Attachment A: Workplan for Lactonase Biocontrol Project – Phase III

Project Title: Hyper-Thermostable Enzyme (Lactonases) for use as Microbial Biocontrol Agents for Plant Diseases – Phase III

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Project Duration

List contract start and end time.
Start: 06/01/2020
End: 05/31/2021

Summary

Many bacterial pathogens infect crop plants, representing major economic burdens. This also limits our ability to feed the world's populations. Current methods for controlling plant diseases due to bacterial infection have had limited success, in part due to bacterial resistance, specificity, and environmental concerns associated with antibiotics. Novel strategies are therefore greatly needed to control microbes. Numerous bacterial pathogens use chemical signaling systems to coordinate virulence factor expression and biofilm formation. A common bacterial communication mechanism called quorum sensing (QS) regulates bacterial gene expression in response to fluctuations in cell density. A common class of QS molecules are acyl homoserine lactones (AHLs). The hydrolysis of AHLs lead to the disruption of bacterial communication, and a subsequent reduction of virulence and biofilm formation. The use of a controlled biologically-derived agent, e.g. a lactonase preparation, to control plant pathogens, is therefore appealing. Our group has isolated and engineered enzymes that are highly proficient and extremely stable, that can be used as biocontrol agents and be active at all times, independently of the ecosystem. Over the last year, we have demonstrated that this approach can protect a variety of plants, including corn, from infection. Our results were exciting as we learned that crop protection is broad, extending from grasses (Corn, Wheat, and Barley) to Dicots (Soybean, Field Beans, and Potato). Over the last year we have: 1) established growth chamber methods to effectively grow and infect corn, and a variety of other plants, 2) developed and evaluated plant infection/protection assays, and 3) tested efficacy of the lactonases to control disease and at the same time examined their influence on leaf microbiota, and 4) carried-out a small scale field trial on corn. While results of this field study looked very promising, we need to address environmental impacts on enzyme adherence to leaves and expand the scale of field trials in multiple locations. Here we propose to 1) improve formulation of this enzyme and 2) examine its ability to reduce damage to crops plants in the field at multiple locations. We also propose to examine how the enzyme functions to prevent disease from atypical bacterial pathogens of corn.

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Background

Many bacterial pathogens infect crop plants causing huge economic losses that also limit our ability to feed the world's populations. Current methods for controlling plant diseases due to bacterial infection, mostly through the use of chemical pesticides, have had limited success, in part due to bacterial resistance, specificity, and environmental, regulatory, and policy repercussions due to pesticide use. Therefore, novel strategies are currently needed to control microbes infecting plants.

Numerous bacterial pathogens use chemical signaling systems to coordinate expression of their virulence factors. These are the same gene systems involved in biofilm formation. A common bacterial communication mechanism called quorum sensing (QS) regulates bacterial gene expression in response to fluctuations in cell density. The QS bacteria produce and release into their environment chemical signal molecules, called autoinducers. A common class of autoinducer-QS molecules are acyl homoserine lactones (AHLs). AHL-mediated communication is critical for expression of bacterial virulence factors and is present in most gram negative and some gram positive bacterial pathogens of a wide variety of plants. Disruption of AHL communication via quorum quenching (QQ) enzymes (lactonases) control pathogens by reducing virulence and has been shown on cell cultures and *in vivo*.

The ability of lactonase enzymes to control bacterial virulence is extremely appealing for crop protection. The first identified lactonase from *Bacillus thuringensis*, AiiA, was used to produce genetically modified plants. The expression of AiiA in tobacco and potato plants significantly reduced maceration area of leaves (tobacco) or tubers (potato), upon infection with *Pectobacterium carotovorum* [1]. In addition to ectopic expression of lactonases in plants, a relatively new emerging quorum quenching technique is the use of bacteria, which naturally employ quorum quenching enzymes as biocontrol agents to manipulate QS pathways. Several studies have demonstrated effective biocontrol activity through the application of bacteria harboring AHL-degrading enzymes to infected plants[2]. However, and to the best of our knowledge, the efficiency of such biocontrol agents has not yet been demonstrated in the field. This is in large part due to: 1) the lack of ability of these agents to adequately express enzymes under field conditions, 2) the fact that enzymes are susceptible to degradation in the environment, 3) QQ bacterial strains do not exhibit rhizosphere competence, and root and shoot colonization ability, or 4) the inability to survive, proliferate and produce enzymes on growing plant roots and leaves in the presence of indigenous microbial population [3].

