Application of Anaerobic Fluorescence Activated Cell Sorting to Isolate and Cultivate Inflammatory Bowel Disease Associated Gut Microbes

Application of Anaerobic Fluorescence Activated Cell Sorting to Isolate and Cultivate Inflammatory Bowel Disease Associated Gut Microbes

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Abstract

The gut microbiome has been an area of intense study over the last decade and numerous studies have demonstrated its profound impact on both health and disease. The microbiome has been implicated in the etiology of chronic inflammatory conditions such as ulcerative colitis and Crohn's disease, collectively referred to as inflammatory bowel disease (IBD). Examination of IgA coated bacteria in IBD patients has led to the identification of specific bacteria that are involved in disease progression. Techniques to isolate and culture viable bacteria anaerobic bacteria from the gut microbiome based on surface phenotype, such as high levels of IgA, are extremely limited. The integration of a fluorescence activate cell sorting (FACS) instrument within an anaerobic culture environment was explored in this study to determine the feasibility of directly isolating IgA+ bacteria from IBD patients. Construction of a custom anaerobic FACS sorting platform enabled the technical development of methods to isolate viable IgA+ and IgA- obligate anaerobes from human fecal material. These methods were deployed to generate a IBD patient IgA coated bacteria culture library containing over 2000 isolates, representing 147 unique species. This collection of IBD patient bacteria can now be phenotypically characterized *in vitro* and *in vivo* to understand their contributions to IBD pathophysiology.

Dedication

To Dadu

Acknowledgements

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Chapter I.

Introduction

The gut microbiome is the collection of trillions of microbes that inhabit our gastrointestinal tract which has become increasingly appreciated as a significant factor in human health and disease. The microbiome has been implicated in the etiology of chronic inflammatory conditions such as ulcerative colitis and Crohn's disease, both of which are collectively referred to as inflammatory bowel disease (IBD). Changes in gut bacterial composition have been observed in IBD patients and certain features of this imbalance or dysbiosis have been correlated to disease state (Kostic et al., 2014). In many cases however, direct causative evidence linking a single bacterium or bacterial ecosystems to disease phenotype has not been adequately demonstrated. This is largely due to both the technical challenges presented by isolating and culturing anaerobic bacteria from patients and a general lack of methods to systematically identify bacteria with phenotypes associated with disease (Maier et al., 2015). A major step toward the goal of identifying disease-causing members of the microbiome will be to develop platforms that allow the prospective isolation and cultivation of anaerobic bacteria from patients for phenotypic characterization. Obtaining viable disease associated gut bacteria will enable experimental investigation into causative links of microbiome composition to the pathophysiology of IBD.

IBD and the microbiome

Inflammatory bowel disease (IBD) is a chronic inflammatory condition that is characterized by periods of active disease and remission. During active disease or flare states, mucosal inflammation occurs leading to ulcerations and other disruptions of the gut epithelial barrier. IBD is an umbrella term for the major forms of chronic gastrointestinal inflammatory conditions, ulcerative colitis (UC) and Crohn's disease (CD). While there are many common features of both UC and CD, some distinctions do exist. CD does seem to have a stronger causal genetic link and can occur in sites along the large and small intestine. UC is restricted to the large intestine and the ulcerations formed are more superficial than those found in CD. One key similarity that does exist among these different forms of IBD is the strong influence of one key environmental factor residing right next door to the gut epithelium, the microbiome (Xavier et al. 2007).

The strong connection between IBD and the microbiome have been intensely studied over recent years. IBD is also viewed as a key opportunity to translate our developing understanding of the microbiome into therapy. Examination of the microbiomes from patients with IBD has revealed certain shifts in the abundance of certain groups of bacteria that correlate with disease. For example, it has been consistently observed that there is an expansion of *Enterobacteriaceae* and a depletion of *Clostridia* species in IBD patients (Gevers et al. 2014). Additionally, studies of

microbiome structure over time in IBD patients suggest that changes in the abundance of specific species is much more dynamic than those from healthy subjects (Halfvarson et al. 2017). These observational studies of IBD patients demonstrate clear microbiome alterations but do not offer any direct evidence that a one species or group of species are responsible for causing IBD pathophysiology. It is unclear if these changes are a cause or result of host inflammation, thus necessitating experimental demonstrations of the disease promoting or disease mitigating effects of the different members of the microbiome. In recent years, the increase in the availability of cultured bacteria from the human microbiome has begun to allow researchers to design studies that address the mechanisms by which different microbes, at the individual and community level, interact with the host to cause disease.

Experimental mouse models of colitis have been critical tools to provide direct links to the microbiome as a causal factor of disease induction. For instance, many genetic mouse models of colitis, in which genes shown to be associated with IBD in humans have been deleted, require the presence of a microbiome for colitis symptoms to manifest (Xavier and Podolsky 2007). The IL-10 knockout mouse model illustrates one such example. IL-10 is an important cytokine for maintaining mucosal health and animals lacking both copies of the IL-10 gene develop spontaneous colitis. However, when IL-10 mice are maintained in germ-free conditions disease development does not occur (Sellon et al., 1998). Conversely, Eun et al. (2014), colonized IL-10 deficient mice with a collection of seven bacteria shown to be associated with IBD through various observational studies of IBD microbiome composition and saw more severe disease onset than their conventional IL-10 knockout mice counterparts. One conclusion from these

studies is that the microbiome is key to activating the intestinal immune system and that disease development will rely on whether the bacteria that initially educate the immune system are inflammatory or positive regulators of immune function.

It is also important to highlight the positive effects the microbiome can have on intestinal health, particularly the effect of beneficial metabolites produced by commensal bacteria that are believed to protect against the development of IBD. Studies of commensal strains of *Bacteroides fragilis* have demonstrated a protective effect in pathogen-induced models of colitis. Further work investigating the mechanism of *Bacteroides fragilis* mediated colitis protection identified polysaccharide A (PSA) as the key molecule responsible for the anti-inflammatory activity of this commensal. To demonstrate the necessity of PSA for this protective effect, Mazmanian and colleagues (2008) tested strains of *Bacteroides fragilis* with and without the ability to produce PSA in pathogen induced colitis models and showed that protection was only conferred by the PSA producing strain. A purified form of PSA was also shown to be protective, providing further evidence of the beneficial properties of this commensal derived molecule.

Communities of commensals as opposed to single microbes have also been shown to work in concert and produce beneficial metabolites that stimulate various anti-inflammatory host functions. A collection of 17 human *Clostridia* strains has been demonstrated to protect mice in several models of colitis. The protective effect was shown to be due to the expansion of regulatory T-cells, immune cells that are important for controlling inflammatory responses and limiting host tissue damage. The increase in the number of regulatory T-cells is in part dependent on the production of short chain fatty acids (SCFAs), particularly butyrate, which were shown to be generated by the

community of *Clostridia* strains (Atarashi et al., 2013). Furthermore, the SCFA induced regulatory T-cell expansion was dependent on the presence of the entire community of Clostridia strains since single strains and subsets of this collection were unable to reproduce this anti-inflammatory phenotype. Interestingly, analysis of bacterial functions that are lost in IBD patient microbiome samples, SCFA production is among the processes that are significantly diminished (Huttenhower et al., 2014). The protective effects of microbial derived metabolites have made the microbiome a new hunting ground for new therapeutic natural products that may provide therapies for IBD and many other diseases linked to inflammation and has highlighted the important balance that exists between commensal and pathogenic gut bacteria in health and disease.

Culture based identification of intestinal microbes

New isolation techniques for anaerobic bacteria are improving our understanding of the human microbiome by allowing researchers to identify species that are not detected by commonly applied molecular technologies and by enabling the experimental manipulation of previously "un-culturable" bacteria. Strategies to expand the repertoire of cultured anaerobes from the human gut are an active area of research. A recent brute force approach employed a seemingly impossible number of culture and selection strategies to isolate new members of the microbiome (Lagier et al., 2012). In this study, the authors picked over 30,000 colonies that grew in their vast set of culture conditions only find that they had identified 340 unique species. Despite the redundant identification of several isolated bacteria, this effort did result in the isolation of over one

hundred species that had never been observed to reside in the human microbiome. More recently, a different group elegantly took advantage of a natural protective function of many bacteria, sporulation, to obtain an increasing number of previously uncultured species (Browne and Forster et al., 2016). This approach that involved an ethanol treatment to enrich for ethanol-resistant spore forming members of the microbiome was much more efficient and yielded 137 unique bacterial species, from approximately 2000 colony picks. Innovative bacterial isolation techniques will continue to expand our understanding of and facilitate the study of microbiome structure and function during health and disease.

Immunoglobulin A (IgA)

IgA is an antibody produced in abundant quantities at mucosal surfaces. In the intestinal epithelium IgA is produced by plasma B-cells in the lamina propria where it then binds to polymeric Ig-receptor (PigR), which is present on epithelial cells, and is transported across the epithelium into the lumen to protect the host from inflammatory activation of resident microbes that exist adjacent to the intestinal barrier (Slack et al., 2012). Recent work has illustrated the importance of this antibody defense response, particularly T-cell dependent IgA production. The production of high affinity IgA is mediated by the innate immune signaling molecule MyD88 in T-follicular helper cells that then present antigen to IgA producing B-cells (Figure 1). T-cell specific deletion of MyD88 reduces the amount of IgA present in the intestinal lumen and makes knockout animals more susceptible to chemically induced colitis (Kubinak et al., 2015). This

evidence suggests that T-cell dependent IgA production is an important immune function that influences the balance of bacteria in the gut and disruption of this pathway results in vulnerability to disease induction.

