2012-01-01

Investigation of the Membrane Interactions of Mycobacterium Tuberculosis Secreted Factors ESAT-6 and CFP-10

Joaquin A. De Leon
University of Texas at El Paso, jadeleon@miners.utep.edu

INVESTIGATION OF THE MEMBRANE INTERACTIONS OF *MYCOBACTERIUM TUBERCULOSIS* SECRETED FACTORS ESAT-6 AND CFP-10

JOAQUIN A. DE LEON III

Department of Biological Sciences

APPROVED:

Jianjun Sun, Ph.D., Chair

Manuel Miranda, Ph.D

Chuan Xiao, Ph.D.

Benjamin C. Flores, Ph.D.
Interim Dean of the Graduate School

DEDICATION

To my brother

Thank you for helping me to realize how important it is to treasure every moment in our lives no matter how small or insignificant those moments may seem

Love You Enrique

INVESTIGATION OF THE MEMBRANE INTERACTIONS OF *MYCOBACTERIUM TUBERCULOSIS* SECRETED FACTORS ESAT-6 AND CFP-10

by

JOAQUIN A. DE LEON III, BS

THESIS

Presented to the Faculty of the Graduate School of
The University of Texas at El Paso
in Partial Fulfillment
of the Requirements
for the Degree of

MASTER OF SCIENCE

Department of Biological Sciences
THE UNIVERSITY OF TEXAS AT EL PASO
August 2012

ACKNOWLEDGEMENTS

First and foremost I give highest acknowledgement to the most Holy Blessed Trinity. For with God many tasks can be accomplished through his good will. I would like to thank my wife and our beautiful children for their patience, understanding, and support. This was not only a journey for me but for you all as well. Love you Angie, Adrian, Julli, Aryana, and Jaleyah. My parents and in-laws have been such great support, all of you with Angie and the kids have been my cheering section giving encouragement and support. I thank all members of Dr. Sun's lab for your support. It has been real fun problem solving with all of you. I thank my mentor Dr. Sun for allowing me the opportunity to work on such a fun project. You have shown and taught me so much and I do genuinely appreciate that. I thank Dr. Das, Dr. Miranda, and Dr. Xiao for serving as my committee in ensuring that my research and writings were successful in telling such a wonderful story. I thank Dr. Walsh and Dr. Llano for being available to talk and discuss matters outside of academia and research. You both have provided me with so much support and I greatly appreciate that. May God Bless all those who have crossed my path throughout this journey, and peace be with each and every one of you.

ABSTRACT

Mycobacterium tuberculosis is the causative agent of the infectious disease tuberculosis, infecting about one-third of human race. Infections are not vast in the United States when compared to many other countries, yet many cases seen in the United States are of those who have immigrated to the United States. The Hispanic/Latino population in the United States contributes to a large fraction of those infected with tuberculosis in the United States. During this study we purified recombinant *Mycobacterium tuberculosis* secreted factors ESAT-6 and CFP-10 from *Escherichia coli.* Here we have introduced a method in which we can refold and purify insoluble ESAT-6 using one column. Our purified proteins when incubated together form a heterocomplex just as previous works have shown. We have subjected each protein, ESAT-6, CFP-10, and the heterocomplex to a K^+ release assay in which we use prepared liposomes filled with K^+ to show protein activity. We have shown that our recombinant proteins are capable of membrane disruption at a low pH. The work presented here builds a base for future biochemical studies in protein-membrane interaction and providing information on how such reactions may contribute to latency and reactivation of *Mycobacterium tuberculosis* infection.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1: INTRODUCTION

1.1 The Disease

Mycobacterium tuberculosis is a weakly gram-positive rod shaped bacterium with a lipid-rich cell wall. This particular *mycobacterial* species is the causative agent of infectious tuberculosis. Tuberculosis is transmitted from person to person via air particulates generated by an infected individual through actions such as coughing, sneezing, and spitting. According to the 2011 annual report by the World Health Organization (WHO) [1], it was estimated that there were 8.8 million reported cases of tuberculosis, of which 1.1 million deaths had occurred during 2010 among non-HIV people, and 0.35 million deaths occurred among those who were HIV positive. According to the Centers for Disease Control (CDC) [2], there were a reported 11,182 cases in 2010. Of those reported cases 30% were Hispanic/Latino including US born and foreign born, of which 37% attributed to those of whom were foreign born (Figure 1.2). This is an important aspect to consider, especially in a large border town like El Paso, TX (Figure 1.1).

Figure 1.1: CDC report of tuberculosis cases during 2010. Texas, Arizona, and California, the three states bordering Mexico, have cases that are above the national average.

