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**Progress Report**

PROJECT TITLE: Hyper-Thermostable Enzyme (Lactonases) for use as Microbial Biocontrol Agents for Plant Diseases

PROJECT NUMBER: 4136-17SP

REPORTING PERIOD: Aug 1, 2018 - Oct 31, 2018

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1. PROJECT ACTIVITIES COMPLETED DURING THE REPORTING PERIOD.

**Goal:** To evaluate hyper-thermostable lactonases as microbial biocontrol agent inhibiting bacterial disease of corn

The project has two aims:

**Aim I: Evaluate the potential of hyperstable lactonases to protect plants in several infection models.** We will use our current and newly produced lactonases to protect plants from a variety of plant pathogens, including *Acinetobacter sp., Aeromonas sp., A*grobacterium sp., *Burkholderia sp.,* Erwinia sp., *Proteus sp., Pectobacterium, Xanthomonas, Pseudomonas sp.,* Ralstonia sp., and Clavibacter sp.strains. Diseases to be evaluated on corn will be bacterial leaf blight, Holcus spot and Stewart’s disease. We will also determine the best mode of enzyme addition to plant surfaces, timing of enzyme addition, humidity and temperature resistance, dosage to be used, and potential carriers.

**Aim II: Assess the ability of hyperstable lactonases to prevent post-harvest loss of crops**. While we initially targeted this technology to *in vivo* protection of plants from a variety of bacterial pathogens, in these studies, we will specifically focus our attention on preventing post-harvest soft rot of corn and other crop by members of the genus Dickeya (previously Erwinia chrysanthemi), Pectobacterium atrosepticum, (formerly Erwinia atroseptica) and Pectobacterium carotovorum subsp. carotovorum, (previously Erwinia carotovora subsp. carotovora). We will also examine the possible effect of the enzyme on fungi infections, as some of these infections also depend on initial bacterial colonization, infection, or maceration. Variation of enzyme concentration, humidity, and concentrations of bacteria and fungi will be three of the several factors examined.

**Procedures and Plans:**

**Bacterial strains and quorum quenching lactonases.**

Eleven pathogenic strains that we are using in this study include *Clavibacter michiganensis subsp. nebraskensis*, *Erwinia chrysanthemi pv. Zeae*, *Pantoea stewartii Mergaert, Erwinia aroideae 8066, Erwinia atroseptica 8064, Xanthomonas campestris pv. campestris, Pseudomonas syringae pv syringae, Pseudomonas syringae pv phaseolicola, Erwinia carotovora, Xanthomonas campestris pv. phaseoli*, and *Pectobacterium carotovorum*. The strains were grown in selected medium at 25-30˚C. There will be two kinds of the quorum quenching lactonases (SsoPox W2631, and GcL WT) used in this study. The characterizations of these lactonases were studied by Dr. Elias groups 1, 2.

We previously reported successful protection of several plant species from bacterial diseases by using lactonase enzyme SsoPox. Here we described our initial assays on corn using *Clavibacter michiganensis* subsp. nebraskensis (Cmn). The goal was to determine if the presence of lactonase on the corn leaf surface (the phyllosphere) alters bacterial community structure, in addition to preventing disease.

**Variation of maize phyllosphere microbiome due to interaction of Cmn and SsoPox**

**Next-Generation Sequencing**

The V4 hypervariable region of 16S rRNA was amplified for bacteria and archaea by using primer set 515F (5’– GTG CCA GCM GCC GCG GTA A –3’) and 806R (5’– GGA CTA CHV GGG TWT CTA AT –3’). Sequencing was done by the University of Minnesota Genomics Center (Minneapolis, MN, USA) as previously described3. Briefly, samples were first amplified by using the following cycling conditions: 95˚C for 5 min, followed by 25 cycles of 98˚C for 20 s, 55˚C for 15 s, and 72˚C for 1 min. Adapters and barcodes were added by using an additional 10 cycles PCR. Amplicons were gel purified, pooled, and paired-end sequenced at a read length of 300 nt on the Illumina MiSeq platform (Illumnia, Inc., San Diego, CA, USA).

**Bioinformatics**

Highly quality sequence data for analyses were obtrained by submitting the raw sequencing data through the SHI7 pipeline for quantity control (QC) 4, 5. In brief, sequencing adaptors were removed, the two paired-end reads were merged, and residual adaptors were further trimmed. Low quality reads were removed to keep QC values > 31 and highly quality reads were converted into the FASTA format and aligned on the basis of the Greengenes ver. 13.86. UCHIME software was used to identify and remove the probable Chimeric sequence7. All sequence data was rarefied to 63,000 sequence reads per sample before sustainable statistical analysis. Raw sequencing data will eventually be deposited in the Sequence Read Archive (SRA) of NCBI (https://www.ncbi.nlm.nih.gov/sra).

**Statistical analysis**

For statistical analysis, Alpha diversity (average species diversity) indices, as well as Good’s coverage, were calculated using the Shannon index and abundance-based coverage estimate through mother program. Visualization of the taxonomic distribution of microbial communities was performed by using the “ggplot2” package in R (Al-Masaudi et al., 2017). Differences in beta diversity among samples was evaluated by using analysis of similarity (ANOSIM), which employs Bray-Curtis dissimilarity matrices (BC)8, 9. Principal coordinates analysis (PCoA) was used to analyze the differences in microbial community structure between various DNA extraction methods. The VennDiagram package in R was used to identify OTUs shared between various DNA extraction methods10.

