TADR ACTIVITY PROJECT (TAP)
Georgia 712009
Project Proposal

Latest Revision: 01 October 2013

Project Title:

Atypical *Y. pestis* strains isolated from natural foci in Georgia: from proteome to virulence and gene expression

Project Summary

The overall goal of this study is to further explore atypical *Yersinia pestis* strains isolated from natural foci in Georgia, to get deeper insight into the functional links between the expression of a defined set of proteins and virulence. The end result will be deeper understanding of the uniqueness of Georgian and Caucasus region *Y. pestis* strains. This project will improve the effectiveness of surveillance in Georgia and decrease bioterrorism threats. Goals will be achieved by a series of experiments using a battery of modern methods and approaches.

Institutes

Primary Institute

Name: Institute of Chemical Biology, Ilia State University
Project Manager: Revaz Solomonia, PhD
Street Address: 3/5 K. Cholokashvili Av. City: Tbilisi
Region: Caucasus Country: Georgia Zip Code: 0162
Name of Signature Authority: Gigi Tevzade
Tel: +995 32 2294257 Fax: +995 32 2231026 Email: gigi@iliauni.edu.ge

Collaborating Institute

Name: National Center for Disease Control (NCDC)/ Richard Lugar Center for Public Health Research (CPHR)
Street Address: Kakheti Highway 16 City: Tbilisi
Region: Caucasus Country: Georgia Zip Code: 0177
Name of Signature Authority: Amiran Gamkrelidze
Tel: +995 32 2239 89 46 Fax: +995 32 2243 30 59 Email: a.gamkrelidze@ncdc.ge

Collaborators

Collaborator

Name: Dr. Stephen Francesconi
Title: Chief Scientist
Institute: Institute Naval Medical Research Center, Frederick, MD
Address: 8400 Research Plaza, Detrick, MD
Tel: +1 301 619 1804 Fax: Email: Stephen.francesconi@med.navy.mil

DTRA Representatives

Name: Gavin Braunstein, PhD — Title: Science Manager
Address: 8725 John K. Kingman Dr., MCS 6201, Ft. Belvoir, VA, USA 22060-6201
Tel: +1 703 767 4862 — Fax: +1 703 526 7794 — Email: gavin.braunstein@dtra.mil
Name: Hall, David CIV — Title: BTRIC COR
Address: 8725 John K. Kingman Dr., MCS 6201, Ft. Belvoir, VA, USA 22060-6201
Tel: +1 703 767 7791 — Fax: — Email: David.Hall@dtra.mil

Integrating Contractor Representatives

Name: Richard J Obiso Jr, PhD — Title: CBR Manager
Address: Attimo Research and Development Group, 4025 Childress Road, Christiansburg, VA 24073
Tel: +1 540 831 9234 — Fax: — Email: rik@attimogroup.com

Name: Magda Metreveli, MD, PhD — Title: Deputy CBR Manager
Address: CH2MHILL, 4 Freedom Square, GMT Plaza, 0105 Tblisi, Georgia
Tel: +995 595 36420 — Fax: +995 322 92 29 — Email: Magda.Metreveli@ch2m.com

Detailed Project Information

I. Project Description (Introduction and Overview)

Background and History (Organism, Technology, etc.)

Yersinia pestis is a Gram-negative rod that is responsible for both bubonic and pneumonic plague, which are high mortality diseases known throughout history. Public health experts recognize plague as a re-emerging infectious disease. This bacteria poses a serious concern for its potential use as a biological weapon (1). In spite of better surveillance methods, the worldwide incidence is approximately 2,000 to 3,000 reported cases each year. There are reports of clinical isolates with single and multi-drug resistant *Y. pestis* (2, 3). All this undermines the effectiveness of current therapeutics. Therefore, with the ever-growing antimicrobial resistance problems worldwide, efforts to identify new therapies for *Y. pestis* infections are of great importance to public health. Deeper understanding of molecular characteristics of various *Y. pestis* strains and their relation to virulence is one of the key ways to solve this problem.

