AGRICULTURAL RESEARCH FOUNDATION
FINAL REPORT
FUNDING CYCLE 2014 – 2016

TITLE: Turnover of protein as a controller of soil nitrogen cycling

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SUMMARY:

During the first year we performed several experiments that measured protease activity in soil. We found that protease activity in several forest soils was inversely related to dissolved organic N. We also were able to measure the turnover rate of native soil proteases and determined that two protease pools with different turnover rates existed, one presumably related to proteases in soil solution and the other to proteases bound to the soil matrix.

During the second year we investigated ways of stimulating protease production to identify conditions that would allow us to examine bacterial versus fungal contributions. We began a bioinformatics study on the phylogenetic diversity of microbial extracellular proteases. Finally, a collaborative proposal was submitted to the National Science Foundation and it was funded.

OBJECTIVES:

The ultimate aim of the proposed research was to gain a better understanding of protein turnover in soil, including the roles of different types of proteases and microorganisms in this process. The immediate, practical goal was to collect convincing data for use in a proposal to NIFA and/or NSF. To accomplish this, three objectives were pursued:

1. Measure the turnover of a model, ^{13}C- and ^{15}N-labeled protein in soil, including the fate of its C and N.
2. Determine the relative contributions of bacteria and fungi to protease activity during protein degradation.
3. Determine the relative activities of different catalytic classes of proteases active in protein degradation.

PROCEDURES:

We focused on Objective 2 for our ARF-supported research. These experiments used two forested soils (Andisol from the Coast in Cascade Head Experimental Forest and Inceptisol from the western Cascades in HJ Andrews Experimental Forest) with ranges in mineralogy and microbial composition. Each site had replicated plots of two tree species (Douglas-fir and red alder) that differ in their impact on soil N cycling. We characterized the protease activity of these soils using two different assays. We subsequently evaluated how these activities responded to the presence of bacterial or fungal protein synthesis inhibitors.

A new, spin-off project was to perform a bioinformatics analysis of the known diversity and genetic relatedness of microbial extracellular proteases, which has been co-supervised by Ryan Mueller of the OSU Microbiology Department.

SIGNIFICANT ACCOMPLISHMENTS:

1. ***Native protease activity.*** Experiments were carried out to characterize soil proteases of soil from the two tree species at the two locations and associated soil characteristics (Fig. 1). Total protease and leucine-aminopeptidase (LAP) activities followed different patterns; total protease activity decreased with microbial biomass whereas LAP activity increased. When activity was normalized to microbial biomass, there was no correlation between LAP activity and measures of N availability ($p=0.50$ for C: N, $p=0.87$ for DON). This suggests that LAP is not regulated by C or N availability. It may be constitutively produced by this microbial community (there was a positive, but not statistically significant, correlation of LAP activity with microbial biomass). In contrast, total protease activity, on either a soil or microbial biomass basis, increased with soil C: N ratio ($p<0.1$) and significantly decreased with dissolved organic N ($p<0.01$). These relationships suggest that exo- and endo- protease production were regulated by N availability, with greater protease in more N-limited soils.

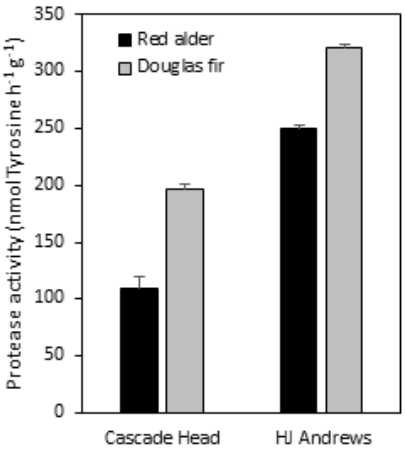

Figure 1. Protease activity of forest soils along a gradient of N availability.

