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Multi-Probe Investigation of Proteomic Structure of Pathogens

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Auspices Statement

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Multi-Probe Investigation of Proteomic Structure of Pathogens
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Introduction and Project Description

Complete genome sequences are available for understanding biotransformation, environmental resistance and pathogenesis of microbial, cellular and pathogen systems. The present technological and scientific challenges are to unravel the relationships between the organization and function of protein complexes at cell, microbial and pathogens surfaces, to understand how these complexes evolve during the bacterial, cellular and pathogen life cycles, and how they respond to environmental changes, chemical stimulants and therapeutics.

In particular, elucidating the molecular structure and architecture of human pathogen surfaces is essential to understanding mechanisms of pathogenesis, immune response, physicochemical interactions, environmental resistance and development of countermeasures against bioterrorist agents. The objective of this project was to investigate the architecture, proteomic structure, and function of bacterial spores through a combination of high-resolution *in vitro* atomic force microscopy (AFM) and AFM-based immunolabeling with threat-specific antibodies.

Particular attention in this project was focused on spore forming *Bacillus* species including the Sterne vaccine strain of *Bacillus anthracis* and the spore forming near-neighbor of *Clostridium botulinum*, *C. novyi-NT*. *Bacillus* species, including *B. anthracis,* the causative agent of inhalation anthrax are laboratory models for elucidating spore structure/function. Even though the complete genome sequence is available for *B. subtilis, cereus, anthracis* and other species, the determination and composition of spore structure/function is not understood. Prof. B. Vogelstein and colleagues at the John Hopkins University have recently developed a breakthrough bacteriolytic therapy for cancer treatment (1). They discovered that intravenously injected *Clostridium novyi-NT* spores germinate exclusively within the avascular regions of tumors in mice and destroy advanced cancerous lesions. The bacteria were also found to significantly improve the efficacy of chemotherapeutic drugs and radiotherapy (2,3). Currently, there is no understanding of the structure-function relationships of *Clostridium novyi-NT* spores. As well as their therapeutic interest, studies of *Clostridium noyii* spores could provide a model for further studies of human pathogenic spore formers including *Clostridium botulinum* and *Clostridium perfringens.*

This project involved a multi-institutional collaboration of our LLNL group with the groups of Prof. T.J. Leighton (Children's Hospital Oakland Research Institute) and Prof. B. Vogelstein (The Howard Hughes Medical Institute and the Ludwig Center for Cancer Genetics and Therapeutics at The John Hopkins Sidney Kimmel Comprehensive Cancer Center).

Summary

The results of this LDRD Project are summarized in the peer-reviewed publications listed below:

1. M. Plomp, T. J. Leighton, K. E. Wheeler, H.D. Hill and A. J. Malkin (2007). *In vitro* high-resolution structural dynamics of single germinating bacterial spores. Proceedings of the National Academy of Sciences of the USA 104, 9644-9649.

2. M. Plomp, J. M. McCaffery, I. Cheong, X. Huang, C. Bettegowda, K. W Kinzler, S. Zhou, B. Vogelstein and A. J. Malkin (2007). Spore coat architecture of *Clostridium novyi-NT* spores. J. Bacteriology 189, 6457-6468.

3. M. Plomp, T.J. Leighton, K.E. Wheeler and A. J. Malkin. (2005). The high-resolution architecture and structural dynamics of *Bacillus* spores. Biophysical Journal 88, 603-608.

4. M. Plomp, T. J. Leighton, K. E. Wheeler, M. E. Pitesky and A. J. Malkin (2005). *Bacillus atrophaeus* outer spore coat assembly and ultrastructure. Langmuir 21, 10710-10716.

5. M. Plomp, T. J. Leighton, K. E. Wheeler and A. J. Malkin (2005).Architecture and high-resolution structure of *Bacillus thuringiensis* and *Bacillus cereus* spore coat surfaces. Langmuir 21, 7892-7898.