Country-specific project background:

Several plague epidemics have been recorded in Georgia over the last century. Since then, the existence of two natural foci has been described. For nearly 100 years, Georgian Anti-Plague stations have conducted active surveillance of these natural foci, collecting over 120 strains of *Yersinia pestis*. Four isolates were chosen for proteomic studies based on genetic analysis and loci along with strains from surrounding regions. Comparative proteomic analysis of these selected *Y. pestis* strains was completed under the CBR GG-18 project using two approaches: (1) Proteomes of each strain were compared in defined physiological conditions; and (2) Proteomes of each strain were compared in different physiological conditions. Four physiological conditions were used to differentially grow *Y. pestis*: 28°C, without calcium; 28°C, with calcium; 37°C, without calcium; and 37°C, with calcium. The life cycle of *Y. pestis* is very complex, and the bacteria has developed impressive adaptive mechanisms for surviving and proliferating in the various environmental conditions it encounters during its life-time; i.e., in the environment (e.g., soil), in various vectors (e.g., fleas), and in various warm-blooded animals which may

serve as reservoirs (e.g., humans and rodents). In that regard, the metabolic pathways of *Y. pestis* are known to change dramatically depending on environmental conditions (e.g., temperature, pH, available nutrients, etc.). The differential expression of various genes enables this species to adapt rapidly to vastly different environments. For example, a change in temperature from 25°C to 37°C induces (within 45 minutes of the temperature shift) a 20- to 100-fold increase in transcription of LCRS operons (4). In addition, F1 protein synthesis is repressed at 25°C, the temperature encountered by bacteria during growth in fleas (5). Many other *Y. pestis* genes are differentially expressed at 37°C and 25°C, and some differences may profoundly influence virulence (e.g., the murine LD_{50} of *Y. pestis* grown at 37°C is significantly less than that of the bacteria grown at 20-25°C (4)). Two-dimensional gel electrophoresis has consistently demonstrated these differentially expressed proteins between the strains. Their subsequent mass spectrometric (MS) analysis (completed under CBR GG-18) revealed the following protein identities: Tellurium resistant protein, Iron binding protein, DNA Binding Protein H-NS, Outer Membrane porin C, Outer membrane porin C2, Outer membrane protein A, and Porin-gram negative type. Increased temperature and calcium ion concentrations unmask other differences between the isolates, especially F1 antigen expression. The results of these studies suggest that these isolates be placed into two groups: (1) 1390 and 1853 and (2) 2944 and 8787 (6). The properties of these differentially expressed proteins suggest that the first group (1390 and 1853) has increased virulence as compared to the second group. Each of these differentially expressed proteins is of particular interest for understanding the physiology and virulence of *Y. pestis* strains.

Tellurium resistance protein. Gram-negative bacteria are particularly sensitive to tellurium salts. Many pathogenic bacteria show tellurite resistance due to the presence of plasmid or chromosome encoded genes. Expression of tellurium resistance proteins does not correlate with direct protein interaction with tellurium, rather they are interfering with some biochemical process triggered by tellurium. Recent data indicates a link between tellurite resistance and calcium signaling in bacteria. Tellurium resistance proteins bear calcium-binding sites and could be calcium-binding proteins. Some forms of calcium signaling plays a crucial role in tellurite resistance. The higher expression of Tellurium resistance protein in strains 1853 and 1390 probably is linked with high resistance to reactive oxygen species and should reflect the higher virulence of these isolates (7).

Iron binding protein. Iron uptake system and iron binding protein are strongly implicated in the virulence of *Y. pestis* (8). Loss of the yersiniabactin (Ybt) siderophore-based iron transport system in mutant strains results in a loss of virulence when injected intradermally, but not intravenously. It is presumed that this system is necessary for providing sufficient iron at the injection site when transmitted by flea bites.

H-NS protein. This protein belongs to a group of nucleoid-associated proteins, which are associated with the chromosome. They possess substantial nonspecific DNA-binding affinity and have two major functions: gene regulation and chromosome organization. H-NS binds tightly to AT-rich sequences, inhibits transcription of genes with high AT content, and increases its thermal stability. The oligomerization of H-NS can promote higher-order DNA structure *in vitro*, potentially through DNA looping, bridging and/or stiffening (9). DNA- Binding Protein H-NS is not detectable or is at a very low level in 8787, but expressed in other 3 isolates.

