“GETTING TO THE ROOT OF PLANT HEALTH: INTERACTIONS OF Sinorhizobium meliloti AND SOIL PROTISTS WITH THE RHIZOSPHERE”

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I. Introduction

The rhizosphere is the region of soil that is directly influenced by plant roots and includes the subterranean plant tissue. Rhizosphere soil forms a physical relationship with plant roots, and remains bound to roots when bulk soil falls off after removal of the plant from the ground. On a cellular level, the rhizosphere is made up of 3 regions; the ectorhizosphere, the endorhizosphere, and the rhizoplane. The endorhizosphere consists of roots cells which secrete exudates into the ectorhizosphere. The endo- and ectorhizospheres are separated by the rhizoplane, which mediates their relationship. Finger-like protrusions of the endorhizosphere, called root hairs, extend into the ectorhizosphere and help to hold the soil matrix together. Exudates secreted from the endorhizosphere consist of sugars, protein, amino acids, as well as other carbon rich molecules. Due to its high concentration of secreted plant exudates, the rhizosphere can contain up to 10^{11} microbial cells per gram of root. According to Berendsen, the rhizosphere is the “greatest reservoir of biological diversity” known in the world.

The complex environment is developed by an intricate relationship between the plant and a diverse community of microorganisms. These specialized interactions occur between roots and soil microbes due to coevolutionary pressures. The additive genomes of the rhizosphere’s microbial community are often considered the plant’s second genome due to its ability to influence and respond to plant derived and community derived pressures. Much like the human gut microbiome influences its host, the soil “rhizobiome” has the ability to control physiological and phenotypic changes in the plant.

The plant and microbes often benefit from each other in the rhizosphere. While the rhizobiome contains members of all branches of the tree of life, the most commonly studied are mycorrhizal fungi, protists, and bacteria. Each member participates in specific niche functions, as well as a cyclical exchange of nutrients with other microbes as well as the plant.

Mycorrhizal fungi are regular inhabitants of the rhizosphere and hold multiple important functions. Fungal hyphae penetrate soils and help to physically structure soil particles. Soil aggregates will form around hyphae, resulting in the formation of channels for water and microbial transportation. Fungi also participate in the translocation of nutrients and minerals from the soil to the plants. Mycorrhizae associated with plant roots effectively increase the surface area of roots, thus increasing water absorption. Soil fungi have also been characterized to suppress soilborne plant pathogens by activating plant defense mechanisms.

Protists are another commonly found member of the rhizosphere microbial community, although they are much less studied and severely underappreciated. The protist branch on the tree of life consists of unicellular eukaryotes which have vast morphological and physiological diversity, and fit many niches in different environments. Generally, protists are organized into 4 morphological groups: ciliates, flagellates, testate amoeba, and naked amoeba; all of which can be found in soil. Although their exact benefit to plant health is unknown, it is recognized that removal of protists from the rhizosphere results in less healthy plants.

Protists prey on bacteria, fungi, and occasionally other protists. Protist predation on other soil dwelling microorganisms is thought to be essential for population control by selective consumption of specific prey, and nutrient cycling by releasing nutrients otherwise stored in biomass. The microbial loop hypothesis, which refers to the turnover of nutrients in soil microorganisms, metabolism of organic matter, and the passage of biomass from one organism to another, assumes protists as a key player. Bacteria and fungi respond to the influx of plant exudates into the soil, especially labile carbon, and actively build new microbial biomass.
Heterotrophic soil protists are attracted to the increase in microbial biomass, and travel towards the roots in response to their food source. Digestion and excretion of the biomass by the protists provides otherwise inaccessible nutrients back into the soil, which can be used by metazoans and plants. This multitrophic interaction trades plant produced carbon for microbial produced nitrogen, phosphorous, and other nutrients. (Figure 1.)

**Figure 1.** The soil food web by Geisen *et. al.* depicting soil protists as a crucial player in soil nutrient cycling. Plants secrete exudates which induce bacteria and fungi growth. Protists consume bacteria, fungi, and other protists, releasing stored nutrients back into the soil. Metazoans and plants benefit from the influx of otherwise stored nutrients. Black arrows: widely assumed nutrient flows to protists; dotted arrows: suggested nutrient flow to higher trophic levels; red arrows: formerly neglected trophic positions of protists. Not all heterotrophic protists are bacterivores, some are mycophages and omnivores.

Geisen *et. al.* 2016
Bacteria are the most frequently studied member of the rhizosphere. While they provide nutrients to the community through their decomposition, bacteria can also facilitate the uptake of scarce resources in soil by the plants. For example, usable iron is often limited to plants, but can be acquired using iron chelated to microbial siderophores. Bacteria are also responsible for much of a plant’s acquisition of nitrogen, a commonly limited nutrient. *Bradyrhizobium, Rhizobium, Burkholderia, Achromobacter,* and *Frankia,* are some of the taxa efficient in biological nitrogen fixation in soil.

*Sinorhizobium meliloti* is one of the most commonly studied members of the nitrogen-fixing soil dwelling microorganisms. *S. meliloti* is a gram-negative alpha-proteobacterium which symbiotically infects nitrogen starved leguminous plant roots. Successful infection by Rhizobia results in a root nodule. Inside the nodule, Rhizobia-produced nitrogenase converts atmospheric nitrogen into ammonia. Ammonia is the usable form of nitrogen for plants, and due to its natural affinity of binding organic molecules and water, is often limited in soil. Production of ammonia within the plant root allows for quick and efficient utilization of nitrogen by the plant.

Rhizobia rely on quorum sensing to respond to environmental signals and shift from a free-living state to an endosymbiotic state to infect legume roots. Quorum sensing is a molecular communication mechanism utilized by bacteria to communicate with their environment. -Hormone-like molecules, called autoinducers, are produced and released by bacteria and accumulate in the environment as the cell density increases. Autoinducers are detected by receptor proteins, and once a threshold is reached they cause a signal-response in gene expression and community behavior.

Soil microbes can use quorum sensing to induce behaviors such as biofilming, motility, sporulation, symbiosis, competence, conjugation, virulence, and antibiotic production. While Rhizobia utilize quorum sensing to form a symbiosis with legume roots, it is also used to communicate with kin and other bacteria.

