

**Record #1826**

John Wertz CRF Application 2009-2010

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<b>Project Title</b>	Cultivation and Characterization of Microaerobes from the Human Microbiome				
<b>Project Abstract</b>	<p>It has become increasingly obvious that microorganisms residing within the human intestine (the gut microbiome) have enormous health impacts. They help digest food and produce sources of energy, excrete vitamins, stimulate a healthy immune system, and prevent disease. However, it is also known that 90% of these microbes have yet to be isolated and studied. Without pure culture isolates, it is impossible to assess the functional relationships these organisms have with each other and their human host, or begin to understand the nature of many intestinal diseases such as Irritable Bowel Disease. I, together with several collaborators, believe these human gut microbes remain uncultivated because many are part of an overlooked but abundant category of microorganisms – the microaerobes; i.e. oxygen-requiring microbes that cannot grow under normal, atmospheric levels of O<sub>2</sub>. Therefore, this proposal seeks to cultivate, quantify, and physiologically characterize microaerobes from the human GI tract by incorporating unique approaches to isolation and cultivation that includes incubation of cultures at low oxygen concentrations and the use of oxygen-detoxifying enzymes. The goals outlined in this proposal will lead to a better understanding of microbial interactions with each other and the human host, and provide insight into the mechanisms of intestinal disease.</p>				
<b>Outside Funding</b>	<p>This Semester CRF application coincides with a submitted NIH grant application of the same name on which I am a co-investigator in collaboration with Dr. Tom Schmidt at Michigan State University, Dr. Vincent Young at the University of Michigan, and Dr. Eugene Chang, MD at the University of Chicago. As of this writing, the NIH application received very high scores, and is expected to be funded. Therefore, this represents a unique opportunity for collaboration with excellent faculty at several institutions, each of us bringing our own expertise to the project with synergistic affect. I will work most closely with Dr. Tom Schmidt at MSU, who was a member of my Ph.D. dissertation committee and with whom I am currently collaborating on another project characterizing several unique bacterial strains isolated from termite guts.</p>				
<b>Project Details</b>	Wertz pd.pdf ← <span style="border: 1px solid red; padding: 2px;">see page 5</span>				
<b>Project Outcome</b>	<p>As part of an NIH grant proposal that is expected to be funded, this proposal is of great benefit to Calvin College, demonstrating that we have the resources, facilities, and faculty to do high-level research bridging the gap between microbial ecology and human health. Provision of this CRF will ensure I have the time I need to successfully complete the research described. This will, in turn, make renewal of this NIH grant easier and give similar NIH grant applications by myself and others at Calvin a greater chance for success in the future. This not only brings in financial gain for Calvin, but also lends prestige to the institution, allowing for recruitment of high-level students and faculty. This also represents a great opportunity for outreach into the community, as millions of people nationwide suffer from intestinal diseases such as Irritable Bowel Disease (IBD). A research program at Calvin that is ultimately geared towards solving a complex dysbiotic disease such as IBD fits well with Calvin's ethos of community</p>				

outreach and engagement - in this case with of a diverse array of health professionals and patients working towards a cure for a currently incurable and not well understood condition.

This proposal also gives Calvin the opportunity to collaborate with researchers at three different and prestigious institutions – Michigan State University, the University of Michigan, and the University of Chicago. Forging good relationships with faculty at these universities can have enormous benefits for future research and funding opportunities at Calvin, as well as provide an “inside track” for our students to attend these institutions as Masters/Ph.D. candidates, or for medical, dental or other pre-professional programs.

Professionally, by obtaining a “library” of novel cultivars from the human microbiome, we are blazing a trail into a relatively new, as yet unnamed, field of biology where microbial ecology meets human health. We will be at the forefront of this field, discovering new ways to cultivate and characterize the microorganisms in the human GI tract, ensuring a better understanding of their function and allowing the proposal of new, directed hypotheses concerning the role of specific microbes in human health and disease. The resources stemming from this grant will be made available to other researchers interested in various human diseases, and hopefully be able to provide new insights into human dysbiosis with potential ramifications far beyond what is imagined now.