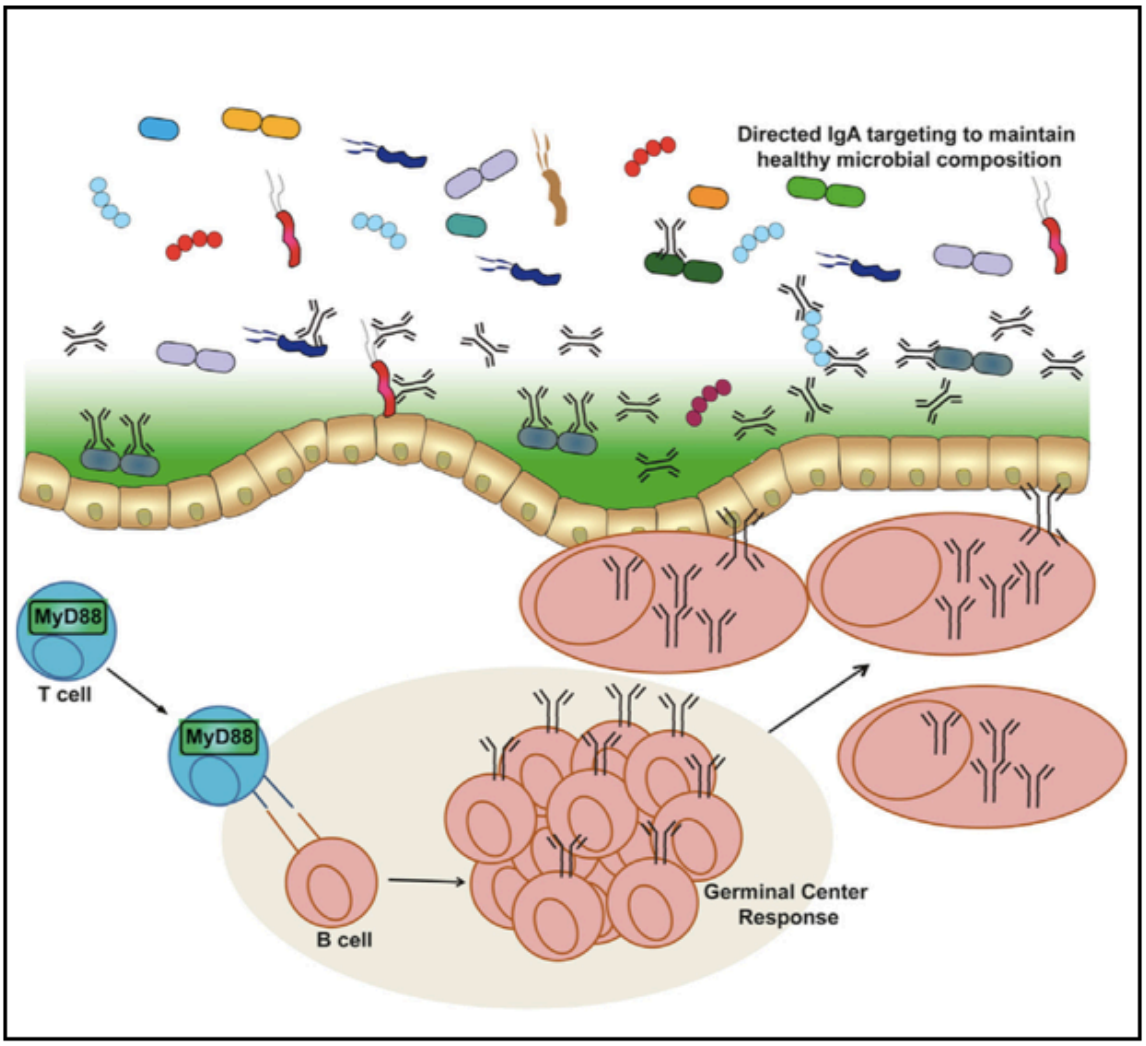

Figure 1: Model of T-cell dependent IgA production.

T-cell dependent IgA production is mediated by MyD88. Kubinak et al. (2015) demonstrated the necessity of MyD88 in T-follicular helper cells for B cell production of IgA molecules targeted by the host to specific bacteria. The MyD88 driven IgA production pathway plays a role in regulating the composition of microbiome.

Palm et al. (2014) took advantage of the IgA response to serve as a guide to identify colitis causing bacteria from patients with IBD. In this study, bacteria were

isolated from IBD patient stool and FACS sorted based on the amount of IgA that was coating a given bacterial cell. After fecal bacteria were sorted from both IBD patients and healthy subjects, the IgA+ and IgA- fractions were sequenced to identify the members of each group and provided a list of bacteria that could be targeted for isolation from the same patient stool samples. Bacteria isolated and identified as a species on the lists of IgA+ and IgA- microbes were compiled into two test consortia to determine if an IgA+ community could cause colitis in mice. To do this, germ-free mice were colonized for two weeks with either the IgA+ or IgA- bacterial consortia and then given dextran sodium sulfate (DSS), a chemical that induces colitis in mice. Remarkably, the mice that received the IgA+ consortium were far more sensitive to DSS induced colitis (Figure 2), showing much more severe features of disease than the IgA- colonized mice and proving that host immune system guided identification strategies can be effective methods to find bacterial drivers of disease phenotypes.

While this study effectively describes a method to systematically identify pathogenic bacteria from IBD patients, it does not describe an approach to directly isolate these bacteria. The oxygen sensitivity of a large proportion the gut microbiome limited the ability of direct isolation of the IgA+ and IgA- bacteria after FACS sorting. Instead, the IgA FACS sorting method was used to identify a list of species that the authors could then search for and isolate when the original stool samples were plated out and cultured under anaerobic conditions. This is an indirect way of isolating IgA+ bacteria and there is no way to be certain the same IgA coated strains in the original sample analysis were the strains being isolated. This method also relies on the ability of IgA coated microbes to grow in competition with their non-IgA coated counterparts. The isolation of IgA coated

microbes from patient samples that have a low frequency of IgA+ cells could potentially be hindered due to outgrowth of more abundant species when plated as a bulk population in the absence of initial enrichment from the complex community. Developing methods to preserve the viability of anaerobic bacteria during the FACS sorting process is an obvious next step to enable the enrichment and direct isolation of antibody targeted bacteria for investigation into disease driving mechanisms of action.

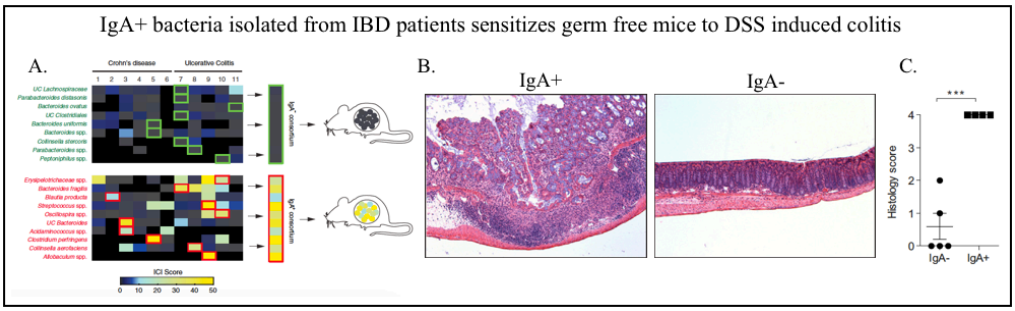

Figure 2: IgA+ bacteria isolated from IBD sensitizes GF mice to DSS-induced colitis.

Demonstration by Palm et al. (2014) that IgA coats colitis causing bacteria in patients with IBD. A. Bacteria identified as being either IgA+ or IgA- by sequencing IBD patient stool samples that were subsequently isolated from the same cultured patient stool samples to assemble both IgA+ and IgA- consortia for experiments in germ free mice. B. Representative histology samples from germ free mice colonized with either IgA+ or IgA- consortia clearly show higher levels of inflammation in response to DSS treatment in the IgA+ group. C. Histology scores for DSS treated IgA+ and IgA- colonized mice show significantly increased histology scoring in the IgA+ colonized mice.

Anaerobic FACS

Fluorescence activate cell sorting (FACS) is a powerful technique to isolate specified cells of interest from a heterogeneous cell mixture. FACS sorting is increasingly being utilized to tease apart and better understand bacterial ecosystems, such as IgA+ bacteria that exist among the rest of the microbiome. Surprisingly, there have not been many examples of FACS systems developed to sort anaerobic bacteria. One group working in collaboration with BD Biosciences Advanced Cytometry group specially engineered a BD Influx sorter with an anaerobic sort chamber and glove box for the viable recovery of various environmental obligate anaerobic species. (Thompson et al., 2015). This group demonstrated the capabilities of their system by examining the single cell recovery rates of a set of four different individual species after the anaerobic sorting procedure. Another example of sorting, mindful of the preservation of anaerobic species, comes from another IgA story. Jeff Gordon's lab has described the role of IgA targeted bacteria in childhood malnourishment. In these studies, immediate transfer of IgA+ bacteria isolated by FACS could transmit the disease phenotype under investigation (Kau et al., 2015). The methods used to preserve viability of anaerobes were based simply on minimizing oxygen exposure. Samples were handled under strict anaerobic conditions as much as possible but FACS sorting was performed under normal aerobic conditions. Despite the observation that the sorted IgA+ bacteria could produce a phenotype, the lack of controlled anaerobic conditions does not exclude the possibility of

lost species due to oxygen exposure, nor does this study provide methods for the systematic isolation of anaerobic IgA+ species for further characterization *ex-vivo*. We are unaware of any published comprehensive efforts to implement anaerobic FACS sorting to generate collections of disease-associated bacteria from the human gut microbiome.

The integration of FACS sorting instrumentation into an anaerobic culture chamber would enable to the direct isolation of viable anaerobic bacteria based on surface marker phenotype. Application of anaerobic FACS sorting to IBD patient stool bacteria will enable the direct isolation, cultivation and banking of IgA+ and IgA- bacteria. Currently, there is a limited set of studies that have described FACS based methods to isolate anaerobic bacteria for subsequent cultivation. Additionally, there are no widely available laboratory instrument configurations that enable FACS sorting compatible with preserving anaerobe viability. This work will describe the construction of a custom anaerobic FACS sorting chamber. This system will be used to develop validated methods that maintain viability of obligate anaerobes isolated from patient samples and FACS sorted based on the level IgA binding. These methods will then be employed to isolate, culture and bank an IgA targeted culture collection from IBD patients. This effort will provide a library of bacteria that will serve as a valuable reagent collection that can be functionally characterized *in vitro* and i*n vivo* to help elucidate bacterial-induced mechanisms of IBD pathogenesis.

Chapter II.

Materials and Methods

The construction of an anaerobic FACS sorting chamber IBD patient stool collection and experimental protocols are described in this chapter. Novel methods were developed and validated to enable anaerobic FACS based bacterial isolation. These techniques were applied to IBD patient stool samples to isolate disease relevant microbes.

