Study of the structure and function of a novel bacterial virulence factor isolated from *Francisella tularensis*

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During my internship at Lawrence Livermore National Laboratory (LLNL), I studied a novel virulence factor of the potential bioterrorism agent, *Francisella tularensis*. This bacterium is a facultative intracellular pathogen with a broad host range, and is known to be the causative agent of the zoonotic disease tularemia (1). *F. tularensis* is highly infectious, requiring only 1 to 10 bacteria to cause severe disease (2). Due to its high rate of infectivity, *F. tularensis* has potential use as a bioterrorism agent. As a result, it has been categorized as a class A biothreat pathogen by the Centers for Disease

Control and Prevention (3).

Although the bacteria is transmitted from vector to host, the bacterial reservoir has not yet been identified (4). Reports indicate that *F. tularensis* is often isolated from water and soil sources, which is a known habitat of environmental amoebas. A previous study demonstrated that pathogenic strains of the bacteria are able to survive and persist within the amoeba, *Acanthamoeba castellani,* which suggests a potential link between *F. tularensis*-amoeba interactions and its ability to persist in a wide range of environments (5). More recently, persistence within *A. castellanii* was characterized by induction of a rapid encystment phenotype (REP) (6). Encystment in amoeba occurs as a survival measure against starvation, desiccation, and other harsh environmental conditions (7). This study found that induction of rapid amoeba encystment by virulent *F. tularensis* strains was essential for its survival and that the pathogenic strains were capable of blocking lysosomal fusion, a highly regulated event essential for the intracellular killing of microorganisms, in *A. castellanii* (6). These findings are direct evidence of the bacteria's ability to persist in the environment. Moreover, seven soluble protein factors from *F. tularensis* were identified as potential virulence factors involved in the induction of rapid encystment.

Of particular importance in the current study is one virulence factor that was found to be an active protease that contributes to cytoskeletal rearrangement, which may be one mechanism by which the bacterium persists within *A. castellanii*. Prior to my arrival, X-ray crystallography was utilized to solve the atomic resolution structure of the native protein.

X-ray crystallography involves bombarding a crystallized molecule with X-rays, and then detecting the scattered radiation from the electron clouds of atoms in the crystal lattice to produce a diffraction pattern (8). This diffraction pattern yields information regarding the amplitude of the diffracted rays, but not their relative phases (8). This is known as the phase problem in X-ray crystallography. In order to generate an electron density map of a molecule, the phases of the diffracted rays must be determined. An electron density map is a three-dimensional representation of the atoms in space, and is vital for structural determination. Crystallographers utilize this map and the known sequence of amino acids, the building blocks of protein, to construct a protein structure. There are many different methods to determine the phases. Some of these methods include Multi wavelength Anomalous Dispersion (MAD) and Molecular Replacement (MR). The MAD method involves the incorporation of heavy atoms, such as selenomethionine, into the molecule of interest so that when bombarded with varying wavelengths of X-rays, the resonance centers of the heavy atoms produce unique scattering patterns that yield the phases of the diffracted rays (8). The most commonly used technique is Molecular Replacement. This technique utilizes previously solved protein structures of homologous proteins to determine the phases, and thus, generates the electron density map for a protein (8).

Once structure determination was complete, comparison of the native protein's atomic resolution structure with other structures of proteases in the same class allowed for identification of a putative active site and catalytic amino acid residues involved in protease activity. Single and double amino acid mutations were made to the putative catalytic residues, and the activity of each of the mutants was tested. Two single amino

acid mutants and one double mutant demonstrated the largest decrease in protease activity.

My role in this project was to use crystallographic data obtained for each of these mutants to solve their atomic resolution structures. The overall goal was to determine whether the decrease in activity for these mutants was due to the fact that the mutations disrupted the catalytic mechanism and not the local or global structure. If the structures remained the same, with the exception of the single/double amino acid mutations, then they would corroborate the evidence that each of these amino acids contribute to the catalytic activity of this protein.

