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DHS Student Report

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DHS Report – Summer Internship at Lawrence Livermore National Laboratory

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In today's society the threat and implementation of bio-terrorism is a distinct possibility. Although there are a variety of organisms that are likely candidates for modification and distribution, *Bacillus anthracis,* responsible for causing anthrax, is at the top of the list. Already in 2001 with the anthrax scare in the mail and congressional buildings, we have witnessed the fear and response of the population to a bacterial-threat: widespread antibiotic treatment, in particular the Fluoroquinolone Ciprofloxacin. With such wide spread use of an antibiotic it is important to understand how the naturally occurring organism may be resistant. In an effort to characterize the Ciprofloxacin resistance mechanisms of *B. anthracis Sterne*, an avirulent strain of *B. anthracis*, the bacteria are introduced to increasing concentrations of antibiotic. Furthermore, it is important to identify what mechanisms the bacteria at different resistance levels employ and whether they are consistently in the same genomic region, identified as the quinolone resistance-determining region (QRDR). Therefore, at each antibiotic level all pertinent regions of the mutant strains' genomes are sequenced in order to determine the QRDR.

My project at Lawrence Livermore National Laboratory (LLNL) consists of two objectives. First, as we repeat selection experiments to isolate resistant *B. anthracis* strains, are we consistently finding that the same mutations we have previously isolated? Second, from other experiments on various avirulent strains of *B. anthracis* we know that two bacteria, which are resistant to different antibiotic concentration levels, may have the same genome in the identified QRDR. Therefore there may be mutation at other sites in the genome responsible for the increase in resistance, possibly within the four genes we are currently studying gyrA, gyrB, parC, and parE.

Throughout this project I have been involved in every step of the protocol. After proper training, I was introduced to the necessary lab techniques for the project. From then on it has been my responsibility to perform the necessary tasks to identify and isolate the mutants. This includes carrying out a detailed protocol of mixing reagents, streaking and incubating plates, inoculating cultures and evaluating any results in order to guide my actions for the next antibiotic concentration level. Simultaneously, I have been running PCR and sequencing reactions on all mutants in order to obtain the genetic sequence of the genes of interest for comparison. Once I have the gene sequences of interest I am able, with the aid of a sequencing program (Sequencher 4.2.2), to analyze the sequences of the mutants against that of a wild type strain. This entails aligning the DNA sequences of a given gene for each of the mutants and locating any base changes from the wild types bacteria's genes. These polymorphisms allow me to identify the QRDR for that particular gene. Depending on whether the polymorphism occurred at a low antibiotic concentration level or high concentration level, we can evaluate whether that change is necessary for low or high-level quinolone resistance. Finally, I will compare the polymorphisms of each mutant at a given antibiotic selection level and evaluate whether *B. anthracis* consistently acquires resistance through the same polymorphisms or whether the resistance mechanism varies with each new mutant strain.

Currently, I am analyzing the sequence data for stage one mutants, while simultaneously continuing the lab work necessary to select for stage two mutants. After I have left, the personnel at the lab that I've been working with at LLNL will continue this project. By the end of this experiment, we hope to corroborate the suggested

mechanisms of resistance typically employed by *B. anthracis* Sterne at different resistance levels. Furthermore, if the mechanism is determined by one of the following genes: gyrA, gyrB, parC, parE we will be able to pinpoint which base pair changes are necessary for acquiring a given resistance level. Hopefully from these data researchers will be better able to determine an appropriate action should quinolone resistant strains of *B. anthracis* arise in either by natural evolution or selection in a laboratory.

Unfortunately, there were a variety of setbacks with respect to the implementation of the initial project protocol. However, these obstacles gave me insight into the problems, as well as the variety of techniques and resources available to solve them, seen by lab researchers everyday. Many of my accomplishments with respect to this project have arisen from these setbacks. For example, for several weeks we tried to grow the bacteria in liquid cultures, which required shaking them in an incubator. However, the bacteria that arose consisted of odd strings and clumps versus the smooth, cloudy culture necessary for the next step. After several attempts, we were regularly growing cloudy cultures having adjusted not only the tubes the cultures grew in but also the position of the tubes in the shaker.