The use of a more controlled biologically-derived disease control agent, e.g. a lactonase preparation, is therefore appealing. Most importantly, the enzyme(s) would be present and active at all times, independently of the ecosystem. However, most enzymes are very unstable under environmental conditions, mainly due to bacterial-produced proteases and unfavorable physical conditions, e.g. pH or water activity.

To overcome these problems, we have isolated a lactonase from an extremophile, and engineered it to be extremely stable. The lactonase *Sso1*, isolated from the hyperthermophilic bacterium *Sulfolobus solfataricus*, exhibits a melting temperature of 106°C [4,5]. We have further engineered *Sso1* to increase its lactonase catalytic activity [6], and it is stable towards aging (several years), detergents, pH, chemicals, organic solvents, proteases, and disinfection methods [5-7]. These properties make *Sso1* a good candidate for scale-up of the protein production and use in the environment. Current lab production yields are typically 1g of pure (>95% purity) compound for every 3L of culture, and numerous application could use partially purified enzyme preparations. Producing enzyme will not be a problem as we have fermentation capacity of 500 L in the BioTechnology Institute (BTI) -enough to make ~250 grams! The *Sso1* QQ enzyme therefore represent a unique candidate to control pathogens and protect plants and crop from bacterial infections.

Figure 1. Control of plant infections by a single spraying of 1mL of lactonase solution (10mg/mL) on plant leaves and crop. (**A**) and (**B**) Soft rot potato assay. Potato slices were infected with $5*10^7$ cells of *Pectobacterium carotovorum* CIR354 and treated with an inactive enzyme (control (**A**)) or with the active enzyme (**B**). The absence of black areas in (**B**) indicates less maceration. (**C**) and (**D**) Barley leaves were infected with 10^8 cells of *Xanthomonas translucens pv. translucens* LMG876 and treated with an inactive enzyme (control (**C**)) or with the active enzyme (**D**). Absence of leaf spots in (**D**) indicates protection. (**E**) and (**F**) Wheat leaves were infected with 10^8 cells of *Xanthomonas translucens pv. undulosa LMG892* and treated with an inactive enzyme (control (**E**)) or with the active enzyme (**F**). Absence of leaf spots in (**F**) indicates protection.

Results from previous years funding:

During this last year, we: **1) examined the potential of our existing hyperstable enzyme and existing mutant enzymes to protect corn plants (and several other plant species) from infection, and 2) examined the potential application of a purified, highly stable lactonase to reduce post- and preharvest damage to plants.**

Specifically, we establish infection systems for corn, wheat, and barley plants, and a crop infection system for potato (tubers and leaves). In all of these systems, we demonstrated that the treatment with a lactonase, consisting of a single spraying of the surface of the leaves with a small volume of enzyme (10mg/mL) was sufficient to protect corn plants from the tested pathogens, including when inoculation was performed with a large numbers of cells (**Figure 1**). Moreover, we specifically established a corn infection assay using the pathogen *Clavibacter michiganensis subsp. Nebraskensis*. After treatment with the lactonase spray, we report here that it protected the plant from showing any symptoms of infection (**Figure 2**).

We believe that these results, obtained by simply spraying a 100% natural, biodegradable, ecological, non-toxic, and biological molecule on plant leaves are spectacular and call for a more comprehensive assessment of

the capacities of this molecule to be used for plant and crop protection under field conditions. Based on these results, we are extremely confident and enthusiastic about the potential of lactonases to be a leading compound for the industry.