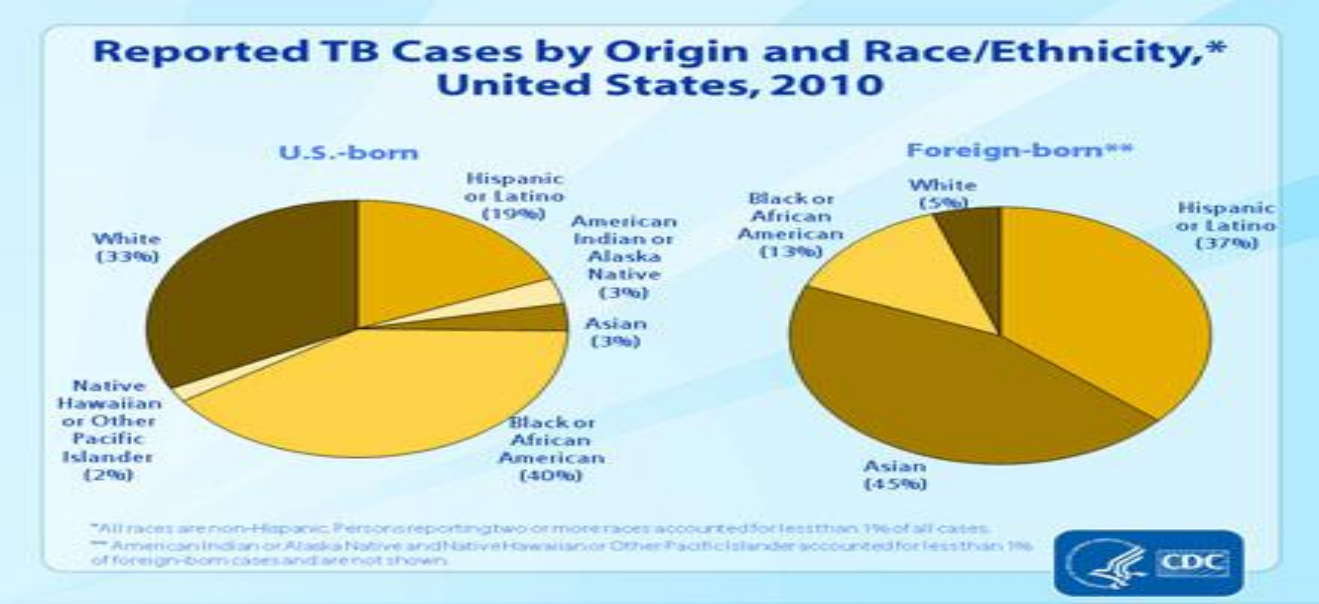

Figure 1.2: CDC reported tuberculosis cases by ethnicity. A high rate of tuberculosis is seen in the Hispanic community, both foreign born and native

1.2 Latency and Reactivation

Persons who have been exposed to *Mycobacterim tuberbculosis* and now harbor the pathogenic bacteria will present themselves in one of the following ways. One way is that the innate immune response of the exposed individuals is activated and effectively inhibits the pathogens. If the innate immune system fails in inhibiting the pathogens, the infected person will develop active tuberculosis within 1-3 years of exposure [3]. Many who have been exposed do not show any signs of infection, but show positive in tuberculin test. These individuals are identified as those with latent infection of tuberculosis, which may be reactivated in to active tuberculosis (Figure 1.3). The mechanism as to how latent tuberculosis becomes active tuberculosis has yet to be elucidated.

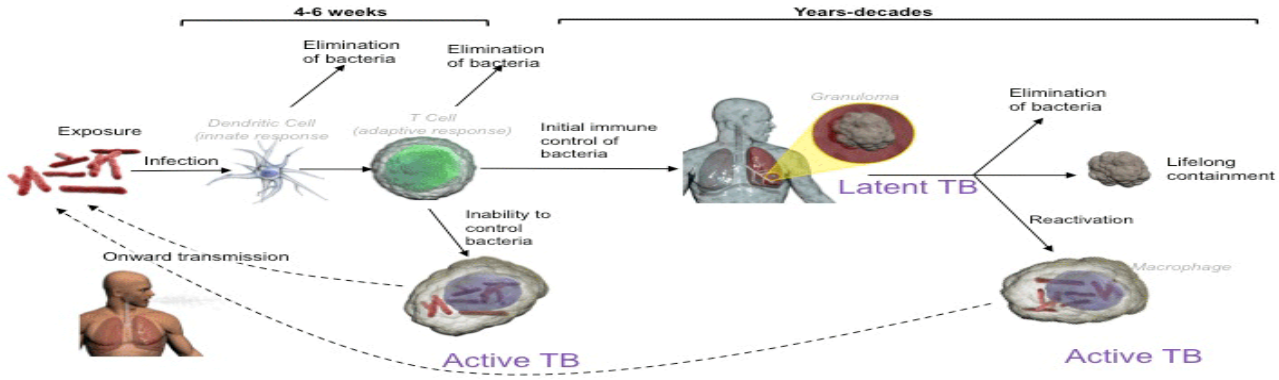

Figure 1.3: Natural history of tuberculosis infection. Here it is shown that after several years of having latent tuberculosis, by means not understood can become active tuberculosis.

1.3 BCG AND TB

Currently Bacillus Calmette-Guerin (BCG), a vaccine discovered by Albert Calmette and Camille Guerin from Pasteur Institute, is used worldwide to combat tuberculosis. BCG is a live, attenuated strain of *Mycobacterium bovis*, which has been used since 1921 with an 80% efficacy. It is widely used in areas where tuberculosis is highly endemic. Currently the United States does not vaccinate.

Despite the regular usage of the BCG vaccine, it had never been investigated as to why and how this attenuated strain of *Mycobacterium bovis* provides protection against tuberculosis and loses its virulence. A research group based out of Seattle, Washington has provided some insight as to why the BCG strain had lost its virulence. A genomic analysis of virulent strains (*M. tuberculosis;* Erdman, H37Rv, and H37Ra), avirulent (*Mycobacterium bovis)*, and vaccine strain (*Mycobacterium bovis;* Connaught, Pasteur, and Brazil) of *Mycobacteria* had identified three genomic differences, commonly referred to as region of difference (RD1, RD2, and RD3), through the use of subtractive genomic hybridization. Of these three, it was found that RD1, a 9.5 kb segment, was conserved in all virulent strains of *Mycobacterium tuberculosis* and *Mycobacterium bovis* [4], while it was absent in all BCG strains, including the BCG vaccine strain.

1.4 RD1

Region of difference 1 encodes a secretion system, termed as ESX-1, which stands for the early secreted antigenic 6 kilo-Daltons (ESAT-6) 1 secretion system. Current understanding of the RD1 region indicate that the following proteins are required for the secretion of virulence factors, also encoded by RD1: trans-membrane protein (Rv3877), and two FtsK-SpoIIIE ATPase like proteins (Rv3870, Rv3871), and secreted virulence factors Rv3875 (ESAT-6) and Rv3874 (culture filtrate protein 10 kilo-Daltons/CFP-10) [5, 6] (Figure 1.4).

To date this secretion system is not well understood as to exactly how *mycobacteria* utilize such a secretion system or even as to why *mycobacteria* need this secretion system. Previous works have shown that ESAT-6 and CFP-10 may be directly involved in membrane disruption [7, 8]), probably through forming pores on the membranes [9].

