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14. ABSTRACT
Nutrient acquisition in the human gut microbiome is a prototype for the mixed microbial systems which profoundly influence health, ecology, energy, and agriculture. The functions effected by microbiota are mediated by the community as whole, and result from synergies between the member cells. Our long-term goal is to develop a more faithful in vitro model of the bacterial communities constituting a microbiome to elucidate the spatial organization and cross-feeding relationships between species. Our objective in this Short-Term Innovative Research project was to develop the tools needed to apply fluorescence microscopy and super-resolution imaging to investigate how

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RPPR Final Report
as of 13-Sep-2017

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STEM Degrees: 1 **STEM Participants:** 0

Major Goals: (1) Determine the conditions under which Bt and Rb can grow in a commensal fashion.
(2) Compare SusG-HT protein dynamics in Bt cells grown in monoculture to Bt cells grown in Bt-Rb co-culture as a function of carbohydrate source.
(3) Measure the similarities and difference between these diffusive properties of SusG-HT in Bt cells imaged in isolation on the microscope and Bt cells imaged within mixed monolayers of Bt and Rb.

Accomplishments:
See Attachment

Training Opportunities: PhD students Chanrith Siv and Hannah Chia worked on the project and became adept in the area.

Results Dissemination: Parts of this work were written up in the PhD thesis dissertation of Dr. Chanrith Siv.
Title: Single-Particle Tracking of Proteins in Living Bacteria:From Single Cells to a Mixed Community
Date: May, 2017

Honors and Awards: Nothing to Report

Protocol Activity Status:

Technology Transfer: Nothing to Report

PARTICIPANTS:

Participant Type: Graduate Student (research assistant)
Participant: Chanrith Siv
Person Months Worked: 6.00 **Funding Support:**
Project Contribution:
International Collaboration:
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Other Collaborators:

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Participant Type: Graduate Student (research assistant)
Participant: Hannah Chia
Person Months Worked: 4.00 **Funding Support:**
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Participant Type: PD/PI
Participant: Julie Suzanne Biteen
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Authors: Chanrith Siv
Acknowledged Federal Support: **Y**

Final Report:
STIR – Capturing the dynamic response of microbes to their environment and their community

Julie Biteen, University of Michigan

Statement of the problem studied. Nutrient acquisition in the human gut microbiome is a prototype for the mixed microbial systems which profoundly influence health, ecology, energy, and agriculture. The important functions effected by all microbiota are mediated by the community as whole, and result from an intricate synergy between the member cells. Our long-term goal is to develop a more faithful *in vitro* model of the bacterial communities constituting a microbiome and to apply microscopy to elucidate the spatial organization and cross-feeding relationships between species.

Our objective in this Short-Term Innovative Research (STIR) project was to develop the tools and techniques needed to apply fluorescence microscopy and super-resolution imaging to investigate how nutrient acquisition by the starch utilization system (Sus) in one prominent gut bacterium, *Bacteroides thetaiotaomicron* (*Bt*), is affected by the other members of its microbial community, in particular the keystone species *Ruminococcus bromii* (*Rb*). During the period of this STIR, we developed the approaches and methodologies needed to investigate molecular- and cellular-scale interactions in a *Bt*/*Rb* mixed microbiome.

Summary of the most important results.

(1) We determined the conditions under which *Bt* and *Rb* can grow in a commensal fashion and measured the extent of cross-feeding under these different conditions;

The human gut microbiota has the capacity to degrade many starch macrostructures through glycoside hydrolases (El Kaoutari et al., **2013**). *Bt* digests many forms of starch, but this bacterium cannot digest resistant starch (RS) (Englyst et al., **1992**). *Rb* is the keystone species for the degradation of RS in the human colon (Abell et al., **2008**). Recent *in vitro* evidence suggests that the byproducts from *Rb* degradation of RS can be further utilized by other gut microbes so that they can grow in the same medium (Ze et al., **2012**). In this project, we used growth curves and cellular imaging to determine the conditions under which *Bt* and *Rb* can grow commensally and we visualized the growth of these two species under the microscope.

Growth of co-cultures in spent media. We grew *Rb* in Ruminococcus Rum media with either corn starch or potato starch—neither of which can be used as carbon sources for growing *Bt* alone—for up to 3 days. This long growth ensured abundant amounts of metabolic byproducts in the media. We then filtered the spent Rum media to remove *Rb* cells and starch. To test the hypothesis that if cross-feeding is present, *Bt* may be able to grow in a media containing the metabolic byproducts of RS degradation by *Rb*, we measured the growth of *Bt*, *Rb*, and *Bt*/*Rb* co-cultures in the spent Rum. *Bt* could not grow in Rum media alone (Fig. 1a) or in RS, but grew in the spent media isolated from *Rb* grown with corn starch (Fig. 1b). We also applied analytical chemistry approaches to identify the byproducts of *Rb* starch consumption. Thin-layer chromatography (TLC) indicated that the byproducts were more

