**Determination of the Route of Bacterial Transfer from adult to larvae in *Odontotaenius disjunctus***

**I. Introduction**

Nitrogen is the largest component of Earth’s atmosphere at ~80% composition. Nitrogen is also a key component in many biological molecules. All life on Earth utilizes this element. However, in the state it exists in the atmosphere, it is entirely too unreactive to be of use to a great number of organisms that need it. This includes plants for which nitrogen is the limiting agent in their growth cycle.10 There are, however, some organisms (known as diazotrophs) that are able to fix atmospheric nitrogen to NH3 using an enzyme named nitrogenase(do I need a citation here or is this considered a known and accepted thing). Ammonia is a form of nitrogen that is more bioavailable. This biological fixing of atmospheric nitrogen was the principle production pathway for ammonia for the better part of Earth’s history. The agricultural revolution and the steady increase in human population created the necessity for a new, quicker, and on demand production of ammonia to fuel the growth of crops.

The Harber-Bosch process is an industrial process used to fix atmospheric nitrogen to ammonia. While biological nitrogen fixing happens at ambient temperature and pressure, the industrial method requires temperatures around 750K and pressures around 10MPa.5 The source of hydrogen for the reaction is methane from natural gas (citation). The requirements for industrial production of ammonia are such that the energy used is very high (get numbers and citation). Understanding how other biological systems fix nitrogen is an important avenue for exploring other ways of fixing nitrogen that have less deleterious effects on the environment and are more energy efficient.

As mentioned above, diazotrophs are organisms that are able to fix nitrogen. There are some that do this by forming a symbiotic relationship with another organism; it is usually observed that they form symbiotic relationships with plants but some have been known to form relationships with insects. Odontotaenius disjunctus is one such insect. O. disjunctus is a beetle that lives in and subsists on woody biomass. It has recently been shown that nitrogen fixation does occur in the gut of this beetle.1 The beetle’s gut microbiome has been characterized revealing a variety of bacteria that are able to fix nitrogen; more specifically, this has revealed the presence of nitrogenase genes that are related to those that occur in several different bacteria such as *Paludibacter propionicigenes, Clostridium cellobioparum, and Bradyrhizobium japonicum* among others1. Furthermore, it has been shown that these genes are being expressed in the gut identifying the gut as the site of nitrogen fixation within the beetle. Now that it has been shown that the beetles harbor nitrogen fixing bacteria, the question becomes how do these beetles acquire the microbes that fix nitrogen? More specifically, how do the larvae acquire these microbes? It is proposed that the larvae obtain at least part of the nitrogen fixing microbial community via consumption of woody biomass processed by adult beetles. The processing is essentially digestion and excretion of the woody biomass. It is already known that larvae subsist solely on this processed biomass3,8,9. Proposed is an experiment that will elucidate if nitrogen fixing bacteria can survive this process and flourish in the gut of the larvae.

**II. Experiment**

The purpose of this experiment is to determine how larval *O. disjunctus* acquire its microbiome. More specifically, the nitrogen fixing bacteria. This will be done by first transforming a fluorescent protein encoding plasmid known as green fluorescent protein (GFP) into the diazotroph *Bradyrhizobium japonicum*. Once transformed into the bacteria, the bacteria will express the GFP gene. As a result, the green fluorescent protein will be synthesized. It has been shown that efficient transformation of plasmid DNA into this diazotroph is possible via electroporation4. A culture of this GFP containing bacteria will be grown and will henceforth be called GFP bacteria. The GFP bacteria will be introduced into the food source of adult *O. disjunctus*. The beetles will consume woody biomass that has been inoculated with the GFP bacteria and it is expected that the bacteria will be present and functioning in the excrement. This GFP bacteria containing excrement will then be used as the food source for new larvae that have not been exposed to any other food source.

II.A. Bacterial Transformation

In order to confirm not only the presence of nitrogen fixing bacteria but also the active expression of genes at various stages in the proposed transfer pathway, a plasmid containing DNA that codes for a protein that fluoresces will be inserted into nitrogen fixing bacteria *Bradyrhizobium japonicum*. The detection of the green fluorescent protein suggests two important things: the bacteria containing the plasmid have survived digestion, and the bacteria are actively expressing genes11. Produced by the jellyfish *Aequorea victoria*, green fluorescent protein is a protein requiring no other cofactors or substrates for its function2,7,11. The ability of the protein to fluoresce on its own is important as it does not require the utilization of cellular resources other than those employed in the construction of the protein. When irradiated with certain wavelengths of UV light, the protein will fluoresce.

Will discuss specific plasmid to be used here

*Bradyrhizobium japonicum* is the bacteria of choice because nitrogenase genes related to nitrogenase genes in *B. japonicum* were detected in the gut of *O. disjuncus*1 and because a transformation protocol for *B. japonicum* has already been elucidated4. Cells of *B. japonicum* will be prepared for electroporation per the methods in the Hattermann & Stacey paper. Electroporation is a process wherein an electrical field is applied to the cellular medium. It is thought that the electrical field causes the formation of temporary pores across the cell membrane. The plasmids enter through these pores. The peptidoglycan layer or cell wall is not affected by this electrical field. This is not a problem as this layer is naturally porous. After electroporation the cells are suspended in medium and given time to recover or allow for the reformation of the cell membrane via the closing of the temporary pores created during exposure to the electric field. Cells that are expressing the plasmid will be selected for by introducing an antibiotic into the medium. Cells that are expressing the GFP gene will also be expressing an antibiotic resistance gene. This means that only cells containing the plasmid will survive and flourish in a medium that contains an antibiotic. It is the case that when the bacterial cells divide chromosomal and plasmid DNA is replicated. This means the fluorescent tag is transmitted to the next generation of bacteria. This allows for experiments that will take longer than the life of a single generation.

