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Summer of DHS-Sponsored Summer Internship
DATE

Description of Summer Project and Research

Introduction

This summer, I spent ten weeks conducting research at Sandia National Laboratories in Livermore, California. I worked with Steven Branda and continued a project previously worked on by Mandy Jokerst & Pam Lane. The project has a long term goal of creating and perfecting a rapid assessment of spore viability, a goal that meshes well with the goals of the Department of Homeland Security (DHS). The interaction of this rapid assessment of spore viability project with the DHS lies in the bio-weapon community and its response to a bio-weapon attack with an agent such as anthrax. Effective response to this type of attack requires rapid assessment of not only the pathogens identity but also its viability.

Currently, the assessment of a pathogen's viability, in particular, has proven problematic to the bio-defense community. The currently available rapid onsite assays for pathogen identification rely upon PCR or immunohistochemistry, and thus cannot distinguish between viable and nonviable spores. The CDC-endorsed "gold standard" assay for spore viability is simple outgrowth of spores in culture, an approach that is time-consuming and not amenable to automation or portability. Recent efforts by other investigators have focused on monitoring the very earliest events of spore germination, in which there is a release of dipicolinic acid (DPA) as well as loss of membrane potential and impermeance to chemicals; these efforts have been fraught with false

positive problems, typically because disinfection treatments often render the spores inviable and yet capable of completing these early stages of germination.

To address this problem, we are developing a field-able microfluidic device for rapid assessment of a spore's identity and viability. Our approach is based on recognition of the spore's proteomic signature. Viability is assessed through detection of early protein synthesis induced upon exposure to a chemical germinant such as DPA or amino acids. The sensitivity of our method should enable assessment of spore identity within minutes, rather than the days required by the outgrowth approach, and importantly, spore viability is assessed simultaneously.

In support of this effort, my research has focused on optimizing conditions for spore germination and its assessment, using SDS-PAGE to detect changes in proteomic signatures induced upon germination. Following a standard protocol for spore germination, manipulations were made at different steps in attempt to optimize every step of the germination process. Through this work we are converging on a standard protocol for rapid, robust, reproducible assessment spore identity and viability.

Methods

As a starting point for my research we focused our efforts on the spores produced by *Bacillus cereus* and *B. subtilis*, two species which are closely related to the causative agent of anthrax, *B. anthracis*. To get us started on this research quickly we purchased pre-existing preparations of *B. cereus* and *B. subtilis* sporesfrom Raven Labs. Based on reports in the literature, preliminary work carried out at Sandia National Labs by Mandy Jokerst and Pam Lane, and my own work this summer, we developed a “consensus” germination protocol.

This protocol included a heat activation step in which the spores are incubated at 70-80˚C for 15-30 min, then let cool on ice for ~15 minutes soon after. This is one step that was manipulated through my experiments in order to find the optimal combination of incubation temperature and time. The spores were then recovered by centrifugation (~10,000 x g for 5 min) and resuspended in either water or a germinant. While the centrifugation and recovery was fairly standard, the germinant was another step in the protocol that was manipulated. Different types of germinant were experimented with to identify the optimal germinant and concentration for spore germination. The spores were then incubated at 37°C for pre-designated amounts of time (typically 5, 20, and 60 min) in order to promote germination. At the pre-designated time-points, the spores were recovered by centrifugation and resuspended in 2X Tris-Glycine running buffer (Invitrogen LC2675) plus a reducing agent. This is a step in the protocol that I manipulated for optimal germination assessment results. Different reducing agents were tested in order to observe which reducing agent was best at enabling the spore proteins to enter the SDS-PAGE gel and separate out crisply, for a robust and characteristic proteomic signature. After resuspension, the spore samples were incubated at 50-100˚C for 10-30 min in order to denature the spore proteins. This combination of denaturation incubation temperature and time was another point in whichmanipulation was not only possible, but also helpful, for assessment of germination. After denaturation the spore proteins were loaded onto a 10-20% Tris-Glycine acrylamide gel (Invitrogen EC61352BOX) in which ~150 volts were applied for ~1 hr in order to separate the spore protein products. This step remained unchanged throughout the experiments, as it was unlikely that any change in the electrophoresis

procedures would have any noticeable effect on the protein signatures. The proteins were then stained in the gel for visualization. Different staining kits were experimented with to determine which combination was the most effective, in terms of both resolution and sensitivity.

My Role

In the work that preceded my internship, the heat activation step had been manipulated to some degree, and the protocol at that point called for heat activating the spores at 80˚C for 15 minutes with no following ice step. After many experiments as well as a closer look at the relevant literature, we decided to experiment with spores that were heat activated at 70˚C for 30 minutes. This also was combined with letting the spores cool immediately after on ice for 15 min. This manipulation did not show an overwhelming improvement in the germination assay;, however it did not have a negative effect on the experiment, and this in combination with the literature led us to continue heat activating the spores at 70˚C for 30 minutes.

The next manipulation came in the type of germinant that was introduced to the spores upon resuspension. Initially, all of my experiments were performed using a 60 mM DPA-$CaCl_2$ mixture, but later on, for the sake of optimization, I tested someamino acid germinants as well. These amino acid germinants included 10 mM L-alanine or L-valine in 25 mM Tris-HCl pH 7.4. This change in germinants was considered due to the fact that the DPA-$CaCl_2$ mixture continuously crashed out of solution, a fact that made us suspect that the spores were not actually experiencing a 60 mM concentration of that germinant. Again, no major differences were detected when amino acid germinants

were used instead, leading us to continue on with the DPA-$CaCl_2$ mixture as our germinant of choice.