I plan to investigate some of the intricate relationships that occur in the rhizosphere, specifically those of protist-plant, protist-*S. meliloti,* and *S. meliloti*-rhizosphere interactions. Members of the rhizosphere work together to function as a succinct unit, responding to pressures which effect the entire community. This research will determine who the key protist players are in the *Medicago* rhizosphere, develop a mechanism for utilizing protists to redistribute bacteria in the rhizosphere, and shed light on how *S. meliloti* communicates with the rhizosphere.

II. Proposed Research

Aim 1. Identify the core “protist-ome” of field grown *Medicago truncatula*

i. Introduction

Plants secrete up to 40% of their photosynthates through their roots and into the soil. The microbial community flourishes in the presence of plant exudates, resulting in a significantly higher population density of microbes in the rhizosphere versus the adjacent bulk soil. Soil bacteria rely on carbon rich root exudates to create new biomass, but are also at risk of being consumed by predators such as protists. Protists release nutrients stored in bacterial biomass, making the rhizosphere a richer environment. The microbial loop hypothesis states that soil bacteria mobilize nutrients, such as nitrogen, in response to carbon rich plant exudates, which are released by protists to be fed back to the plant. However, not all microbes are equally as affected by the photosynthates. It is thought that plants have the ability to selectively enrich or repress certain microbes by exuding specific compounds.
Protozoa are of critical importance in the microbial loop of many ecosystems, yet are the most sensitive to chemical shifts in the soil. Application of nitrogen fertilizer, a very common additive in agricultural soils, significantly reduces protist diversity. One could imagine that changes to the chemical properties of soil due to selective release of exudates by plants may strongly influence the protist community. Protists shape the rest of the microbial community through their ecologically significant traits linked to morphology, nutrition, motility, and habitats. These traits have been shown to affect plant health, control bacterial populations, affect hormone balance and reproductive fitness, and allow protists to act as vectors for plant antagonists.

In Arabidopsis thaliana rhizosphere, the presence of Acanthamoeba castellanii and bacteria increases the carbon and nitrogen levels, resulting in a subsequent increase in shoot rosette diameter when compared to bacteria-alone or sterile plants. A. thaliana produces more carbon exudates which causes an increase in the bacterial population, which the protists (here, amoeba) prey on and release stored nutrients back into the rhizosphere for plant consumption.

Rhizosphere bacteria can change behavior to evade predation by protists. Bacteria have been seen to alter their size, shape, swimming speed, biofilm formation, or produce toxic compounds in the presence of protist predators. Bacteria may respond to physical interaction with predatory protists, or sense excretory products in the environment which lead to the physiological changes. It has also been seen that bacteria can return to “normal” once the threat of predation is removed. Altered bacteria behavior leads to abiotic and biotic changes to the soil composition.

It was originally thought that plant genotype had the strongest influence on community composition, but recent data has shown otherwise. Simonin et. al. illustrated that the wheat rhizosphere microbiome, specifically including protists, is determined by soil and agricultural practices more so than plant genotype. Although, they state that the wheat plants were not fully mature, and that the microbiome may shift with mature roots. Walters et. al. showed similar results in a large scale replicated field study of maize rhizosphere, however their study focused on bacteria, not protists.

I propose to study the effect of plant genotype, plant developmental stage, and soil on the protist-ome of field grown Medicago, a model plant for leguminous research. 18S rRNA is used to study molecular phylogeny of organisms because they are functionally equivalent in all organisms and are rare to laterally transfer. Specifically, the V9 region is used often in protist research due to its ability to be amplified by universal primers. I plan to use 18S rRNA V9 community analyses to characterize the protist-omes of Medicago truncatula and Medicago sativa, inbred and outbred relatives, respectively. Comparison of Medicago sp. with Zea mays community analyses from the same field in the same time period will show which factor has the strongest influence on protist-ome selection and if a core protist community exists.

Hypothesis: Core protist-ome is determined by the environment, not by plant species.
ii. Preliminary Data

Peptide Nucleic Acids (PNAs) have been developed to form stable, sequence-specific duplex or triplex structures with DNA.\textsuperscript{23} Their lack of sugar phosphate backbones and uncharged nature results in protease, nuclease, and high temperature resistance. PNA Clamps have been used in research to suppress host DNA amplification in studies of eukaryotic symbionts and microbiome response to diets.\textsuperscript{24,25} Our collaborators, Taerum \textit{et. al.}, validated the use of PNA clamps to reduce the overwhelming amplification of plant DNA in 18S rRNA rhizosphere sequencing.\textsuperscript{26} Addition of a \textit{Z. mays} 18S rRNA specific PNA clamp to maize rhizosphere analyses substantially decreased maize amplicons and increased the number and diversity of non-maize eukaryotic sequence variants.

I designed a \textit{Medicago} specific PNA clamp that binds to the homologous region of the \textit{Z. mays} 18S rRNA gene in both \textit{Medicago sativa} and \textit{Medicago truncatula}. (Figure 2.) The \textit{Medicago} PNA clamp was tested for acceptable efficacy using the PNA Tool on the PNAbio website (https://www.pnabio.com/support/PNA_Tool.htm). (Figure 3.) It was also run against the SILVA rRNA gene database Probe Search (https://www.arb-silva.de/search/testprobe/) to check for offsite targets that would effect sequencing results, of which there were none.\textsuperscript{27}

**Figure 2.** \textit{Medicago} PNA clamp designed to bind the homologous region of the 18S rRNA gene of both \textit{M. sativa} and \textit{M. truncatula} as the \textit{Z. mays} PNA clamp designed by Taerum \textit{et. al.} binds \textit{Z. mays} 18S rRNA. \textit{Medicago} sp. 18s rRNA genes differ from \textit{Z. mays} by 3 basepairs, which are reflected in the sequence of the \textit{Medicago} PNA clamp.