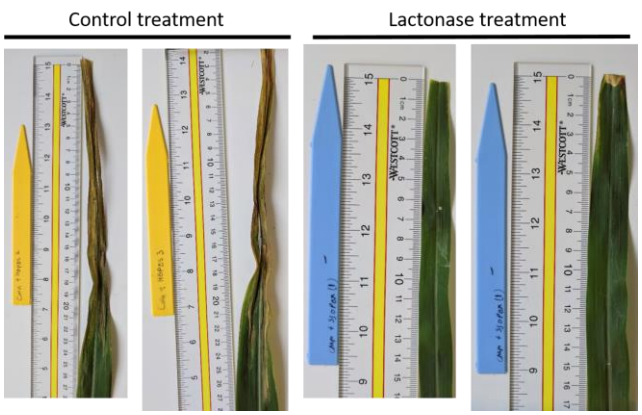


Figure 2. Control of plant infections by a single spraying of 1mL of lactonase solution (10mg/mL) on corn leaves. The Viking maize seed (40-30UP) were grown in Euro pot (diameter 8 inch) with sterilized soil mixture (50 standard soil/ 50 Germinating Mix) in a green house under diurnal conditions with 16 hours lights at 22 ˚C and 8 hours dark at 18˚C. Plant leaves were infected with *Clabibacter michiganensis subsp. nebraskensis* by dipping the clipped leaf into cell suspension (10^5cell/mL). Shown are duplicates plants for the control treatment (left) and lactonase treatment (right).

Based upon our successful demonstration that this approach can protect plants, including corn, wheat, barley, soybean, and potato, from bacterial infections, we propose to take advantage of this success in year 2 and to: 1) characterize the potency of this treatment and improve its formulation to protect corn plants from infection under field-like conditions, and 2) determine the range of pathogens that are controlled by the treatment and required concentrations of the lactonase and bacteria required for control; 3) determine the ability of lactonases to prevent post-treatment diseases of corn. Our studies are built upon the hypothesis that *Sso1* lactonase and derivative enzymes inhibit the pathogenicity of a variety of bacterial pathogens on a large variety of crop plants. In doing so diseases are suppressed.

During past half year, we : 1) quantified the protection levels of our existing hyperstable enzyme and existing mutant enzymes from corn infection, and 2) did studies to understand the influence of our highly stable lactonase on phyllosphere microbiota when *Clavibacter michiganensis* subsp. nebraskensis infecting corn leaf.

Specifically, on the basis of our established corn infection systems, we further quantified the protection levels of hyperstable enzyme on corn disease as showing on Table 1 and Figure 3.

Table 1. Distribution of disease levels

Treatment	Disease levels
Negative control (no additions)	1
PBS Buffer control	1
Lactonase only (enzyme only)	1
Cmn only (bacteria only)	9
Cmn + Lactonase (bacteria plus enzyme)	3

Figure 3. Application of Biological control Strategy

Moreover, we performed qPCR assay to evaluate the corn infection systems. The results elucidated presenting our hyperstable enzyme significantly decreased the concentration of Cmn on the corn leaf, which are corresponding with the disease level.

Through the use of next generation DNA sequencing, we also identified that there was a dramatic difference in microbial diversity when enzyme was present on leaf surfaces. (Figure 5, and Figure 6).

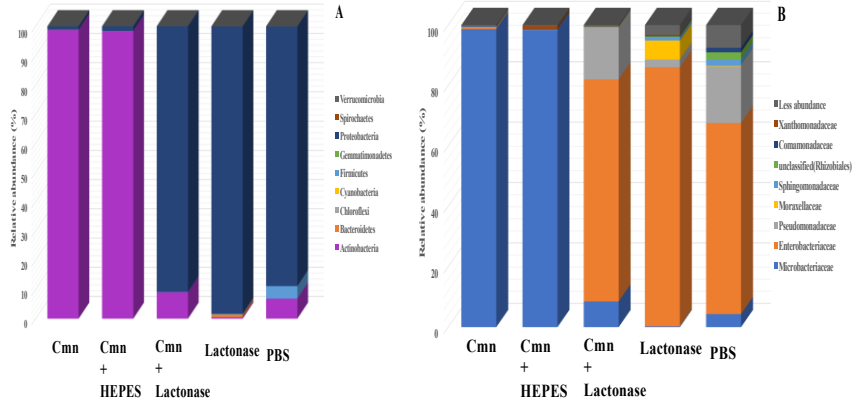


Figure 5. Distribution of microbial composition at (A) phylum level and (B) family level on the corn leaf phyllosphere under the influence of Cmn and lactonase.

Lastly, leveraging studies done in the field by Prof. Nathan Springer we also evaluated the effect of lactonase on two corn plant varieties in the field and initially characterized the leaf microbiota. While there was limited impact on commensal leaf bacteria this experiment will need to be redone this year with care paid to replication and use of plants infected with Cmn.