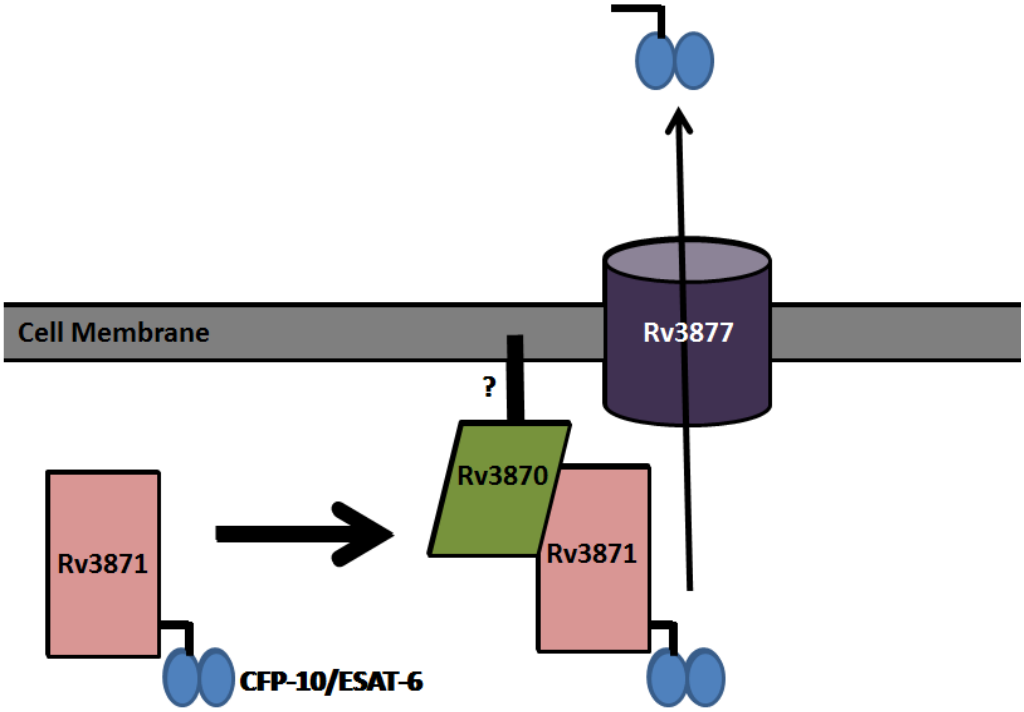

Figure 1.4: Secretion of CFP-10/ESAT-6 as proposed by Patricia Champion. [5]

1.5 Secreted Factors

The secreted factors ESAT-6 and CFP-10 are encoded within the RD1 region of *Mycobacterium tuberculosis*. These two secreted factors are small molecular weight proteins composed of on average 100 amino acids. The average size of these proteins and related proteins share an additional characteristic where they contain the motif tryptophan-X-glycine residue. Thus these families of proteins are commonly referred to as the WXG family of proteins [10].

1.6 Hetero-dimeric Structure of ESAT-6/CFP-10

In 2002, a group reported that the secreted factors ESAT-6 and CFP-10 of *Mycobacterium bovis* expressed in *Escherichia coli* interacted with each other [11]. Utilizing methods such as far UV circular dichroism, fluorescence spectroscopy, NMR spectroscopy, and various binding assays the group "conclusively" showed that ESAT-6 and CFP-10 form a hetero-dimeric complex.

In 2005 the same group was able to determine the solution structure of the hetero-dimeric complex by NMR spectroscopy [12]. The group showed that the complex formed a four-helix bundle, in which each protein upon complex formation adopted a helix-turn-helix hairpin structures and that the two proteins lied antiparallel to each other (Figure 1.5). The group also showed that the N- and C- termini of both proteins in the complex are unstructured and flexible. The unstructured C-terminal end maintains a helical structure, for which they hypothesized that this region may be involved in interacting with proteins associated with host cells. In addition, the group has identified two salt bridges between the two proteins, which may aid in stabilization of the hetero-dimeric complex.

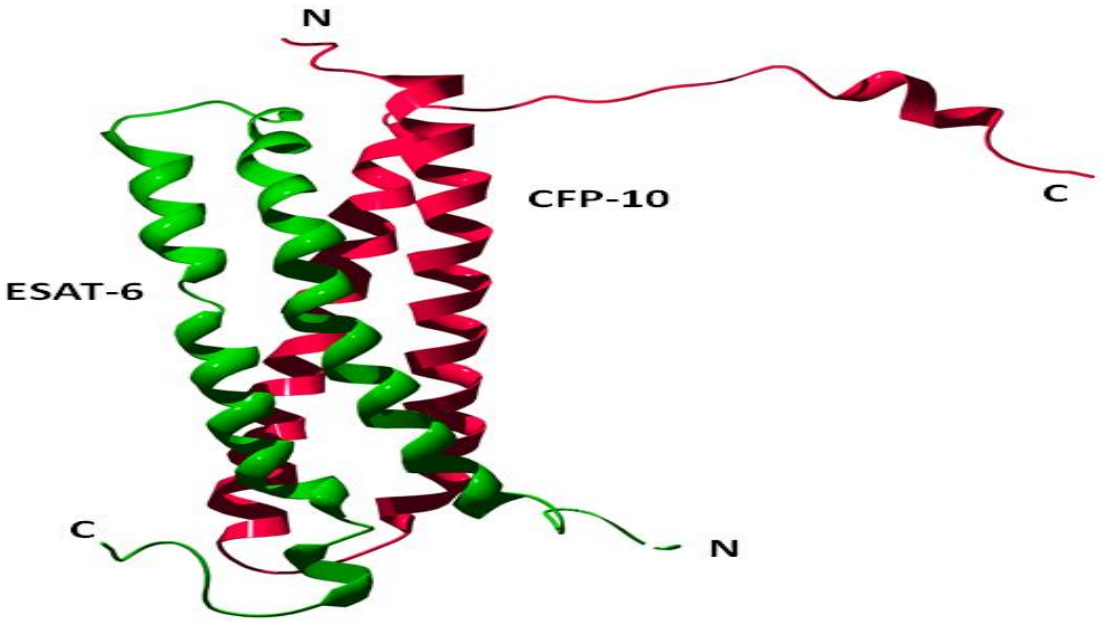

Figure 1.5: Cartoon ribbon **r**epresentation of CFP-10/ESAT-6 complex. This representation shows how the two proteins interact when bound to each other. Here it is shown the antiparallel association and flexible N- and C- termini.