Another project achievement was selecting the proper plating technique. Since the bacterial cultures were 10 ml, simply pouring the liquid cultures onto a selection plate was not an option. In order to concentrate the bacteria onto the plate and remove excess media we evaluated a variety of filtering techniques. These included using vacuum filtration, multiple syringe filtering techniques, as well as simply gravity-based filtration. The task consisted of concentrating the bacteria evenly onto the filter paper, then placing the filter onto a selection plate. However, achieving this ideal with any of

the filtering options proved extremely difficult, if not impossible. This is partially due to *B. anthracis* being a high-risk infectious bacterium and therefore bio-safety level 2 safety precautions are necessary. Options that would work for filtering a precipitate out of a solution weren't possible because of the biological risk. Once we began to explore techniques other than filtering and growing the bacteria on the filter, we decided to concentrate the culture into a smaller volume, around 1 ml, and plate that culture.

Several other project accomplishments include achieving certain project milestones. We were able to successfully select for ciprofloxacin resistance in the wild type *B. anthracis* st. Sterne (dugway). Thus far we have selected for mutants at two Ciprofloxacin concentration levels, the lower concentration level is referred to as stage 1 and is characterized as roughly 3x the wild type bacteria's MIC. The higher concentration level, stage 2, is characterized as 3x the highest MIC values seen in stage 1 mutants. Specifically we have isolated 13 stage 1 mutants from the first selection concentration. Now we are in the process of isolating second stage mutants, i.e. plating stage 1 mutants on the higher stage 2 Ciprofloxacin plates and isolating any mutants, which appear. One concern with this project was its ability to increase exponentially at any selection stage, i.e. each one of those 13 mutants could yield another 24 mutants meaning stage three selections would need to be conducted on 312 mutants. I believe my primary accomplishment with respect to this project has been in keeping an organized record of all mutants and easily being able to trace their lineage. As the project continues this organization will be essential to understanding when and how high-level resistance is acquired and which polymorphisms are most likely responsible for them.

As far as sequencing the genes of the resistant mutants, the primers that were designed and ordered prior to my arrival appear to accurately isolate the exact section of the four genes of interest for Ciprofloxacin resistance. My personal achievements in this area have been in efficiently and accurately performing PCR reactions so as to amplify the required gene sequences without contamination. Likewise, the sequencing of the PCR products requires careful technique and organization to obtain quality data.

Beginning this project, I had a very basic understanding of bacteriology and sequencing. As I have completed various steps in the project I have been able to research the background on *B. anthracis* in general as well as specific techniques using journal articles and textbooks as my resources. Now, I have an understanding of the accepted theories on microbiology, resistance mechanisms, antibiotic mechanisms, and genetics that I consider equivalent to having taken a college seminar.

I consider myself extremely fortunate to be completing my internship with LLNL not only because of my project but also because of the seminars and opportunities that a large, well-respected national lab has to offer. Throughout the past 10 weeks I have attended a minimum of one if not several seminars in addition to the weekly DHS intern briefings. These seminars have ranged in topic, including understanding and geographically tracking *Yersinia Pestis*, the plague, across the United States as well as building genetic databases of viruses which are quickly accessible for comparison allowing researchers to determine whether the virus is novel. Other seminars are more bio-terrorism focused, for example, I attended a very intriguing lecture on micro-fluidics based bio-detection. That particular lecture was geared towards the student engineers at the lab and demonstrated how the technology we are developing is being

implemented across the country in order to increase security. Since this is such a diverse lab though, there has been ample opportunity to attend lectures outside of my area of specialty. Some examples include explosives, nuclear weapons, lasers, x-ray detection devices, global warming, and the energy crisis. It was very interesting to hear the Secretary of the California Environmental Protection Agency speak about the actions her office is organizing in order to reduce pollution and energy consumption. What was fascinating was her emphasis that regardless of the technology the real struggle for energy-conscious states has been implementation and enforcement of regulations.