As a new faculty member at Calvin, successful publications and presentations at national meetings can pave a pathway that will make obtaining future sources of funding much easier, and set my research on a path for success for many years to come. It is extremely likely that this research will result in one or several publications, as well as a presentation at a major national meeting. This research will also lead to future opportunities to work on aspects of this project or research stemming from this project during the summer months, allowing me to engage future students in exciting mentored research experiences. Therefore, not only would this give me the opportunity for publications and meeting presentations, but also give those same opportunities to future students, ensuring they have the skills and background to be successful in their own careers.

**Pedagogical Impact** This proposal presents an opportunity for me to gain insight and understanding into a completely new system – the human microbiota. Though my research on termite gut microbes has similarities to the proposed research, currently my understanding of much of human microbiology and the interaction of microbes with the human immune system is limited to that described in textbooks. This research will push my understanding far beyond textbooks into the most current literature, increasing my own comprehension of the microbial ecology of the human gut and its interaction with the immune system. This will have significant pedagogical implications since my primary teaching responsibilities are the Microbiology and Medical Microbiology courses in the Biology Department. In these courses, students often enter with an “us (humans) vs. them (germs)” idea of the world. However, a significant portion of these courses should be devoted to discussion of the “normal” microbes that protect us from disease such as the human intestinal microbiome. Students in these courses can also become directly involved with the proposed research - some of the physiological characterization studies will make perfect honors projects or independent investigations for the laboratory portion of these courses. Finally, the most exciting pedagogical implication of this

research to me is that it is a fantastic example of how God has designed and continues to sustain his creation through extremely complex interactions, in such a way that our health and continued vitality is dependent upon the activity of some of the smallest and often overlooked forms of life.

**Itinerary/Work Schedule** My collaborators and I anticipate that the NIH grant upon which this proposal is based is extremely likely to be funded, given the high scores it received upon review. The starting date for the grant would be the first of the year, 2009, and therefore much of the initial media preparation and plating experiments can be done during the summer of 2009. These will be done by myself and a Calvin student working in conjunction with Dr. Thomas Schmidt at Michigan State University. This will provide a Calvin student the opportunity to work, at least part of the summer, in a major research lab. Once the bacteria are growing, and comparisons between cultivation conditions can be made (which often takes 1-2 months), the task of obtaining bacterial isolates begins. Therefore, though the initial experiments will take place during the summer of 2009, the actual analysis and selection of bacterial isolates, which I anticipate may be in the range of several hundred, will require a significant amount of time beginning at the end of summer and continue into the Fall Semester, 2009.

Once promising organisms have been isolated in pure culture, research goals #2 and #3 can occur in tandem. In my experience, neither of the methodologies for these goals are difficult, but they are time consuming. The Q-PCR method (research goal #2) often requires optimization and troubleshooting before an accurate result can be obtained. This will need to be done for each individual isolate, and is therefore done most efficiently when I can have large "chunks" (for lack of a better term) of time to focus solely on those experiments. This is why I am requesting a 2 credit reduction during the fall semester, 2009.

The physiological characterization of the isolates, again, is not difficult, but is time consuming. Setting up media and cultivation conditions to test substrate utilization, oxygen tolerance, and assays for enzyme activity require large slots of time during the day. This is especially true since each test will require growth of the organism under microoxic conditions – requiring the extra step of reducing the concentration of oxygen in the medium and headspace of the vessels in which the test is done. Again, the gassing station that is available in my laboratory makes this readily achievable, but is not something that can be performed between classes and appointments – it often requires one or several days of focus to accomplish. Measurements of growth, such as the determination of optimum growth rate and cell yield under different concentrations of oxygen, often requires the taking of optical density readings in 2 – 6 hour intervals over the course of a week or more. These tests will need to be done on each individual isolate (though tests can be coordinated to include multiple isolates at the same time), and in order to determine statistical significance, several of these tests will also need to be repeated. Again, this will require large portions of uninterrupted time during the day when I can be focused on the task at hand and work quickly and efficiently to ensure the success of the experiments. Therefore, I believe a 2 course reduction during the fall semester is the best way to achieve the large portions of contiguous time needed to complete these experiments.

**Budget** As part of an NIH proposal, supply money has been requested in that budget. Hence, the expenses incurred for the experiments described in

this proposal will be paid from those monies.

