Abstract

*Clostridium difficile*, an intestinal bacterium causing a disabling diarrheal disease, causes ~25% of all antibiotic-induced diarrhea cases. We have been investigating the use of “bacteriotherapy”, via a process termed fecal transplantation, as a treatment for recurrent *C. difficile*-induced diarrheal disease. The treatment has been shown to be effective. Since both practical and aesthetic considerations make “fecal transplantation” an undesirable therapy, we propose to identify the critical microorganisms in the normal intestinal flora that can be used to protect humans against *C. difficile* infection. To achieve our goals, we propose to use high-throughput DNA sequencing of intestinal tract microorganisms present in feces before and after fecal transplantation to characterize key microbial populations present in the human intestinal tract, and by doing so, to ultimately develop a standardized formulation that would be easy to administer to patients. Results of these studies will provide preliminary data to support submission of NIH RO1 proposal.

[150 words]
Grant-in-Aid of Research, Artistry and Scholarship

A Gene Sequencing and Taxonomic Approach to Determine Intestinal Microflora Useful for the Treatment of Clostridium difficile Diarrheal Disease in Humans
PI: Michael Sadowsky, Soil, Water and Climate, University of Minnesota, Twin Cities

This application is a Category 6 funding request for a faculty member moving into a significantly different area of research. This represents a major shift in focus for myself, an environmental microbiologist with over 25 years of experience working in the areas of plant-microbe interactions, environmental E. coli, and biodegradation. As required, additional justification for this category is found in the attached cover letter.

Present State of Knowledge:

Resident bacterial communities inhabiting the lower gastrointestinal (GI) tract of warm blooded animals have co-evolved to form a mutualist relationship with their hosts. These microorganisms make essential contributions to gastrointestinal function, the host’s nutrition, development, immune defense mechanisms, and metabolism. One dramatic example of the consequence of disrupting the resident human intestinal flora is Clostridium difficile colitis, which often is caused by exposure to antibiotics. Clostridium difficile, is a spore-forming, anaerobic, bacterium that was first described in 1935 (6). Although the bacterium is part of the resident population of GI microorganisms, the presence and extent of growth of this bacterium is usually held in check by the presence of other bacteria. In the late 1970s, this bacterium was found to be associated with antibiotic-related diarrhea (2). This is chiefly due to the overgrowth of C. difficile in the human GI tract following antibiotic therapy for unrelated problems, leading to loss of other microorganisms that control the growth of this bacterium. Moreover, the bacterium has been shown to produce two toxins, that are correlated with disease symptoms (11).

Antibiotic-induced C. difficile diarrhea disease may lead to prolonged hospitalization, at additional costs of up to $10,000/case (12, 16). C. difficile has been reported to be the cause of approximately 25% of all antibiotic-associated diarrhea cases (3). In the U.S., there are approximately 300,000 C. difficile-associated diarrhea disease cases/year, many of which occur in hospitals (12). Recently, the incidence, severity, and mortality associated with infection by this bacterium have increased (10).

While infants and young children may harbor C. difficile in their feces, they typically remain asymptomatic (9). The situation in adults, however, is much different. Colonization of adults occurs by the fecal-oral route, ingested C. difficile spores, which are persistent and released into the environment, germinate to vegetative cells in the colon causing inflammation and diarrhea (9). Most cases of C. difficile infections can be treated by antibiotics such as vancomycin or metronidazole. However, recurrence of C. difficile is observed in about 20% of cases (10), and represents a particularly challenging aspect in care of these patients. Antibiotics only kill vegetative cells, and not the spores that are produced by the bacterium. Furthermore, antibiotics may also perpetuate disruption of normal resident flora, which may further promote re-establishment of the infection. Therefore, alternative approaches to therapy are often desperately needed.

The re-establishment of the normal composition of the intestinal flora has long been hypothesized to be a curative therapeutic approach. This idea is supported by the reported
success of “bacteriotherapy”, the addition of new fecal bacteria to the patient via a process termed fecal transplantation, which is currently one of the last resort treatment options for recurrent *C. difficile* colitis (1,13,17). My collaborator on this proposed project, Dr. Alex Khoruts, M.D., recently performed this treatment on one patient who suffered from severe recurrent *C. difficile* diarrhea. The patient had diarrhea for over a year, failed to respond to multiple treatments of antibiotics and probiotics, lived in diapers, and was confined to a wheelchair because of debilitation. The procedure performed involved seeding of the patient’s colon with fecal matter taken from her husband (the donor) by way of a colonoscopy. Remarkably, the patient reported virtually immediate improvement in her abdominal discomfort and was free of diarrheal symptoms by day 2 post procedure! She has now remained free of *C. difficile* and has had no diarrhea for about 6 months. However, while this procedure was successful, both practical and aesthetic considerations make “fecal transplantation” an undesirable therapy to treat a large number of patients with this severely disabling disease.