Data convincingly demonstrate that outer membrane proteins (Porin C, Outer membrane porin C2, Outer membrane protein A) determine bacterial virulence (10). For example, *E.coli* K1 lacking OmpA is incapable of inducing bacteremia in neonatal rats (11).

F1 antigen is widely accepted as a virulence factor of *Y. pestis*. This is a capsule-like antigen, fraction 1 (F1), at 37°C. F1 is encoded by the *caf1* gene located on the large 100-kb pFra plasmid, which is unique to *Y. pestis*. F1 is a surface polymer composed of a protein subunit, Caf1, with a molecular mass of 15.5 kDa. The secretion and assembly of F1 require the *caf1M* and *caf1A* genes, which are homologous to the chaperone and usher protein families required for biogenesis of pili. F1 has been implicated to be involved in the ability of *Y. pestis* to prevent uptake by macrophages.

II. Scientific Goals

This project is designed to provide comprehensive knowledge of the molecular basis of variable virulence between selected strains of *Y. pestis*. The collaborative project will involve Ilia State University and National Center for Disease Control (NCDC). The Institute of Chemical Biology of the Ilia State University will carry out all validation experiments with gene and protein expression, while NCDC will carry out all work requiring Biosafety Level 2 (BSL-2) containment. The research proposed will cover "Analysis of the gene expression of BTRP priority pathogens *in vivo*," research priorities cited among the specific research priorities described in the CBEP Research Agenda (Please see: CBEP Research Guidelines/Attachment-1/Specific Research Priorities).

The proposed research will focus on the following objectives:

(1) Validation of previously identified differentially expressed proteins in Georgian isolates of *Y. pestis* using Western Blotting and RT-PCR methods

(2) Characterization of virulence of selected Georgian isolates of *Y. pestis* by determination of cytotoxicity *in vitro*

III. Technical Approach and Methodology

The duration of this project will be 12 months.

Objective 1: Validation of previously identified differentially expressed proteins in *Y. pestis* Georgian isolates

Rationale: These studies will validate results obtained by previous proteomic approaches (CBR GG-18 project) and will provide important information regarding gene regulation pathways and changes in protein expression in *Y. pestis* strains on the whole genome level. Two dimensional electrophoresis, silver staining and relative protein expression analysis by Platinum 2-D software are not highly quantitative approaches. In spite of the significant expression differences between strains, we are defining this group of proteins as "candidate" proteins. The verification of the differences requires strong quantitative approaches. The "gold standards" for the validation of differences in the gene expression are mRNA quantitative real time polymerase chain reaction (RT-PCR) and Western immunoblotting of proteins.

Yersinia pestis cell growth in varying temperature and calcium concentrations. Cells will be grown overnight at 28^{o}C. The next morning 0.1 ml of the cells from each strain in quadruplicate will be inoculated into 5ml of growth media and grown at the same conditions for 4 hours. Two tubes from four for each strain will then be transferred to the 37 ^{o}C and one of them will be supplemented with a 0.4 M $CaCl_2$ solution (15 ul) to a final concentration of 4 mM. An equal volume of water will be used for controls. All cultures will be grown for an additional 4 hours. Optical densities of cultures will be measured to harvest the same number of bacteria from each treatment. RNA isolation will be

performed using the TRIzol Max (Invitrogen) protocol, while protein will be extracted by adding 5% sodium dodecyl sulfate (SDS).