UC Donor stool collection and processing

Fecal collections from UC patients were arranged through the biological research material service provider, BioReclamationIVT. An acceptable donor was undergoing a UC flare that had not taken antibiotics for at least 6 weeks. Fresh fecal material was collected from flare state UC patients and a healthy control donor. Each stool sample was sealed in a donation container sealed tightly with parafilm and shipped overnight on ice from the collection site to Seres Therapeutics. Upon receipt, stool was immediately transferred to anaerobic chamber for processing to maintain viability of oxygen sensitive microbes. Fecal material was assigned a Bristol Score (Thabit and Nicolau 2015) and homogenized by mixing thoroughly with a disposable spatula. In cases in which stool was watery (Bristol Score=7), donation container was swirled to mix. Stool was then weighed-out into tubes and resuspended in 15% pre-reduced glycerol in PBS to make a 20% w/v fecal suspension and aliquots were stored at -80°C. Two additional fecal

samples used in this experiment were obtained from collaborators at University of Pennsylvania collecting stool from patients with pediatric IBD. These samples were frozen after collection and shipped to Seres Therapeutics on dry-ice. These samples were thawed in the anaerobic chamber and processed for storage as described for fresh fecal donations. Pediatric IBD patients had been newly diagnosed and had not been treated with antibiotics. All samples used in this study were collected under IRB approved protocols and donor identity has been protected by sample providers. All handling of human fecal material and its derivatives were conducted under BSL-2 conditions.

Customized anaerobic FACS chamber

A custom anaerobic chamber was constructed by COY Laboratory Products to house a BIO-RAD S3e FACS sorting instrument (Figure 3). The instrument was selected for its compact dimensions and relative ease of use, designed for "walk-up FACS sorting". The anaerobic chamber was outfitted with additional gloves to provide ability to access critical FACS instrument components and enable maintenance of anaerobic conditions, 0% Oxygen and 3% Hydrogen, while troubleshooting technical issues that arise in flow cytometry, such as replacing a clogged tip or correcting flow stream alignment. Additional plug ports were added in the area surrounding the FACS instrument to deliver extra electrical plug and fluidics tubing access points. A large removable access port was placed behind the instrument to allow easy access for planned routine service and repairs.

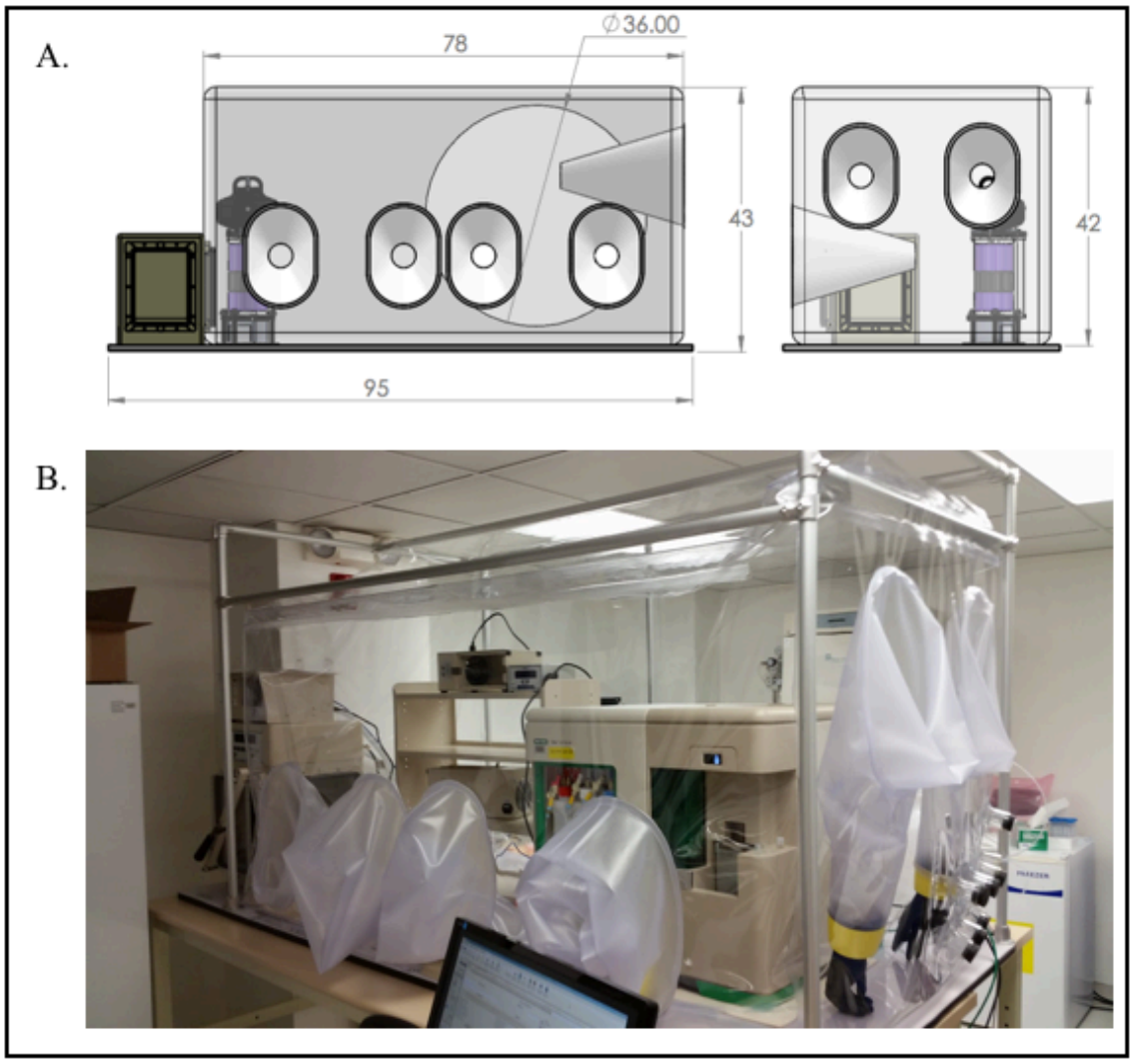

Figure 3: Custom built anaerobic FACS sorting chamber.

A. Schematic drawing of custom anaerobic chamber designed to accommodate BIO-RAD S3e FACS instrument. B. Picture of constructed anaerobic FACS chamber ready for use.

Anaerobic FACS sorting of immunoglobulin A (IgA) bacteria from UC donor stool

Glycerol stored UC donor stool was thawed in the anaerobic chamber. Stool material was transferred to a 1.5ml conical tube and centrifuged for 10 minutes at low speed (~100rcf) to sediment larger pieces of fecal material. Supernatant was collected and transferred to a fresh 1.5ml conical tube and spun at 10000rcf to pellet fecal bacteria. Bacteria were washed by resuspending pellet in 1ml pre-reduced wash buffer, phosphate buffered saline (PBS) containing 0.5% bovine serum albumin. Bacteria were washed a total of 3-4 times until the supernatant appeared clear. Bacteria were blocked for 20 minutes on ice in wash buffer containing 20% mouse serum and then split into three tubes and incubated for 30 minutes with either PE conjugated mouse anti-human IgA or a PE conjugated mouse IgG isotype control diluted 1:20 in wash buffer or wash buffer alone. After incubation in IgA-PE or isotype control staining solution, bacteria were washed 3 times in wash buffer and passed through a 40μm cell strainer to remove large clumps of cells before FACS sorting. Unstained bacteria in wash buffer and isotype control stained bacteria were analyzed on the FACS to determine background levels of PE signal against side scatter. IgA-PE stained sample was then analyzed to identify positively IgA-PE labeled cells or events. Selection gates were set up to sort IgA-PE hi+ and IgA- cells into collection tubes using the enrich sort setting. Sheath fluid was collected from sort stream immediately after bacteria sorting for plating to determine background levels of bacteria in FACS instrument fluidics. IgAhi+ and IgA- bacteria

collected with the enrich sort setting were then re-sorted in purity mode to further purify each population. The purity mode sort setting enhances sample enrichment by only sorting high confidence positive events, whereas the enrich setting sorts faster but has more permissive sorting criteria. Double sorted IgAhi+ and IgA- bacteria were collected for plating and subsequent anaerobic culture.

Anaerobic bacterial culture

Anaerobic FACS sorted bacteria were serially diluted prior to plating as follows. 150µl of sorted bacteria containing approximately 45,000 events were used as the top plating concentration and then 50µl were added serially to wells containing 100µl PBS to make a 3-fold dilution series of six concentrations of sorted bacteria for each sample. Dilution series for both IgAhi+ and IgA- bacteria were plated on three of the four different media used in this study to provide a diverse set of growth conditions and to target the recovery of certain bacterial phyla. The media used were brain heart infusion (BHI), M2GSC, Brucella Laked blood agar with kanamycin and vancomycin (LKV), and *Bacteroides* bile esculin (BBE). Table 1 provides a more detailed description for each of these media. 100µl of each bacterial dilution were spread per plate and incubated at 37°C under anaerobic conditions for 2-3 days. After the growth period, bacterial titers were calculated for IgAhi+ and IgA- samples grown on each media condition. Individual colonies were then picked for growth in liquid cultures in 500µl BHI media in deep-well 96 well plates to build culture collections of the isolated IgAhi+ and IgA- species from each patient. 200-500 colonies were picked per patient and cultured for 2-3 days until

cultures became turbid. The culture collections were then sampled for microbe identification as described below and then prepared for storage by addition of 125µl of pre-reduced 75% glycerol for a final concentration of 15% glycerol. Plates were stored at -80°C for future use.

Table 1: Bacterial culture media used to isolate IgA+ and IgA- bacteria

Medium	Rich or selective medium	Comments	Source
BHI (Brain Heart Infusion)	rich	Nutrients provided from brain heart infusion and specific vitamin supplements to support the growth of most anaerobes	Anaerobe Systems
M2GSC	rich	Rumen fluid based rich medium that supports the growth of most anaerobes	Prepared in house (Eeckhaut et al. 2011)
LKV (Laked Blood Agar with kanamycin and vancomycin)	selective	Contains kanamycin to inhibit growth of facultative anaerobes and vancomycin to inhibit gram-positive bacteria	Anaerobe Systems
BBE (Bacteroides Bile Esculin)	selective	Contains gentamicin to inhibit facultative anaerobes, bile to inhibit bile sensitive gram-negative anaerobes	Anaerobe Systems

Identification of isolated IgA+ and IgA- bacteria

To identify the species isolated from cultivation of IgA+ and IgA- sorted bacteria, 2µl of liquid cultures were sampled from 96 well culture library plates and added to a corresponding 96 well plate containing lysis buffer solution and submitted to Genewiz (Cambridge, Massachusetts) for full length 16S ribosomal RNA (rRNA) sequencing using the 27F (5'-AGAGTTTGATCMTGGCTGAG-3') and 1492R (5'-

CGGTTACCTTGTTACGACTT-3') primer set. These primers are designed to target regions of the 16S rRNA gene that are highly conserved across the kingdom of Bacteria and flank hypervariable regions that are unique among different species of bacteria. These variable regions are sequenced and enable microbe identification by matching the resulting sequence to existing sequences in databases of known bacterial species (Clarridge III 2004). The amount of overlap of the sequenced sample to the closest matching reference sequence provides a measure of accuracy for a given species call.