**Research Funding History** As stated, this proposal is part of an already submitted and reviewed NIH grant proposal that has received high scores and therefore has a good likelihood of being funded. I also received a 2008 Summer Calvin Research Fellowship, which resulted in some very successful, interesting and surprising results that will provide insight into the experiments proposed here. We were able to isolate and characterize two completely novel bacteria from the guts of termites – one almost certainly belonging to a yet-to-be named (by us) genus and species, and the other a member of as yet unnamed species. These two organisms display a commensal behavior never before described in the termite ecosystem, with the possibility of giving great insight into the ecology of the termite gut microbiota as well as the host interactions with its gut symbionts. Thus, the results from that CRF-supported project will, with a few additional experiments, lead to the submission of a manuscript to Applied and Environmental Microbiology, a highly regarded journal published by the American Society for Microbiology. This same data will also be used for a future NSF grant application focused on the symbiotic interactions of microorganisms within the termite gut, a large component of which will be a focus on the cultivation and isolation of microaerophiles and O<sub>2</sub>-consuming primitive eukaryotes.

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## Background and Significance of Project

In the past 10 years, it has become increasingly obvious that the microorganisms residing within the human intestinal tract (collectively known as the gut microbiome) have an enormous impact on our health. The human microbiome represents approximately 100 trillion organisms in as many as 800 distinct species - 10X the number of non-bacterial cells found in our human body. The numerous and essential functions of the human microbiome for the continued health of the individual cannot be overstated. Among other functions, the “normal” symbiotic microbial community in the gastrointestinal tract helps digest food and produce utilizable sources of energy, excrete vitamins, maintain a balanced environment, stimulate a healthy immune system, and prevent disease.

Some of the most common intestinal diseases are Crohn’s disease (CD) and ulcerative colitis (UC), collectively grouped under the term inflammatory bowel disease (IBD). A pervasive disease, it is characterized by an exaggerated host cell inflammation in digestive tract tissue causing abdominal pain, diarrhea, malnutrition, and increased risk of colon cancer. While the specific cause is unknown, there is increasing evidence it is due to a breakdown in the balance of the beneficial microbiota vs. harmful bacteria, a phenomenon known as dysbiosis. However, before we can begin to understand complex dysbiotic diseases such as IBD, we need to first better understand the functions of the normal intestinal microbiota.

Recent studies have begun to characterize the intestinal microbial community and document changes that occur with IBD. However, our current knowledge of the organization, fluctuations and function of the individual microbial populations in the human GI tract is severely limited. A major factor for the paucity of data on the gut microbiome has been the general inability to grow, as pure culture isolates on “plates” in the laboratory, 90% of the microbes that are detected via DNA sequence analysis – an irony often referred to as the “the great plate count anomaly.” We believe that one of the principal reasons for the inability to retrieve, in pure culture, many significant members of the gut microbiome is because routinely used culture methods have been inattentive to factors such as oxygen concentration and the control of toxic by-products of oxygen metabolism.

Molecular oxygen ( $O_2$ ) is one of the most abundant and pervasive chemical factors affecting life on earth. However, the utilization of oxygen by microbes (as well as any other living being) comes with a curse, and that is the inescapable production of toxic by-products such as superoxide anion ( $O_2^-$ ), hydroxyl radical ( $OH\cdot$ ) and hydrogen peroxide ( $H_2O_2$ ). These compounds often critically damage DNA, inhibit the function of essential proteins and enzymes, and are toxic at extremely low concentrations. These compounds will inhibit the growth of microbes unless cells have enzymes such as

superoxide dismutase, superoxide reductase, catalase or peroxidase to detoxify them. In the past, use of techniques such as incubation in low-O<sub>2</sub> environments, provision of oxygen detoxifying enzymes, and use of low nutrient medium (which also prevents damage from toxic oxygen by-products) has met with much success in the isolation of unique termite gut microorganisms during my time as a graduate researcher at MSU as well as in my laboratory at Calvin during my summer 2008 CRF-funded research.