6. A. J. Malkin, M. Plomp, T. J. Leighton, A. McPherson and K.E. Wheeler (2005). Unraveling the architecture and structural dynamics of pathogens by high-resolution in vitro Atomic Force Microscopy. Microscopy and Microanalyses, 32-35. doi: 10.1017/S1431927605050828, Published online by Cambridge University Press 30 Dec 2005.

7. M. Plomp, T. J. Leighton, K. E. Wheeler and A. J. Malkin (2005). Bacillus spore coat ultrastructure, assembly and environmental dynamics. Scanning 27, 2, 97-98.

8. C. M. Schaldach, G. Bench, J. J. DeYoreo, T. Esposito, D. P. Fergenson, J. Ferreira, E. Gard, P. Grant, C. Hollars, J. Horn, T. Huser, M. Kashgarian, J. Knezovich, S. M. Lane, A. J. Malkin, M. Pitesky, C. Talley, H. J. Tobias, B. Woods, K-J. Wu, S. P. Velsko (2005). State of the art in characterizing threats: Non-DNA methods for biological signatures. In: Microbial Forensics. (Eds. S. Schutzer, R.G. Breeze, B. Budowle). New York, Elsevier Academic Press, 251-294.

The results of this LDRD Project were presented in invited talks at eight national and international conferences, including conferences of Materials Research and American Physical Societies. These also include plenary lectures at the international conferences “Seeing at Nanoscale IV” (Philadelphia, PA, July 2006) and 3d Latin American Conference on Scanning Probe Microscopy (Ouro Preto, Brazil, Arril 2005).

The development of AFM-based immunolabeling approaches and experimental results are described in the manuscript entitled “Mapping of proteomic composition on pathogen surfaces using atomic force microscopy-based immunolabeling”, by M. Plomp, T. J. Leighton, K. E. Wheeler and A. J. Malkin. This manuscript is currently in the final preparation stage for the submission to the Biophysical Journal.

The short summary of our accomplishments on the ER LDRD Project is presented below (also see attached reprints). We have utilized *in vitro* AFM to address spatially explicit bacterial spore coat protein interactions and their structural consequences at nanometer

resolution under physiological conditions (4-11). We have directly visualized, for the first time, species-specific high-resolution native structures of bacterial endospores including the exosporium and crystalline layers of the spore coat of four *Bacillus*. (4-8) and *Clostridium novyi-NT* (10) spore species in their natural environment, namely air and fluid. We found that strikingly different species-dependent spore coat structures are a consequence of nucleation and crystallization mechanisms that regulate the assembly of the outer spore coat (4-6). We have also developed a unifying mechanism for outer spore coat surface self-assembly (5,10). We have also confirmed the formulation-specific self-assembly of the spore coat by demonstrating distinctive spore coat structures of *B. thuringiensis* spores grown under different sporulation conditions. These findings establish that AFM imagery can provide species/preparation-specific signatures and structural attributes of considerable bioforensic value.

We have demonstrated that the spore coat assembly of *Clostridium novyi-NT* is guided by dislocations (10), which presents the first demonstration of non-mineral crystal growth patterns for a biological organism. The similarities in the mechanisms controlling the assembly of spore coat structures and the growth of inorganic and macromolecular crystals revealed in our work provides an unexpected example of nature exploiting fundamental materials science mechanisms for the morphogenetic control of biological ultrastructures.

The direct visualization of individual *B. atrophaeus* spores has shown that upon dehydration, spore dimensions decreased by ~12% (4), followed by a nearly complete recovery in size upon rehydration. We have proposed that the observed decrease in bacterial spore size and concomitant alteration of spore coat surface morphology following dehydration are due to the contraction of the internal spore core and/or cortex (4). These studies establish that the dormant spore is a dynamic physical structure and provide an experimental platform for investigating spore structural dynamics, germination and response to decontamination regimes.