II.B. Determination of microbe transmission pathway

This culture will be introduced to the food source of adult beetles with the expectation that the GFP bacteria will flourish in the *O. disjunctus* gut. It is expected that the bacteria will show up in the excrement of the adult beetles. This will be tested for. Because the hatched larvae will feed solely on the woody biomass that is excreted by the adults, they will also be introduced to the GFP bacteria that passed through the gut of the adults. They will be tested for GFP bacteria presence in their gut. It is expected that the GFP bacteria will be present and active. Another group of larvae will have uncontaminated excrement material to feed on but the soil in their environment will contain GFP bacteria in concentrations similar to conditions observed in the wild (find citation). It is expected that environmental exposure will not be a major source of microbes.

II.C. Visualization of Green Fluorescent Protein

Visualization of GFP is achieved via irradiation with UV light. Find protocol

**III. Discussion**

In the case that the expectations are met, GFP bacteria will be observed in the gut of larvae that have been exposed to GFP bacteria containing excrement. The larvae that were fed normal excrement and exposed to GFP bacteria via the environment should not have communities of GFP in their guts.

It may be the case that the group of larvae exposed to the GFP bacteria environmentally does show GFP bacteria activity in their gut. This would be unexpected but not implausible and might suggest a mix of transfer mechanisms.

No difficulty is expected in the transformation of *B. japonicum* with the GFP containing plasmid. The plasmid will be no larger than 10 Kb. The procedure outlined in Hattermann & Stacey 1990 used plasmids around 30 kb. As stated earlier *B. japonicum* was chosen because nitrogenase genes were found in the gut of *O. disjunctus* beetles that had high sequence similarity with the nitrogenase gene of *B. japonicum.* The relatedness of this one gene does not lend credence to the idea that *B. japonicum* as an entire organism will be able to survive and function in the gut of *O. disjunctus.* In the case that the microbe does not flourish, the bacterium used will have to be reconsidered. There are several other candidates and transformation protocols may be elucidated with trial and error electroporation varying the applied voltage and pulse time.

Further studies may be conducted on the expression of nitrogenase genes in the GFP bacteria.

**References**

1. Ceja-Navarro, J. a, Nguyen, N. H., Karaoz, U., Gross, S. R., Herman, D. J., Andersen, G. L., … Brodie, E. L. (2013). Compartmentalized microbial composition, oxygen gradients and nitrogen fixation in the gut of Odontotaenius disjunctus. *The ISME journal*, *Advanced O*, 1–13. doi:10.1038/ismej.2013.134
2. Chalfie, Martin; Tu, Yuan; Euskirchen, Ghia; Ward, William W.; Prasher, D. C. (1994). Green fluorescent protein as a marker gene expression. *Science*, *263*(5148), 802–805. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/18051416
3. Gray, E. (2013). Observations on the Life History of the Horned Passalus, *35*(3), 728–746.
4. Hattermann, D. R., & Stacey, G. (1990). Efficient DNA transformation of Bradyrhizobium japonicum by electroporation. *Applied and environmental microbiology*, *56*(4), 833–6. Retrieved from <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=184308&tool=pmcentrez&rendertype=abstract>
5. Ito, Y., & Goto, T. (2005). Electrochemistry of nitrogen and nitrides in molten salts. *Journal of Nuclear Materials*, *344*(1-3), 128–135. doi:10.1016/j.jnucmat.2005.04.030
6. Leff, L. G., & Leff, a a. (1996). Use of green fluorescent protein to monitor survival of genetically engineered bacteria in aquatic environments. *Applied and environmental microbiology*, *62*(9), 3486–8. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=168148&tool=pmcentrez&rendertype=abstract
7. Ma, L., Zhang, G., & Doyle, M. P. (2011). Green fluorescent protein labeling of Listeria, Salmonella, and Escherichia coli O157:H7 for safety-related studies. *PloS one*, *6*(4), e18083. doi:10.1371/journal.pone.0018083
8. Nardi, J. B., Bee, C. M., Miller, L. A., Nguyen, N. H., Suh, S.-O., & Blackwell, M. (2006). Communities of microbes that inhabit the changing hindgut landscape of a subsocial beetle. *Arthropod structure & development*, *35*(1), 57–68. doi:10.1016/j.asd.2005.06.003
9. Pearse, A., Patterson, M., Rankin, J., & Wharton, G. (1936). The ecology of Passalus cornutus Fabricius, a beetle which lives in rotting logs. *Ecological Monographs*, *6*(4), 455–490. Retrieved from http://www.jstor.org/stable/10.2307/1943239
10. Smith, B. (2002). Nitrogenase reveals its inner secrets. *Science*, *297*(5587), 1654–1655. Retrieved from http://www.jstor.org/stable/3832311
11. Southward, C. M., & Surette, M. G. (2002). The dynamic microbe: green fluorescent protein brings bacteria to light. *Molecular microbiology*, *45*(5), 1191–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12207688