1.7 Hypothesis and Specific Aims

Does the ESX-1 system and its secretion of ESAT-6 and CFP-10 trigger reactivation of tuberculosis through membrane disruption/pore formation on the phagosomal membrane? To understand the mechanism of reactivation, the goal is to study the early secreted antigens and how they interact not only with each other but with lipid membranes. Previous works have been successful in studying and getting a better understanding of pore formation with pore forming toxins [14] in a K^+ release assay. During this study we will investigate whether the K^+ release assay is a plausible tool for future studies involving

Mycobacteria tuberculosis secreted factors and their involvement in pore formation.

Specific Aim 1:

Purify recombinant *Mycobacterium tuberculosis* ESAT-6 and CFP-10 proteins expressed in *Escherichia coli.*

Specific Aim 2:

Determine membrane activities of ESAT-6 and CFP-10 using the K^+ release assay on liposomes.

Specific Aim 3:

Compare membrane activities of ESAT-6 proteins from *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*, a non-virulent *Mycobacteria* strain.

CHAPTER 2: MATERIALS AND METHODS

2.1 Expression and Purification of CFP-10

Escherichia coli BL21(DE3) transformed with pGEX-4T-1-CFP-10 were grown in 4 L of LB containing 100 µg/mL carbenicillin at 37°C. At OD_{600} 0.8, Isopropyl-β-thiogalactoside (IPTG) was added to reach a final concentration of 1 mM. Cells were harvested after three hours of induction. Cell pellet was then resuspended with 1X PBS (pH7.3), 3 mL/gram of cell, lysozyme, $MgCl_2$, and phenylmethanesulfonyl fluoride (PMSF) were added to final concentration of 0.2 mg/mL, 1 mM, and 1 mM, respectively. Lysis was carried out at 4°C for 15 minutes with constant stirring, after which cells were centrifuged at 32, 000 X g for 1 hour at 4°C. The soluble fraction was then loaded onto glutathione sepharose 4B column equilibrated with 1X PBS. Proteins were eluted using a linear gradient with elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione pH 8.0). Thrombin (1 U/mg) was added to the elute and incubated at room temperature for 14 hours. The thrombin-treated proteins were then applied to Ni^{2+}-charged sepharose column equilibrated with binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). The bound proteins were eluted with binding buffer containing 250 mM imidazole using a linear gradient at a flow rate of 5 ml/min.

2.2 Expression and Purification of ESAT-6

BL21 (DE3) cells transformed with pET-22B-esat-6 were grown using BioFlo 115 bio-reactor with 2.5 L of ECPM1 media containing 100 µg/mL carbenicillin. Culture was induced with IPTG (1mM) at OD_{600} of 1.0 and incubated for 5 hours at 37°C, after which cells were pelleted, resuspended and lysed (4°C, 30 min, with constant stirring) with B-Per, Bacterial lysis buffer, (4 mL/gram of cell) supplemented with EDTA (0.5 mM) and PMSF (100 µM). The inclusion body was isolated from cell lysate by centrifuging at 12, 100 X g for 15 mins at 4°C. The resulting pellet was then washed three times with wash buffer (50 mM Tris, 10 mM EDTA, 0.5% Triton X-100, pH 8.0). Pellet was then solubilized with 8 M urea (pH 7.4) containing 1 mM EDTA and 100 µM PMSF for four hours at room temperature and then centrifuged at 15,200 X g for 40 min at 4°C. The solubilized inclusion body was then loaded onto a Ni^{2+}-charged sepharose column equilibrated with 8 M urea, 20 mM Tris (pH 7.4), 20% glycerol buffer. The bound proteins were then refolded on the column using a linear gradient 0-88% refolding buffer (20 mM Tris, pH 7.4, 500 mM NaCl, and 20% glycerol) at a flow rate of 5 ml/min for ten column volumes. Proteins were then eluted using a linear gradient (0-100%) with refolding buffer containing 250 mM imidazole at flow rate of 5 ml/min.

2.3 Purification of CFP-10/ESAT-6 Complex

Purified ESAT-6 and CFP-10 were mixed at molar ratio 1:3 and incubated for two hours at room temperature. The mixture was then transferred to the binding buffer (20 mM phosphate, 500 mM NaCl, pH 6.8) through buffer exchange using a concentrator with (molecular weight cut off 3 kDa). The sample was then loaded onto 5 ml HisTrap column and washed 3 times using binding buffer with 20 mM imidazole. The heterocomplex was eluted with binding buffer containing 250 mM imidazole. Elution was carried out at a flow rate of 5 ml/min using a linear gradient from 0-100% for 20 column volumes.

2.4 Gel Filtration

All of the purified proteins (ESAT-6, CFP-10, CFP-10/ESAT-6) were further clarified by gel filtration using a Superdex 75 column at flow rate 0.5 ml/min in the buffer containing 20 mM Tris-HCl, 150 mM NaCl, at a pH of 7.4.

2.5 Protein quantitation

Purified proteins were quantified using BCA Protein Assay Kit (Thermo Scientific Pierce).

2.6 Native gel shift Assay

Increasing concentrations (1-2 µM) of purified ESAT-6-His6 were mixed with 1 µM of purified CFP-10-His6 and incubated at room temperature for 2 hours. Samples were then run on 8.5% Native PAGE.