**Figure 3.** PNA Tool results for \textit{Medicago} PNA Clamp. Melting temperature (Tm = 81.4°C) ensures binding of clamp during PCR primer annealing and DNA elongation. The \textit{Medicago} PNA clamp received a “good” comment, meaning it does not form hairpins or other inefficient structures that the tool would have alerted about.
iii. Proposed Research

A field experiment is currently underway at a plot at Lockwood Farm in Hamden, CT. Two sets of *M. truncatula*, *M. sativa*, and *Z. mays* (B73) were planted in June 2020 in adjacent plots. In the first plot, 6-week-old seedlings, previously grown in sterile sand in pots, were planted in three rows. *Z. mays* seedlings were planted six inches apart in the western most row to reduce shading effects on the legumes. In the remaining two rows, *M. truncatula* and *M. sativa* seedlings were alternately planted six inches apart. This planting design was repeated in the second plot using seeds. *Z. mays* and *M. sativa* seedling root samples and *M. truncatula* seedling root samples were taken at 5, 10, and 15 weeks post transplanting. *Z. mays* seed root samples were taken at 7 and 15 weeks post planting. Bulk soil samples were taken at all time points. Week 15 time point for both seedling and seed samples will be pulled mid-September 2020. (Supplemental Table 1.) This experimental design allows for comparison of plant species, plant developmental stage, and soil as influencers on the protist-ome. This field experiment may be repeated at the same field in Summer 2021 to track any changes to the protist community.

Upon pulling from the field, loosely attached soil is removed from roots by rigorous shaking. Roots are stored at 4°C until ready to process. The protocol for processing of the roots was adapted from McPherson et. al. Roots are suspended in 35mL phosphate buffer (minus surfactant) and shaken for 2 minutes to release all rhizosphere organisms into the suspension. Roots are removed from the suspension, saved in separate conical tubes, and are stored at -80°C. The suspension is centrifuged at 3,700rpm for 10 minutes, supernatant is removed, and the pellet is stored at -80°C. Bulk soil samples are also stored at -80°C. Once all time points have been collected, DNA will be extracted from the pellets and bulk soil using the DNeasy PowerSoil Kit (Qiagen, Germantown, Maryland, USA).

The concentration of *Medicago* PNA Clamp needed to reduce unwanted host amplification from *M. truncatula* and *M. sativa* during sequencing will be determined using varying concentrations of the clamp in separate PCRs with *M. truncatula* and *M. sativa* gDNA. Protist gDNA will be used as a control to confirm target amplification is not hindered in the presence of the clamps. The minimum concentration of PNA Clamp that results in the complete reduction of plant 18S rRNA V9 amplification will be used during community 18S rRNA V9 PCRs.

V9 libraries will be assembled using two PCRs. In the first PCR, 18S rRNA V9 sequences will be amplified using the Earth Microbiome Project primers 1391F (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTACACACCGCCCGTCACT) and EukBr (GTCTCGTGGGCTTCAGATGTGTATAAGAGACAGGTACACAGGTTCACCCCACTAC) in the presence of the PNA clamps at the appropriate concentrations. Amplicons will undergo a second PCR to be barcoded with Nextera DNA CD Indexes (Illumina Inc., San Diego, California, USA) before being sequenced on the Illumina iSeq 100 platform using 2 x 150bp sequencing.

Sequences will be analyzed following Taeurn et. al. bioinformatics pipeline. Assembled contigs will be screened to include sequences between 100 and 200bp. Filtered contigs will then be aligned to the SILVA SSU database, and chimeric sequences will be removed. Remaining sequences will be assigned Amplicon Sequence Variants (ASVs) instead of operational taxonomic units (OTUs) due to their higher sensitivity and specificity on taxonomical diversity. ASVs will be classified against the SILVA SSU reference database and the Protist Ribosomal Reference
These taxonomical classifications will be used to compare protist abundance between plant root samples.

**Aim 2. Develop the use of soil protists as carriers of beneficial bacteria in the rhizosphere**

1. **Introduction**

Though the complex microbial community contributes to the microbial loop of nutrients in soil, nitrogen is often a limited resource. Due to the lack nitrogen atoms to form the nitrogenous core of chlorophyll, nitrogen starvation in plants causes chlorosis. The lack of chlorophyll reduces photosynthesis resulting in the inability of the plant to grow tall, branch, and produce a fruit. Although there is a brief time in which plants can recover with exogenously delivered nitrogen fertilizer, nitrogen starvation leads to death of plants.

*S. meliloti* acts as a natural fertilizer in nitrogen starved legumes. Roots of legumes undergoing nitrogen stress release flavonoids into the soil which induce the cascade of the *nod* genes in *S. meliloti*. The suite of *nod* genes encode for approximately 25 proteins which produce and export Nod factor. Nod factor initiates the morphological changes a root hair undergoes to form a symbiosis with *S. meliloti*. A bacterium attaches to a root hair at the growing root tip and is encapsulated by the root hair as it curls around the bacterial cell. Division of the bacterium results in an infection thread, which continues to extend into the root cell before reaching the opposite cell wall. At this junction, the invagination fuses with the opposite cell wall, the bacteria cross the intercellular space, and invade the neighboring root cell. This invasion leads to the creation of a symbiosome, a host-membrane encapsulation of bacteria. Presence of the bacteria within the nodule primordium cells helps to the develop a true nodule. The nodule is seen as a pink growth on the exterior of legume roots. Within the nodule, *S. meliloti* produces nitrogenase, the enzyme responsible for the conversion of atmospheric nitrogen (N$_2$) into ammonia (NH$_3$) through the process of nitrogen fixation (N$_2$ + 8H$^+$ + 8e$^-$ + 16ATP $\rightarrow$ 2NH$_3$ + H$_2$ + 16ADP + 16P).

Beneficial bacteria, such as *S. meliloti*, have been delivered to plants via seed inoculum for some time. However, the bacterial population along the roots declines from seed to root tip since soil bacteria are often found in biofilms, thick mats of immobilized cells. For this reason, growing roots may come into contact with the bacterial inoculum early in escape from the seed, but soon lose almost all contact with the intended microbial population. Soil protists, however, can travel orders of magnitude faster than bacteria, and specifically use the water film on plant roots as a “highway” for travelling long distances.

Due to their feeding behaviors, heterotrophic protists may act as microbial buses for members of the rhizosphere. Ciliates commonly eat by means of filter feeding. Food particles and prey which are smaller than the ciliate will enter the oral funnel and be packaged into a phagosome. Soluble nutrients are removed via pinocytosis, and the remaining meal either undergoes digestion or egestion, a decision which is not well characterized.

