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Investigating the"Trojan Horse" Mechanism of Yersinia pestis Virulence

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LDRD 04-ERD-101

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Introduction

Yersinia pestis, the etiological agent of plague, is a Gram-negative, highly communicable, enteric bacterium that has been responsible for three historic plague pandemics [1,2]. Currently, several thousand cases of plague are reported worldwide annually, and *Y. pestis* remains a considerable threat from a biodefense perspective. *Y. pestis* infection can manifest in three forms: bubonic, septicemic, and pneumonic plague. Of these three forms, pneumonic plague has the highest fatality rate (~100% if left untreated), the shortest intervention time (~24 hours), and is highly contagious. Currently, there are no rapid, widely available vaccines for plague and though plague may be treated with antibiotics, the emergence of both naturally occurring and potentially engineered antibiotic resistant strains makes the search for more effective therapies and vaccines for plague of pressing concern.

The virulence mechanism of this deadly bacterium involves induction of a Type III secretion system, a syringe-like apparatus that facilitates the injection of virulence factors, termed *Yersinia* outer membrane proteins (Yops), into the host cell [3]. These virulence factors inhibit phagocytosis and cytokine secretion, and trigger apoptosis of the host cell [4]. *Y. pestis* virulence factors and the Type III secretion system are induced thermally, when the bacterium enters the mammalian host from the flea vector, and through host cell contact (or conditions of low Ca^{2+} *in vitro*) [5]. Apart from the temperature increase from 26°C to 37°C and host cell contact (or low Ca^{2+} conditions), other molecular mechanisms that influence virulence induction in *Y. pestis* are largely uncharacterized.

This project focused on characterizing two novel mechanisms that regulate virulence factor induction in *Y. pestis*, immunoglobulin G (IgG) binding and quorum sensing, using a real-time reporter system to monitor induction of virulence [6]. Incorporating a better understanding of the mechanisms of virulence and pathogenicity into detection systems, may allow us to anticipate both natural and engineered evolution of infectious diseases while laying the foundation for next-generation detection of biothreat agents.

Mission Relevance

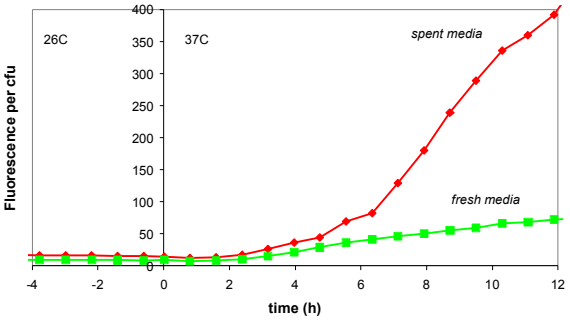
This project represents a national security investment that ultimately will help detect the presence of biological warfare/bioterrorist threat agents and genetically modified organisms. Our research complemented existing LLNL programs in counterbioterrorism and built on LLNL's expertise in genomics, proteomics, biocomputing, instrumentation, and national security. The project firmly established a novel virulence regulatory mechanism in *Y. pestis* and methods will be useful for future applications relevant to national security, environmental management, and biotechnology missions in the Department of Homeland Security and the DOE Office of Science.

Accomplishments and Results

Specifically, we proposed that *Y. pestis* is able to evade host defenses by using the host's immune response to the pathogen's advantage by binding antibodies in the reverse orientation. Thereby, the pathogen is able to present as "self" rather than "foreign" long enough for a quorum of bacteria to coordinate a full attack on the host. This was based on preliminary evidence from real-time characterization of virulence demonstrating that quorum sensing molecules and human antibodies increase virulence factor levels. Under this LDRD project, we firmly established the involvement of IgG and quorum sensing in *Y. pestis* virulence; have 2 manuscripts in preparation [7, 8]; have established a Systems Biology view of virulence through bioinformatics combine with ongoing "Omic" studies of *Y. pestis*; and have submitted an NIH proposal [9].