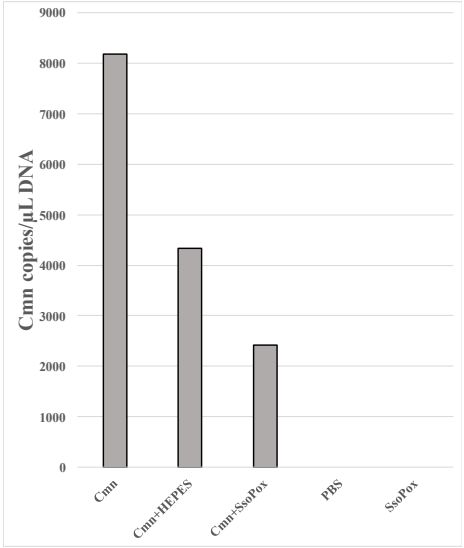
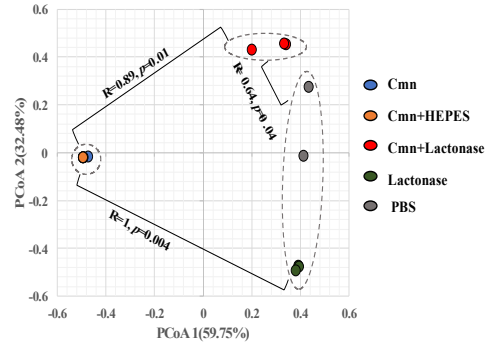


Figure 4. Quantification of Clavibacter michiganensis subsp. nebraskensis (Cmn) under different treatments by using qPCR through CMN_1184.



Principal coordinate analysis of maize phyllosphere among three categories treatments: control (lactonase and PBS), Cmn treatment (Cmm and Cmn+HEPES), influence of lactonase (Cmn+lactonase). The relationship between the ordination plot and the distance matrix had a r^2 value of 0.99. 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).

We believe that these results, obtained by simply spraying a 100% natural, biodegradable, ecological, non-toxic, and biological molecule on plant leaves are spectacular and call for a more comprehensive assessment of the capacities of this molecule to be used for plant and crop protection under field conditions.

Based on these results, we are extremely confident and enthusiastic about the potential of lactonases to be a leading compound for the industry.

The specific aims of this proposed research are:

Aim 1: Continue the characterization of the amount of lactonase required for treatment of corn leaves and different plants. We will use our current (and newly produced lactonases) as well as our existing plant infection models for corn (and others) to determine the dose-response and the maximal efficacy of the enzymatic treatment on corn in the field. We will also evaluate different formulations of the enzymatic treatment by testing the addition of emulsifiers and surfactants (polyglycerol esters, lecithin, and monoglceries, carriers (organic vs inorganic), and detergent-surfactants (tweens, nonidet P40 and others). We will also determine the best way to apply lactonase to plants. Based on initial studies it looks as if spraying is the best technology and now we will further investigate carriers other than water and other application methods.

Aim 2: Perform RNAseq technology on Cmn to determine the mode of action of lactonase. Previous studies produced an unexpected results, the lactonase enzyme protected corn plants from disease due to *Clavibacter michiganensis* subsp. Nebraskensis. This bacterium was thought to specific AHLs to communicate with bacteria. We will use the RNAseq method to examine what bacterial genes and proteins ate involved in signaling with AHLs. We will also see how this enzyme influences expression of bacterial virulence genes in other non-target gram positive plant pathogens.

Aim3: Determine if Cmn disease in field-grown plants can be suppressed by lactonase and examine its effect on other leaf microbes. The ultimate test of this technology is to see that it functions to suppress bacterial disease under field conditions. With help from Dr. Malvick, we will test the efficacy of this enzyme on *Clavibacter michiganensis* subsp. Nebraskensis in the field, at three location.