Flagellate feeding behaviors are more selective than ciliates, and follow a set of feeding phases. (Figure 4.) When flagellates come into contact with possible prey, they process whether or not the meal will be consumed. The prey is either ingested or rejected. If ingestion occurs, a food vacuole forms and the prey is taken in by the flagellate. Ingested food can be completely digested, or stored in vacuoles to be egested from the protist cell. Egested food in flagellates is typically due to particles being indigestible.
Protist Facilitated Transport is a developing technology in which preselected protists and bacteria can be delivered simultaneously in a seed inoculum. Upon delivery, protists consume the readily available bacterial inoculum, as well as swim through biofilms and physically pick up bacteria on their cell bodies. As the seed germinates, protists travel along the root, and have the opportunity to egest undigested bacterial meals. Physical contact with the roots can also transfer protist-bound bacteria onto the roots. As protists explore the rhizosphere, the likelihood of bacteria being delivered to regions of the root that they would otherwise not have come into contact with increases. (Figure 5.) In the case of *S. meliloti*, which can only infect root hairs near the growing root tip, protists may deliver the symbiotic bacteria to distal regions of the root system which would otherwise be untouched by *S. meliloti*.

Protists have been shown to transport fluorescently labelled beads in emulated soil micromodels. For the soil micromodels, and my own research, I isolated protists from bean rhizosphere. The ciliate used in all experiments was identified as *Colpoda sp.* using 18S rRNA sequencing. In my research, I propose to use *Colpoda sp.* to demonstrate its ability to transport fluorescently labelled *S. meliloti* down plant roots. Furthermore, I plan to illustrate the capability of *Colpoda sp.* to release live *S. meliloti* at infectible root hairs, resulting in an increase in nodulation and plant shoot biomass.

**Hypothesis:** Soil protists can be used to transport beneficial bacteria, such as *Sinorhizobium meliloti*, to distal regions of plant roots. Deposited *S. meliloti* on distal roots will increase nodulation rates and shoot biomass.
ii. Preliminary Data

Vetting Protist Isolates

Two rhizosphere protists, *Colpoda sp.* and *Cercomonas sp.*, were isolated from bean rhizosphere and identified using ciliate and cercomonad, respectively, specific 18S rRNA primers. To test their likelihood of interacting with *M. truncatula* roots, pure protist cultures were inoculated onto a grown root tip and observed under the microscope. *Colpoda sp.* and *Cercomonas sp.* were both seen preferentially travelling close to root hairs. (Figure 6.)

Protist isolates were fed fluorescently labelled *S. meliloti* and observed using fluorescent microscopy for uptake and egestion of *S. meliloti*. Both protists were observed ingesting the bacteria and picking up mats of biofilms on their cell bodies as they explored the environment, an unexpected but welcomed result. (Figure 7.) *Colpoda sp.* was chosen to move forward in experiments since they are larger, likely ingest and pick up more bacteria, swim faster, and can swim freely in liquids, unlike *Cercomonas sp.* which need to adhere to a surface for movement.

![Figure 6. Microscope image of Cercomonas sp. moving along root hair. Cercomonas sp. remains adhered to a surface for movement, unlike Colpoda sp. which is a free swimmer. Similar behavior was seen in Colpoda sp., but they are much harder to photograph due to their fast motility.](image)
Transport in μ-Rhizoslides

3D-printed soil microcosms, termed μ-rhizoslides (adapted from their macro relative) were designed and printed by Chris Hawxhurst, Shor Lab, Department of Chemical and Biomolecular Engineering. A spacer is placed between two glass slides with a filter paper wick between the spacer and back slide. (Figure 8A.) The wick acts to move water from bottom to top of the slide in order to prevent unwanted downward movement. Sterile 1:2 125-250µm:63-125µm sand and fine vermiculite mix were added to each μ-rhizoslides. A sterile, pre-germinated M. truncatula seed was planted on the top of each enclosure. μ-Rhizoslides were incubated on an angle forcing roots to grow against the glass slide. (Figures 8B and 8C.) Slides were kept in a growth chamber on a 16:8 hour light:dark cycle for the length of the experiment. One day post planting, slides were inoculated on the root-shoot interface with 10µL of one of three treatments: 1) Page’s Saline Solution (Negative Control), 2) 10^6 Rm1021/pSEVA551::mRFP (Bacteria-Alone Control), or 3) Treatment 2 + 1.5x10^3 encysted Colpoda sp. (Bacteria + Protists). Slides were imaged immediately post inoculation (“Week 0”) and each week following, for 3 weeks.

Two weeks post inoculation, fluorescence was seen to accumulate in the top 5mm of the soil when inoculated with Rm1021/pSEVA551::mRFP when compared to the negative control where no fluorescence was detected. In the presence of Colpoda sp. fluorescence was detected up to 50mm away from the site of inoculation. (Figure 9., Supplemental Figure 1.) These results were replicated in subsequent experiments, where the protists were ex-cysted prior to inoculation and slides were inoculated tilted backward, making any movement toward the roots, instead of just down the roots, significant. (Figure 10.)

Bacteria and protists were recovered from top and bottom sections of each μ-rhizoslide at the end of the experiment to ensure presence/absence of transport was due to the presence/absence of protists. Bulk soil was removed and roots were divided into top and bottom fractions, 5cm from the root-shoot interface. Root fractions were suspended in Page’s Saline Solution and shaken to remove bacteria and protists from roots. 10-fold of the serial dilutions were plated on TY+Tc plates, and rfp fluorescent colonies were counted after 3 days. 3-fold serial dilutions were also carried out in 96-well plates to estimate protist abundance. Eight replicate dilutions were done for each root section. Plates were incubated for 1 week, then checked for presence of protists.
Presence of protists assumed that “at least one” protist made it into that dilution well. The Minimum Recovered Number (MRN) was back calculated as follows:

\[ \text{MRN} = 10 \times 3^{(\text{farthest positive dilution factor} - 1)} \]

As expected, protists were only recovered from the protist treated rhizoslides. Bacteria was recovered from the top section of the bacteria-alone treated rhizoslides, as well as the top and bottom sections of the bacteria and protists treated rhizoslides. (Figure 11.) Similar results were seen in ex-cysted protist replicate experiments for both bacteria and protist recovery.