The weekly seminars organized by the University Relations department designed for the DHS interns highlighted a number of DHS projects around the lab, some of which students are actively working with. I found this to be a valuable source of information on the current research projects of interest to DHS, as well as, noting the variety of specialties involved in each project. From these lectures, I have determined that regardless of my major in college, there is a large variety of projects I may find myself working on in the next five or ten years.

Beyond lectures, I was able to participate in two field trips and plan on attending a third. The first trip was to an estate owned by Jacques Littlefield. Mr. Littlefield is the owner of roughly 250 restored military tanks. All of the tanks are mobile, though unable to participate in combat due to safeguards in the weapons, yet the few tour groups admitted are encouraged to climb on and into the tanks. It was fascinating to learn about the advances in technology made since World War I and II, as well as what tanks were particularly suited for warfare in a region or against a specific military attack tactic.

Once again, I was reminded of the interplay between the research lab and real-world implementation that is necessary in order to produce a product or knowledge base that is useful. A tank with treads that last for hundreds of miles on a track in a lab, may last only 50 miles out in the desert heat and sand. Likewise, a bio-agent detection device may operate within an acceptable error limit when fed pure samples in a sterile lab, but in the city streets such an environment is not feasible. It is our job as scientists to recognize and incorporate environment and the end usage into our prototypes, which I feel has been appropriately emphasized throughout my time at LLNL.

My second field trip was to the Joint Genome Institute (JGI) and relates directly to my project. After each antibiotic selection stage I sequence the mutant's genes of interest and the purpose of JGI is to sequence organism's entire genomes. They select these genomes from a variety of requests from scientists around the country who are able to justify why a certain bacteria or plant's genome is of special interest. Although the JGI's general process for mapping the genome is the same process I employ, they have essentially taken a manual, time consuming system and automated it. Their assembly line organization combined with the widespread use of robots has exponentially increased their production. While touring the lab, I was constantly surprised by the degree of automation and how extremely high-throughput their system truly is. Although the explanation of the science behind the sequencing was familiar to me, the last presentation of the field trip was interesting as it detailed the projects, such as bioengineering trees so that they may be planted as a fertile crop that will later be harvested and used as bio-based fuel. Lastly, even though I am familiar with the online

genetic databases, I was pleased to note their continued dedication to publishing all genomes they sequence allowing the public access to the information.

Finally, the third field trip I am schedule to attend is to LLNL's Site 300. This is the experimental explosives test site. Primarily the site was established in order to support nuclear weapons research, currently though there is ongoing research regarding other high-level explosives as well as their components. For instance, site 300 tests explosive material designed by the chemistry department in Livermore. In addition, the site is responsible for conducting different stress tests on weapons components such as operational temperature ranges, vibration thresholds and shock recovery rates. This research allows researchers to predict the lifetime of these components and develop technology to extend that lifetime given specific operating conditions. Like the tank farm field trip, my particular project at LLNL is not related to weapons research, but since many of the lab's directives are weapon's based, I feel as though attending this trip will allow me to gain a better understanding of the variety of research at LLNL.

I have been exposed to a range of projects throughout my time at LLNL between attending seminars and chatting with the other DHS scholars interning at the lab this year. The majority of these projects are already funded in part by DHS grants, giving me a sense of the categories of projects, which fall under the DHS mission. I believe that the next set of projects that should be encouraged will emerge from smaller, basic science projects such as the one I am currently working on. The results of these experiments give other researchers the background information to develop detection

devices, weapons, defense systems, and treatment protocols necessary to protect the United States.

Overall, I consider my time at LLNL to be well-spent. I have been exposed to the cutting edge research on across the scientific spectrum. Much of which due to my course load, I would never be able to learn about by taking a seminar on at school, like explosives and micro-fluidics. Furthermore, my research project regarding antibiotic resistance has exposed me to a variety of new lab techniques including culturing bacteria and sequencing genomes. I now understand the biological mechanisms of *B. anthracis* and why *B. anthracis*, both wild type and resistant forms, are the focus of so many research projects. Finally, unlike with research labs at school, working at the national lab level has exposed me to the procedures and projects common to labs outside of academia. As a DHS scholar, I have been able to conduct research for the past 10 weeks with potential for real-world application, while simultaneously supporting the DHS mission directive.