underscore the importance of a team effort that involves clinician investigators, microbial ecologists, and biostatisticians in the design of microbiome-IBD studies in compiling, reviewing, and analyzing these important patient factors.

### TECHNICAL NUANCES AND CHALLENGES OF MICROBIOME RESEARCH RELATED TO IBD

Researchers who are considering embarking on studies of the microbiome in IBD need to consider numerous factors before initiating their studies. The planning and design phase are essential and arguably the most critical aspects of any research endeavor. The explosion of microbiome-based technologies provides numerous research opportunities and emphasizes the need for collaboration between clinical researcher and microbiologist, particularly in the early stages of study development.

These considerations can be divided into two main categories. The first involves the clinical (e.g., patient-oriented) aspects of the research. The second major category involves consideration of the technical aspects of microbiome research. Although most researchers who study clinical aspects of IBD might consider the latter category to be of prime importance, it is critical to realize that the nature of microbiome research requires re-evaluation of the patient-oriented components of IBD research.

### CLINICAL CONSIDERATIONS (SAMPLING, REPLICATES, ETC.)

Microbiome research involves characterizing complex microbial communities that inhabit a particular ecological niche. A major concern with regards to this question is exactly what samples should be studied. Current evidence suggests that in a genetically susceptible individual, IBD results from an abnormal interaction between the indigenous microbiota and the host epithelium/immune system (Sartor, 2008; Round and Mazmanian, 2009; Garrett et al., 2010). As such, consideration has to be given as to whether it is appropriate to sample luminal contents versus the microbes that are associated with the mucosa. It has been proposed that examination of fecal material is an appropriate surrogate for all of the microbial communities that are upstream of the rectum. In this manner, stool can be considered to be the "summary statement" of the entire gastrointestinal (GI) tract. However, while many of the organisms that inhabit the GI tract can be detected

in feces, the relative abundance of these organisms in fecal material is likely to be quite different from that found either luminally or mucosal-associated in more proximal sections of the GI tract. Since the relative abundance of microbes in a community is important to the actual function of that community, stool may not be the most appropriate analyte for microbiota research in all types of IBD (Lepage et al., 2005). For example in patients with ileal Crohn's disease, analysis of fecal material is likely to be a poor surrogate for the microbial community found in the terminal ileum.

Given the realization that fecal material may not be the most suitable sample for assessing microbial communities associated with IBD, despite the ease of obtaining this material and its relative abundance, many microbiome studies related to IBD utilize material retrieved via endoscopy. With regards to endoscopically harvested material, it should be noted that there is also an axial variation in the microbiota. Studies have demonstrated that the intestinal microbiota have both a structural and spatial organization that may be altered in patients with IBD and vary with disease activity (Swidsinski et al., 2002; Hu et al., 2010; Wang et al., 2010a,b). In addition to the axial variation, the variation in communities along the GI tract, coupled with the known regional variation in IBD from region to region, makes it important to take these variables into consideration when designing studies. A comprehensive experimental design might therefore involve sampling from both mucosal and luminal communities.

Another important consideration arises when obtaining samples via endoscopy; the standard bowel preparation regimens can have a significant impact on the gut microbiome. The washout of luminal intestinal contents can skew or abolish both the longitudinal and axial gradients that are normally present within the GI tract (Wang et al., 2010a,b) and thus affect the entire gut microbial community. Although bowel preparation is required for standard diagnostic and interventional endoscopic procedures, its routine use may adversely affect research endoscopies performed to understand the role of the indigenous microbiota in IBD. Even local endoscopic washes prior to sample collection could alter the community structure of the associated microbes and should be avoided or taken into consideration when interpreting findings. Furthermore, the exact sampling technique needs to be considered. Biopsies are commonly used, but cause local trauma (which may affect subsequent longitudinal samples) and survey a limited area. Brushings can be used, but it needs to be established whether these two techniques yield similar samples. Regardless of what types of specimens are obtained, investigators should carefully follow established protocols for collection, handling, and storage of samples. Inconsistencies in these steps may dramatically alter microbial communities thereby decreasing the reliability and accuracy of the results.