![Figure 8](image)

*Figure 8.* (A) Set up of µ-Rhizoslide. 3D-printed spacer between two glass slides with a filter paper wick between the spacer and back slide to wick water from bottom to top of enclosure. (B) Illustration of µ-Rhizoslide with roots growing against glass and shoot growing out the top of the enclosure. (C) Actual image of the µ-Rhizoslide taken 2 days post planting of germinated seed.

Hawxhurst & Micciulla, in preparation

![Figure 9](image)

*Figure 9.* Fluorescent images of soil in µ-rhizoslides two weeks post inoculation. No fluorescence seen in negative control. Fluorescence seen in top 5mm of bacteria-alone control. Fluorescence seen as far as 50mm in presence of Colpoda sp.

Hawxhurst & Micciulla, in preparation
**Figure 10.** Fluorescent images of soil in μ-rhizoslides two weeks post inoculation with excysted protists and *rfp*-labelled Rm1021. The shoot is emerging from the right side of the μ-rhizoslide in this image. Minimal fluorescence is seen at time 0 (white). Fluorescence is detected on the roots one week post inoculation (green), and is seen to have travelled farther down the roots two weeks post inoculation (yellow). In this μ-rhizoslide, roots hugged the wall of the slide. The fluorescence follows the roots, not the μ-rhizoslide wall.

Hawxhurst & Micciulla, in preparation

<table>
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<th>Rhizoslide Protist Recovery</th>
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**Figure 11.** Recovery of bacteria and protists from top and bottom fraction of roots. Bacteria were recovered from the top section of the bacteria-alone treated rhizoslides, and from the top and bottom sections of the bacteria and protist treated rhizoslides. Protists were only recovered from the bacteria and protist treated rhizoslides. Error bars represent one standard deviation from the mean.

Hawxhurst & Micciulla, in preparation
Transport in Greenhouse Pots

In order to test for viability and functionality of egested *S. meliloti*, the transport experiments were adapted for larger scale greenhouse experiments to observe nodulation of roots. Sterile, pre-germinated *M. truncatula* seeds were planted in sterile, nitrogen free soil in the greenhouse. Plants were grown for two weeks prior to inoculation to allow for root systems to establish in the soil. Three concentrations (8000, 800, and 200) of *Colpoda sp.* (UC1), *Cercomonas sp.* (UC5), and *Acanthamoeba castellanii* (ATCC, Manassas, Virginia, USA) were inoculated onto the root-shoot interface with $10^6$ *S. meliloti* Rm B1. Bacteria alone and protist alone controls were also run. Plants supplemented with nitrogen fertilizer were used as positive controls for how well plants can grow in the greenhouse in the presence of complete nutrients. Otherwise, all plants received nitrogen free fertilizer and were grown for 8 weeks post inoculation. *S. meliloti* infection of nitrogen starved roots can take up to 3 weeks to form nodules. Upon completion of the experiment, plants were carefully removed from their pots and dissected at cotyledons into root and shoot fractions. Root length was recorded, then binned into 5cm sections from the top of the root. Number of nodules in each bin was also recorded. Shoots were dried for 3 days at 80°C, then weighed to record biomass.

When compared to plants treated with Rm B1 alone, the only treatment which had a significantly higher average shoot weight was the highest concentration of *Colpoda sp.* (8000) + Rm B1. All other protist treatments did just as well as plants treated with bacteria alone when comparing shoot weights. Plants treated with *Colpoda sp.* (8000) + Rm B1 had a slightly higher average shoot weight (42.87mg) than plants supplemented with nitrogen containing fertilizer (41.51mg). (Figure 12.) This increase in plant shoot weight when treated with *Colpoda sp.* (8000) + Rm B1 can be attributed to the increase in average total nodules seen on the roots. (Figure 13.)

Similar nodulation is seen in the next highest concentration of *Colpoda sp.* treated plants, *Colpoda sp.* (800) + Rm B1, and although the average shoot weight is slightly higher than plants treated with Rm B1 alone, 27.40mg and 24.93mg, respectively, it is not as high as plants treated with 10-fold more *Colpoda sp.* Unfortunately, the time at which a nodule is formed is unable to be recorded in these experiments. Therefore, it is safe to assume that the nodules in the *Colpoda sp.* (8000) + Rm B1 treated plants developed sooner than the nodules in the *Colpoda sp.* (800) + Rm B1 treated plants. Had the experiment lasted longer, it is possible that the *Colpoda sp.* (800) + Rm B1 shoot weights would have greatly outcompeted the Rm B1 alone treated plants in shoot weight.

It is also important to note that the farther root bin (5cm sections) travelled in the *Colpoda sp.* (8000) + Rm B1 treatment was Bin 3 (10-15cm from the root crown). *Cercomonas sp.* (8000) + Rm B1, *Colpoda sp.* (800) + Rm B1, and *Colpoda sp.* (200) + Rm B1 treated plants also showed nodules in Bin 3, but the frequency was much less for *Cercomonas sp.* (8000) + Rm B1, 2 nodules, and *Colpoda sp.* (200) + Rm B1, 1 nodule. *Colpoda sp.* (800) + Rm B1 had 8 nodules in Bin 3, which is comparable to *Colpoda sp.* (8000) + Rm B1 Bin 3’s 10 nodules. However, as stated above, the length of time these nodules have been present is unknown. It is likely that the nodules found in *Colpoda sp.* (800) + Rm B1 were younger than those found in *Colpoda sp.* (8000) + Rm B1 since the average shoot weight of those plants was much lower.
Figure 12. Average shoot weight (mg) versus treatment. Plants treated with bacteria alone have an average shoot weight of 24.93mg. Addition of nitrogen fertilizer nearly doubles the average shoot weight. Plant treated with *Colpoda sp.* (8000) + Rm B1 have a similar effect on shoot weight as plants treated with nitrogen fertilizer.

Figure 13. Average total nodules versus Treatment. Nodules are only seen in bacteria treated plants. On average, plants treated with bacteria alone had 4.95 root nodules at the end of 8 weeks. Three protist treatments, *Colpoda sp.* (8000) + Rm B1, *Cercomonas sp.* (8000) + Rm B1, and *Colpoda sp.* (800) + Rm B1 had higher average root nodules than bacteria alone, with 7.14, 6.30, and 6.23 nodules, respectively.
iii. Conclusions

Transport of *S. meliloti* by *Colpoda sp.* is seen in both short, small scale experiments in µ-rhizoslides, and longer, large scale experiments in the greenhouse. Most importantly, viability and functionality of egested *S. meliloti* is undisturbed, as demonstrated by nodulation on distal roots in the greenhouse experiment.

