

AD_____

Award Number: DAMD17-03-1-0491

TITLE: Structural Studies on Toxins and Virulence Factors of Yersinia Pestis

PRINCIPAL INVESTIGATOR: S. Swaminathan, Ph.D.

CONTRACTING ORGANIZATION: Brookhaven National Laboratory
Upton, NY 11973

REPORT DATE: July 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 01-07-2006		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 1 JUL 2005 - 30 JUN 2006	
4. TITLE AND SUBTITLE Structural Studies on Toxins and Virulence Factors of Yersinia Pestis				5a. CONTRACT NUMBER DAMD17-03-1-0491	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) S. Swaminathan, Ph.D. E-Mail: swami@bnl.gov				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Brookhaven National Laboratory Upton, NY 11973				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT: In this annual report we present our continuing efforts on two or three fronts. We are working on the expression, purification and crystallization of complexes of YopB and YopD with other proteins, like their chaperones. We have made progress in purifying YopB:YopD:SycD complex. If successful, this will help in understanding the translocation mechanism of effector proteins. Our crystallization efforts on YopH and SycD are continuing.					
15. SUBJECT TERMS Yersinia pestis, Yop Complexes, Macromolecular Crystallography, 3D Structure					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			USAMRMC
			UU	11	19b. TELEPHONE NUMBER (include area code)

Table of Contents

Introduction.....	4
Body.....	4
Key Research Accomplishments.....	11
Reportable Outcomes.....	11
Conclusions.....	11
References.....	11
Appendices.....	None

Structural Studies on Toxins and Virulence Factors of *Yersinia pestis*

Progress Report for the Period ending June 2006

Introduction

This project is on no-cost extension and we are reporting our progress in this period.

The overall goal of this project is to determine the three-dimensional structures of proteins and virulence factors from *Yersinia pestis* to understand the mechanism of translocation of effector proteins. *Yersinia pestis* is responsible for the plague and is a potential and emerging biowarfare threat. This organism has evolved a powerful method of delivering effectors into cells of the host immune system. The effectors act to completely circumvent the immune system. Understanding the molecular mechanism of this system is important to get an insight into the process of phagocytosis and inflammation. The effector proteins are secreted into the cells by a type III secretion system which involves a number of proteins. Some of them are actively involved in the delivery system and others are required for pore formation. Information derived from this study will help in developing vaccines and therapeutics for the plague. It will also help in identifying genetically engineered threats. The goal is to study the three-dimensional structures of Yop proteins and their corresponding chaperones. By studying the three-dimensional structures of effector proteins we can get an insight into the mechanism of toxification of the cells which would eventually lead to design of inhibitors to block the toxicity.

Body

Work on YopH:

YopH is a key translocated effector of *Y. pestis* that exhibits tyrosine phosphatase activity. The 50 kDa YopH protein is organized into two modular domains separated by a linker region. The N terminal domain has phosphopeptide binding activity and functions as a substrate-targeting domain for the C-terminal catalytic domain. The two separate domains of YopH have been crystallized and their structures solved (1,2). However, the structure of the native protein has not been determined. It is believed that

two domains are connected by a flexible loop or region which prevents crystallization of the intact YopH. It has recently been shown that both the domains have substrate binding sites. We wanted to take advantage of this and design a peptide containing two phosphorylated tyrosine far enough both in sequence and in three-dimensional space to bind to each of the domains. Our idea is to crystallize the intact or mutant YopH with a synthetic peptide. In the last year we have been trying to crystallize these complexes but have had no success yet. We believe that determining the structure of the native protein is important, as it will provide clues into potential functional interactions between the two domains. We have expressed large quantities of YopH for this purpose and are working on crystallization of these with peptides. Particularly we are concentrating on the following synthetic peptides:

Cas peptide (342-371): GSQDI**p**YDVPPVRGLLPNQYGQEV**p**YDTPPMA

Cas peptide (258-287): PATDL**p**YQVPPGPGSPAQDI**p**YQVPPSAGTGH

These peptides are based on the natural substrate, Crk-associated substrate, p130 (p130Cas) of epithelial cells. The p130Cas is one of the proteins with many phosphorylated tyrosines that are critical for many functions like cell-cell interaction, metabolism and immune response. The phosphorylated tyrosines (pY) are shown in bold in the sequences above. Work is still in progress.