In addition to these considerations regarding sample acquisition from an individual patient, there are multiple considerations relating to overall planning and establishing a study population. It is becoming abundantly clear that although certain features of the gut microbiome are encountered in many normal individuals, there is also considerable person-to-person variation (Costello et al., 2009; Benson et al., 2010; Willing et al., 2010; Walker et al., 2011). As such, considerations related to replication, study size, and selection of controls related to microbiome research require significant attention. The analytic methods related to processing microbiome data involve statistical methods that previously were not commonly applied to biomedical research (see below). Therefore, the standard power analyses that are applied to clinical research are not always easily translated to microbiome study design. Given that this field is in its infancy, there is no consensus yet on which statistical methods and power analyses are most appropriate. However, given the ongoing work in this area, clarification of which statistical methodology to use is forthcoming. In addition, because many early microbiome studies have been aimed at finding associations between aspects of the microbiome and health or disease, these studies have been "hypothesis generating"; once they are completed, appropriately powered confirmatory studies can be designed.

Experimental replication also requires significant consideration (Prosser, 2010). Replication can be in the form of multiple samples from the same region from a given individual at a specific time, longitudinal sampling from an individual patient, or obtaining samples from multiple individuals stratified by specific clinical criteria. Each form of replication may be important depending on the actual clinical question posed. Selection of appropriate controls can also be problematic given the inherent individual variation in microbiome structure. Comparative studies of the microbiome in IBD have used "healthy" controls that included patients with irritable bowel syndrome (IBS), diverticulosis, acute self-limited colitis, and a variety of other gastrointestinal conditions. Clearly many of these controls may also have perturbations in their microbiome, which must be accounted for in the data analysis and interpretation of results. In some cases, patients might be able to serve as their own controls, which will be useful for understanding how the microbiota changes correlate with the course of disease. With such a study design the status of the microbiome within a given patient prior to a particular intervention, for example treatment with a biologic agent or antibiotics or surgery, could serve as the controls for subsequent samplings that were done in a longitudinal manner.

Finally, although study of human patients is a necessary component of IBD research, there is a distinct utility for using animal models of disease (Wirtz and Neurath, 2007). Animal models, including the wide variety of murine models of IBD, can be used in conjunction with human clinical and translations studies to address questions mechanism and causality in IBD pathogenesis. Specific hypotheses that arise from observations in human patients can be directly tested in appropriate animal models. The same considerations with regards to sampling need to be applied to animal model studies, especially to provide comparability with observations made in patients.

## ANALYSIS OF THE MICROBIOME: WHERE TO BEGIN?

For investigators who have not conducted or even considered performing microbiome research the variety of analytic methods that are available can appear daunting (Robinson et al., 2010). Many of the culture-independent methods for studying microbial ecology have benefited from the advances in next-generation sequencing platforms (Andersson et al., 2008; Huse et al., 2008). Additionally advances in mass spectroscopy and other means for large-scale analysis of complex mixtures of proteins and metabolites have been applied for microbiome research in order to try and make sense of which methods might be appropriate for specific

biomedical questions. Investigators can start by understanding what types of questions each specific method is best suited to address. One way to consider the suite of methods for microbiome analysis available is to divide them into groups based on the specific types of information they provide about a given microbial community. There are several useful reviews that describe the available technologies (Zoetendal et al., 2008; Simon and Daniel, 2011). The first type of information available is information about the structure of a specific consortium of microbes. This can be thought of as a census of microbes both in terms of the number of different types of microbes and their relative abundance. The next type of information goes beyond community structure and provides a cataloging of functional capacity of the entire community. The final type of information that can be gained by certain analytic methods gives information regarding the *in situ* activity of the given microbial community. We will discuss each of these platforms in more detail as well as describe in general terms specific analytic methods that can provide each of these types of data.