As expected, there is a positive relationship between number of root nodules and shoot weight. (Figure 14.) This graph displays which treatments have higher nodulation and higher shoot weight than bacteria alone treated plants (green), *Colpoda sp.* (8000) + Rm B1, *Cercomonas sp.* (8000) + Rm B1, and *Colpoda sp.* (800) + Rm B1. It also illustrates the strikingly similar shoot weight seen in *Colpoda sp.* (8000) + Rm B1 treated plants to the nitrogen fertilizer treated plants (red). High concentrations of *Colpoda sp.* inoculated with *S. meliloti* may act as a sustainable and environmentally friendly alternative for nitrogen fertilizers in legumes.

Interestingly, the addition of protists alone had a beneficial effect on plants when compared to the true negative control plants inoculated with Page’s Saline Solution. (Figure 12.) It is unclear whether it is the addition of protists, their bacterial endo- and ecto-symbionts, or the combination of bacteria with their protist host that provides the added benefit to plant shoot weight.

![Figure 14](image)

Figure 14. Average shoot weight versus average total nodules. There is a positive relationship between number of nodules and shoot weight. Nitrogen fertilizer increases shoot weight without the need of nodules, hence their high shoot weight with 0 nodules.

Key: Blue: Pages or protists alone (no Bacteria); Green: Bacteria alone; Purple: Treatments with protists plus bacteria; Red: Treatments with nitrogen fertilizer.

iv. Proposed Research

A replicate of the greenhouse experiment is underway and will be pulled at the beginning of September 2020. I expect to find similar results to this greenhouse experiment. A replicate of the greenhouse experiments may be conducted in the field at Lockwood Farm, Hamden, CT in Summer 2021, if the organisms are allowed in the soil. A field study will be the true test of whether *Colpoda sp.* will transport *S. meliloti* down root systems in a non-sterile, competitive environment.
If similar benefits to shoot weight are seen in the protist alone treated plants at the end of the replicate greenhouse experiment, a follow-up experiment will be conducted to determine who, the protists, their symbionts, or the combination of both, are responsible for the added benefit. Protists will be reared in antibiotics to remove endo- and ecto-symbionts, and be applied as the protist only treatment to plants. Bacteria will be concentrated from lysed protist cells and collected as the bacteria only treatment. Regularly cultured protists will be applied to plants assuming to have their co-cultured symbionts. Results from this experiment should clarify which factor benefits plant health and contributes to increased shoot weight.

The rhizoslides will be used to test the ability and efficiency of transport by other rhizosphere isolated protists. To date, Gage Lab has 18 identified protists which were isolated from various rhizospheres, and I have 80 maize-rhizosphere isolated protists. Of particular curiosity is the efficiency of a flagellate, Cercomonas sp. (UC5) and a larger Colpoda, Colpoda maupasi. (UC15).

**Aim 3. Characterize LuxS-like protein in Sinorhizobium meliloti**

i. **Introduction**

Complex microbial communities require inter- and intra-species communication to relay and interpret information about the environment. Quorum sensing is a mechanism of molecular communication employed by bacteria to regulate gene expression in response to cell-cell signaling. First described in marine bacterial species Vibrio fischeri and Vibrio harveyi, which respond to the accumulation of a secreted molecule at high cell density by expressing bioluminescence genes, quorum sensing has since been confirmed in the vast majority of bacteria. V. fischeri is considered the paradigm for quorum sensing in most gram-negative bacteria, and its gene complexes are used to describe the molecular pathways, although there are species specific homologues.

Analysis of the population density sensing apparatus has shown that two independent quorum sensing systems exist, each composing of a sensor-autoinducer pair. In the first system, LuxI produces the acyl-homoserine-lactone (AHL) autoinducer (autoinducer-1, AI-1), which freely diffuses into the environment. As cell density increases, so does the extracellular concentration of AI-1. LuxR, the cytoplasmic autoinducer receptor, binds AI-1 at a critical threshold and confers transcriptional activation of the lux operon. The lux operon encodes for both the bioluminescence genes (in V. fischeri) and luxI, which produces more AI-1, thus creating a positive feedback loop.

In the second system, LuxS produces AI-2 in the final step of methyl recycling from S-adenosyl methionine (SAM). Demethylation of SAM results in S-adenosyl-homocysteine (SAH). SAH is converted by PfS into S-ribosyl-homocysteine (SRH), which is catalytically cleaved at the thioester bond by LuxS, resulting in homocysteine and (S)-4,5-dihydroxyxypentane-2,3-dione (DPD) DPD spontaneously rearranges to form autoinducer-2 (AI-2). Like AI-1, AI-2 accumulates extracellularly, but is bound by a periplasmic binding protein, LsrB, before being transported back into the cytoplasm. Imported AI-2 is phosphorylated by kinase LsrK to keep it intracellularly locked. Phosphorylated AI-2 (AI-2-P) binds LsrR, alleviating its repression of the lsr operon. Freed expression of the lsr operon increases the production of the Lsr transporter complex, stimulating further internalization of AI-2. Internalization and phosphorylation of AI-2 in the cytoplasm removes AI-2 from the environment. (Figure 15.)
Processing AI-1 and AI-2 from the extracellular matrix allows for a bacterium to distinguish between self and non-self, and understand how to respond to its environment. Bacteria able to sense and respond to their surroundings have the advantage of expressing genes relevant to their fitness. In soil, one of the most intricate environments, quorum sensing influences species specific interactions which are crucial for survival.

*S. meliloti* relies on quorum sensing for multiple behaviors in the soil, including the formation of the symbiosis with nitrogen starved legumes. Separate mutations of the *sinI* and *sinR* genes, homologues to *luxI* and *luxR* AI-1 synthase and receptor, severely reduce exopolysaccharide (EPSII) production in *S. meliloti*. In fact, a disruption in *expR*, a gene encoding for a transcriptional regulator in the ExpR/Sin quorum-sensing system, results in no EPSII production by *S. meliloti* strain Rm1021. These data suggest that EPSII synthesis may be partially controlled by quorum sensing. *S. meliloti* biofilm formation also relies on the production of the low molecular weight fraction of EPSII. Therefore, there is a link between quorum sensing and behaviors related to fitness in *S. meliloti*.