Western immunoblotting. For protein determination, we will use the micro-BCA kit (Thermo Scientific). Protein will be determined in quadruplicate. Aliquots of equal volume containing 30 micrograms of protein will be applied to the gels. SDS gel electrophoresis and quantitative Western blotting will be carried out on Bio-Rad apparatus. After protein samples have been transferred onto nitrocellulose membranes, the membranes will be stained with Ponceau S solution to confirm transfer and the uniform loading of the gels. The membranes will be washed with phosphate-buffered saline containing 0.05% Tween 20 and stained by standard immunochemical procedure using peroxidase-labeled secondary antibodies and SuperSignal West Pico Chemiluminescent substrate (Pierce). The blots will then be exposed to X-ray film pre-flashed with Sensitize (Amersham) using intensifying screens. The optical densities of corresponding bands will be measured using LabWorks 4.0 (UVP).

RT-PCR. RNA concentration will be measured using spectrophotometry (Nanodrop), and cDNA will be synthesized by reverse transcription for RT-PCR using random primers (Retroscript). Internal RNA standards will also be used to produce cDNA for accurate quantization of amplified products. Primers will be designed for the following genes of *Yersinia pestis*: *Porin C, Outer membrane porin C2, Outer membrane protein A, Tellurium resistance protein, HN-S, Iron Binding protein, Porin-gram negative type.* RT-PCR will be carried out with SYBR Green PCR master mix.

Antibody production. We are planning to produce polyclonal antibodies against protein H-NS to be used for Western analysis. This protein is involved in chromosome organization, adaptation of bacteria to low temperatures, and could be an important factor for virulence. This protein has not yet been fully characterized and therefore we would like to investigate its comparative expression in up to 50 strains from the NCDC collection in addition to strains already selected for this project. The duration of project is long enough to select the most immunogenic and specific domains of protein, synthesize the corresponding peptide, and raise and purify polyclonal antibodies in rabbits. We plan to utilize AbD Serotec (http://www.abdserotec.com/custom-antibody-services.html) for the production of high quality antibodies within the timeline of the project. This objective complements the goals set forth in CBR GG-23 toward the development of antibodies to be used for diagnostic purposes.

Objective 2: Characterization of the virulence of selected Georgian isolates of *Y. pestis*

Rationale: Differential protein expression between strains does not provide direct evidence for differences in virulence. This assumption must be validated with appropriate experimentation.

***In vitro* cytotoxicity.** *In vitro* cytotoxicity assays will be completed using *Y. pestis* lysates from selected strains applied to the HEp-2 cell line. The use of lysates will mitigate the danger of culturing virulent *Y. pestis* in macrophages and will eliminate the need for *in vivo* animal studies. Protocols for the use of monolayer cell culture for cytotoxicity assays have been well described (12). Lysates from each strain will be added to culture media in wells of subconfluent cell monolayers. Toxicity will be measured by observing morphological changes and by using vital staining (13).

Determination of apoptosis. During infection, *Y. pestis* LPS induces apoptosis in naïve macrophages, contributing to the virulence of this organism (14). For this project, determination of apoptosis will rely on a commercially available kit (ApoDETECT Annexin V-FITC Kit, Life Technologies), which incorporates the use of immunofluorescent cytometry for the detection of apoptotic cells. We will also measure apotosis by enzymatic assay of caspase-3 activity based on conventional spectrophotometry.

Contingencies: If the proposed cytotoxicity assay is insufficient for determining virulence, the use of co-cultivation would need to be considered. In addition, future studies could evaluate these strains in a mouse infection model. Both of these techniques would require available BSL-3 containment and would need to be reviewed under separate biosafety and biosecurity WIPTs with DTRA.

IV. Expected Results

The quantification of selected proteins by Western blotting and analysis of their corresponding mRNA by RT-PCR will validate the differences between the selected *Y. pestis* strains established through previous proteomic analysis. Determination of cytotoxicity *in vitro* will clarify possible differences in virulence factors between strains. The results obtained will be an important contribution to our understanding of the uniqueness of Georgian and Caucasus region *Y. pestis* strains. These experiments may also lead to insights in vaccine development, and contribute to the understanding of mechanisms of macrophage action in the infection process.

The successful implementation of this project will lead to completely new data about differences between Y. pestis strains on a proteome level and will shed new light on the uniqueness of Georgian and Caucasus region Y. pestis strains.