2.7 Liposome Preparation

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) were solubilized in chloroform (20 mg/ml) and purged to dry under nitrogen gas. The resulting lipid film was further dried in vacuum overnight and rehydrated with 10 mM HEPES (pH 7.4) and 150 mM KCl. The lipid solution was then subjected to 6 freeze-thaw cycles and then extruded 20 passages through a filter with 200 nm pore size using a mini-extruder. The liposome solution was then loaded onto HiTrap desalting column equilibrated with 10 mM HEPES (pH 7.4) and 150 mM NaCl and eluted which resulted in liposomes having Na^+ outside and K^+ inside.

2.8 K^+ Release Assay

A 100 µl of prepared liposomes was added to 5 ml of reaction buffer (pH ranging from 4.0-9.0) with constant stirring. The purified proteins, ESAT-6 or CFP-10 or the heterocomplex, were then added to the reaction buffer containing

liposome. Release of K^+ ions from liposomes was monitored by K^+ ion selective electrode and recorded using DATAQ software.

2.9 Aggregation Assay

In a 96-well plate, 50 µg of desired protein was mixed with the buffer (pH 7.0) used in gel filtration to bring to a volume of 100 µl. An initial reading at a wavelength of 340 nm was done, this reading would then be subtracted from reading after either pH 4.0 buffer or pH 7.0 buffer was added to mixture. 50 µl of the pH buffer, 1 M NaAc or 1M Tris was added to each and mixed thoroughly by shaking for one second every five seconds for 30 seconds on shaker after which a reading was taken.

CHAPTER 3: RESULTS

3.1 Expression and Purification of CFP-10

CFP-10 is reported to be co-secreted with ESAT-6 [15, 16], so it would be necessary to purify CFP-10 and test its binding to ESAT-6. GST-CFP-10 was shown to be expressed as a soluble product of ~36 kDa, after a three hour induction, in *Escherichia coli* BL21 DE3 (Figure 3.1).

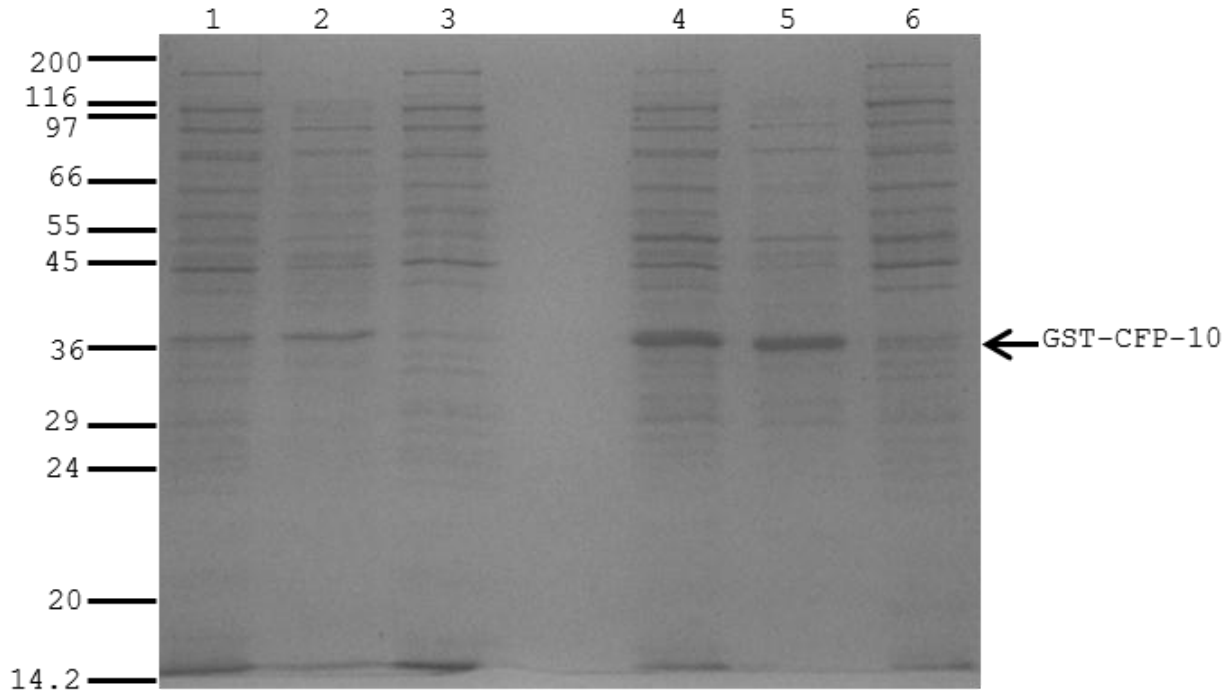

Figure 3.1: Induction and expression of CFP-10. Lanes 1-3 are whole cell lysate (WCL), soluble fraction, and insoluble fraction, respectively. Lanes 4-6 are samples which have been induced with IPTG, showing WCL, soluble fraction, and insoluble fraction, respectively.

GST-CFP-10 was then treated with thrombin while bound to the column. After a 14 hour incubation period at room temperature we

were able to detect cleaved CFP-10 and GST (Figure 3.2). About 75% of CFP-10 was cleaved and recovered (Figure 3.3). Using this approach, we were able to obtain 17 mg of CFP-10 per gram of cell weight.