Experiments

Determining the viability and consistency of IgA+ sorted bacteria

Working in the anaerobic FACS chamber, bacteria were isolated from UC002 and the healthy control glycerol preserved stool samples. Both samples were stained with anti-human IgA-PE. Unstained and mouse IgG-PE isotype control samples were set up to define the background signal of the FACS instrument PE detector. Once background PE levels were established, anti-IgA-PE stained samples were analyzed on the FACS and positive events were identified as belonging to populations with PE signal well above background. IgA+ events were sorted according to the previously described protocol (see methods) and collected for plating. A total of three IgA+ sorts were performed from each sample to provide replicates to analyze the consistency of the recovered bacteria. Six-point dilution series were set up for the IgA+ sorted bacteria from the healthy donor and the UC002 patient samples. IgA+ dilutions from each donor were plated on BHI and grown under anaerobic conditions at 37°C for 2 days. After 2 days of growth, titers were

calculated for UC002 IgA+ sort 1-3 and healthy control IgA+ sort 1-3. Then 92 colonies were picked for 16S rRNA sequencing based microbe identification from cultures initiated with sorted material from UC002 IgA+ sort 1-3 and healthy control IgA+ sort 1-3. The similarity of titer and the abundance of identified bacteria present in each set of replicate sorts were compared to evaluate of the consistency of the anaerobic FACS sorting method.

Measuring differential abundance of bacteria in IgA+ and IgA- sorted populations

Bacteria from the healthy control donor was collected from aliquoted glycerol preserved fecal material and anti IgA-PE, unstained and isotype control samples were prepared for anaerobic FACS sorting. IgA+ and IgA- populations of bacteria were sorted simultaneously to enrich each population. To maximize the percentage of positive events in each of the sorted populations of bacteria, enriched IgA+ and IgA- samples were subjected to a second purification IgA+ or IgA- sort. The resulting IgA+ and IgA- samples were then set up in a dilution series for culture on BHI and LKV media. Cultures were grown anaerobically for 2 days and colonies were picked for microbe identification. The ability to isolate and identify IgA coated bacteria by anaerobic FACS sorting was analyzed by comparing the abundance of isolated species in each the IgA+ and IgA- cultures.

Generation of IBD patient IgA+ and IgA- culture collections

IgA+ and IgA- bacteria populations were isolated from glycerol preserved IBD patients sample utilizing the anaerobic FACS sorting protocol. Bacteria were double

sorted to maximize purity, set up in a dilution series and cultured under anaerobic conditions at 37°C. IgA+ and IgA- populations were grown on at least 3 of 4 media to enhance the variety of cultured microbes isolated by FACS. BHI and M2GSC served as rich media used to grow a wide variety of anaerobic gut bacteria. Selective media, LKV and BBE, were used to target diversity within certain groups of gram-negative obligate anaerobes, such as *Bacteroidetes*. After anaerobic culture for 2-3 days colonies were picked from both IgA+ and IgA- for growth in BHI liquid culture media. Culture plates were returned to 37°C and grown for an additional 4 days enabling colonies of slower growing bacteria to emerge. If new colonies of interest were observed, a second round of BHI liquid cultures were inoculated to capture additional species in the culture library that may not have been isolated following the initial 3-day growth period. Liquid cultures were incubated anaerobically at 37°C for 2 days, then 2µl of liquid cultures were used as samples for microbe identification and the cultures were mixed with glycerol for storage at -80°C. 200-500 colonies were picked from each patient to provide a sample size large enough to determine if a given identified species was more abundant in either the IgA+ or IgA- cultures directly isolated from IBD patients. Figure 4 summarizes this direct isolation strategy of IgA coated bacteria employing anaerobic FACS and provides a comparison to the approach used by Palm et al. (2014) to generate IgA+ and IgA- microbial consortia from IBD patients.

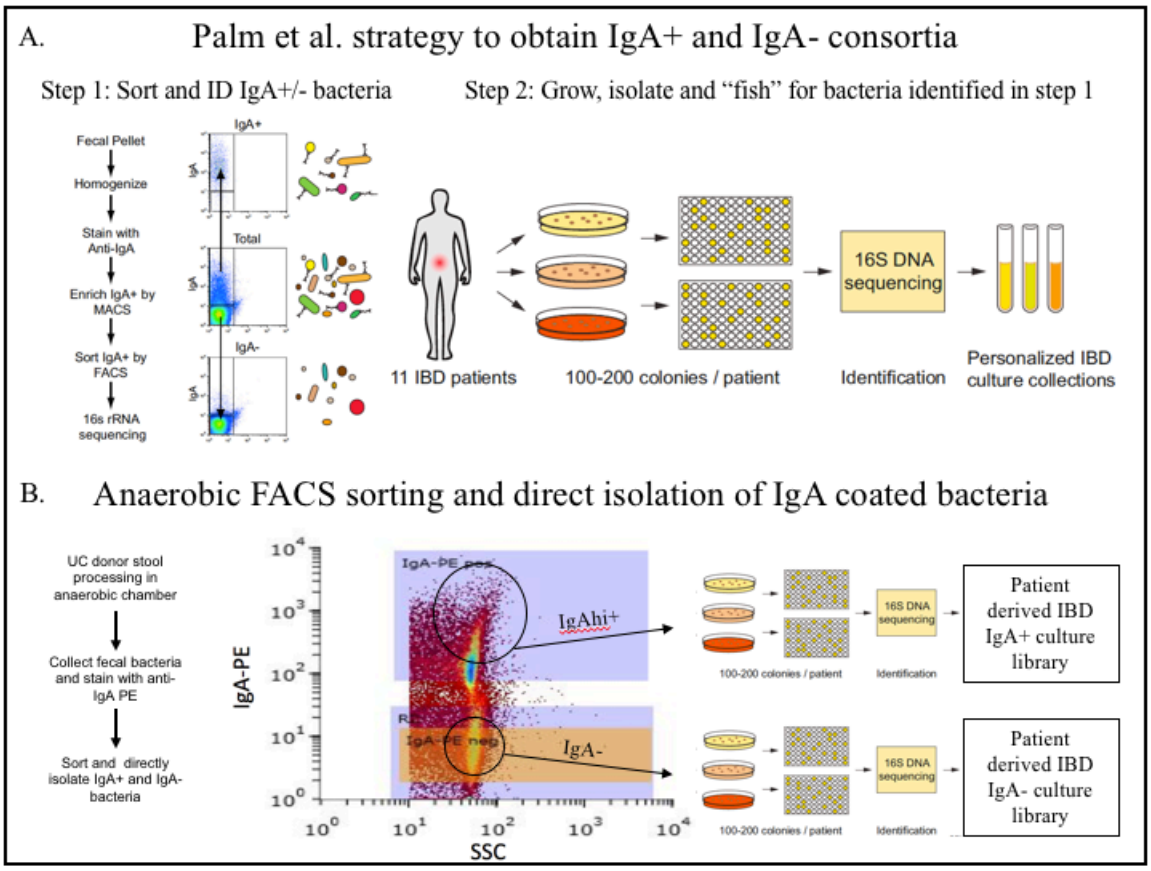

Figure 4: Comparison of IgA associated bacteria isolation strategies.

A. Palm et al. (2014) workflow for generation of IgA+ and IgA- bacteria from IBD patients. The first step identified microbes in IgA+ and IgA- FACS sorted populations (IgA-SEQ). Then the authors cultured the same samples, isolated hundreds of colonies per patient and selected bacteria identified by their initial IgA-SEQ experiment to be included in their IgA+ and IgA- consortia for in vivo experimentation. B. Workflow for direct isolation strategy of IgA+ and IgA- bacteria utilizing anaerobic FACS sorting. Anaerobic sorting of microbes enabled subsequent anaerobic culture of both IgA+ and IgA- sorted populations from a set of 6 IBD patients. Directly isolated bacterial colonies were picked and identified by 16S sequencing. Bacteria that were predominantly abundant in cultures initiated from either IgA+ or IgA- would be candidates for inclusion in consortia to be used for phenotypic characterization in vitro and in vivo.

Chapter III.

Results

Anaerobic FACS methods developed in this study successfully enabled the isolation and subsequent cultivation of obligate anaerobes based on surface phenotype. This section describes the experiments performed to validate this approach and the application of anaerobic FACS sorting to assemble an IBD patient bacterial strain library.

IBD patient stool collection

IBD patient stool was collected from two different sources to be used as starting material for development and implementation of anaerobic FACS methods for isolating host targeted, disease associated bacteria. The first source, BioReclamationIVT, provided fresh samples shipped overnight to our laboratory following collection during at the doctor's office. For this study, we requested UC patient donors be experiencing a flare, the period of active disease in which symptoms are most severe. Donors were only accepted for this study if they had not taken antibiotics in the 6 months prior to donation date to eliminate the major microbiome shifts caused by these drugs from consideration. In addition to current medication, age, body mass index, and gender information, we also

received Mayo scores for each patient that donated for this study. A Mayo score describes the severity of various features of UC, such as physician assessment, frequency of bowel movements, presence of bloody stool, and superficial appearance of the colonic mucosa viewed by endoscopy (Vucelic 2009). Upon receipt, samples were scored according to the Bristol stool scale (Thabit and Nicolau 2015), a diagnostic assessment of stool consistency (Figure 5). All freshly received samples scored at 5 or greater, skewing the samples toward the diarrhea side of the scale (Table 2).