Differences between Georgian *Y. pestis* isolates are described by a single nucleotide polymorphism analysis (15), but these studies are most useful for the construction of phylogenetic trees and do not provide information regarding differences in physiology or virulence. Data from this project will be integrated with SNP analysis data to create a comprehensive picture about the molecular markers of *Y. pestis* strains.

V. Tasks

Task Description:			
Project management, reporting, and administration			
	Description of Deliverables	Responsible Party	Schedule for Completion
1	Procurement of all equipment and chemicals	CH2M HILL	Q1
2	Selection and synthesis of immunogenic peptide region from H-NS protein sequence, starting production of antibodies	Ilia State University	Q1-Q2
3	First stage of verification of proteomic analysis results - Real time PCR experiments	Ilia State University and NCDC	Q2
4	First stage of verification of proteomic analysis results – Western immunoblotting experiments	Ilia State University and NCDC	Q2-Q3
5	Cytotoxicity and apoptosis assays	Ilia State University and NCDC	Q2-Q4
6	Analysis of results	Ilia State University and NCDC	Q3-Q4
7	Preparation of paper and final report	Ilia State University and NCDC	Q4

* Note: Schedule for Completion starts with initiation of the project

VI. Project Management

This project will be managed by the Ilia State University in the close cooperation with NCDC and Dr. Stephen Francesconi. Quarterly reports and short summaries will be prepared by the lead institution with inclusion of corresponding parts from NCDC.

We are planning to prepare the first conference abstract in the third quarter and to start the preparation of a manuscript in the fourth quarter.

The scientific leader of the project will be in regular communication with Dr. Stephen Francesconi. The project manager will participate in quarterly project calls and, if necessary, will organize more frequent calls. Local meetings will be also organized for the discussion of project progress.

All financial management on the project will be the responsibility of the project manager. He will also ensure active participation in international conferences and meetings and presentation of the data obtained by this project.

VII. Meeting Goals and Objectives of CBR Program

Yersinia pestis belongs to the list of selected agents and toxins (see http://www.selectagents.gov/Select%20Agents%20and%20Toxins%20Exclusions.html); the main goal of this project is the further characterization of gene expression at transcriptional and translational levels. Therefore, this proposal addresses the CBEP goal: "intermediate and long-term priorities on human diseases: Analyze gene expression of BTRP priority pathogens *in vivo*." Please see: CBEP Research Guidelines/Attachment-1/Specific Research Priorities.

The proposed study is important because it addresses pertinent needs of Georgia. Plague is recognized as a re-emerging infectious disease and Georgia has two natural loci for *Y. pestis*. The obtained data (verified list of potential virulent genes) will be the basis for fast screening of new isolates and existing strains from the NCDC collection for virulence markers. It is also internationally relevant as it explores molecular differences between the strains of *Y. pestis* from the same geographical region. The results of the project could lead to new directions for the molecular and physiological characterization of *Y. pestis* and it should lead to the preparation and publication of a manuscript in a peer-reviewed microbiological journal.

The design of the project is such that it requires collaboration of Ilia State University and NCDC.

Safety Issues: Pathogen work will be carried out at NCDC/Richard Lugar Center for Public Health Research in the museum / BSL-3 facility, which is equipped with BSL-2 and BSL-3 containment laboratories. Total RNA and protein fractions will be isolated at NCDC, tested for sterility (using DTRA-approved methodologies [also used in CBR GG-18]), and will then be transferred to Ilia State University. This approach was used during the GG-1 and GG-18 projects and is highly reliable.

Pathogens

	Biosafety Level (Diagnostic Quantities)			
Pathogen	**1**	**2**	**3**	**4**
Yersinia pestis		X		

Approximate Budget

Please see the Form B, which includes a detailed budget and break-out of all proposed costs.

Relevance to DoD Program Objectives

In a region endemic for *Y. pestis*, it is important to understand how the organism survives not only in the environment, but also in its reservoir and host. Earlier studies have determined natural foci, or pockets in the environment that have animals and fleas perpetually infected with plague. Human populations that overlap these foci are inevitably infected. Understanding the nature, course, and ultimately the method to prevent and cure this disease, especially in a region that contains phenotypically and genotypically different strains from the norm is important for DoD as well as public health in general.