Figure 1. Quorum sensing molecules induce virulence in *Y. pestis*

The effect of spent media on the induction of virulence in *Y. pestis* was tested with the expectation that spent media contains molecules secreted by the bacteria that are involved in quorum sensing. Here, spent media is defined as a 1:1 dilution of fresh:used media. The rate of increase of the induction of virulence factor genes was greater when the experiment was performed in spent media than when performed in fresh media (**Fig. 1A**). This raised the possibility that quorum sensing molecules may be present in spent media and might further play a role in the expression of virulence factors in *Y. pestis*. Therefore, the presence of acylhomoserine lactone (AHL) quorum sensing signaling molecules in spent media was analyzed using liquid chromatography and tandem mass spectrometry. The known quorum sensing molecules N-3-oxo-hexanoylhomoserine lactone, N-3-oxo-octanoylhomoserine lactone, N-hexanoylhomoserine lactone (C6-HSL) and N-octanoylhomoserine lactone (C8-HSL) were found in the extracts of spent media [7]. The effect of purified AHL molecules on the induction of virulence factor genes was then tested in the real-time reporter assay. Fresh media was supplemented with either C6-HSL (**Fig. 1B** blue, C8-HSL (**Fig. 1B** green), or a combination of both molecules (**Fig. 1B** red). Media supplemented with AHLs augmented the thermal induction of virulence factors to a greater extent than fresh media lacking these molecules.



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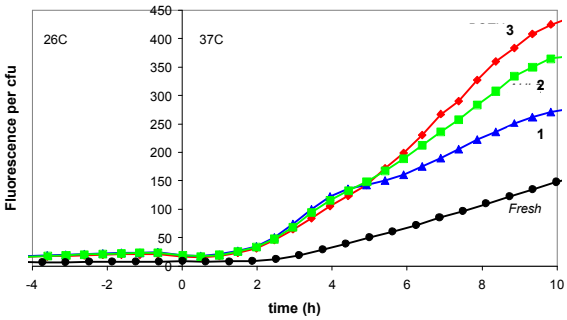

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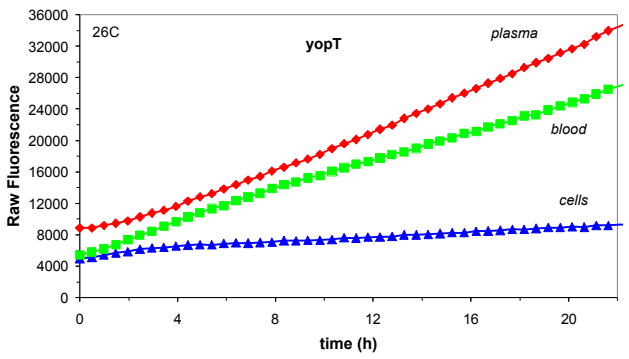
Figure 2. Serum IgG induces virulence in *Y. pestis*

The effect of human blood components on the induction of virulence in *Y. pestis* was also rigorously tested using the real-time reporter system. Strikingly, the induction of virulence was observed with addition of whole blood to *Y. pestis*, unlike previous reports of virulence induction in bacterial media observed only after temperature shift from 26°C to 37°C. In order to determine the component of whole blood responsible for the induction at 26°C, blood was fractionated into plasma and cellular components by centrifugation. The effect of the addition of either whole blood, plasma, or blood cells was assayed. This experiment demonstrated that plasma, rather than the cellular fraction of whole blood, stimulated virulence factor induction at 26°C (**Fig. 2A**). This effect was independent of the addition of 4mM $CaCl_2$ to the media. Though the composition of human plasma is extremely complex, one of the most abundant classes of plasma proteins are immunoglobulin molecules. Thus, the effect of human IgG compared to human IgM on virulence induction was analyzed using the real-time reporter assay. We determined that human IgG but not IgM stimulates virulence induction (**Fig. 2B**). Preliminary results were confirmed demonstrating that *Y. pestis* is able to bind antibodies in the "reverse" orientation, thus presenting as "self" rather than "foreign", which may explain the "Trojan Horse" attack capability of this pathogen. Detailed mechanistic studies have been proposed to the NIH [9] to further address this novel virulence mechanism.