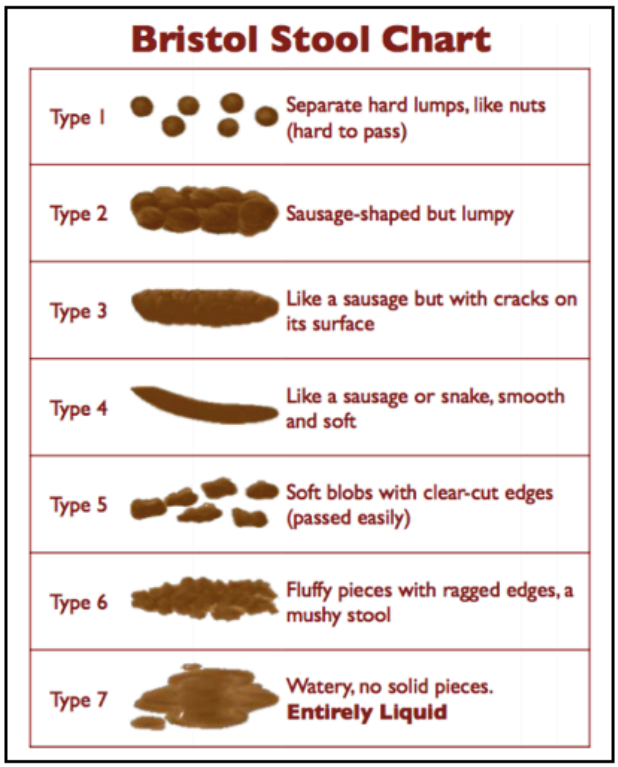

Figure 5: Bristol stool chart. (Thabit and Nicolau 2015).

The second source of material for this study was through a collaboration with researchers at University of Pennsylvania that had been collecting stool samples from patients with pediatric Crohn's disease. These samples were frozen at the time of collection and were therefore not fully processed using our protocol designed to minimize oxygen exposure. However, we were interested in including these samples because they

were collected at a specialized university research center and the cases had been recently diagnosed. These patients tended to have quite severe disease and had not been given antibiotics as part of their treatment regime. Bristol scores for these two samples were 6 and 7 and these patients had high scores on the Pediatric Crohn's disease activity index (PCDAI), one of these two patients also had a high Pediatric Ulcerative Colitis disease activity index (PUCDAI) score. Like the Mayo scale, the PCDAI and PUCDAI are scores used by physicians to stratify pediatric IBD by the severity of their symptoms (Vucelic 2009). Together, the samples obtained from BioReclamationIVT and University of Pennsylvania provided a suitable initial stool collection to explore the applicability of anaerobic FACS to the isolation of disease associated members of the microbiome.

Table 2: IBD stool donor information

Sample ID	**Source**	**Diagnosis**	**Bristol Score (1-7)**	**Comments**
UC002	BioReclaimationIVT	Ulcerative Colitis	5	Mayo Score=7 Processed anaerobically
UC003	BioReclaimationIVT	Ulcerative Colitis	5	Mayo Score=6 Processed anaerobically
UC004	BioReclaimationIVT	Ulcerative Colitis	7	Mayo Score=3 Processed anaerobically
UC006	BioReclaimationIVT	Ulcerative Colitis	5	Mayo Score=2 Processed anaerobically
UPenn 1	Garry Wu (UPenn)	Pediatric Crohn's	6	PUCAI=85, PCDAI=57.5 Received frozen, processed anaerobically
UPenn 2	Garry Wu (UPenn)	Pediatric Crohn's	7	PUCAI=25, PCDAI=62.5 Received frozen, processed anaerobically

Validation of anaerobic FACS sorting method

The identities of isolated microbes were kept confidential for proprietary purposes.

Two samples, a healthy control and UC002, were used to develop anaerobic FACS sorting methods. Once FACS sorting conditions were optimized, IgA+ sorted bacteria were plated and both samples reproducibly had titers of $3x10^3$ CFU/ml (Figure 6). Based on the concentration of IgA+ events present in the sorted sample used for plating, these titers represent a 10% recovery rate. Since we did not utilize a viability marker in our FACS sorting scheme, exclusive isolation of live IgA+ bacteria could not be guaranteed. Therefore, the unknown percentage of non-viable bacteria present in the sorted material prevented an accurate measure of the true recovery rate of viable bacteria. Despite this limitation, we were encouraged by the observed robust recovery of several species of obligate anaerobes from the phylum *Bacteroidetes*.

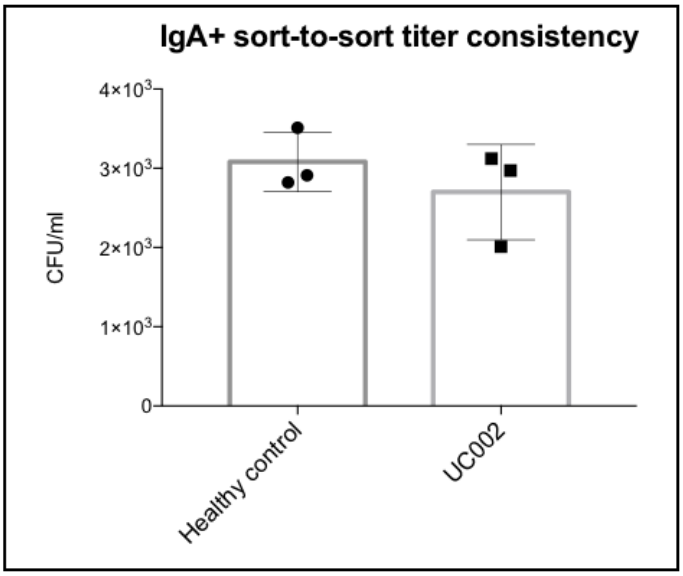

Figure 6: IgA+ sort consistency.

Titers of anaerobically sorted IgA+ bacteria from a healthy donor stool sample and an ulcerative colitis stool sample are similar in cultures initiated from three independent sorts in both samples tested.

Comparison of the species identified in cultures generated by three replicate IgA+ sorts from both the healthy control and UC002 samples showed a high degree of overlap within each sample. When comparing isolated species identified at high frequency (4 or more colonies for a given species) 7 out of 8 were present in bacteria cultured from all three replicate sorts of the healthy control sample (Figure 7). Using the same criteria for species overlap, 3 of 3 high frequency species were present in all the UC002 replicate sorts. Species were also found to be identified in all IgA+ replicate sorts but with a frequency of less than 4 colonies per sort sample that were exclusive to either the healthy control and UC002 samples. When comparing the species isolated from both donors tested in these validation experiments, only 3 of the 39 species identified were common to the IgA+ populations of the IBD donor and the healthy donor.

To evaluate the differences in species identified through culture of IgA+ and IgA- sorted fractions from a given input microbial community, bacteria isolated from the healthy control donor were compared. A total of 15 strains species were isolated at a frequency of 3 or more colonies in either the IgA+ or IgA- cultures (Figure 8). Of these 15 species, 11 were at least 3-fold more prevalent in the IgA+ population with 7 of these species being exclusively found in the IgA+ population. 3 of the 15 species were more abundant in the IgA- population, 2 of which were at least 8-fold more abundant and the remaining IgA- species was 3-fold more abundant than in the IgA+ population. Only 1 species identified in this experiment was found at high abundance in both the IgA+ and the IgA- populations. These results, together with the data confirming consistency of sorted microbes isolated within donor samples, demonstrate the utility of anaerobic FACS sorting to directly isolate gut bacteria based on levels of IgA coating and validate the expansion of this approach to the entire IBD donor stool collection.

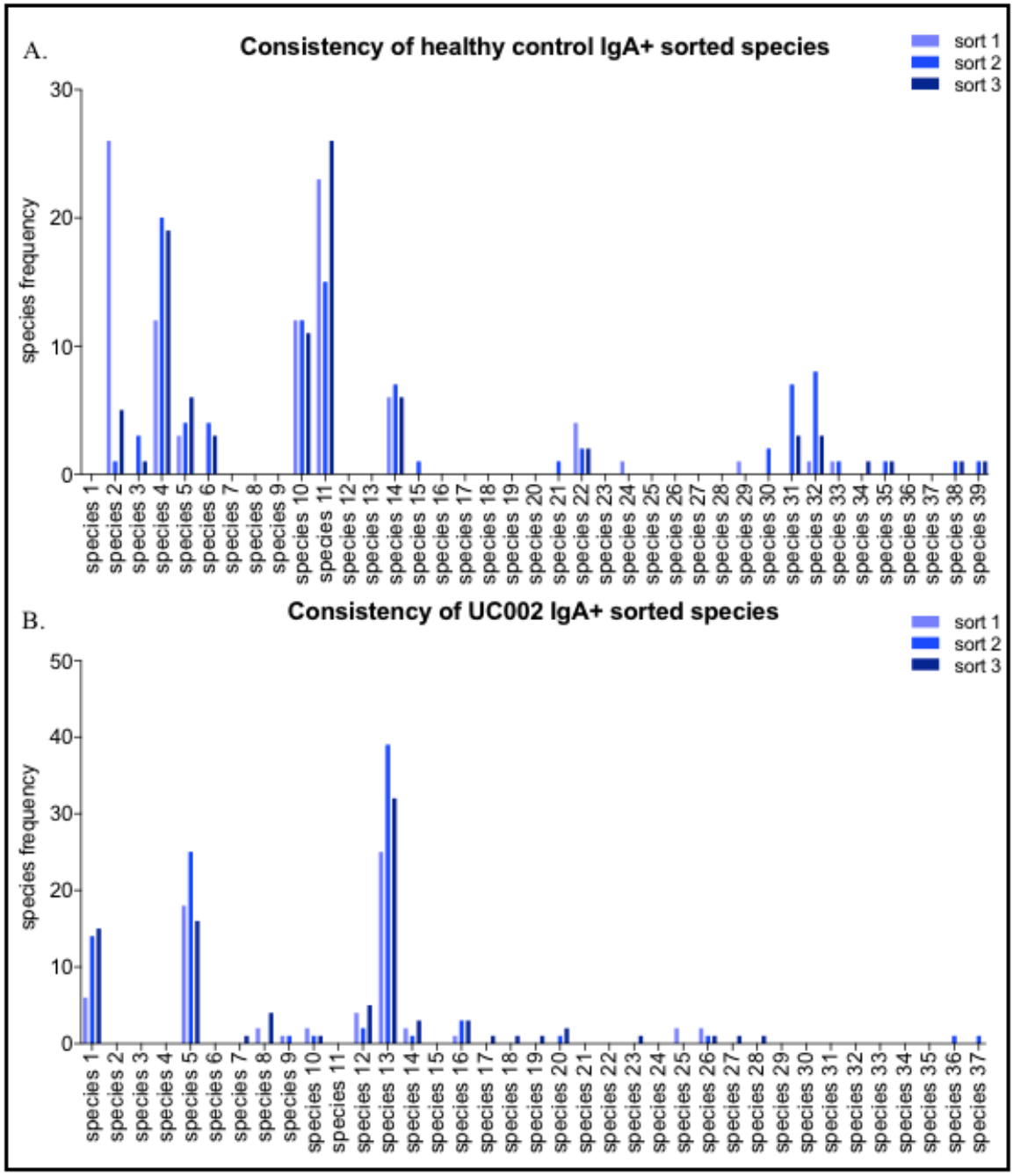

Figure 7: Common IgA+ bacteria isolated within donor samples.