2. ***Protease turnover.*** Continuous chloroform exposure during incubation disrupted cellular activities, preventing microbial re-growth, and inhibiting CO_2 respiration compared to control samples. The decline in protease activity is an indication of protease turnover and could be fitted with a double-exponential curve, suggesting that there are two pools of proteases: one that turns over rapidly (half-life <1 day) and one that turns over more slowly (half-life of ~1 month). The fast pool may represent free protease enzymes and the slow pool proteases associated with the soil matrix. Besides microbial regulation of protease production, matrix factors may also contribute to the level of protease activity in soils.
3. ***Fungal and bacterial contributions to protease activity.*** The purpose of this experiment was to manipulate the microbial regulation of protease turnover. Five treatments were applied: (1) a non-amended positive control, (2) a negative control, where chloroform fumigation was used to stop all microbial activity, (3) bacteria suppressed with streptomycin, (4) fungi suppressed with cycloheximide, and (5) bacteria and fungi suppressed with a mixture of streptomycin and cycloheximide. Respired CO_2, protease activity, inorganic N, dissolved organic N and C were measured in Day 0, 1, 2, 4, 8, 16 and 32 of the incubation. Two standard methods for protease activity were used: casein hydrolysis, which measured both endo- and exopeptidase activities, and L-aminopeptidase, which measured a specific exopeptidase activity. Positive control respiration rates declined from 34.6 to 13.9 ng CO_2-C g^{-1} soil d^{-1} during the 32 days of incubation as soil carbon availability decreased. Chloroform depressed respiration of microbial community by 62 to 77%

compared to positive control, indicating that it effectively inactivated respiration of bacterial and fungal populations. The bacterial inhibitor had little effect on respiration, possibly because this is a fungal-dominated soil. Surprisingly, the fungal inhibitor increased respiration, perhaps because of overflow respiration from fungi that were blocked in protein biosynthesis, or because bacteria used carbon from fungi that were killed. Soil protease activity was also suppressed by chloroform, depressing LAP activity from 74.4 to 24.4 nmol MC g^{-1} soil h^{-1} (Fig. 2) and casein degradation activity from 106.7 to 71.9 nmol tyrosine g^{-1} soil h^{-1}. There was little effect of the antibiotics on protease activity; however, this may be because soil conditions were not conducive to protease production. Our next experiment will be to repeat the experiment with the addition of a purified protein to stimulate protease production.

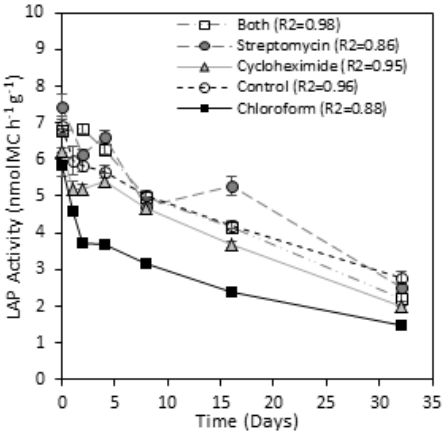

Figure 2. Effect of various inhibitors on soil protease activity.

4. ***Induction of protease activity.*** Zein, a protein from corn, was used as a substrate to stimulate the soil protease production. Taking advantage of zein's higher $\delta^{13}C$ isotopic signature (-14.8‰) compared to soils we are using (-27‰), we were able to calculate the fractionation of zein carbon in different pools and determine how fast the zein was mineralized by the microorganisms. Two treatments were applied (control and zein addition at the rate of 400 µg g^{-1} soil, which potentially could double the microbial biomass) to the four soils from Cascade Head and the H.J. Andrews Experimental Forest under red alder and Douglas-fir. Soil samples were incubated for 8 days. Respiration (CO_2), total potential protease activity, leucine amino peptidase activity, and mineralized nitrogen were measured after Day 0, 1, 2, 4 and 8. Zein started to be decomposed 2 to 4 days following its addition. After 8 days, the carbon mineralization rate of zein began to stabilize and reach roughly 30 µg C-CO_2 g^{-1} soil and about 30% of the initial added zein was calculated to be utilized by the microorganisms. Zein did not seem to induce the leucine amino peptidase production after 8 days in and soil but stimulated total protease activity in the H.J. Andrews soils but not the Cascade Head soils. Extracted ammonium and nitrate concentrations tended to be higher in the control samples than the zein added treatments, suggesting that the N in the zein was being assimilated and not being mineralized. We will need to do further work to identify an amendment that will stimulate protease production.
5. ***Protease diversity.*** This study's objective was to determine the phylogenetic distribution of bacterial secreted proteases across taxa. Peptidase subfamilies were extracted from the MEROPS database and 944 bacterial genomes of interest were obtained from the SILVA database. Secreted peptidases were identified using the SignalP 4.0 algorithm. The Bray-Curtis similarity index was used to compare the secreted peptidase profiles for all the bacterial genomes. A distance matrix between the profiles was calculated using the Vegan package in R studio. Phylogenetic analysis of the 16S rRNA sequences was done using PHYLIP. A Mantel test in the Analysis of Phylogenetic and Evolution package in R was used