### 16S SEQUENCE RETRIEVAL: CHOOSING THE APPROPRIATE PLATFORM

Landmark ideas and research from Woese et al. (1990) and Pace (2009) established a common metric for identifying microbes – the nucleotide sequence of the small subunit (SSU) ribosomal RNA. The gene encoding the SSU rRNA has a sedimentation coefficient of 16S which is unique to bacteria and archaea and allows distinction from the SSU rRNA from human eukaryotic cells. Initially, SSU sequences were obtained by amplification and sequencing of SSU genes from complex microbial communities and then compared to databases containing more than 2 million aligned rRNA gene sequences (DeSantis et al., 2006; Pruesse et al., 2007; Cole et al., 2009) to provide a census of microbes in each sample. More recently, the application of "next-generation" sequencing platforms has increased the number of sequences that can be obtained, as well as lowering the cost of analysis (Sogin et al., 2006; Huse et al., 2008).

Analysis of the data obtained by SSU sequence analysis continues to evolve, but two general approaches are used to bin or classify the sequences into microbial populations. Sequences can be compared to reference taxonomic outlines and binned based on similarity to references sequences ("phylotyping") or the sequences can be assigned to operation taxonomic units (OTUs) based on similarity to other sequences within a given dataset. There are relative advantages and disadvantages of each method (see Schloss and Westcott, 2011 for a discussion). It should be noted however, that the desire to "name" a given community member based on SSU analysis can be complicated by the fact that most existing taxonomies for bacteria are based solely on cultured organisms.

The number of sequences required to assess microbial communities depends both on the questions being asked as well as the spatial and temporal variability in a community. Deeper sequencing will uncover less common members of a community, which may be necessary to enumerate a particular pathogen, but shifts in overall community structure can be detected identified with many fewer sequences (Young and Schmidt, 2004; Antonopoulos et al., 2009). A critical factor in determining the depth of sequencing required to address a question is to assess variability within replicate samples and determine if the variability is less than that found in treatment level comparisons. Pilot studies with either clone libraries or high-throughput sequencing methods are essential to identify the degree of variability and will establish the extent of sequencing required in a full-scale experimental design.

The next step is to consider how the structure of the microbial community might relate to its function. 16S sequences on their own do not provide specific functional information. However, if there is a genome sequence available corresponding to a bacterium with a given 16S with a known function, it may be possible to infer the functional capacity. It should be noted that inference of the metabolic potential of an organism based on its SSU rRNA gene sequences may also be complicated by the lateral transfer of genes between microbes.

### LOOKING AT THE "BIG PICTURE": METAGENOMES, METATRANSCRIPTOMES, AND *IN SITU* ANALYSIS

Rather than inferring metabolic potential from 16S rRNA gene sequences, the genetic diversity of the microbiome can be accessed directly through shotgun metagenomes (Handelsman, 2004; Riesenfeld et al., 2004; Streit and Schmitz, 2004; Gill et al., 2006). In this approach, DNA extracted from a sample of the microbiome is sequenced directly, rather than following amplification of a specific gene (e.g., 16S rRNA). The absence of a specific amplification step to recover microbial genes often means that suitable amounts of DNA from microbial communities are difficult to obtain, particularly without interference from host DNA. Physical methods for separating microbial communities from host tissue, including the user of lasers to remove attached microbes from epithelial cells in the GI tract (Wang et al., 2010a), can be effective, but typically provides insufficient DNA for direct sequencing. Fortunately, there are approaches for whole genome amplification that can be employed to produce sufficient DNA for metagenomic sequencing (Binga et al., 2008). Understanding the biases and variability introduced by each of these steps is essential for a meaningful analysis of the resulting sequences.