Work on YopB, YopD and their complexes

Delivery of secreted effectors such as YopH into host cells infected with *Y. pestis* is controlled by a set of secreted “translocator” proteins. Three proteins are required for the translocation process, LcrV, YopB and YopD. A fourth protein, YopK, appears to function as a negative regulator of translocation. The structure of a mutant form of LcrV has recently been determined. However, the structures of YopB, YopD and YopK have not been determined. YopB (42 kDa) and YopD (33 kDa) contain predicted transmembrane domains and are thought to form a channel in the host cell membrane. YopK (21 kDa) does not have any recognizable features, and its function remains mysterious. Attempts to overexpress recombinant forms of YopB and YopD in *E. coli* have met with no success, due to their hydrophobic characteristics. As an alternative approach, we have engineered *Yersinia* to secrete these proteins into growth media, and we have investigated the possibility that the secreted forms of these proteins could be

used for structural determinations. Currently we are working with relatively small volumes of culture (less than 100 ml) and proteins secreted into *Yersinia* growth media are concentrated by filtration and analyzed by native polyacrylamide gel electrophoresis. Although yields of protein are small (e.g. 10 micrograms) this procedure does provide enough protein for initial studies. The results of these experiments showed that all three proteins, YopB, YopD and YopK, were secreted as soluble proteins. However, all three proteins migrated as multimeric forms on the gels. YopB and YopD formed multimeric ladders of bands on the gels, and YopK ran as a broad band at a very high molecular weight. Thus, although the secreted forms of YopB, YopD and YopK are soluble, the multimeric forms of these proteins will make protein crystallization and X-ray determination challenging. Preliminary results indicate that YopB and YopD are forming homo- and hetero-oligomers. We plan additional studies to determine if the hetero-oligomeric forms of YopB and YopD are suitable for crystallization studies. However, we are also trying to express YopB and YopD individually as membrane proteins.

Since it was very difficult to express and purify YopB and YopD alone, we set out to work on complexes of YopB and YopD with their chaperone, in this case SycD. We have cloned YopB and YopD in pET vector. However, expression of these proteins in *E.coli* is still a challenge. The quantity that we obtain is not enough for our crystallization trials. In addition to getting YopB:YopD complex by co expression, we are also trying another technique. SycD is an intrabacterial protein chaperone. It has been shown that SycD binds to YopB. We are now attempting to co-express YopB with SycD. For this we have cloned YopB with and without histag and SycD with and without histag. When they are expressed together neither of them is toxic to the cell unlike when YopB alone is expressed.

As reported in the last annual report, though we get some expression of the complex, the amount of protein expressed is not enough for crystallization. We are optimizing the conditions expression for better yield. In addition to continuing our work from last year we have undertaken to express YopB-YopD-SycD complex as described below.

Expression of His-SycD-YopB-YopD

A new construct has been made including LcrH/SycD, YopB and YopD ORFs together (say SycDyopBD) in pET28a vector. It has N-terminal His tag attached to SycD (His-SycDyopBD). This construct was expressed in *E. Coli* cells. The cells were grown in two different methods, one with IPTG induction and other with auto-induction (3). Chloramphenicol and Kanamycin were used as antibiotics in both the procedures.

In the auto-induction protocol cells were expressed in MDG medium initially and transferred to ZYP 5052 medium. The cells were grown at 37°C until the OD reached 0.6 and the temperature was reduced to 20°C for further growth overnight. Harvested cells were lysed and purified with Ni affinity and size exclusion chromatography columns.

Purified protein showed three faint bands in SDS-PAGE corresponding to the proteins, SycD, YopB and YopD (Fig. 1). The intensity of the three bands implied that the proteins were not expressed in a stoichiometric ratio. SycD band is stronger than the other two indicating larger population followed by YopD. The band corresponding to YopB is the lightest. Though it is a poor estimate, the ratio of SycD:YopB:YopD may be deduced as approximately 4:1:2 (Fig. 1).

The IPTG induction protocol also showed the same trend but the overall yield of proteins is less than that of the auto-induction method. So the expression was carried out with auto-induction in most of the experiments.

In order to confirm the expression of the protein of interest, Western blotting was done for YopB and YopD separately using the corresponding antibodies. The Western blot showed the presence of YopB and YopD proteins matching to the bands marked in SDS-PAGE (Figs. 2 & 3).

The size exclusion chromatography has three significant peaks. Peak 1 contained the SycDyopBD complex. The second peak is about half the size of peak 1 and may contain SycD and YopD. Thirdly, a huge peak corresponding to SycD is obtained that is about 100 times stronger than the peak 1. It may be possible that the SycD expression is much more than the complex or the complex formation is poor so that the His-tagged SycD binds to the Ni column more than the complex.

It can be concluded with these experiments that the SycDyopBD complex is expressed but very low in quantity. In fact the crystallization experiments were also made

using the complex and SycD samples obtained from size exclusion purification trials. Since the protein concentration is considerably low the crystallization experiments have not been successful, so far.

Improving the yield of proteins especially the SycDyopBD complex is our focus for the next year. If the *E.Coli* cells are not able to express these proteins, a suitable alternative medium will be identified.