to test the correlation between the phylogenetic and the secreted peptidases distance matrices. Secreted peptidase subfamily distribution was visualized on the phylogenetic tree, based on the count of gene content in each strain using iTOL. This resulted in a total of 22 bacterial phyla and 20 proteolytic subfamilies (Fig. 3). The phylogenetic distribution of secreted peptidases belonging to 944 bacteria was found to significantly correlate with the taxonomic distribution, inferring an evolutionary relationship (Fig. 4). Different peptidase subfamilies had different patterns of clustering on the phylogeny. Further study will be done to expand the method to include fungi and to determine the conservation of the functional (peptidases) and phylogenetic distributions in the fungal community.

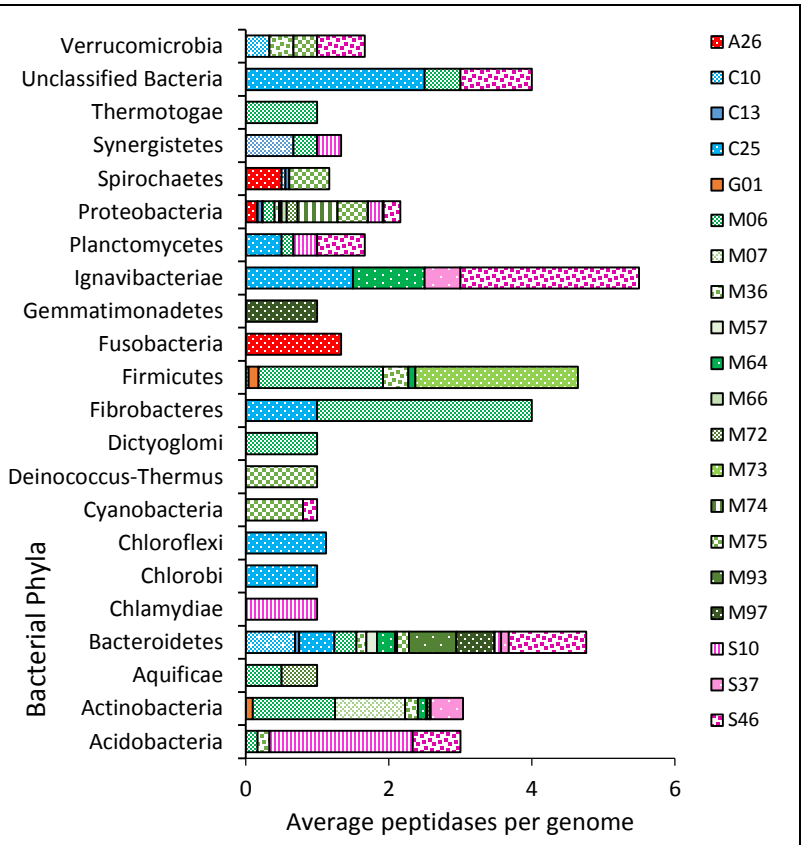

Figure 3. Phylum-specific average secreted peptidase gene content across 944 bacterial genomes. Different colors represent genes from different peptidase subfamilies (A, C, G, M, and S are respectively Aspartic, Cysteine, Glutamic, Metallo, and Serine peptidase families).