Materials and Methods

<u>Aim 1: Characterize the potency of the lactonase treatment, and improve its formulation to protect corn plants from infection.</u>

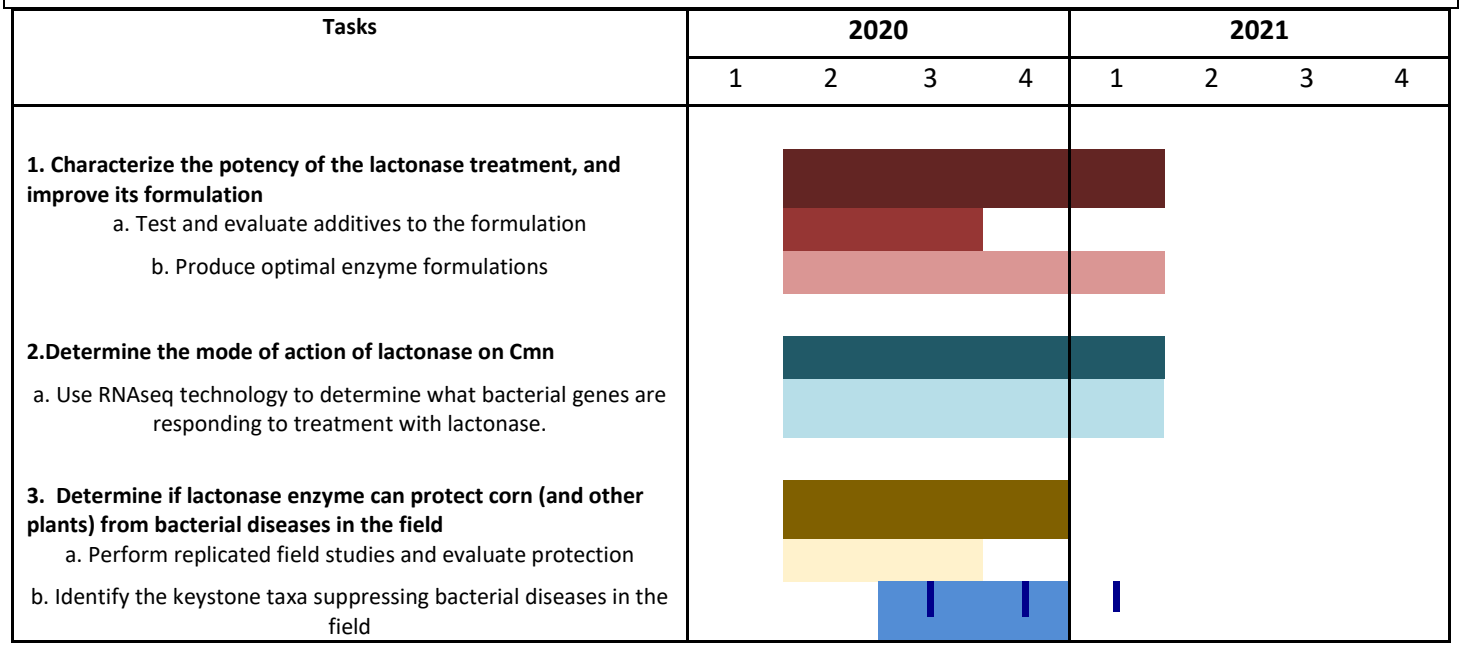
Using our existing plant infection model (Figure 1), we will test the concentration of enzyme needed (from 0.05 - 5 mg/mL), and days after application for disease protection (from 1-30 days) in field conditions or in potted greenhouse-grown corn plants. These parameters will be key to evaluate the ability of these enzymes to be used for treatment. Additionally, we will examine potential carriers (buffer, solvent (ethanol), with or without wetting agents or lecithin, on the efficacy of the treatment. We will also test different emulsifiers and surfactants (polyglycerol esters, lecithin, and monoglycerides, carriers (organic vs inorganic), and detergent-surfactants (tweens, nonidet P40 and others). This aim will be performed done mostly with corn, but for expediency reasons we will test these on soybean in the growth chamber as well.

<u>Aim 2: Determine the mode of action of lactonase on Cmn</u>. Here we will use RNAseq technology to determine what bacterial genes are responding to treatment with lactonase. This data is crucial to see if a general mechanism

exists, other than AHL quenching, to suppress bacterial diseases of plants caused by gram positive bacteria. We will extract mRNA from Cmn exposed (or not) to lactonase in the lab and on corn plants. We will then do differential gene expression assays to determine which bacterial genes specifically respond to enzyme. In addition to testing this on *Clavibacter michiganensis* subsp. Nebraskensis, we will also test this on *Xanthomonas vasicola* (syn. *X. campestris* pv. zeae).