We have recently performed the first probe microscopy *in vitro* visualization of the structural dynamics of a single pathogen (bacterial spore) during its replication cycle (germination) (11). Here we have demonstrated that AFM can reveal previously unrecognized germination induced dynamic alterations in spore coat architecture, topolology and the disassembly of outer spore coat rodlet structures. Our results (11) suggest that the spore coat rodlets are structurally similar to amyloid fibrils, which have been associated with neural degenerative diseases (i.e Alzheimer and prion diseases). AFM analysis of the nascent surface of the emerging germ cell revealed a high-resolution cell wall peptidoglycan structure, with the thickness of thinner fibers approaching 1 nm. We have demonstrated that *in vitro* AFM has the capacity to provide important insight into peptidoglycan architecture and the biological role of the cell wall in critical cellular processes and antibiotic resistance. This study was published in the high-impact *Proceeding of the National Academy of Sciences of the USA* in June 2007 and has received secondary coverage in many national and international press releases (i.e. http://www.sciencedaily.com/releases/2007/06/070604164928.htm). This new capability allows molecular-scale physical analysis of bacterial and cellular life cycles and provides important insights into their structure-function relationships.

AFM-based dynamic germination experiments of *Clostridium novyi-NT* spores (10) allowed us to reconstruct the complete architecture of the spore coat, thus establishing the first model for the structure of *Clostridium novyi-NT* spores. A comprehensive

understanding of spore architecture and the structural dynamics of germinating *Clostridium novyi-NT* spores may improve useful for enhancing the design of experiments employing *Clostridium novyi-NT* spores in bacteriolytic therapies for cancer treatment.

AFM provides a unique capability to image high-resolution architecture and structural dynamic of pathogens (mainly viruses, bacteria and bacterial spores) at near molecular resolution in native conditions. Further development of AFM in order to enable the correlation of pathogen protein surface structures with specific gene products is essential to understand the mechanisms of the pathogen life cycle. We have developed for the first time an AFM-based immunolabeling technique for the proteomic mapping of macromolecular structures through the visualization of the binding of antibodies, conjugated with nanogold particles, to specific epitopes on a pathogen's surface (12). This information is generated while simultaneously acquiring the surface morphology of the pathogen.

We have established (12) the validity (strength of antigen-antibody binding and avidity) of immunochemical labeling of the exosporium of *Bacillus anthracis* and the spore coat of *Bacillus atrophaeus* spores through various control experiments. We have further established the immunospecificity of labeling, through the utilization of specific anti-*B. atrophaeus and B. anthracis* polyclonal and monoclonal antibodies, which were targeted to spore coat and exosporium epitopes. The AFM immunolabeling experiments have confirmed that bclA glycoprotein is the immuno-dominant epitope on the surface of *B. anthracis* spores.

This new experimental capability allows for correlation of specific gene products with high-resolution architecture, which extends the specificity and range of structural information that AFM can currently provide. The ability to directly visualize antigen-antibody complexes on the native surfaces of single pathogens has broad-ranging implications for the fundamental understanding of the pathogens' life cycle and development of medical countermeasures, and microbial forensics.

As illustrated here, we have developed probe microscopy-based approaches for elucidation of high-resolution architecture and assembly of microbial and cellular systems, their structural consequences, structural dynamics in response to environmental changes and life cycle of biological systems at a near-molecular resolution under physiological conditions. In particular, a comprehensive understanding of the germination process is paramount both for practical applications related to the prevention of a wide range of diseases caused by spore-forming bacteria, and for fundamental studies of cell development. In the case of *Clostridium* spores, molecular-scale models of the germination response in the presence of chemotherapeutics, drugs and inhibitors will be pivotal for the implementation of a bacteriolytic cancer therapy.

EXIT PLAN

We have established correlations between spore morphological signatures and structural attributes as a function of preparation procedures and environmental changes. This data could provide an enabling platform to identify proteins targets for development of vaccines, spore detection, attribution and decontamination technologies. The proteomic and immunochemical imaging methods developed for *Bacillus* spores could now be applied to other pathogen threats.