I was also given the task of refining the structure of the native protein covalently linked to a known protease inhibitor. Activity assays demonstrated that protease activity is abrogated in the presence of the inhibitor. Therefore, solving the atomic resolution structure of the protein bound to this inhibitor is essential for validating the experimental data that describes this novel virulence factor as a protease. Additionally, knowing how the inhibitor fits into the protein's active site would reveal binding pockets and information about binding specificity, which is essential for design of a novel drug inhibitor against this virulence factor. Structure refinement occurs after the preliminary structure has been built, and is the process in which the atomic resolution model is adjusted in order to improve its agreement with the measured diffraction data. The tricky part about this structure was modeling the covalent link of the inhibitor to the amino acid residue in the active site. Dr. Geoffrey Feld, a post-doctoral fellow at LLNL, is still working on modeling the inhibitor into the active site. I primarily worked on structure

refinement by adjusting amino acid sidechain rotamers and by placing solvent (water molecules) into the model.

As a side project, I also worked with Dr. Amy Rasley, an immunologist, on some preliminary experiments to visualize the protein interacting with cytoskeletal filaments using fluorescence microscopy. We labeled a native and mutant form of the virulence factor of *F. tularensis* with a red fluorescent dye, AlexaFluor568, and then incubated each of them with human epithelial cells containing green-fluorescent protein (GFP) labeled actin, a cytoskeletal filament. Fluorescent microscopy was utilized to observe any protein-actin interactions over a period of time. Observing protein-actin interactions in a human cell-line would provide evidence of the virulence factor's target in the human host. This information would lead to the definition of the protein's mechanism of action, which is vital for novel drug or vaccine design against the pathogen.

I came into this project having previously solved only one, low resolution structure of a very large protein using X-ray crystallography. Through this project, I learned how to use a different crystallography program suite, PHENIX, to solve high atomic resolutions structures using a technique called Molecular Replacement, and how to refine these structures with this same software. Geoffrey taught me a lot about the PHENIX program suite that I was using for structure refinement. He also was a tremendous help in tackling the crystallographic data of the virulence factor in complex with its inhibitor. He taught me several new refinement and modeling techniques for placing the small molecule inhibitor into the protein's active site.

I have been able to polish the skills I gained in my undergraduate research

experience to a level where I feel comfortable working with crystallographic data independently. This experience has boosted my confidence in my knowledge of X-ray crystallography, and will certainly allow me to excel in graduate school as I begin laboratory rotations with other crystallographers. Additionally, working on the fluorescent microscopy experiments refreshed my knowledge of mammalian cell culture—a skill I learned as a first year in college.

On the topic of macromolecular crystallography, Dr. Mark Hunter, another post-doctoral fellow at LLNL, taught me the theory behind crystallization, several new protein crystallization techniques that I had not known before, and how to use a robot to set up crystallization trials. I have gained knowledge on not only the subject of macromolecular crystallography but also on the subject of nanocrystallography. Traditionally, macromolecular crystallography uses macrocrystals of a protein to determine its macromolecular structure. However, a majority of proteins that can be crystallized are unable to be crystallized as macrocrystals, which presents a major bottleneck in crystallography. Recently, this obstacle has been overcome through the use of a hard X-ray free-electron laser (XFEL), the Linac Coherent Light Source (LCLS) at the SLAC National Accelerator Center. A revolutionary study demonstrated that by using a fully hydrated stream of nanocrystals, which are easily generated for most proteins, single-crystal X-ray diffraction snapshots were collected using femtosecond pulses from the XFEL (9). After collecting more than three million diffraction patterns, a three-dimensional data set was assembled from these nanocrystals. This study presents a new approach for the structure determination of important and interesting macromolecules that do not yield large enough crystals for traditional macromolecular

crystallography. The group I worked with this summer aims to use this novel technique to capture “molecular movies”, or atomic-resolution dynamical imaging of various biomolecular interactions.