Species with matching microbe identification are consistently isolated from culturing anaerobic FACS sorted IgA+ fecal bacteria from a healthy donor sample (A) and from a donor with ulcerative colitis (B). Species 1-39 are codes for matching microbial species in both samples.

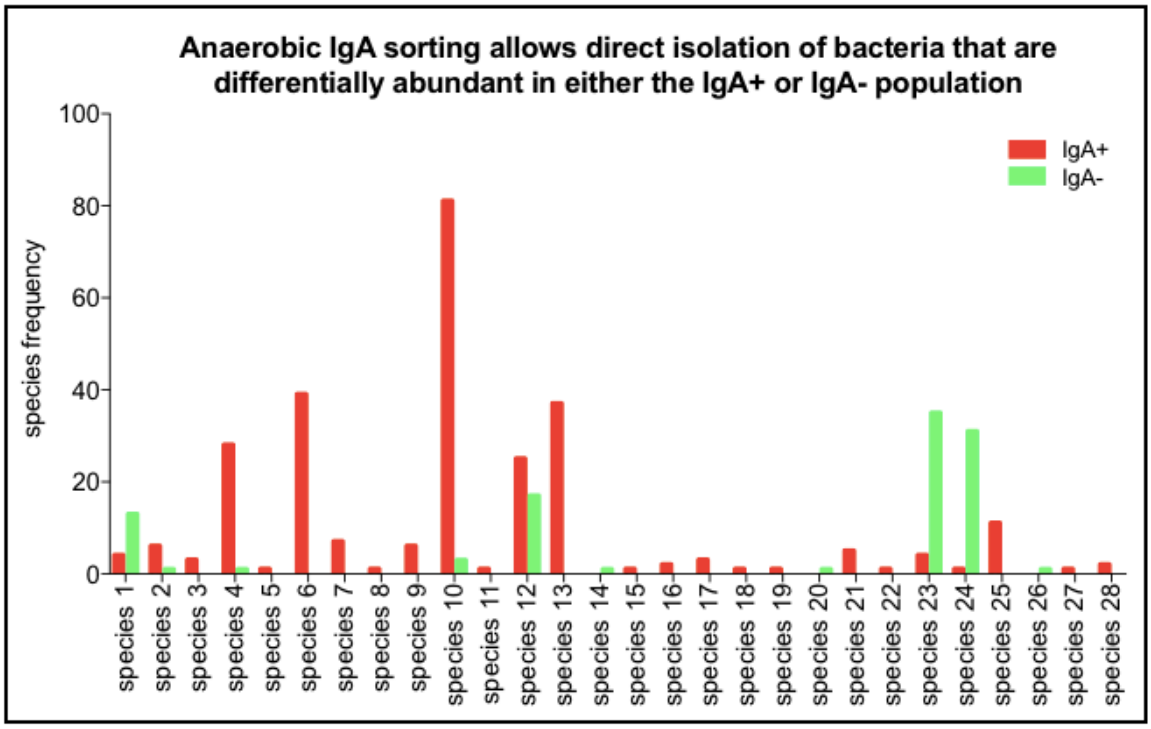

Figure 8: Differential abundance of IgA+ and IgA- sorted bacteria.

Species cultured from IgA+ sort population are in most cases present exclusively or at a much higher frequency than those cultured from the IgA- sort population and vice versa.

IBD patient IgA targeted bacteria library generation

Bacteria from all collected IBD donor samples were subjected to anaerobic IgA FACS sorting. A wide range in the percentage of IgA+ cells present in each sample was observed. Donor UPenn 1 had the largest fraction with 44% IgA+ cells compared to just 2% IgA+ cells in UC002 donor bacteria. Further analysis revealed the segregation of the IBD samples into two groups, the first group includes three samples with less the 3% IgA+ cell, whereas, the remaining three samples all had greater than 20% IgA+ cells (Figure 9). Interestingly, the samples with elevated numbers of IgA+ cells also had the higher Bristol scores of all the samples in the IBD stool collection. Further analysis is

needed to determine if a true bimodal distribution exist among IgA coating levels in a larger cohort of IBD patients.

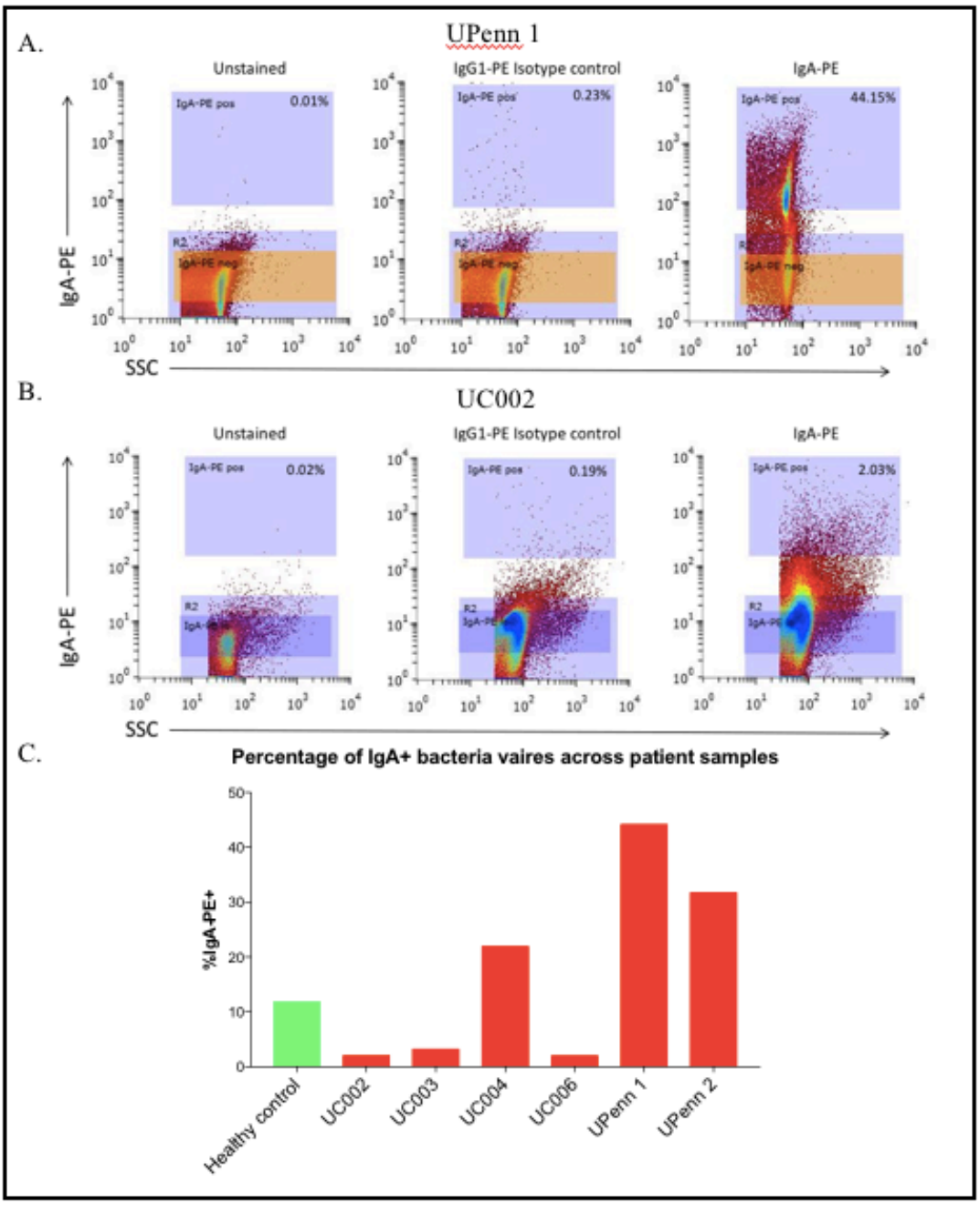

Figure 9: IgA-FACS profiles of IBD donor fecal bacteria.

FACS plots generated from IgA-PE stained bacteria from UPenn 1 donor sample (A) is >20-fold higher than from UC002 sample (B). The percentage of IgA-PE bacteria varies among the set IBD donor samples used in this study.

After all samples were processed, 1841 colonies were picked and identified to yield 147 unique species in the resulting IBD IgA sorted bacteria library. Isolated species from the five phlya of bacteria reported to reside in the human gut were recovered. 23 of the species matched with those on the Human Microbiome Project most wanted list of previously uncultured microbes that were isolated by Browne et al. (2016). Approximately 75% of the species collected belonged to the *Firmicutes* and *Bacteroidetes* (Figure 10), the major constituents of the microbiome which are known to include many obligate anaerobes (Maier et al. 2015). In this study, anaerobic IgA FACS sorting applied to a single stool sample that was collected and processed under aerobic conditions led to the recovery of only facultative anaerobes belonging to the *Enterococcus* genus (Fisher and Phillips 2009) that are capable of surviving in oxygen rich conditions. The failure to recovery any obligate anaerobes in this sample underscores the need to minimize oxygen exposure to human microbiome samples intended for cultivation. Further analysis is required to quantify the effect oxygen exposure to recovery of obligate anaerobes but such experiments were outside the critical path of this project focused on generating a collection IBD associated bacteria.