When sequences derived from metagenomes are compared to previously characterized genes, using platforms such as MG-RAST (Glass et al., 2010), a picture of the metabolic potential of a community emerges. Millions of sequences from shotgun metagenomes from the human GI tract (Qin et al., 2010) have been generated in an effort to identify those that are consistent with health and various disease states. It has been suggested that while the taxonomic structure of microbiomes can fluctuate considerably, the composition of metabolic genes remains consistent (Turnbaugh et al., 2009). The definition of OTUs for both rRNA genes and protein-encoding genes will certainly influence this interpretation of the data: defining the appropriate level of resolution in sequence analysis is central to future analysis of microbiome sequences.

A logical extension of the metabolic potential suggested by community metagenomic sequencing is insight into the actual activity of a community gained through metatranscriptomic sequencing (Gilbert and Hughes, 2011; Gosalbes et al., 2011). In this case, total RNA is isolated and structural RNAs removed to enrich for mRNA, which is then reverse transcribed into cDNA for sequence analysis. Rather than just revealing the potential activity, this will indicate which of the potential metabolic

pathways are actually being used on the basis of their transcription within the community. To move even closer to actual function, metaproteomics employs high-throughput, high-resolution mass spectroscopy to determine which proteins are actually present in a given community (Verberkmoes et al., 2009). This approach generally requires some knowledge of the coding potential of a community in order to make predictions about potential proteins based in mass/charge ratios, and thus is often combined with metagenomic sequencing. A final approach used to assess *in situ* function, often via mass spectroscopy, is to measure the complement of metabolites (e.g., short chain fatty acids, lipids, small molecules) associated with a community. This so-called metabolomics or metabonomics approach assesses function based on the presence of metabolites, many of which will be produced by specific members of the community (Martin et al., 2007; Kinross et al., 2011).

The three dimensional structure of microbial communities in the GI tract, particularly those in close proximity to epithelial cells may also provide useful information about the function of the community, including cell–cell interactions among microbes and between microbes and their host. Extraction and purification of DNA for microbiome analysis obliterates the architecture of microbial communities, but fortunately the sequence data gathered as part of a SSU microbial census can be used to design fluorescently labeled probes that permit visualization of the structural organization of microbes in preserved samples. The recent application of combinatorial labeling of probes and spectral imaging (Valm et al., 2011) offers the potential to visualize dozens of microbes in a community and holds considerable promise for microbiome studies.

### SELECTING THE APPROPRIATE METHODOLOGY; AN ARGUMENT FOR THE TEAM APPROACH

With this immense armamentarium of tools for microbiome analysis, the decision as to which method to employ must return to the most basic considerations, namely, what is the scientific and/or clinical question(s) to be addressed? In some cases, associations with disease based on 16S sequence retrieval are an appropriate first step, in an exercise as we discussed earlier that can be thought of as being hypothesis generating. However, in order to specifically test a given hypothesis or to monitor the physiologic effects of specific microbiome alterations, functional assessments via metagenomics or metabolomics might be more appropriate. To help in such decision making, a "team science" approach is often necessary, bringing together clinicians with expertise in IBD with microbial ecologists, bioinformatics specialists, statisticians, and microbial physiologists. As demonstrated by the NIH Human Microbiome Project (HMP) and the European MetaHIT projects, collaborative teams of scientists from a broad range of disciplines working together to address questions of the microbiome in health and disease are an important and effective approach. Similarly, the study of IBD using a "systems science," with interdisciplinary teams and expertise will be essential for discovering the etiopathogenesis of these diseases, novel therapies, and potentially a cure.

### SUMMARY

We propose that collaboration between microbial ecologists and clinician investigators is critical and should be considered an essential component for translational studies of the role of the microbiome in IBD. We caution against overzealous claims about the significance of the findings based on our current evidence. Existing descriptive studies should be used to generate hypotheses and help us move toward mechanistic studies that will truly help us discover the causes and potential cures for IBD and other gastrointestinal diseases. As our molecular techniques for evaluating the microbiome evolve and become more refined, the field needs to move beyond the descriptive studies which constitute the current state of microbiome-IBD research and toward mechanistic studies that will fundamentally improve and expand our understanding of IBD.

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