The ultimate goals of our studies are to identify the critical microorganisms of the normal intestinal flora that can protect the host against *C. difficile* infection, and by doing so, develop a standardized formulation that would be easy to administer to patients. The reason this kind of work could not be done in the past is because standard microbiological methods are unable to adequately characterize the microbiota residing in the intestinal tract, the overwhelming majority of which cannot be cultured on microbiological growth media. This is due, in part, to: 1) the vast diversity of resident microorganisms present, 2) many of the microorganisms are strict anaerobes, 3) many of these microorganisms show physiologic interdependence within the bacterial community, 4) some of the microbes may require growth factors provided by the host, 5) the growth requirements of many of the resident intestinal microorganisms are currently unknown.

The importance of microorganisms for human health has been realized by the scientific community for decades, and has recently received resurgent attention. In response to their importance, the NIH has initiated the Human Microbiome Project (HMP), with the ultimate goal of comprehensively characterizing the human microbiota and the role it plays in human disease and human health (http://nihroadmap.nih.gov/hmp/). This has been done chiefly by exploiting large scale DNA sequencing efforts of total microbial DNA (often referred to as the microbial metagenome or microbiome), or sequencing of the taxonomically-significant portion of microbial genomes, the 16S ribosomal DNA (rDNA). Over the past decade, investigators have utilized full-length sequence analysis of rDNA for taxonomic studies of gut microorganisms. However, this approach is still prohibitive in its intensity of required resources, it is very expensive, and suffers from poor detection and estimation of the frequency of moderate- and low-abundance microbial taxa present in the GI tract.

In the studies proposed here, we and my collaborator on this project will determine whether high-throughput, massively-parallel, DNA sequencing of short hyper variable regions of fecal rDNA from the metagenome of intestinal tract microorganisms has the necessary resolution and accuracy to characterize key microbial populations present in the human intestinal tract. Our analyses will be done before and after fecal transplantation. This technique has been reported to give similar information as traditional full length 16S rDNA sequencing efforts, but has higher-throughput, lower costs, and provides more information than traditional sequencing. This will
allow us to use less invasive and rational sampling strategies for determining microbial population structural changes associated with fecal transplantation in future patient studies, and determine if key microbial populations are involved in gut recolonization following fecal transplantation.

As a preliminary start to our studies, we examined excreted fecal material and tissue biopsies from the patient case above using terminal restriction fragment polymorphism analysis (T-RFLP) of bacterial rDNA. While this analysis showed efficient colonization of the recipient’s intestinal tract by donor microorganisms, T-RFLP analysis does not have the resolution needed to identify specific microorganisms responsible for establishment of the bacterial community needed for restoration of the patient’s health. Thus, more robust analyses are needed to detect moderate- and low-abundance microbial taxa that may be contributing to recolonization of the gut, disease suppression, and to answer questions concerning sampling and analysis strategies.

Recently, an important technological advance has been described by Sogin et al. (14) who used massively parallel pyrosequencing of hypervariable regions from SSU rRNA genes and assigned taxonomy based on the best match in a Global Assessment for Sequence Taxonomy process (GAST). Subsequently, Huse et al. (8) showed that taxonomic classification based on tags from the V6 or V3 variable regions of 16S rDNA is ~99% concordant with that obtained using full length SSU rRNA sequencing. This new pyrosequencing technology is orders of magnitude more economical and more powerful in revealing the true diversity of complex bacterial communities, such as those found in the human gut. Clearly, this is the best current methodology for our project.

**Plan of Work:**

In the studies proposed here, we will collect freshly voided fecal samples from up to four individual patients, before and after fecal transplantation. Samples will also be collected from fecal donors. We have identified two patients with *C. difficile* diarrheal disease that have agreed to fecal transplantation, and two additional will be recruited during the course of these studies. Dr. Khoruts has already applied for IRB approval for collecting data from a series of patients using this minimally invasive procedure. DNA will be extracted from fresh feces using a MoBio DNA extraction kit and the V3 and V6 hypervariable regions of the full-length 16S rDNA will be amplified by PCR using primers 338F and 533R, and 967F 1046R as described by Huse et al.(8). The V3 and V6 amplicon libraries from fecal samples will be sequenced on a Roche Genome Sequencer GS-FLX system by the University of Minnesota Biomedical Genomics facility using standard protocols. A total of 256,000 reads will be made from all amplicon libraries, sequences will be compared to V6 and V3 reference databases (4) and taxonomic classification of PCR products will be assigned using the GAST taxonomic classification tool (14). The taxonomic signature of microorganisms in each sample will be compared and statistically analyzed as described by Dethlefsen et al. (4).