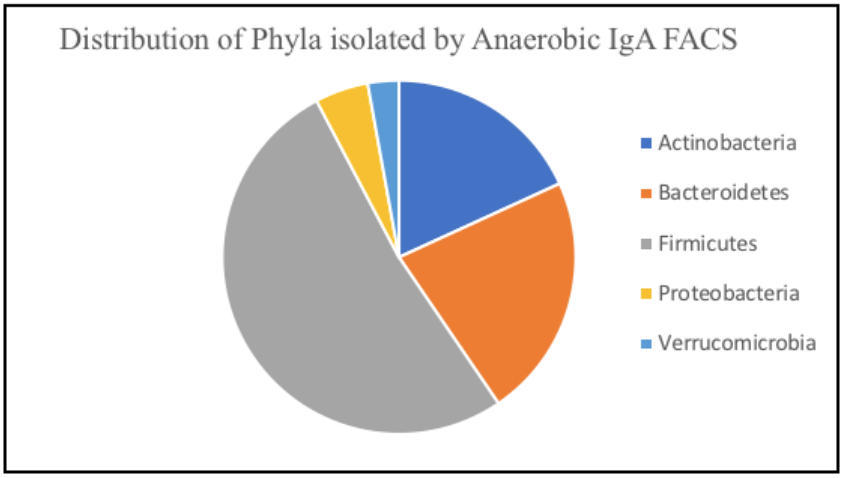

Figure 10: Phyla of IgA FACS sorted isolated species

During the campaign to isolate and bank IgA sorted bacteria, a lack of diversity among the isolated IgA- gram negative bacteria was noticed. We had employed LKV media, which is selective for gram-negative obligate anaerobes, but decided to add BBE media as an additional selective growth condition for IgA sorted cells. The use of BBE resulted in the isolation of 15 gram-negative Bacteroidetes species from just two patients. Among the newly isolated IgA- gram-negatives were two species matching those from the IgA- consortium assembled by Palm et al. (2014). Inclusion of the selective media LKV and BBE greatly aided the recovery of species from the phylum Bacteroidetes. We did notice that a small fraction of the species isolated on LKV were of the phylum Firmicutes (Figure 11), which are mainly gram positive and should be sensitive to vancomycin. Upon further inspection, the Firmicutes identified on LKV were all isolates belonging to one species of Negitivicutes, a group of gram-negative Firmicutes and explains how it would be able to evade the selective culture conditions. As expected, species from all five phyla isolated by anaerobic IgA sorting could be cultured on the rich media, BHI and M2GSC.

When analyzing the species isolated across the IBD patient set, it is clear that each patient is unique. Heterogeneity exists among the profiles of cultured microbes derived from the IgA+ and IgA- populations from one patient to another (Figure 12). For example, there are 8 instances in which a species is IgA coated in one patient but abundant in the IgA- population of another patient. A possible reason for these discrepancies is that strain level differences beyond the resolution of the microbe identification methods used in this study may exist within a species causing one but not the other to induce an IgA response.

Ecological conditions such as nutrient abundance may also explain differences in behavior of the same organism in different microbiomes.

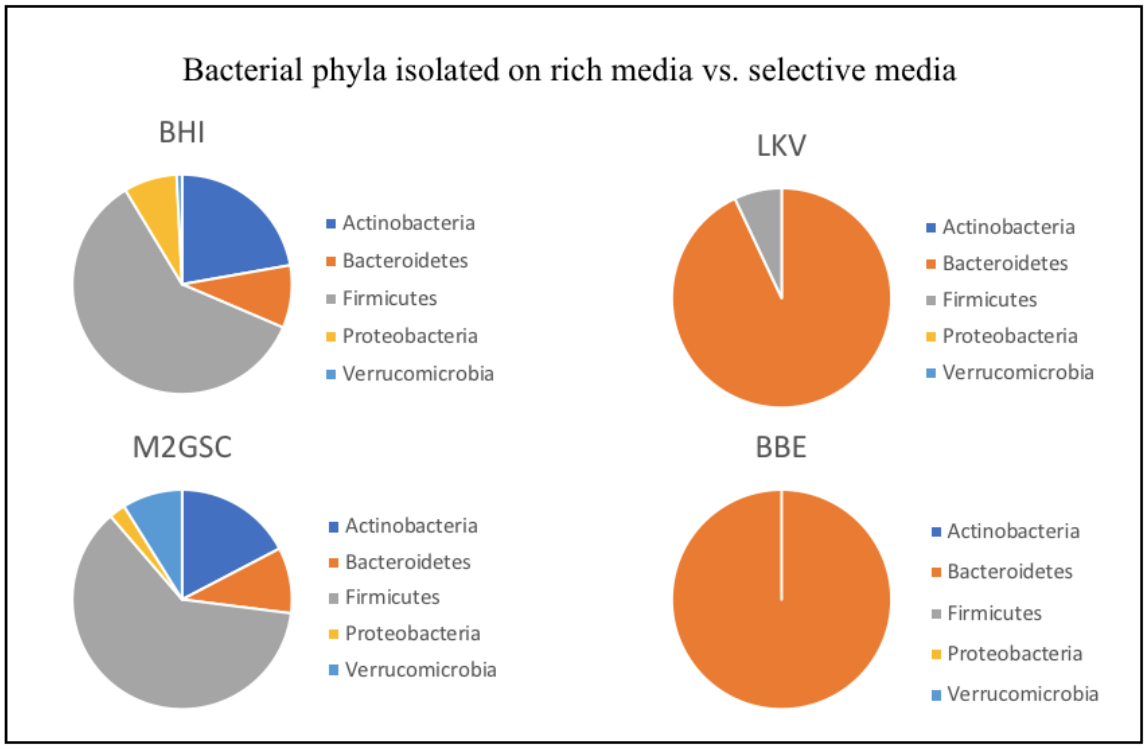

Figure 11: Bacterial phyla isolated on rich vs. selective media

Pie charts displaying the diversity of phyla isolated on the different culture media used in this study. Bacteria from 5 different phyla were isolated on BHI and M2GSC, two different rich media. Strains isolated after growth on the selective media LKV and BBE were almost exclusively from the phylum *Bacteroidetes*.

When we compared isolated organisms within each patient sample, we observed that many species had differential abundance in IgA+ and IgA- libraries indicating that the sorting conditions established during the method optimization phase of the project were robust. The number of species identified that were observed at least four times more frequently in either the IgA+ or IgA- derived cultures were as follows: 10 species in UC002, 9 species in UC003, 7 species in UC004, 6 species in UC006, 12 species in

UPenn 1 and 8 species in UPenn 2. In 17 instances a species was present at 10-fold or greater abundance in one of the two populations (Figure 12). We next focused on the species that were identified to be exclusively overabundant in either IgA+ or IgA- patient cultures. Bacteria that are only present in the IgA+ patient populations would be hypothesized to possess inflammatory properties that contribute to IBD pathogenesis. Conversely, bacteria only found among the IgA- isolates would not be among the disease-causing members of the microbiome and would perhaps represent commensal organisms that provide benefit to the host. This analysis resulted in a catalog of 18 IgA+ dominant and 9 IgA- dominant bacteria (Figure 13). Among this collection, 9 of 18 IgA+ species are present in more than one patient analyzed and 1 species is present in 4 of 6 IBD donors suggesting that certain species may play a role in disease across donors. The IgA+ dominant and IgA- dominant groups isolated in this study include 7 species that were also found in the IgA+ and IgA- consortia described in the report by Palm et al. (2014), in which the IgA+ consortium sensitized mice to DSS-induce colitis. Thus, the IgA+ and IgA- bacteria identified in this work provide an expanded reagent set to enable phenotypic characterization of the metabolic, inflammatory and immunomodulatory capacity of these microbes *in vitro* and *in vivo*. Additionally, these results demonstrate the successful application of the anaerobic FACS method developed to directly isolate antibody bound bacteria from patients while maintaining the viability of obligate anaerobes.

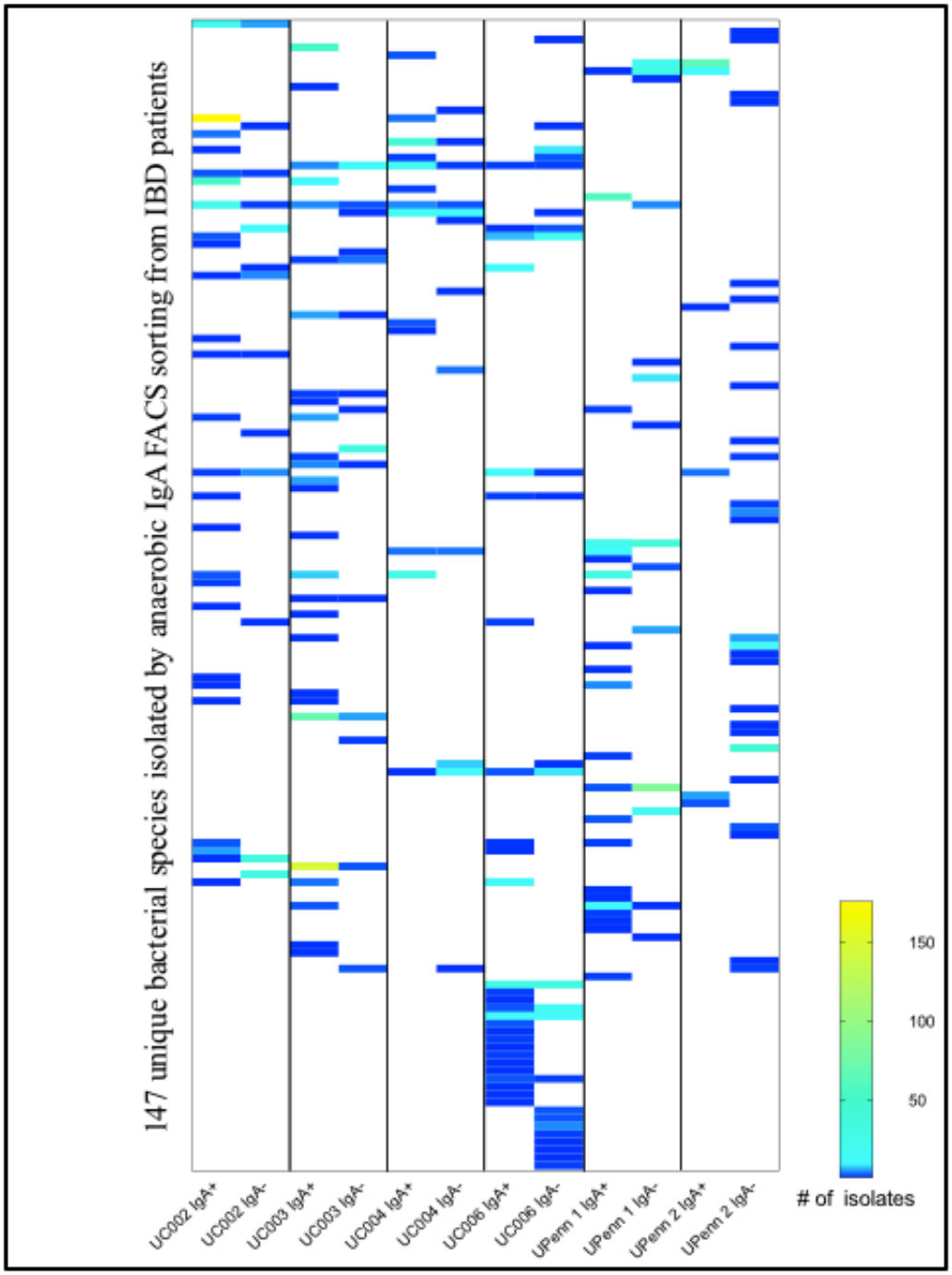

Figure 12: Bacteria isolated by IgA-FACS from IBD donor stool.