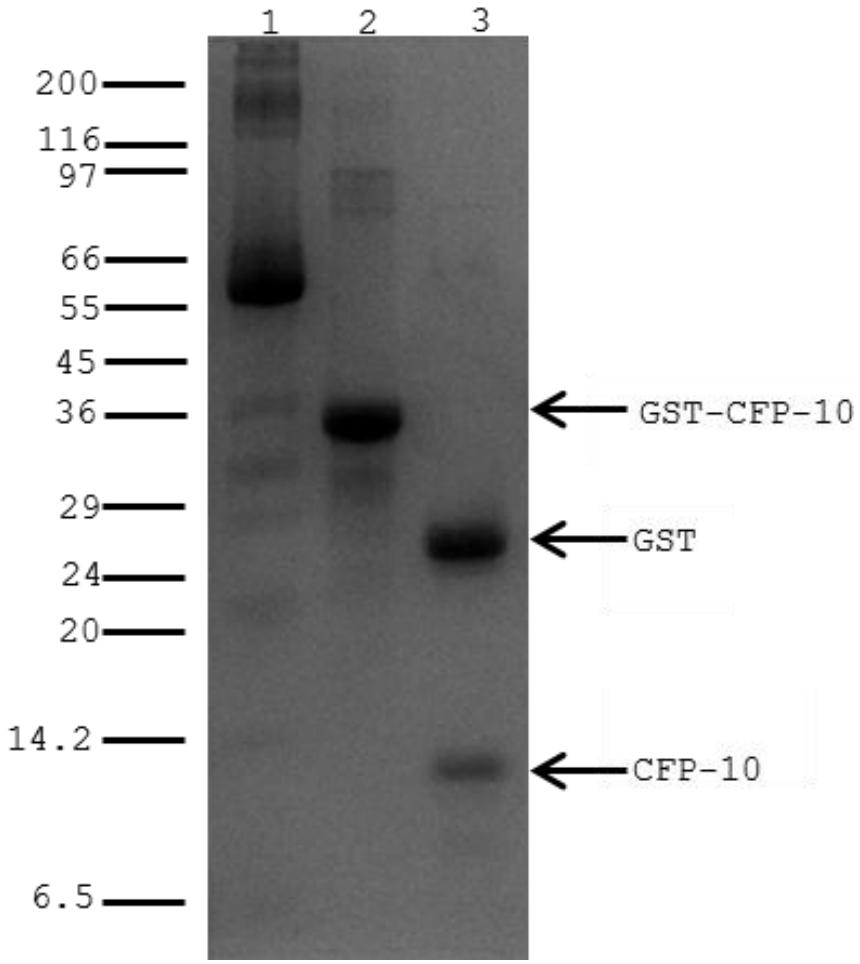

Figure 3.2: Thrombin cleaves GST moiety from CFP-10Cleavage of GST moiety from CFP-10. Lane 1 shows thrombin to ensure thrombin is not found in elutions, lane 3. Lane 2 is GST-CFP-10 prior to thrombin treatment.

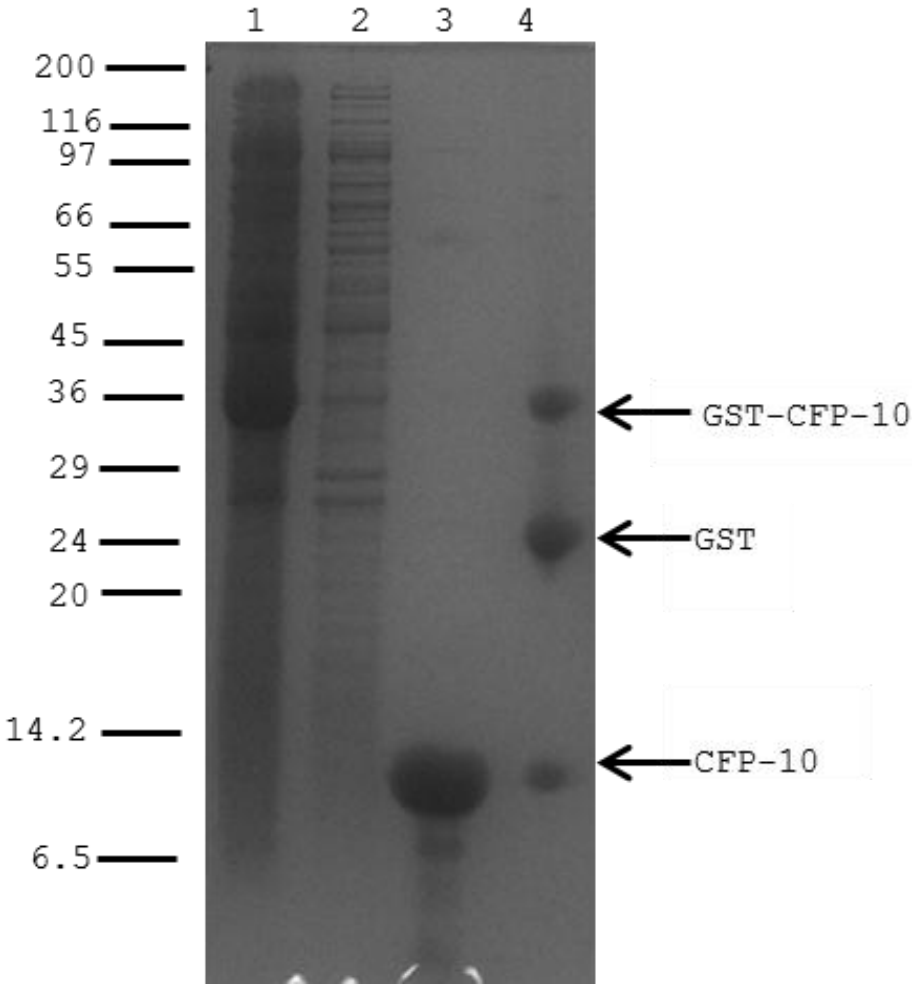

Figure 3.3: Purification of CFP-10 after thrombin cleavage. Lane 1 shows the soluble fraction. Lane 2 shows the wash after binding to GS4B column. Lane 3 shows CFP-10 purified following thrombin treatment. Lane 4 shows that GST was cleaved from CFP-10.

3.2 Expression Purification and Refolding of Insoluble ESAT-6

Previous attempts to purify GST-hexa-histidine-ESAT-6 using the same approach as described for CFP-10 resulted in expression of soluble product, yet unspecific cleavage of hexa-histidine-ESAT-6 during thrombin cleavage, which resulted in complete loss of protein.