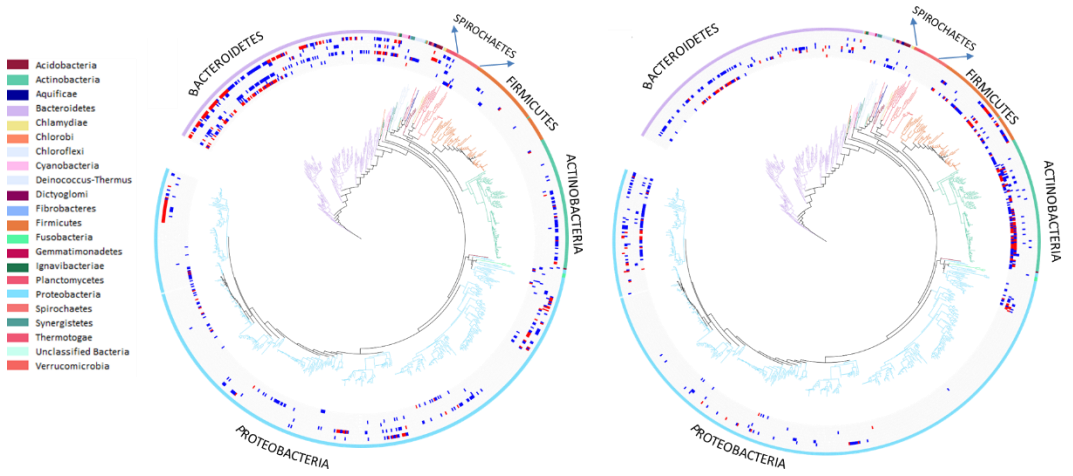

Figure 4. Strain-specific secreted peptidase distribution in bacteria. The consensus phylogenetic tree was based on a 16S rRNA alignment retrieved from the SILVA database and constructed using PHYLIP (distance matrix F84, neighbor joining). The outer circles show the absence (white) or the presence of one copy (red) or multiple copies (blue) of genes from a secreted peptidase subfamily in each genome. From inside out of the outer circles: A26, C10, C13, C25, G01, S10, S37, S46 peptidase subfamily and Phyla classification *(left figure); and* M06, M07, M36, M57, M64, M66, M72, M73 peptidase subfamily and Phyla classification *(right figure)*. This tree was displayed using ITOL.

BENEFITS & IMPACT:

The results of this research were presented by Ms. Nguyen at the annual meeting of the Soil Science Society of America in November 2014. The laboratory research will form the basis of the first chapter of her thesis and the bioinformatics exercise will be the core of the second chapter.

In the long-term, a practical application that could grow out of this research would be a better understanding of the decomposition of organic N amendments that are commonly used in organic farming, such byproducts of agricultural (e.g., feather meal) or bioenergy (e.g., algal meal) production. A major challenge in using organic fertilizers is predicting the dynamics of nutrient release from these materials in relation to crop demands. Such an understanding could result in improved practices of nutrient management, particularly of N, that would optimize availability during crop growth while minimizing losses of nutrients into the environment.

ADDITIONAL FUNDING RECEIVED DURING PROJECT TERM:

A related project on the fertilizer value of algal meal was funded by the Sun Grant program and is supporting Shannon Andrews as part of her Ph.D. program.

We were successful in receiving a grant for “Turnover of proteins as a controller of soil nitrogen cycling” from the National Science Foundation ($600,000 for 3 years). This is a collaborative project with Markus Kleber and Ryan Mueller at OSU and Bob Hettich at Oak Ridge National Laboratory. It will support the final years of Trang Nguyen’s Ph.D. program. (This ARF grant provided research funds during her first two years.)

FUTURE FUNDING POSSIBILITIES:

Proposals to the EMSL user facility to try a new approach for isolating proteases from soil is in preparation as is a collaborative proposal to DOE on organic N as a regulator of soil C turnover.