Heat map of bacteria isolated by anaerobic FACS sorting of IgA+ and IgA- microbes from six different IBD patients. Each row represents a unique species. Lines separate columns of IgA+ species (left) and IgA- species (right) from the indicated patient sample.

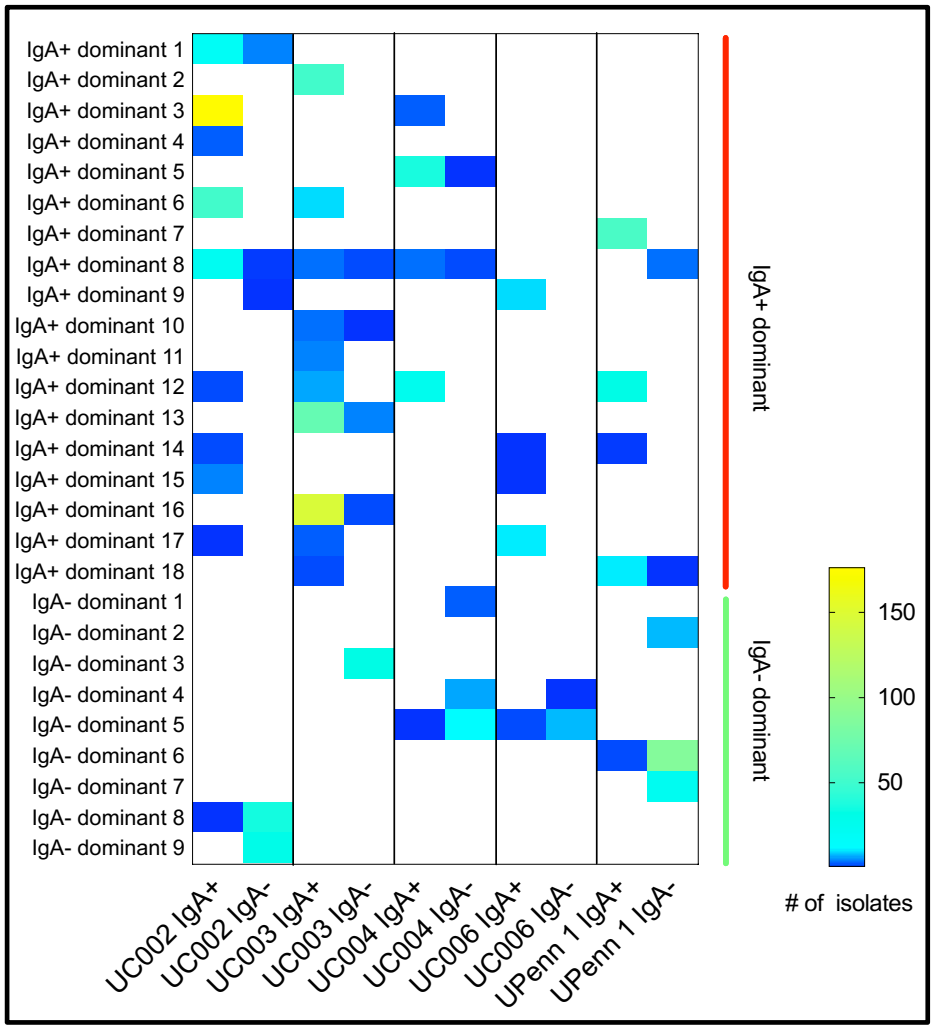

Figure 13: IgA+ and IgA- dominant bacteria isolated from IBD donor stool.

Heat map of species predominantly isolated from cultures initiated with populations of IgA+ bacteria or populations of IgA- bacteria in one or more IBD patients.

Chapter IV.

Discussion

Recent studies have demonstrated that a large percentage of the human microbiome can be cultured (Browne et al. 2016) *ex vivo,* facilitating the functional characterization of gut bacteria. Developing culture collections for human intestinal bacteria is crucial for enhancing our understanding of the microbiome. Many of the studies that have led to the increasing appreciation of the impact of the microbiome on health and disease have relied on culture independent methods to characterize microbiome composition in human populations and make correlations to the health status of the donor. The ability to isolate and culture intestinal bacteria makes them available for experimental manipulation and the ability to demonstrate pathogenic or commensal effects in mouse models. The goal of this study was to develop an anaerobic FACS sorting platform to permit the isolation of viable anaerobes from a complex bacterial ecology based on a specific surface phenotype. These capabilities enable the direct cultivation of any live bacteria that can be labeled with a fluorescent probe and survive the physical stress inflicted on a cell during flow cytometry. IgA is an abundantly produced host antibody that is secreted into the intestinal lumen to provide a layer of defense against the many bacteria that inhabit our intestines. IgA has been shown to target pathogenic bacteria in the context of disease (Kau et al. 2015). Palm et al. (2014)

used culture-independent identification methods to characterize IgA+ and IgA- bacteria in IBD patients and found that bacteria identified as IgA+ could cause disease in mouse models of colitis. However, that effort required the generation of a sizable microbiome sequencing dataset and intensive computational effort to produce a list of IgA relevant bacteria for further study. The anaerobic FACS sorting platform developed and described in this study enabled a simplified approach for the direct isolation and subsequent cultivation of IgA coated bacteria from IBD patient microbiome samples, providing a culture collection to the study disease driving activity of pathogenic bacteria.

In vitro and *in vivo* characterization of IgA-coated and non-coated bacteria collected from IBD patients may reveal microbe driven mechanisms underlying the pathophysiology of conditions such as ulcerative colitis and Crohn's disease. Phenotypic screening of the IBD IgA+ patient culture collection in assays that measure the induction of various features of host inflammation such as cytokine secretion will be a step toward the identification of microbes responsible for chronic disease. Screening the IgA- portion of culture library for production of health associated bacterial metabolites and other natural products may help identify species with beneficial host functions, such as butyrate induced T-regulatory cell expansion (Atarashi et al. 2013). Importantly, direct isolation of bacteria from patients provides the opportunity to address each of Koch's postulates by directly testing their pathogenic potential by measuring disease induction and transmissibility in mouse models (Bloom et al. 2011). Construction of IgA+ and IgA- consortia with species purified from the anaerobic FACS sorted IBD patient bacteria library will provide appropriate tools to confirm or deny the finding that IgA targeted bacteria contain the colitis causing members of the microbiome (Palm et al. 2014). If

IgA coated microbes do in fact create a microbiome driven model of colitis, it would have the potential to represent a new platform for the testing of IBD therapeutics.

Anaerobic FACS can also aid future studies to understand the IgA-microbiome axis in IBD and other states of health and disease. IgA-guided isolation and profiling across large IBD cohorts will enable researchers to identify trends in frequently IgA-targeted bacteria across patients perhaps leading to biomarkers used to stratify patients into sub-types of UC or CD. Characterizing the changes in IgA coated bacteria that are labeled over time and in response to certain stimuli may increase our understanding of the IgA antibody production pathway during different phases of disease, such as a UC flare. Extension of the IgA-FACS isolation to other diseases will provide the opportunity to understand how this immune response is related to the microbiome in different situations. At the time of writing this thesis, a report has been published demonstrating that *Proteobacteria* species in the microbiome can stimulate serum IgA production to provide systemic immune protection against translocated bacteria in the context of sepsis that results from compromised intestinal barrier integrity (Wilmore et al. 2018). Using IgA-FACS, one could isolate and study the common features of gut bacteria that are targeted by serum IgA to gain insight into the mechanisms of *Proteobacteria* induced immune development.

The IBD microbiome has been intensely studied and many datasets, generated by sequencing populations of gut bacteria using technology that on average provides a genus level resolution of strain identification, exist to describe the bacteria whose presence is correlated to disease. A common finding among these studies is that the percentage of *Fimicutes* is reduced in UC patients versus healthy individuals (Gevers et al. 2014). IgA-

SEQ (Palm et al. 2014) allowed identification of IgA+ and IgA- bacteria in UC patients revealing the various species of bacteria that were directly targeted for neutralization by the host immune system. Interestingly, the species *Bacteroides fragilis* was identified in IgA+ and IgA- populations by IgA-SEQ. Without the capability to directly sort viable IgA+ and IgA- *Bacteroides fragilis*, an obligate anaerobe, it is impossible to assign IgA status to the *Bacteroides fragilis* isolated from bulk cultures of the donor microbiome. In contrast, anaerobic IgA-FACS sorting enables the unequivocal assignation of IgA status to isolates of the same species that might be present in both the IgA+ and IgA- populations. Subsequent higher resolution strain identification, such as whole genome sequencing, could possibly lead to the discovery of disease causing genes. Furthermore, phenotypic characterization of species present in IgA+ and IgA- sorted populations might reveal functional differences that exist between different isolates of the same species and facilitate our understanding of strain level differences that exist among many species. The IBD IgA-FACS sorted bacteria library generated in this study contains several species of bacteria isolated in both the IgA populations from the same patient (Figure 12), all now amenable to investigation of strain level divergence.

The anaerobic FACS sorting method described in this study was quickly deployed to isolate a collection of IBD associated bacteria, yielding a patient culture collection of 1841 isolates representing 147 unique species in just 6 months. As discussed above, these bacteria are now available for experimentation aimed at identification of the causal roles they play in IBD. Using this isolation platform, gut bacteria targeted by other antibodies or proteins secreted by the host immune system could be isolated. Recently published findings suggest other candidate targets that can be used to “fish-out” and

isolate host targeted bacteria including IgG and lypd8. IgG is another host produced antibody that has been shown to bind bacteria in the microbiome (Kim et al. 2016). However, the significance of IgG coating is less characterized than that of IgA and application of immune guided isolation can help define this biology. Lypd8 is a protein secreted by the colonic epithelium and provides mucosal protection by neutralizing flagellated bacteria residing adjacent to host tissue, and the lack of Lypd8 has been shown to make mice more susceptible to DSS-induced colitis (Okumura et al. 2016). Culture based characterization of Lypd8 bound microbes will permit further *in vitro* study of this newly discovered mechanism of host defense, perhaps in co-culture systems with epithelial cells of the gastrointestinal tract. Continuing to develop novel isolation approaches such as anaerobic FACS sorting of viable microbes targeted by the host immune system will allow understanding of microbiome-disease associations to move from correlation to causation in a patient specific manner.

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