After the pre-designated germination times, the spores were recovered and resuspended in a 2X sample-loading buffer along with a reducing agent. This is an important step in the protocol, as it helps determine how efficiently the spore proteins enter the SDS-PAGE gel and separate out during electrophoresis. I experimented with the reducing agents BME, DTT, and TCEP. In this case, significant differences were seen between spores with no reducing agents and spores with reducing agents; clearly, a reducing agent is necessary for this step. However, there were not significant differences between the different reducing agents. Based on this work this we decided that TCEP was a suitable reducing agent and continued on using TCEP.

In the denaturation step, similar to the heat activation step, a combination of temperature and time was manipulated with. Again, based on the available literature, we decided to experiment with different combinations that ranged from 50-100˚C for 10-30 min. The most effective combination seemed to be a denaturation temperature of 100˚C for a time of about 15 minutes.

The last manipulation in the protocol came in the staining of the proteins in the gel. Initially the gels were being stained with a Coomassie blue staining solution (Invitrogen LC6060). After realizing that the a silver staining kit (Thermo Scientific 24597) had the potential to give much better sensitivity than the Coomassie blue staining, we gave that a try and found that it substantially helped the protein signature results in terms of both sensitivity and band resolution.

Milestones and Accomplishments

Through ten weeks of experimentation, many conclusions can be made about the early spore germination step of the rapid assessment of spore identity and viability project. Due to the fact that not many differences were discovered between spores that were subject to a germinant and spores that were not, it is possible that the spores are simply not being germinated and that the heat activation step was ineffective. This is a major point in this experiment due to the fact that it could in fact be the spores themselves that need to somehow be manipulated rather than the germination protocol. Another major conclusion that was discovered was the fact that although the silver staining was much more time consuming and finicky than the Coomassie blue stain, it was well worth the extra effort in order to produce a much more sensitive and defined protein signature. Another accomplishment in all of this research was our ability to minimize the amounts of spores per experiment. Initially we were using two types of spores, and each at 100 ul per treatment. We are now able to produce similar results with only 50 ul of a single type of spore, cutting our spore usage down to a quarter of the original, a big cost savings.

Impact on Academic/Career Planning

Prior to beginning my internship at Sandia National Labs this summer I had not really been exposed to a laboratory setting other than at my university. I quickly found out how different a laboratory setting can be when the experiments aren't as structured and definitive as they are in college labs. In my college labs I did not enjoy experimentation so much only because the results were known, and rather than observing new developments and attempting to figure out the cause of them, you simply had to restart an experiment that was not going according to plan. In the laboratory

setting that I was exposed to here at Sandia National Labs I was pleased to realize that new developments are not only very common, but also fully supported. Inventiveness and curiosity thrive in this type of setting where results are unknown as well as deeply prized. This freedom in my research helped me realize what a laboratory setting is really like outside of a structured class setting.

Through most of my academic career I have wanted to follow the medical path of science and practice as a medical doctor. I was lucky to have a father that works in the field which exposed me to the nature of this career. I had never been exposed to the laboratory setting and the research aspect of science, and thus had never really shown much interest in pursuing research as a career, however my internship this summer was a very eye-opening experience for me. This internship gave me the exposure and experience that I lacked throughout my academic career and opened up a whole new window of opportunity for me and my future direction. Being that I am half way through my undergraduate career, I feel that I am in perfect position, with exposure to not only the medical aspect of science, but now also the research aspect, to decide how I want to continue my education and in which direction I which to point my future career.

During my time here at Sandia National Labs I was able to attend a lecture on the history of nuclear weapons. Not only was this extremely interesting, but it was also very educational as it made me realize the potential that there is for advancements of weapons. Since the first nuclear bomb there have been tremendous advancements in modern weapons and there doesn't seem to be an end in sight thus far. I found it interesting how this lecture tied into my project as well, however instead of nuclear weapons I was dealing with a potential bio-weapon. I could relate to the potential and

danger that nuclear weapons have with the potential and danger that a bio-weapon such as anthrax spores have, and the types of preventative regulations and responses needed. I learned about the many security measures that are in place, all brought on by the Department of Homeland Security, in order to protect our country.

In addition to this lecture, Sandia was helpful in networking with all types of people with interests in many different areas from across the country. Networking plays a major role in future planning, and I believe that nothing has been more helpful from these ten weeks than then people I have come across throughout my experience.

My mentor throughout this summer internship was Steven Branda and I credit all of my optimism towards research as well as my pleasure throughout this summer to him. There were many informal discussions between the two of us that I feel had the biggest impact on me. Steve and I discussed his academic path in great detail as well as the options open for myself and different routes that are possible in my future. It was very helpful to be able to level with someone who understands what it means to be in my position. Steve was available for help in every way that I needed and really went out of his way to make sure that my summer internship was not only a success for Sandia National Labs, but also a success for me and a person. I am still unsure as to what my academic and career future has in store for me, but Steve has been a great resource for me, and hopefully this is not the last time we cross paths.

Benefits

The benefits of this summer internship at Sandia are endless and immeasurable. I now have experience that I would never have had to help influence my future. I was able to work at a top level laboratory and get a feel for what exactly it means to do

research. Not only did I benefit with personal experience, but also with technical experience in the lab, andI am sure that these will prove helpful in my future in science. I have gained some expertise in different scientific techniques that are useful in all types of research and laboratory settings. Again, the networks that I gained through all of the Sandia sponsored events, as well as just through everyone that made this possible, have been the biggest influence on me. I thank the Department of Homeland Security for providing me the resources and opportunity to have this summer internship, and I sincerely hope that this is not the last experience that I share with them.