Although homologues to the LuxS protein, AI-2 Synthase, have been found in many bacteria, one has yet to be identified in alpha-proteobacteria. While *S. meliloti* falls under that umbrella, homologues of other components of AI-2 internalization, including those of the Lsr system, have been identified. It is possible that *S. meliloti* eavesdrops on AI-2 secreted from other bacteria, a behavior characterized by the interference of AI-2 signaling of other species and disruption of group behaviors. However, due to its use of quorum sensing to initiate species
specific symbiosis with legumes, it is also possible that an orthologue to LuxS has yet to be discovered in *S. meliloti*.

In a study to identify the function of LuxS, genome analyses showed that, although the *luxS* gene does not consistently reside in a particular location on the chromosome, *luxS* is sometimes found in an operon with *metK* and *pfs*.\(^9\) Genome analysis of *S. meliloti* indicates an unidentified conserved gene, *smc00396*, upstream of *pfs* which encodes for a conserved hypothetical protein. (Supplemental Figure 2.) I propose to identify the function of this hypothetical protein in experiments using previously characterized AI-2 reporter bioassays. I also plan to determine the role of AI-2 signaling in biofilm formation and nodulation by *S. meliloti*.

**Hypothesis:** *S. meliloti* has a LuxS-like protein which functions as AI-2 synthase. AI-2 signaling is utilized by *S. meliloti* to mediate specific behaviors involved with fitness, such as biofilm formation and nodulation.

### ii. Preliminary Data

**Protein Domains**

Alignment of the amino acid sequences illustrates how different the sequences are for proteins characterized as LuxS. *V. harveyi* and *Escherichia coli* LuxS share 76% sequence identity. However, *Bacillus subtilis* LuxS shares 35% sequence similarity with both *V. harveyi* and *E. coli* LuxS. The SMe00396 hypothetical protein only shares 16%, 15%, and 10% sequence similarity with *V. harveyi*, *E. coli*, and *B. subtilis* LuxS, respectively. (Supplemental Figure 3.) However, identification of the catalytic domains present in the SMe00396 hypothetical protein sheds light on its possible function.

LuxS catalytically cleaves the sulfur-carbon bond in SRH to produce AI-2 and homocysteine. (Figure 16.) A protein BLAST of the translated *S. meliloti smc00396* hits thioesterases, enzymes with the ability to cleave sulfur-carbon bonds. Although the SMe00396 hypothetical protein is not similar to known LuxS proteins at the amino acid sequence, both proteins have the same catalytic domains, and possibly the same function.

![SRH Cleavage](image)

**Figure 16.** LuxS thioesterase activity on SRH (S-(5-deoxy-D-ribos-5-yl)-L-homocysteine) which results in L-homocysteine and (S)-4,5-dihydroxypentane-2,3-dione, which spontaneously rearranges into AI-2. *S. meliloti* putative LuxS-like protein has thioesterase catalytic domain in common with that characterized LuxS protein. Uniprot.org


*smc00396* was amplified from *S. meliloti* Rm1021 gDNA in two separate PCRs to prevent non-specific amplification. The initial PCR was done using primers (Forward Primer: CCAGTGTCCGAAAACTC, Reverse Primer: CGGCCATCACATAGAGGATG) that sit outside of *smluxS* CDS. The first PCR product was cleaned and used as template in the second PCR with primers (Forward primer: GAATAACATATGGATGAGCGATCCC, Reverse Primer: AAAGAATTCTCATTCTCGATACCCTCGC) containing restriction enzyme tails for downstream cloning.

The *smc00396* PCR product with restriction enzyme tails was initially A-tailed, ligated into pGEM, and electroporated into NEB10B cells. This clone was sequenced using M13 and REV primers, and 100% sequence match was found between the cloned *smc00396* and *S. meliloti* Rm1021 genome. *smc00396* was digested from pGEM with restriction enzymes NdeI and EcoRI and ligated into pre-digested pET28a+. pET28+::*smc00396* was electroporated into NEB10B cells for screening. Upon positive screen, pET28+::*smc00396* was electroporated into BL21-DE3/pLysS cells (strain JLM42).

JLM42 liquid culture was grown to OD₅₉₅=0.2, then induced with 0.5mM IPTG and incubated overnight at 37°C. Cells were lysed with lysozyme and incubated with halt protease inhibitor. Total, supernatant, and pellet samples were taken from lysed cells and run on an SDS-Page gel to determine solubility. (Figure 17.) Smc00396 protein can be seen in all samples, illustrating that it is soluble, although some of the protein is in inclusion bodies in the pellet.

His-tagged SMc00396 in the supernatant was purified over a nickel column, then eluted into fractions. Fractions were run on an SDS-Page gel to visualize purification. His-tagged SMc00396 is 167 amino acids and 18.03 kDa. It runs between 15 and 20 kDA on the SDS-Page gel, and seems to be purified. (Figure 18.)

![Figure 17. Protein samples run for each stage of inducing and lysing protein. His-tagged SMc00396 is 18.03 kDa. No protein seen in overnight (O/N) sample. Some protein seen before induction (BI), indicating that expression in this plasmid is a little leaky. Protein is seen after induction (AI), and in all three fractions post cell lysing, total (T), supernatant (S), and pellet (P). Some protein in seen in column wash (CW). Protein seen in supernatant means it is soluble. The large amount of protein seen in the pellet indicates incomplete cell lysing or inclusion bodies.](image-url)
iii. Proposed Research

Extracellular catalysis of SRH by SMc00396 into AI-2 and homocysteine will be visualized using *V. harveyi* strain BB170. BB170 is a sensor 1+ sensor 2+ reporter strain that luminesces in the presence of AI-2, but not AI-1. SRH synthesized by hydrolyzing the adenosine from SAH will be incubated with purified SMc00396 protein for 1 hour at 37°C. After incubation, the protein will be filtered from the medium and the filtrate will be used in the BB170 assay to check for luminescence. In this assay, diluted BB170 culture is inoculated with the filtrate in a microtiter plate and light production is measured every 30 minutes. Light production is normalized to colony forming units (cfu) and AI-2 activity is reported as the fold induction of the reporter strain over background, when inoculated with medium alone. Fold induction of BB170 in the presence of SRH + SMc00396 filtrate would indicate the ability of SMc00396 to cleave SRH into AI-2 and homocysteine. (See Supplemental Table 2 for all controls that will be run in this experiment.)