The mammalian gut lumen has traditionally been considered to be an environment devoid of oxygen. More recently however, the role of oxygen in this ecosystem has been re-examined. Recent studies have suggested there exists a low-oxygen environment adjacent to host tissues in the human GI tract created by diffusion of O<sub>2</sub> from host tissues into the intestine. This results in a gradient of oxygen in the intestine, with the highest concentrations at the intestinal surface decreasing to anoxia in the interior, luminal space. This oxygen gradient creates ecological niches for a specific type of bacteria known as microaerobes – microbes that either require low concentrations of O<sub>2</sub> or whose growth is retarded or completely suppressed by normal, atmospheric concentrations of O<sub>2</sub>. Due to their proximity to host tissue, the microbes in the low-oxygen portion of the intestine are most likely to have biologically significant interactions with their human hosts. Again, I suspect such organisms have remained largely obscure because the techniques required to cultivate them (e. g. provision of O<sub>2</sub> at a *constant but low concentration*, use of oxygen-detoxifying enzymes, and care in obtaining and preparing clinical samples to ensure they remain at below-atmospheric oxygen levels) have never been employed in experiments designed to isolate bacteria from the human intestine.

Accordingly, this proposal seeks to integrate refined procedures for microbial enrichment and isolation that includes incubation of the samples in low oxygen conditions from the moment they are collected. I believe that addressing the “oxygen problem,” as this proposal seeks to do, will enhance the retrieval of pure cultures of microbes from the human microbiome. This will facilitate genetic, biochemical and physiological experiments that will lead to a better understanding of microbially-catalyzed processes in the gut, as well as to the rational development of treatments that exploit microbial activities for enhanced human health. It is with these convictions that the following study is proposed.

**Research Goal 1:** Exploit microoxic atmospheres and novel cultivation strategies to isolate microaerobes from the mucosa of the GI tract.

**Methods.** We hypothesize that significant undiscovered microbial diversity in the human GI tract is represented by microaerophilic microbes. Samples will be obtained from the unprepped colons of healthy individuals (3 male and 3 female participants per year over the course of three years) by Dr. Eugene Chang, MD, at the University of

Chicago. Using unprepped colons will preserve the integrity of the microbes close to the intestinal wall and any microoxic zones contained therein. Upon collection, the specimens will immediately be placed in a low-oxygen storage and transfer medium that will preserve the viability of the bacteria upon transfer to Michigan State University.

Initial cultivation efforts will be carried out at Michigan State University by myself and a Calvin student (if at all possible) in collaboration with Dr. Tom Schmidt, whose laboratory at MSU contains two Coy-type atmosphere-controlled glove bags outfitted for a microoxic environment (2.5% O<sub>2</sub>, 5% CO<sub>2</sub>, and 92.5% N<sub>2</sub>). Efforts are underway to obtain a similar glove box at Calvin, and once that occurs, similar initial isolation and cultivation experiments can be performed here, without the need to travel the short distance to MSU (which I anticipate would occur, initially, on a bi-weekly basis). Samples obtained from the University of Chicago will be serially diluted into liquid media, thereby enabling quantitative estimates to be made of population levels and accommodating the possibility that as-yet-unknown interspecies interactions may also be important for growth in the lab. From each liquid culture dilution, solid media plates will also be prepared for direct isolation attempts of bacterial colonies by using low-nutrient complex and defined media containing a mixture of potential energy sources. Media will be supplemented with one of several oxygen-detoxifying enzymes such as catalase, superoxide dismutase, etc. A certain amount of trial-and-error will likely be necessary initially to identify the best cultivation conditions, however, as stated before, I, together with the Schmidt lab have had experience coaxing difficult-to-grow organisms into pure culture and do not anticipate an inordinate delay in this aspect of the project.

Once inoculated, one set of replicate liquid and solid media cultures will remain within the low-oxygen environment in the microoxic glove box, and the second set will be removed and placed within incubation chambers under an atmosphere of 5% CO<sub>2</sub>-enriched air. The cultures will be monitored for growth, with attention directed towards morphological and numerical comparisons between those in microoxia vs. those in CO<sub>2</sub>-enriched air. Numerically dominant organisms found under microoxic incubation that are not present in CO<sub>2</sub> enriched air will be selected for further isolation, cultivation, and physiological analyses. It is this precise methodology that led me to isolate and cultivate a microaerophile belonging to a novel genus from the termite gut, which we named *Stenoxybacter acetivorans* (see recent publications in attached CV).