We plan to develop our work on recurrent *C. difficile* colitis and diarrheal disease into an NIH RO1 proposal. In order to do this, we require preliminary data that will use SSU rRNA hypervariable tag sequencing to confirm the feasibility of this approach. Previous studies utilizing even relatively limited explorations of microbial diversity suggested some differences in
composition according to anatomic distribution along the surfaces in the human hindgut (7,15). Therefore, Dr. Khoruts has applied for a small amount of funding from the Minnesota Medical Foundation to explore the feasibility of using pyrosequencing of intestinal biopsy samples from different segments of the lower intestinal tract to describe the intestinal microbiome. However, there is good reason to believe that at least some critical bacteria can be identified simply from excreted fecal material since that is what is used to seed the patient’s colon during the ‘fecal transplantation’. Another critical question in development of the RO1 proposal is variability of microbial populations between different individual samples from the same subject and the extent of differences between patients and donors. These preliminary data will be used to estimate how many patients will be required for the larger study, and how many samples will be needed per time point. The RO1 proposal will be built on these results and use the least invasive and least expensive sampling strategy for determining microbial population structural changes associated with fecal transplantation on a larger number of patients. Ultimately, this work will allow us to determine if key microbial populations are involved in gut recolonization following fecal transplantation, and in the future to develop a standardized, easily administered formulation to treat *C. difficile* colitis and diarrheal disease in patients.

This research direction represents a major shift in focus for myself, an environmental microbiologist with over 25 years of experience working in the areas of plant-microbe interactions, environmental *E. coli*, and biodegradation research. Thus, working on a human disease project is a totally new and very exciting area of research for me. I believe this is an incredible opportunity for developing a truly translational research program with remarkable potential for growth and the ability to enhance patient life. The host microbiome represents an entirely new frontier in gastroenterology and medicine. In addition, the research described here represents a shift in the research focus of my collaborator Dr. Khoruts, a well-known a clinically active gastroenterologist and a basic immunologist. He is the ideal collaborator for this project. Our collaboration represents a major strength of this proposal, and a chance to put together a unique interdisciplinary Minnesota team that would be a real player in this field. The seed funds provided by this Grant-in-Aid of research are essential to generate the momentum for this work.

**Budget Justification:**

Funds ($26,266) are specifically requested to sequence metagenomic fecal DNA from freshly voided feces from four patients and donors. The DNA sequencing efforts will be carried out by the University of Minnesota Biomedical Genomics Center, located on the St. Paul Campus, using the GS FLX System, an ultra-high-throughput, next-generation DNA sequencing platform. Approximately 8 metagenome samples will be analyzed in our studies, each producing 32,000 DNA sequences. Sequencing costs ($18,400) are broken down as follows: Preparation of DNA Libraries $2,400, DNA Titration / emPCR set-up $2,500, and DNA Pyrosequencing $13,500 (1.5 sequencing plates). Funds ($7,866 - $6,666 salary + $1,2000 fringe) are also requested for partial support (2 months, 100% time) of a post-doctoral researcher to analyze the many thousands of DNA sequences generated during this project. The postdoc will also be responsible for taxonomically classifying microorganisms based on their sequence data and comparing the taxonomic status of microorganisms in feces from control and experimental groups.
Need Justification:

While I currently have research funding to determine sources of fecal bacteria and pathogens in the environment, to examine the biodegradation of atrazine and related herbicides, to examine nitrogen cycling in ecosystems exposed to elevated atmosphere CO₂ and O₃, and develop soil metagenome libraries for isolation of new enzymes, I do not have any discretionary funding to carry-out large scale sequencing efforts of intestinal tract or other microorganisms or funding that is directed towards treating human diseases. As is indicated above, this research represents a major shift in focus for me, an environmental microbiologist with over 25 years of experience working in the areas of plant-microbe interactions, environmental E. coli, and biodegradation research. The requested funds will be used to obtain preliminary data to develop our work on recurrent C. difficile-induced diarrhea disease into an NIH RO1 proposal. This preliminary data is critical to obtain NIH support for this project. I currently have University support for one full time technician (all these funds are committed), and no other available funds that are not directed towards specific projects. Since I came to the University of Minnesota in 1989, no start-up funds are available for this proposed research. I have not applied for, or received, a Graduate School Grant-in-Aid of Research since 1990, more than 18 years ago.

[1,914 words – sections 2 and 3]

Literature Cited