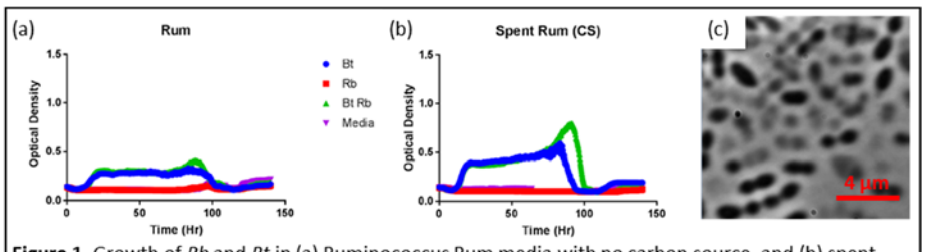

Figure 1. Growth of *Rb* and *Bt* in (a) Ruminococcus Rum media with no carbon source, and (b) spent Rum isolated from *Rb* grown with corn starch. (c) Microscope image of *Rb/Bt* co-culture.

simple carbohydrates and that the amount of byproduct increased with longer growth times; ongoing work is using mass spectrometry and high-pressure liquid chromatography (HPLC) to more definitively determine the identities of the sugars released by *Rb*, and determine which of the released sugars is subsequently consumed by *Bt* in cross-feeding.

Microscopy of co-cultured cells. Due to the slower growth rate of *Rb* relative to *Bt*, we found the ideal conditions for co-culture of these species: we inoculated *Rb* cells into fresh starch-containing media, and let these cells grow for 2 days before adding in log-phase *Bt* cells. The co-culture growth rate was characterized, and at various time points, aliquots were prepared for microscopy according to our previous protocol for anaerobic live-cell imaging (Karunatilaka et al., **2013**). In this way, we visualized live anaerobic bacterial co-cultures for the first time to our knowledge (Fig. 1c).

(2) We cloned into *Bt* two novel fluorescent protein markers, UnaG and CreiLOV, and characterized these proteins for use in anaerobic cell imaging;

Having grown mixed *Bt*/*Rb* cultures and imaged monolayers of these communities, we sought a method to more selectively identify different cells, even in a living 3D biofilm. However, traditional fluorescent proteins such as GFP and mCherry require an oxygen cofactor to fluoresce (Tsien, **1998**). These probes are therefore incompatible with bacteria that live in anaerobic environments such as the gut as these species are typically oxygen-sensitive; for example, *Bt* goes dormant (suspends its growth) in the presence of oxygen and *Rb* dies in even partially anoxic conditions. We therefore pursued an alternative approach and tried labeling *Bt* with two alternative, genetically encodable tags: flavin-based fluorescent proteins (FbFPs), which utilize the metabolic byproduct flavin as a cofactor to produce fluorescence (Mukherjee et al., **2015**), and the fatty-acid binding protein UnaG (Kumagai et al., **2013**).

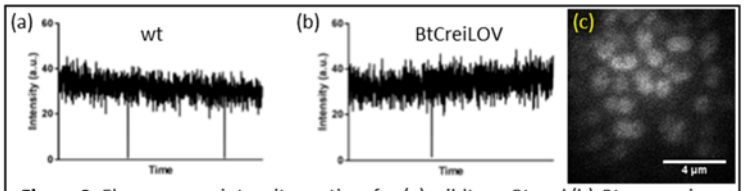

Figure 2. Fluorescence intensity vs. time for (a) wildtype *Bt* and (b) *Bt* expressing BtCreiLOV. (c) Fluorescence image of *Rb* stained with *anti-Rb* antibodies.

Flavin-based fluorescent proteins. We acquired the genes for two FbFPs codon-optimized for *Bt*: the original *btFbFP* and the engineered *btCreiLOV* in an *E. coli* S17 strain, and conjugated them into *Bt* with homologous chromosomal recombination to place the genes under the promoter usBT1311 for constitutive expression. We evaluated the fluorescence signal from *Bt* cells expressing BtCreiLOV and BtFbFP theta in aerobic and anaerobic conditions (Fig. 2ab). We observed no significant fluorescence over the wt autofluorescent background. The *ecCreiLOV* gene was additionally cloned into *E. coli* under control of the arabinose-inducible pBAD vector, but again no significant fluorescence was observed. We attributed the low FbFP signal to the low quantum yield of these FPs relative to GFP.