The methodologies developed here will enable the modeling of pathogen architectures and function for applications in structural biology, bioforensics, cancer research and medical pathology. In particular, these findings establish that AFM imagery can provide preparation-specific signatures and structural attributes of considerable bioforensic value. AFM analysis enables the reconstruction of pathogen formulation and processing conditions which is of interest to intelligence and law enforcement US government agencies. We have received funding for FY 06 from the Federal Bureau of Investigations for FY 06 and from the National Counterproliferation Center for FY 07–FY08 for AFM-based microbial forensics projects. In FY 08 we have also submitted proposal on probe microscopy-based microbial forensics to the Department of Homeland Security. A scientific basis for the development of probe-microscopy forensic systems for programmatic applications was developed in this LDRD Project.

REFERENCES

1. Dang, L.H., Bettegowda, C., Huso, D.L., Kinzler, K.W. and Vogelstein, B. (2001). Combination bacteriolytic therapy for the treatment of experimental tumors. Proceedings of the National Academy of Sciences of the USA **98**, 15155-15160.

2. Bettegowda, C., Dang, L.H., Abrams, R. et al. (2003). Overcoming the hypoxic barrier to radiation therapy with anaerobic bacteria. Proceedings of the National Academy of Sciences of the USA, **100**, 15083-15088.

3. Agrawal, N., Bettegowda, C., Cheong, I. et al. (2004). Bacteriolytic therapy can generate a potent immune response against experimental tumors. Proceedings of the National Academy of Sciences of the USA, **101**, 15172-15177.

4. M. Plomp, T.J. Leighton, K.E. Wheeler and A. J. Malkin. (2005). The high-resolution architecture and structural dynamics of *Bacillus* spores. Biophysical J. **88**, 603-608.

5. M. Plomp, T. J. Leighton, K. E. Wheeler, M. E. Pitesky and A. J. Malkin (2005). *Bacillus atrophaeus* outer spore coat assembly and ultrastructure. Langmuir **21**, 10710-10716.

6. M. Plomp, T. J. Leighton, K. E. Wheeler and A. J. Malkin (2005).Architecture and high-resolution structure of *Bacillus thuringiensis* and *Bacillus cereus* spore coat surfaces. Langmuir **21**, 7892-7898.

7. A. J. Malkin, M. Plomp, T. J. Leighton, A. McPherson and K.E. Wheeler (2005). Unraveling the architecture and structural dynamics of pathogens by high-resolution in vitro Atomic Force Microscopy. Microscopy and Microanalyses, 32-35. doi: 10.1017/S1431927605050828, Published online by Cambridge University Press 30 Dec 2005.

8. M. Plomp, T. J. Leighton, K. E. Wheeler and A. J. Malkin (2005). *Bacillus* spore coat ultrastructure, assembly and environmental dynamics. Scanning **27**, 97-98.

9. C. M. Schaldach, G. Bench, J. J. DeYoreo, T. Esposito, D. P. Fergenson, J. Ferreira, E. Gard, P. Grant, C. Hollars, J. Horn, T. Huser, M. Kashgarian, J. Knezovich, S. M. Lane, A. J. Malkin, M. Pitesky, C. Talley, H. J. Tobias, B. Woods, K-J. Wu, S. P. Velsko (2005). State of the art in characterizing threats: Non-DNA methods for biological signatures. In: Microbial Forensics. (Eds. S. Schutzer, R.G. Breeze, B. Budowle). New York, Elsevier Academic Press, 251-294.

10. M. Plomp, J. M. McCaffery, I. Cheong, X. Huang, C. Bettegowda, K. W Kinzler, S. Zhou, B. Vogelstein and A. J. Malkin (2007). Spore coat architecture of *Clostridium novyi-NT* spores. J. Bacteriology **189**, 6457-6468.

11. M. Plomp, T. J. Leighton, K. E. Wheeler, H.D. Hill and A. J. Malkin (2007). *In vitro* high-resolution structural dynamics of single germinating bacterial spores. Proceedings of the National Academy of Sciences of the USA **104**, 9644-9649.

12. M. Plomp, T. J. Leighton, K. E. Wheeler and A. J. Malkin (2008). Mapping of proteomic composition on pathogen surfaces using atomic force microscopy-based immunolabeling. In preparation for submission to Biophysical J.