**Results**

**Composition of microbial communities on maize phyllosphere**

Results in Table 1 show the mean coverage of all three treatment categories was greater than 99%, without significant difference between Cmn, Cmn+SsoPox, and control groups, respectively. Mean Shannon diversity indices for individual categories ranged from 1.04 to 1.64, and Shannon diversity differed significantly between Cmn groups to Cmn+SsoPox and control. The ranking of diversity was as follows: Cmn+SsoPox > Control > Cmn. However, there was no significant different between Cmn to Cmn+SsoPox in Chao1 and *S*obs.

Bacterial community analyses found that the Cmn group was primarily composed of members of the phyla Actinobacteria (>60%), Cyanobacteria (>20%), and Proteobacteria (>5%). The composition in Cmn+SsoPox groups ranked from Proteobacteria (mean 57.4%) > Actinobacteria (mean 30.8%) > Cyanobacteria (11.7%). This compared with the dominant Cyanobacteria (>50%) and Proteobacteria in the control groups (Figure 1A). Microbacteriaceae, which is the family level for *Clavibacter michiganensis* subsp. *nebraskensis*, was found as the major composition (mean 64.8%) in the Cmn group, but decreased significantly to 30.6% in the Cmn+SsoPox group (Kruskal-Wallis test, *P*<0.001), and rarely presented (ca. 0.2%) in control groups (Figure 1B). Notably, SsoPox treatment significantly increased the composition of *Enterobacteriaceae* in the treatments (Kruskal-Wallis test, *P*<0.001).

**Shifts in bacterial community composition following treatments.**

Ordination analyses via PCoA (Figure 2) showed ehe separation of samples by three categories, and these clusters were significantly different by ANOSIM (*P*<0.001) and AMOVA (*P*<0.001). Inoculation with Cmn significantly shifted the bacterial community (beta diversity) from control groups to the treatment groups. For example, significant differences were observed in control *v.s.* Cmn (R=0.98, *P*<0.001) and control v.s. Cmn+SsoPox (R=0.93, *P*<0.001), respectively. Among samples treated with Cmn, difference in beta diversity, evaluated by ANOSIM and AMOVA were also significantly different between Cmn and Cmn+SsoPox (R=0.74, *P*<0.001). Notably, in the control group, PBS and SsoPox were not significantly different (P>0.05). Similarly, Cmn and Cmn+HEPES communities did not significantly differ (P>0.05) from each other.

**Reducation of Clavibacter and *Clavibacter michiganensis* subsp. *nebraskensis***

Analysis of *Clavibacter* from abundance data at the genus level revealed that Clavibacter had a mean value less than 0.3 % in all of treatments, which was ranking as follows: Cmn (0.18%$\pm $0.02%) > Cmn+HEPES (0.17 %$\pm $0.02%) > Cmn+SsoPox (0.06%$\pm $0.02%) > SsoPox = PBS (0%), respectively (Figure 3). In the Cmn Groups, the relative abundance of *Clavibacter* was not significantly different between Cmn and Cmn+HEPES treatments (ANOVA, *P*>0.05). However, with treatment with the lactonase SsoPox, *Clavibacter* was significantly reduced in the Cmn+SsoPox groups (ANOVA, *P*<0.05).

**TABLE 1** Coverage and alpha diversity of bacterial communities on the maize phyllospere within various treatments.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Treatment | No. samples | Coverage (%) | *S*obs† | Shannonindex | Chao1 |
| Cmn | 9 | 99.80±0.05A | 43±11A | 1.04±0.16A | 112±38A |
| Cmn+SsoPox | 6 | 99.71±0.09A | 68±20AB | 1.69±0.25B | 167±72A |
| Control | 5 | 99.70±0.10A | 73±30B | 1.44±0.29B | 138±49A |

† *S*obs: number of OTUs observed at 97% similarity.

\* Sample groups sharing the same letter did not vary significantly (*P* ≤ 0.05) by ANOVA followed by Tukey’s post-hoc test.





Figure 1.Distribution of microbial composition at (A) phylum level and (B) family level on the maize phyllosphere under influencing of Cmn and SsoPox.

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Figure 2. Principal coordinate analysis of maize phyllosphere among three categories treatments: control (SsoPox and PBS), Cmn treatment (Cmm and Cmn+HEPES), influence of SsoPox (Cmn+SsoPox). The relationship between the ordination plot and the distance matrix had a r2 value of 0.93. Analysis of similarities (ANOSIM) described the differences of beta diversity among three treatments (the threshold of *p* value is 0.017 on the basis of Bonferroni pairwise comparison).



**Figure 3.** Distribution of *Clavibacter* from NGS under difference treatments. Sample groups sharing the same letter did not vary significantly (*P* ≤ 0.05) by ANOVA followed by Tukey’s post-hoc test.

**References:**

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2.) IDENTIFY ANY SIGNIFICANT FINDINGS AND RESULTS OF THE PROJECT TO DATE.

We have just started to evaluate plant model infection assays.

3.) CHALLENGES ENCOUNTERED. (*Describe any challenges that you encountered related to project progress specific to goals, objectives, and deliverables identified in the project workplan.*)

 We initially had difficulty getting strains to test until we obtained a license from APHIS, and this took time. We are now approved to test all pathogens, after 2 rounds of requests It also took time to get plant disease assays (Stalk, leaf, and tuber) up and running in a repeatable fashion. We have achieved this and are now versed in testing on corn, wheat, barley, soybean, and potato.

4.) FINANCIAL INFORMATION (*Describe any budget challenges and provide specific reasons for deviations from the projected project spending.*)

There are no budget challenges.

5.) EDUCATION AND OUTREACH ACTIVITES. *(Describe any conferences, workshops, field days, etc attended, number of contacts at each event, and/or publications developed to disseminate project results.)* None yet. We just started to get results and are now considering filing patent protection before submitting results for publication.