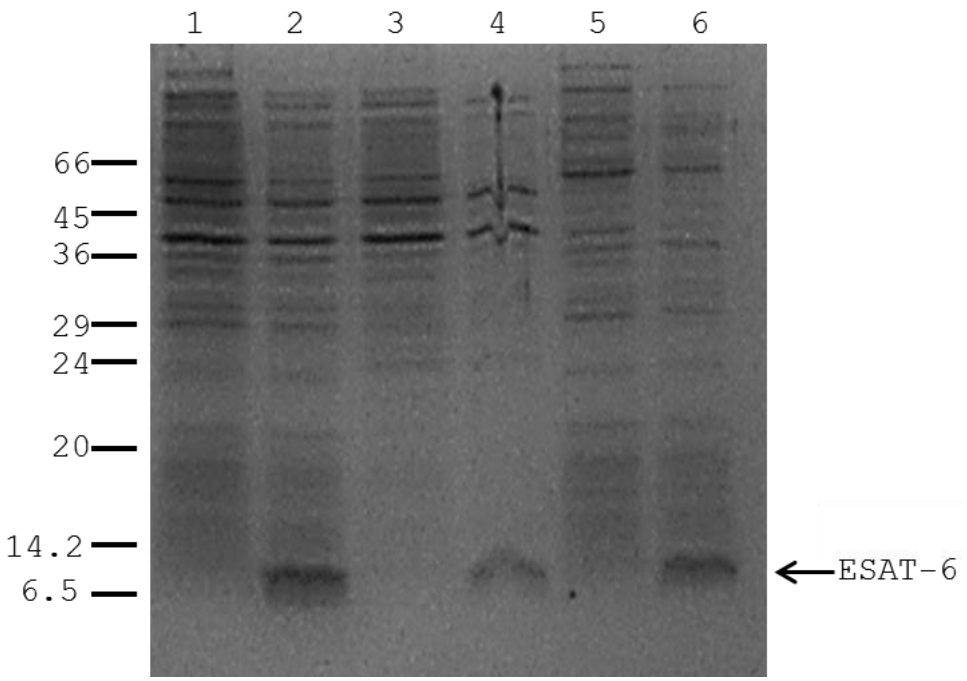

Figure 3.4: Expression of pET 22b-ESAT-6. Lane 1 and 2 are whole cell lysates prior to and after induction with IPTG, respectively. Lanes 3-6 are soluble and insoluble fraction before induction with IPTG and after induction, respectively.

Sub-cloning of *esat-6* from expression vector pET-15b into pET-22b, we were able to express ESAT-6 in BL21 cells as before with the exception that ESAT-6 was found to be expressed as an insoluble inclusion bodies (Figure 3.4), which is consistent with other reports. ESAT-6 is expressed as an insoluble protein in *Escherichia coli*. Here we developed a more efficient purification strategy so as to not lose product due to changes of various purification columns. The pellet containing insoluble inclusion body was solubilized with 8 M urea; the soluble extract was then loaded onto a Ni^{2+} charged column. Prior to elution urea was slowly diluted out using a linear

gradient, 0-88%, over a period of 2.5 hours, ensuring proper folding of ESAT-6. During elution the remainder urea was removed and the yield of the refolded ESAT-6 was 26 mg per gram of cell weight, and purity is about 80% (Figure 3.5 lane 4 and lane 5).

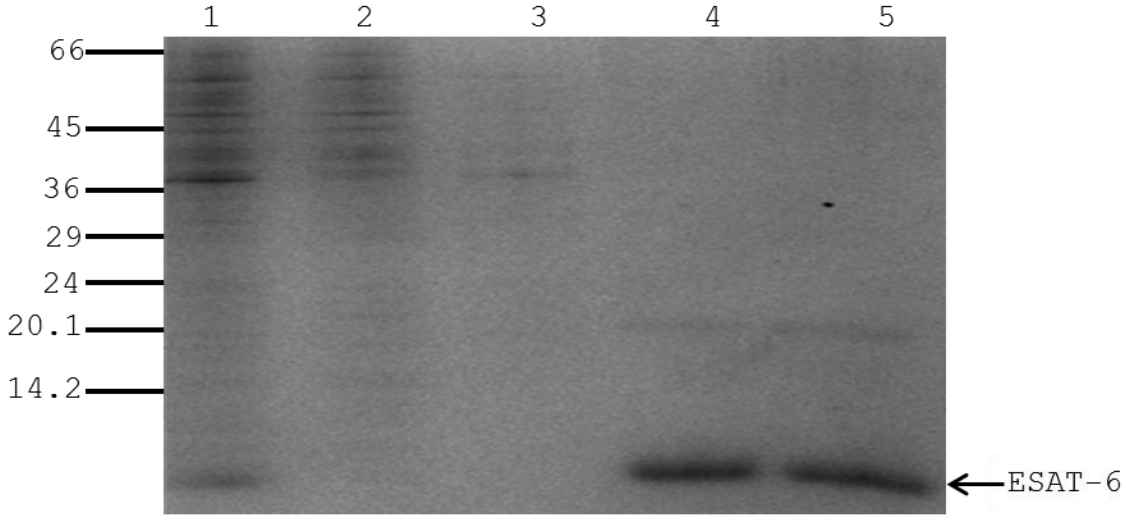

Figure 3.5: Purification of ESAT-6 after on- column refolding. Lane 1 showing insoluble fraction after treatment with 8 M urea. Lane 2 is the flow through after urea solubilized fraction was loaded onto the HisTrap column. Lane 3 is after 3 column volume of washing. Lane 4 and 5 are the elution of refolded ESAT-6.

3.3 ESAT-6 Refolded on Ni^{2+} Charged Column Binds CFP-10

Previous studies have indicated that ESAT-6 and CFP-10 form a heterodimer [11] and Renshaw et. al. have "conclusively" shown that these two proteins form a 1:1 complex. To evaluate whether the refolded and purified ESAT-6 is functional, we looked at the binding of the two purified proteins. On an 8.5% native gel the

migratory patterns of ESAT-6 and CFP-10 alone differed significantly (Figure 3.6).