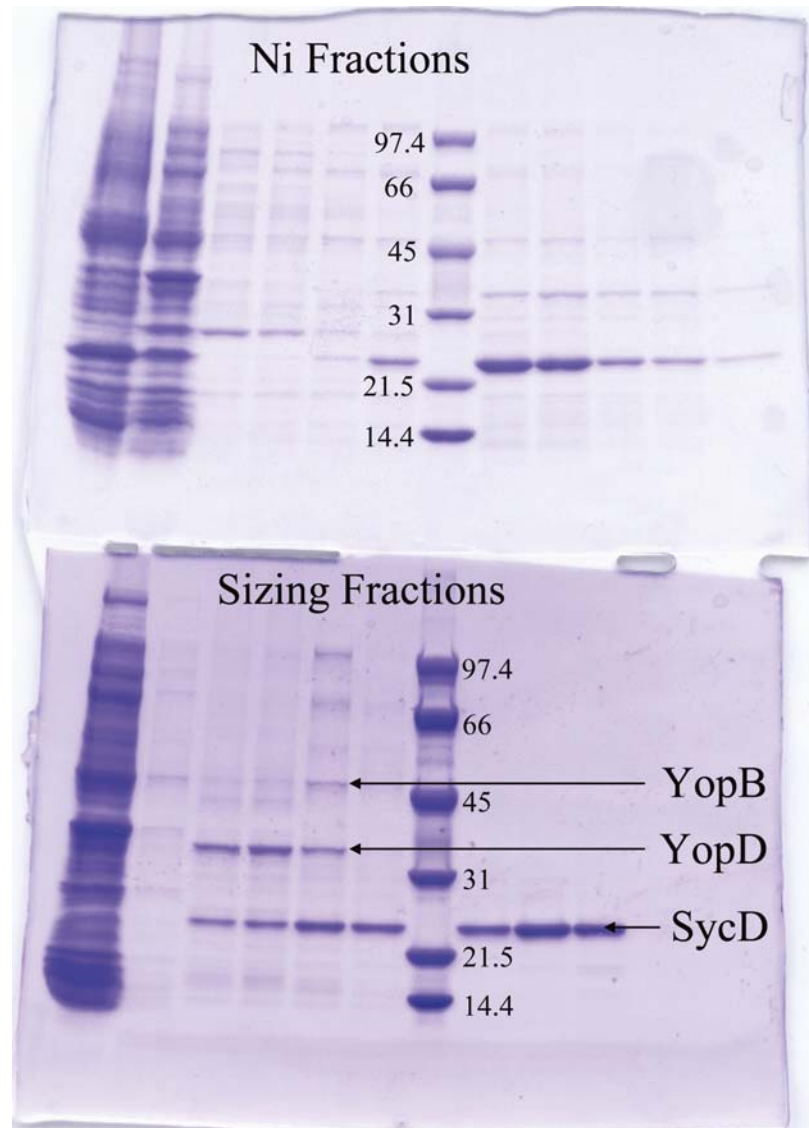


Fig. 1. SDS PAGE gel with the Molecular weight marker. YopB, YopD and SycD are shown with arrow marks.