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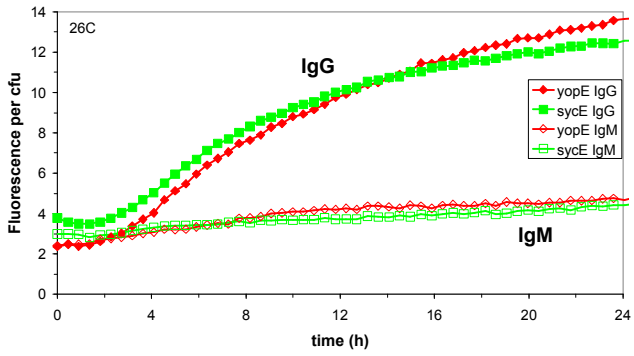

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Summary

This LDRD project supported 1) bioinformatics and advanced statistical analyses for "Omic" virulence studies, 2) technical molecular biology support for the real-time expression studies, and 3) technical writing for manuscripts and an NIH grant submission. All proposed goals were accomplished and are expected to result in externally funded projects. Success of this project is expected to have far-reaching and considerable impact on the study of *Y. pestis* specifically, virulence mechanisms in general, and biodefense preparedness from the practical aspect of detection.

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References

1. Perry, R.D. and Fetherston, J.D. Yersinia pestis--etiologic agent of plague. *Clin Microbiol Rev*. (1997). 10: 35-66.
2. Inglesby, T.V., Dennis, D.T., Henderson, D.A., Bartlett, J.G., Ascher, M.S., Eitzen, E., Fine, A.D., Friedlander, A.M., Hauer, J., Koerner, J.F., Layton, M., McDade, J., Osterholm, M.T., O'Toole, T., Parker, G., Perl, T.M., Russell, P.K., Schoch-Spana, M., and Tonat, K. Plague as a biological weapon: medical and public health management. Working Group on Civilian Biodefense. *Jama*. (2000). 283: 2281-2290.
3. Cornelis, G.R. Molecular and cell biology aspects of plague. *Proc Natl Acad Sci U S A*. (2000). 97: 8778-83.
4. Cornelis, G.R. Yersinia type III secretion: send in the effectors. *J. Cell Biol.* (2002). 158: 401-408.
5. Cornelis, G.R., Boland, A., Boyd, A.P., Geuijen, C., Iriarte, M., Neyt, C., Sory, M.P., and Stainier, I. The virulence plasmid of Yersinia, an antihost genome. *Microbiol Mol Biol Rev*. (1998). 62: 1315-52.
6. Forde, C.E., Rocco, J.M., Fitch, J.P., and McCutchen-Maloney, S.L. Real-time characterization of virulence factor expression in Yersinia pestis using a GFP reporter system. *Biochem. Biophys. Res. Commun.* (2004). 324: 795-800.
7. Clatworthy, A., Forde, C.E., Fitch, J.P., and McCutchen-Maloney, S.L. Quorum sensing regulates virulence-factor expression in *Yersinia pestis*. In preparation for *Journal of Bacteriology* (planned submission March 2005)
8. Clatworthy, A., Forde, C.E., Fitch, J.P., and McCutchen-Maloney, S.L. The 'Trojan Horse' mechanism of *Yersinia pestis* virulence. In preparation for *Science* (early stages of preparation)
9. Clatworthy, A., and McCutchen-Maloney, S.L. NIH grant "Novel Virulence Regulatory Mechanisms of *Yersinia pestis*". Submitted to the National Institute of Allergies and Infectious Diseases, February 2005.