Aim 3: Determine if lactonase enzyme can protect corn from bacterial diseases in the field, at three locations. Last year had a small field-level project that demonstrated that our technology has the ability to suppress bacterial disease of corn in the field. This was done at Rosemount with the help of Dr. Dean Malvick at UMN. This year we propose to test the efficacy of our lactonase enzyme to suppress disease caused by *Clavibacter michiganensis* subsp. Nebraskensis in replicated field trials done at three UMN Agricultural Experiment Stations located at Lamberton, Rosemount, and Waseca. If enzyme quantities and field space is available, we will also test this enzyme to suppress disease symptoms caused by *Xanthomonas vasicola* (syn. *X. campestris pv. zeae*) on corn.

Deliverables



Collaborators, Partners, In-Kind,

Michael Sadowsky will lead and manage the project, analyze data, and write reports.

Dr. Sadowsky is an expert in plant-microbe interactions, soil microbiology and microbial ecology, as well as applications technology. Dr. Sadowsky will help perform all experiments, and provide key expertise in the required assays, in plant handling as well as using plant infection models. As PI, Dr, Sadowsky will supervise the entire project, co-mentor the research associate Dr. Zhang, and produce reports.

Mikael Elias, Dr. Elias is the inventor of the technology central to this proposal, and an expert in molecular engineering, protein evolution, structural biology and bacterial signaling. Dr. Elias will aid in all experiments, be responsible for co-mentoring the postdoc and for production of all enzymes for plant infection assays.

Education and Outreach Activities

All aspects of the technology as it pertains to plant protection from bacteria pathogens will become part of the curriculum for Microbial Ecology and Applied Microbiology Course (Bio4121) and course packets and powerpoint presentations describing the technology will be provided to Debby Samac, Linda Kinkel, Dean Malvick, and James Bradeen in the Plant Pathology Department. Results from the proposed research will be disseminated via: 1) publications in peer-reviewed journals; 2) extension bulletins (working with Carl Rosen); 3) the quarterly BTI Gateway magazine, and 4) via workshops given to the MN Corn Growers, Soybean Growers, and Wheat Growers Associations. In addition, we will closely work with the Minnesota Area II Potato Growers Research and Promotion Council and The Northern Plains Potato Growers Association to aid in dissemination of our research results and eventual filed testing once state regulatory approval is obtained.

Budget

Total Project Costs: **$76,664**

Table 1. Annual itemized budget.

Item	**FY2020-21**
1. Consultants and Subcontractors	$0
2. Personnel Salaries and Fringe	$59,664
3. Supplies and Materials	$13,000
4. Equipment	$0
5. Travel	$500
6. Communications (Telephone/Mail)	$0
7. Laboratory Analysis (soil, plant, water, manure, nutritional value, etc.)	$500
8. Education/outreach related activities	$500
9. Other costs – field fees	$2,500
Total Funds Requested:	**$76,664**

Personnel. A postdoctoral research associate (Dr. Qian Zhang) will be supported, for 1 year. The researchers will create enzymes and characterize them, assay them on plants and crop infection models, analyze data, and write reports. Full-time salary (100% effort) for a total of 1 years = $48,000. Fringe benefit calculated using current negotiated fringe rate of 24.3% = $11,664

General supplies: $13,000 for the project period (tips, microfuge tubes, gloves, PCR reagents, general chemicals, protein purification, etc.); media. Microbiological analyses, Cell/Enzyme Production costs – The Bioresource (BRC) of BTI will aid in purification of all enzymes for plant assays.
Travel costs ($500): within state travel for presentations, outreach.
Field use and supply fees ($2,500): these funds will be used to grow plants and test our enzymes on infection models.

Reporting and Invoice Schedule

Invoicing and quarterly reporting are linked in the MN Corn contract. Late or missing quarterly reports will delay processing of invoices on the project.

For Activity	**Invoice/Report Date**	
April 1 to June 30	July 31	1st Quarter
July 1 to September 30	October 31	2nd Quarter
October 1 to December 31	January 31	3rd Quarter
January 1 to March 31	April 30	4th Quarter/Final Report

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