Analysis of the intracellular function of SMc00396 will be done using *E. coli* strain LW12 (*E. coli* W3110 ΔluxS::Kan), a mutant of non-pathogenic *E. coli* K-12. IPTG induction of LW12 complemented with pGem::smc00396 should confer expression of the SMc00396 protein. If SMc00396 has the ability to cleave endogenously produced SRH, AI-2 activity in the filtrate will be detected in the BB170 AI-2 bioassay described above.

To test for the effect of AI-2 quorum sensing on biofilm formation and nodulation of *Medicago* by *S. meliloti*, a large portion of smluxS will be deleted from the chromosome. The 3’ end of the gene will remain intact due to its overlap with the start site of *pfs*. To untangle the relationship between AI-2 and EPS & II on *S. meliloti* biofilm formation and nodulation, a suite of Δsmc00396 “clean deletion” mutants will be constructed using varying EPS producing parent strains. (Supplemental Table 3.) *S. meliloti* Δsmc00396 strains will be grown on low phosphate solid media to test for EPS production. EPS production is visualized by a mucoid phenotype on agar plates. Halted EPS production in Δsmc00396 mutants may indicate a relationship between AI-2 signaling and EPS production. *S. meliloti* Δsmc00396 strains will also be inoculated onto *M. truncatula* seedlings in slant tubes to test for nodulation. Successful nodulation is indicated by large, pink growths on the roots 3 weeks post inoculation. If small, white nodules are seen when inoculated with the Δsmc00396 mutants, it is likely that AI-2 is an important signal in *S. meliloti – Medicago* symbiosis.
III. Significance

i. Aim 1

Understanding the functionality of plant-microbe interactions and the plant as a meta-organism will shed light on how plants benefit from their microbiome. As challenges in crop production increases due to the growing population and climate change, there is an increasing demand to develop sustainable agricultural practices. Microorganisms have shown potential in being applied as biofertilizers and biopesticides in place of chemical products. Unveiling which protist taxa are members of a core microbiome in specific plants or environments will help mitigate the often deleterious effects of introducing non-indigenous microbes to a new agroecosystem. In order to safeguard plant productivity, as well as human health, an appreciation for the rhizosphere microbiome is essential.

ii. Aim 2

Nitrogen fertilizers are used extensively in crop fields to provide plants with the limited resource and increase yields. However, unused fertilizer is mobilized to the water table and accumulates in large bodies of water fueling microbial growth. Microbes utilize much of the dissolved oxygen, resulting in hypoxic, or “dead,” zones. In these hypoxic zones, the endogenous flora and fauna are at risk of being killed off due to the lack of dissolved oxygen. The number of global dead zones doubles every decade.

Soybean is one of the top four global food crops. Approximately 100 million hectares of arable land is used to farm soybeans. Being of the leguminous family, Soybeans are naturally infected by nitrogen-fixing Rhizobium. Usage of soil protists to deliver nitrogen fixing Rhizobium to infectible Soybean root hairs may help reduce the need for excessive nitrogen fertilizer in Soybean fields.

iii. Aim 3

The importance of AI-1 mediated quorum sensing by S. meliloti has already been illustrated. Identification of an AI-2 Synthase protein in S. meliloti will shed light on its ability to produce AI-2 in participation with AI-2 mediated quorum sensing. The ability to recognize AI-2 produced by other species may allow for eavesdropping of signals from the environment, but identifying an AI-2 Synthase gene in S. meliloti will permit downstream studies on if, when, and how this signal is produced. The Δsmc00396 mutants which would be constructed in this research have the opportunity to illuminate the role of AI-2 in various behaviors exhibited by S. meliloti, such as biofilm formation, nodulation, motility, and more.
References


Supplemental Figures and Tables

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**Supplemental Table 1.** Schedule for Summer 2020 field experiment stating when each set of plants are pulled, and what is able to be concluded from each sample.
Supplemental Figure 1. Cumulative fluorescent intensity in the soil of μ-rhizoslides calculated from shoot to root tip two weeks post inoculation. Fluorescence is not seen in negative control. Fluorescence is seen in the first 5mm of the bacteria-alone rhizoslides, but not in the rest of the soil (indicated by the straight line following the spike in fluorescence at 5mm). Fluorescence accumulates in the soil as you move from shoot to root tip in the presence of Colpoda sp.
Supplemental Figure 2. Genome analysis of *S. meliloti* indicates an unidentified conserved gene, *smc00396*, upstream of *pfs* which encodes for a conserved hypothetical protein. This gene possibly codes for a LuxS-like protein in *S. meliloti*.

Supplemental Figure 3. Alignment of LuxS amino acid sequences from *V. harveyi*, *E. coli*, and *B. subtilis*. *S. meliloti* Rm1021 LuxS-like (SMc00396) amino acid sequence shares 16%, 15%, and 10% sequence similarity with the above organisms.
### Experiment

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<td>BB120 filtrate</td>
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### Supplemental Table 2.

**Experimental design for AI-2 assay using *V. harveyi* BB170 luminescent report strain.** Controls will be run to ensure that any positive results in SRH + SMc00396 experiment are true and not caused by another factor.

*V. harveyi* strain BB120 is the sensor 1+ sensor 2+ parent of BB170 and is used in the literature to demonstrate 100% AI-2 activity.

### Supplemental Table 3.

**Experimental design for biofilm and nodulation assays.** Addition of Δsmc00396 deletion to these strains will help illustrate the effect of LuxS on biofilm formation and nodulation by *S. meliloti*.

Rm1021 does not make EPSII because of a disruption in expR, a gene encoding for a transcriptional regulator in the ExpR/Sin quorum sensing system.

Strains constructed by Dr. Reed Goodwin using Rm8530 as the parent. EPSI mutants were constructed by the deletion of exoY. EPSII mutants were constructed by the deletion of wgaAB. Rm1021 and RG33 are different genotypes, therefore both are used in this assay.