Based on the physical and chemical characteristics of the mucosa, it is expected that microaerobes will thrive in this environment by taking advantage of the increase in energy that can be obtained by using oxygen during metabolism. However, it is also conceivable that this habitat is occupied by microbes that are tolerant of oxygen, but are not microaerophiles (i.e. they do not utilize oxygen and are not dependent on its presence for growth). If this is the case, the focus of the project will be shifted to the cultivation, characterization, enumeration, and localization of these populations. This

requires only minor modification of the methods proposed to include strictly anoxic cultivation after short term incubation under microoxic conditions to eliminate microbes that are completely intolerant of oxygen.

**Research Goal 2:** Determine the abundance of the isolated microaerobes in the GI tract.

**Methods.** As described in research goal 1, numerous microaerobic organisms are expected to be isolated from samples of the low-O<sub>2</sub> portion of the human intestine. Selected organisms will proceed to further physiological characterization (research goal 3). In order to prioritize the organisms that will progress to this level of additional characterization, a molecular technique known as Quantitative Polymerase Chain Reaction (Q-PCR) will be done to assess the abundance of the organism in the human GI tract. This information will permit the selection of organisms that have the greatest potential to impact our understanding of the role of microaerobes in the human microbiome.

An accurate assessment of the relative dominance of a specific microaerobe in the human mucosa can be done via Q-PCR. This work can be done at Calvin, as the necessary software and computers are available in my laboratory, and Dr. Randall DeJong (Biology) has recently purchased a Q-PCR machine for use by individuals at Calvin. For Q-PCR, gene sequences specific to the organism of interest will be identified, and short sequences of DNA, termed “primers” that target these sequences will be designed using software available in my laboratory. After testing these primers to ensure they target only the organism of interest, they will be used in a Q-PCR reaction using DNA purified from the intestinal mucosa by members of the lab of Dr. Vincent Young at the University of Michigan. In this experiment, the more abundant the target DNA is in the sample, the sooner it is detected by the Q-PCR machine. The results will be normalized to the amount of host DNA using a human-specific gene which has been used by Dr. Vincent Young with success in the past.

**Research Goal 3:** Provide a physiological characterization of numerically dominant isolates.

**Methods.** As described above, metabolic models of bacteria based solely on DNA sequence fail to capture many aspects of the actual function of an organism in its environment. However, an infinite amount of metabolic information can be readily measured with pure culture isolates. Conducting biochemical and physiological characterization of the isolates will give invaluable insight into the possible function and ecological role these organisms have within the human GI tract.

Growth rate and yield will be determined for each isolate over a range of oxygen concentrations (using equipment in my laboratory at Calvin) to assess the influence of

molecular oxygen on the rate and efficiency of growth. Metabolite production from the cultures will also be assessed, with particular attention to products that may be used as metabolites or signaling and communication molecules to the host tissues, including vitamins B12 and K, nitrous oxide, and butyrate – a molecule that has anti-inflammatory capability and is used as an energy source for the host tissue. Further biochemical characterization such as the presence of catalase, superoxide dismutase, or other peroxidases will also be done. Growth temperature and pH optima will also be performed. All of these biochemical studies are currently in use in my lab, and can be readily and easily performed at Calvin. Some may even make excellent “mini-projects” for interested honors students or for independent investigations in the laboratory sections of my medical microbiology and upper level microbiology courses.

### **Concluding remarks**

The mammalian gut represents a complex ecosystem with multiple interactions between the host and the indigenous community of microorganisms. However, there is a great paucity of data regarding the function of the microorganisms because of the lack of representative bacteria that have been obtained in pure culture isolation to date. This proposal seeks to cultivate and characterize the subset of microbes most closely associated with the intestinal wall, and hence are most likely to be those that interact with the human cells and immune system. The microenvironment of the mucosa suggests that these organisms are most probably microaerobes and are thus missing from the current set of human cultivars because of their unique requirement for some, but not too much, oxygen. I believe the techniques outlined in this proposal will result in a large set of novel isolates that can then be quantified and further characterized, with a focus on their probable function in the GI tract. This information will leave me, in association with the collaborators mentioned above, poised for future research endeavors that will further explore the interactions of these bacteria with their human host and suggest mechanisms, and possible courses of treatment, when these interactions break down during disease.