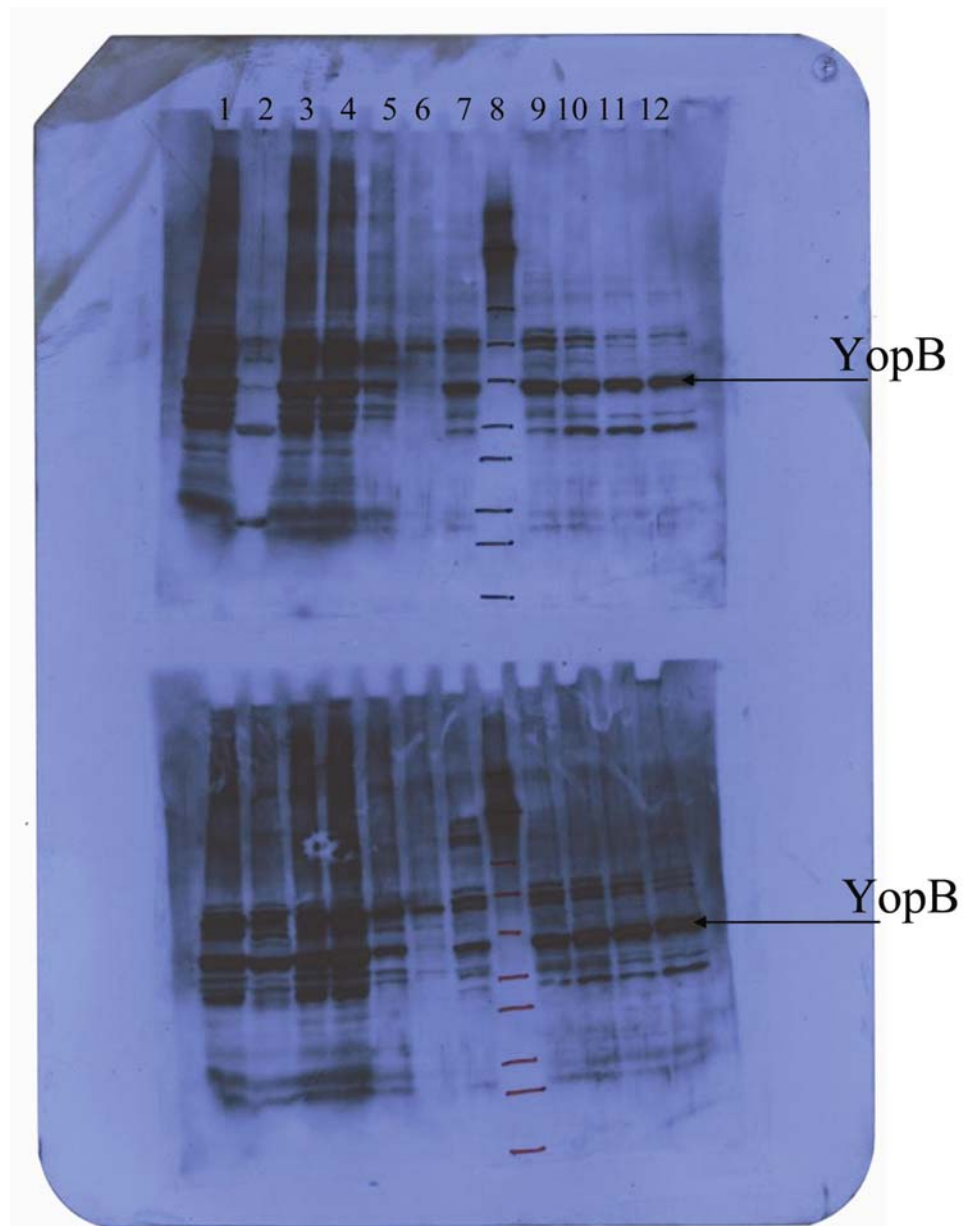


Fig. 2. Western blot showing YopB



Fig. 3. Western blot showing the presence of YopD.

Work on YopB-YopD complex:

Preliminary studies indicate that YopB and YopD are secreted into growth media by *Yersinia* in a soluble form. These proteins appear to be forming homo- and hetero-oligomeric complexes. It is possible that the hydrophobic regions of these proteins are buried in the oligomers, which allows them to be soluble in aqueous solutions. Although the homo-oligomeric forms of these proteins may not be suitable for crystallization, we anticipate that the hetero-oligomeric complexes may represent distinct species that could be isolated in pure form and used for crystallization studies. To investigate this possibility, we will purify a secreted form of YopB that contains an N-terminal 6X his tag. We have confirmed that this tagged form of YopB retains full biological activity. The purified YopB will be run on native gels to determine the percentage of the protein

that is in a homo- or hetero-oligomeric form. If we are able to identify a specific YopB-YopD complex on native gels, we will then attempt to obtain sufficient amounts of this complex for structural studies. So far not much success has been achieved towards this. However, this work is being continued with some modifications.

Crystallization of SycD:

SycD is the intrabacterial chaperone of YopD that directs the translocation of the secreted Yop effector proteins across the target membrane. Crystallization of this protein, SycD is also in progress. Expression and purification of this protein is successful and the initial crystallization trials are in progress.

Key Research Accomplishments: We are able to express SycD-YopB-YopD complex. However, the yield is low and we are currently optimizing the expression condition.

Reportable outcomes

None.

Conclusions

We have achieved partial success in purifying Yop B, D and SycD complex.

Plans for the next year:

We will continue and complete the work in progress.

Personnel in the Project

1. S. Swaminathan (PI)	Scientist	20% effort
2. S. Eswaramoorthy	Associate Scientist	30% effort

Sub-contract to State University of New York at Stony Brook

1. J. Bliska	Professor	10% effort
2. M. Ivanov	Technician	100% effort

References:

1. Smith, C. L., Khandelwal, P., Keliikuli, K., Zuiderweg, E. R., and Saper, M. A. (2001) *Mol Microbiol* **42**, 967-979
2. Stuckey, J. A., Schubert, H. L., Fauman, E. B., Zhang, Z.-Y., Dixon, J. E., and Saper, M. A. (1994) *Nature* **370**, 571-575
3. Studier, F. W. (2005) *Protein Expr. Purif.* **41**, 207-234