Fatty acid-binding proteins. The protein UnaG has shown fluorescence excitation and emission similar to GFP (Kumagai et al., **2013**), and this protein can also be used in anoxic conditions. We acquired the *unaG* gene and cloned it into *E. coli* as described above. When these cells also didn't display measureable fluorescence at a range of inducing conditions and bilirubin cofactor concentrations, we revisited all of our constructs. Unfortunately, sequencing revealed a frameshift error in the BtCreiLOV sequence that introduced premature stop codons, and an SDS-PAGE gel stained with Coomassie for all soluble proteins extracted from the cell lysate indicated that the *E. coli* pBAD promoter was unresponsive to arabinose induction. Fortunately, this means that the CreiLOV and UnaG are still viable possibilities for anaerobic fluorescence imaging in a microbiome, and work is ongoing to remake all strains.

Alternative approach: antibody labeling. In addition to the selective labeling of *Bt* via genetically encoded FPs, we used immunofluorescence to probe a culture of *Rb* with antibodies against starch degrading enzymes on *Rb* and a fluorescent secondary antibody (Fig. 2c). This labeling was successful under anoxic conditions, and we found only minimal non-specific labeling of *Bt* cells in a mixed *Bt*/*Rb* culture. Ongoing experiments will further optimize antibody labeling to minimize nonspecific binding and eliminate free, unbounded dyes in the sample.

(3) We developed protocols for *Bt*/*Rb* biofilm growth and we built a light-sheet microscope for imaging thick biofilms with high resolution.

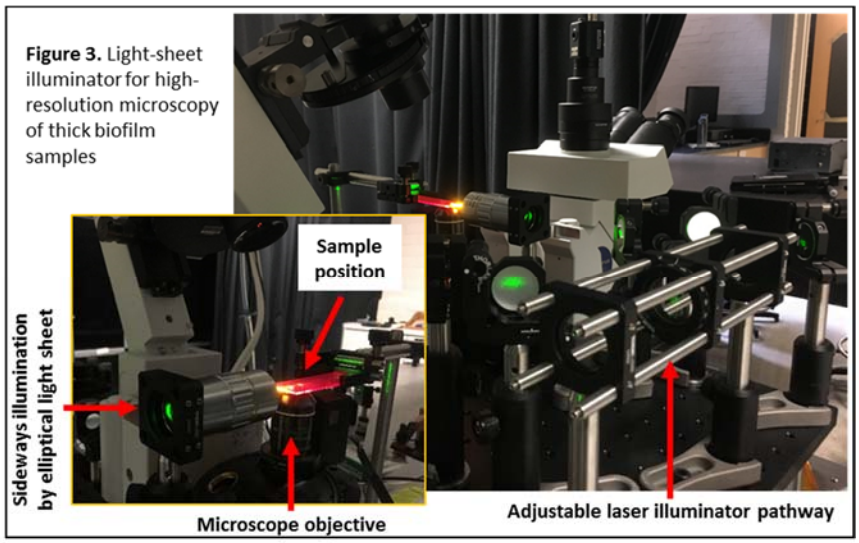

Figure 3. Light-sheet illuminator for high-resolution microscopy of thick biofilm samples

Biofilm growth. After visualizing monolayers of *Rb*/*Bt* cells from a co-cultures, we aimed to create 3D biofilms of these cells. We deposited co-cultures in glass-bottom wells for 24 hours to create static biofilms, but the adherence was poor. We then used a BioFlux microfuidic system to circulate cells in a pneumatic flow (Samarian et al., **2014**); this temperature-controlled environment more closely mimics the human body. Though the conditions need to be further optimized, this experiment yielded thick, dense samples of *Rb* and *Bt*.

Building a light-sheet illuminator. The 3D structure and thickness of the biofilms posed a new technical challenge: our super-resolution microscope relies, which relies on through-objective epifluorescence illumination, is idea for single-cell fluorescence imaging, but thick samples like a biofilm produce strong out-of-focus fluorescence that precludes high-resolution imaging. To address this challenge, we built a light-sheet illuminator (Fig. 3), which creates a thin, elliptical light sheet and addresses the sample from the side to selectively illuminate only one plane of a thick sample and avoid out-of-focus emission (Lee et al., **2016**). Custom built to fit onto our microscope, we have validated the light-sheet illuminator with test samples and are beginning now to image biofilms on it.

Conclusion. Overall, these technical advances and preliminary results will enable broader scientific progress toward our overarching goal of measuring microbe-microbe interactions on the cellular level within mixed microbiota.

Key Personnel. The work here was designed by PI Dr. Julie Biteen who also assisted with data analysis and interpretation. Experimental progress was accomplished by two PhD students: Chanrith Siv, who was supported by this project, and Hannah Chia, who was supported on a departmental fellowship. Dr. Siv graduated with his PhD in May 2017; this work was included as a chapter in his thesis. The work was done in consultation with our long-time collaborator, Prof. Nicole Koropatkin in the UM Medical School; Chanrith and Hannah grew cells in Dr. Koropatkin's anaerobic chamber. *Bt*/*Rb* biofilm growth was done in consultation with a new collaborator, Prof. Alexander Rickard in the UM School of Public Health, who gave Chanrith access to his BioFlux microfluidic flow cell system.

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