In addition, weekly project group meetings I attended allowed me to practice public speaking and presentation skills. I learned how important it is to collaborate with various research groups, specialized in different fields, to maximize the depth of information gleaned from a research project. I was also given insight into the intensive planning and group effort it takes to prepare a project proposal to receive funding. The weekly Department of Homeland Security (DHS) seminars demonstrated the strong connection between the missions of DHS and LLNL. Early in the summer, I attended a movie presentation called the “History of Engineering” that helped me understand the original purpose and goals of research conducted at the laboratory, and how those goals have changed over time.

Aside from attending meetings and talks, I also participated in an hour-long tour of the National Ignition Facility (NIF) housed on-site at LLNL. NIF became operational in March of 2009 after more than ten years of planning and building. It is the site of the world’s largest laser. It consists of 192 laser beams that are capable of directing almost two million joules of ultraviolet laser energy to a target—60 times the energy of any other laser system. When these lasers focus onto millimeter-sized targets, unprecedented temperatures and pressures are generated within the target materials. These temperatures far exceed 100 million degrees and pressures more than 100 billion times the Earth’s atmosphere. These conditions are similar to those found in

stars, planets and nuclear weapons. One of the main goals of the facility is to produce fusion ignition. Currently, nuclear power plants utilize fission, or the splitting of heavy atoms, to generate energy for electricity. Fission produces greenhouse gas emissions as well as radioactive byproducts. Fusion, on the other hand, fuses atoms of deuterium and tritium, isotopes of hydrogen that are readily available in the environment, to generate energy. Because fusion does not produce hazardous byproducts, it is a key factor in our nation's long-term energy plans. Other experiments conducted at NIF aim to study the complex physics of nuclear weapons, particularly how the materials that compose these weapons perform under extreme conditions. During my tour I was able to get a glimpse of the assembly and control rooms along with the outside of the target chamber. Our tour guide was a very knowledgeable NIF employee, and provided us with in depth information about the facility and its inner workings. A couple of weeks after my tour, NIF achieved a 500-trillion-watt laser shot, the largest ever of its kind. This result serves as a testament that nuclear fusion is not far behind. It was certainly a privilege to have the opportunity to tour a facility conducting research at the frontier of nuclear technology!

Overall, I have helped solve four high atomic resolution structures of virulence factor mutants and have helped refine one structure of the native virulence factor in complex with an inhibitor. These structures will be deposited into the Protein Data Bank (PDB), and will be used for a publication describing this novel virulence factor. I found that the structures were conserved between the four mutant proteins, meaning that any change in protease activity can likely be attributed to the amino acid mutations made to the active site. The structure of the protein in complex with the inhibitor is still a work-in-

progress. I also began preliminary experiments attempting to visualize the virulence factor interacting with intracellular cytoskeletal components using fluorescent microscopy. These results will form the basis for future experiments studying the function and activity of this protein.

After working at LLNL, I feel like I could fit into the national laboratory setting. I believe I would bring a sense of urgency to the laboratory, which would positively impact the productivity of research projects. My internship also helped me realize that I enjoy working in a “wet” laboratory more than working in a “dry” laboratory (on the computer). Although I still enjoy the field of crystallography, working with Dr. Rasley has inspired me to further explore the fields of microbiology and immunology. I now plan to add a laboratory rotation involving pure microbiology/immunology to my graduate training. Furthermore, my internship has made me realize that I would like to work at a national or federal lab more devoted to the biosciences. I believe that a places like the National Institutes of Health (NIH), the United States Army Medical Research Institute for Infectious Disease (USAMRIID), or the Centers for Disease Control and Prevention (CDC) would better suit my research interests. In all, my experience at Lawrence Livermore National Laboratory has been invaluable. Not only have I gained insight into working at a national laboratory, but I have also gained a better understanding of my skills, future research interests, and career goals.

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