Relationship to Other On-Going or Planned BTRP Projects

The approaches used in this proposal could be important also for the CBR GG-19 project, which will begin in the summer of 2013. In addition, this project links back to the CBR GG-18 project and builds on the extensive proteomic data that was obtained in the project. The data generated from this project will be combined with the CBR GG-18 project data and published in peer-reviewed literature. In addition, this project directly links with the proposed CBR GG-23 project, with a key collaborator in ABD Serotec (Germany).

References

1. **Hawley RJ, Eitzen EM Jr**. 2001. Biological weapons--a primer for microbiologists. Annu. Rev. Microbiol. **55**:235–253.
2. **Welch TJ, Fricke WF, McDermott PF, White DG, Rosso M-L, Rasko DA, Mammel MK, Eppinger M, Rosovitz MJ, Wagner D, Rahalison L, Leclerc JE, Hinshaw JM, Lindler LE, Cebula TA, Carniel E, Ravel J**. 2007. Multiple antimicrobial resistance in plague: an emerging public health risk. Plos One **2**:e309.
3. **Levy SB**. 2005. Antibiotic resistance-the problem intensifies. Adv. Drug Deliv. Rev. **57**:1446–1450.
4. **Perry RD, Fetherston JD**. 1997. Yersinia pestis--etiologic agent of plague. Clin. Microbiol. Rev. **10**:35–66.
5. **Cavanaugh DC**. 1971. Specific effect of temperature upon transmission of the plague bacillus by the oriental rat flea, Xenopsylla cheopis. Am. J. Trop. Med. Hyg. **20**:264–273.
6. **Solomonia R, Nozadze M, Mikeladze E, Zhgenti E, Chanturia G, Kekeklidze M, Obiso, Jr. R, Francesconi S, Bakanidze L**. 2012. Characterization of atypical Y. pestis strains isolated in natural foci of Georgia, p. 99. *In* San Francisco, California, USA.
7. **Suprenant KA, Bloom N, Fang J, Lushington G**. 2007. The major vault protein is related to the toxic anion resistance protein (TelA) family. J. Exp. Biol. **210**:946–955.
8. **Sebbane F, Jarrett C, Gardner D, Long D, Hinnebusch BJ**. 2010. Role of the Yersinia pestis Yersiniabactin Iron Acquisition System in the Incidence of Flea-Borne Plague. Plos One **5**.

9. **Wang W, Li G-W, Chen C, Xie XS, Zhuang X**. 2011. Chromosome organization by a nucleoid-associated protein in live bacteria. Science **333**:1445–1449.
10. **Krishnan S, Prasadarao NV**. 2012. Outer membrane protein A and OprF: versatile roles in Gram-negative bacterial infections. Febs J. **279**:919–931.
11. **Khan NA, Goldsworthy GJ**. 2007. Novel model to study virulence determinants of Escherichia coli K1. Infect. Immun. **75**:5735–5739.
12. **Borenfreund E, Puerner JA**. 1985. A simple quantitative procedure using monolayer cultures for cytotoxicity assays (HTD/NR-90). J. Tissue Cult. Methods **9**:7–9.
13. **Ekwall B, Silano V, Paganuzzi-Stammati A, Zucco F**. 1990. Toxicity tests with mammalian cell culture. *In* Short-term Toxicity Tests for Non-genotoxic Effects*,* Bourdeau *et al.* (Ed.) Wiley, Hoboken, NJ.
14. **Bergsbaken T, Cookson BT**. 2009. Innate immune response during Yersinia infection: critical modulation of cell death mechanisms through phagocyte activation. J. Leukoc. Biol. **86**:1153–1158.
15. **Hu P, Zhgenti E, Chanturia G, Tsereteli D, Kekelidze M, Zangaladze E, Stokowski R, Andersen G, Torok T**. 2012. Application of a SNP-genotyping high density microarray to study Yersinia pestis isolates from the Caucasus region, p. 164. *In* San Francisco, California, USA.