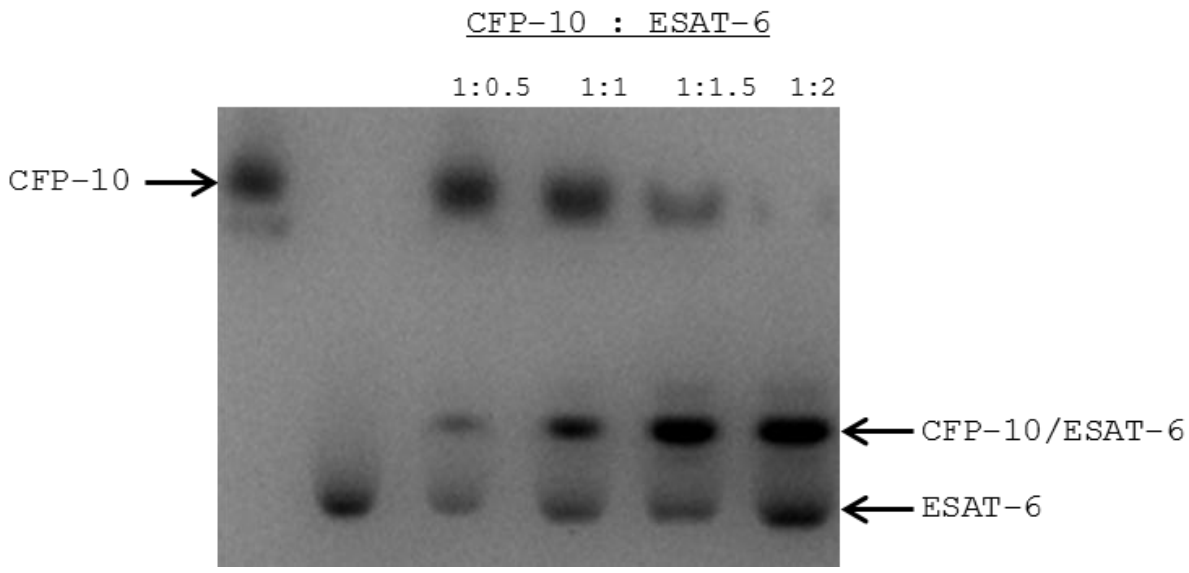

Figure 3.6: Native gel shift binding assay. On-column refolding resulted in properly folding of ESAT-6 so as to bind CFP-10. Lane 1 and 2 show 2 µg of CFP-10 and ESAT-6, respectively. Lanes 3-6 show formation of heterodimer while maintaining a constant CFP-10 concentration ESAT-6 binds in able to bind in a dose dependent manner as evident by the steadily increase in formation of heterodimer.

The differing migrating patterns of both proteins allowed for unambiguous distinction and thus it was possible to visualize binding upon mixing the two monomers together. CFP-10 concentration was held constant while mixing with various concentrations of ESAT-6 it was apparent that binding had occurred in a concentration dependent manner, as seen by the decrease in CFP-10 and an increase in the complex.

3.4 Isolation of Esat-6/Cfp-10 complex shows 1:1 binding

In order to carry out any further biochemical studies on the heterodimer, it would be necessary to further isolate the complex from any unbound monomers, which have been shown to have membrane disrupting activity individually [17]. ESAT-6 was mixed with an excess of CFP-10 (1:3), and after 2 hours of incubation, the mixture was then loaded on to a 5 ml HisTrap column. Using this approach we were able to isolate the heterocomplex, which was further confirmed by gel filtration (figure 3.7).

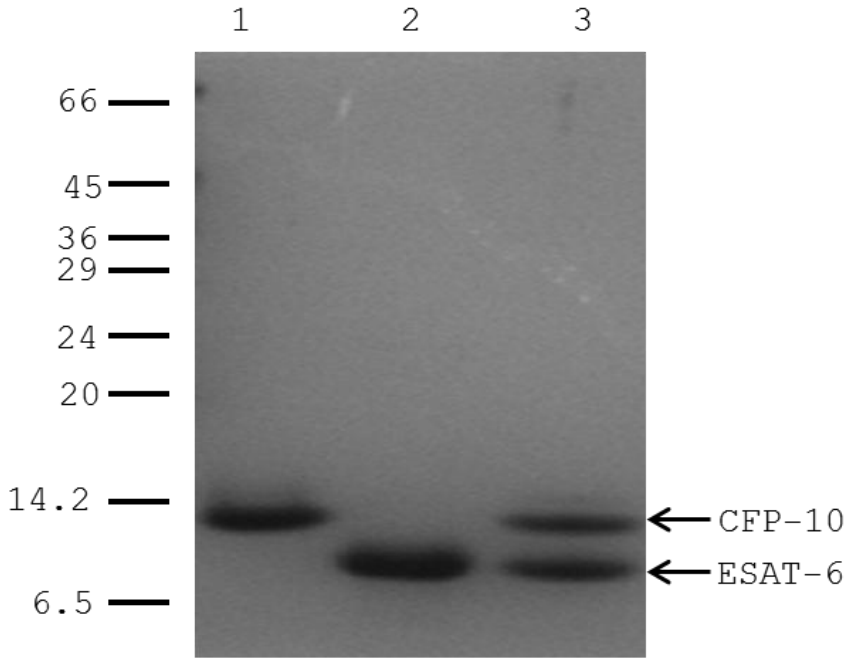

Figure 3.7: Final purification step of all proteins. All proteins were subject to gel filtration. Lane 1 is purified CFP-10. Lane 2 is purified ESAT-6. And lane 3 is the purified 1:1 heterocomplex.

3.5 Disruption of Liposomal Membranes at Low pH

Previous works have utilized liposomal membranes to study bacterial toxins and their ability to cause membrane disruption [14]. de Jonge et al. had suggested that interaction of ESAT-6 with liposomal membrane is pH dependent. We have utilized liposomes which contained KCl and upon liposomal disruption a release of K^+ could be detected using a K^+ ion selective electrode. Here we are able to show our approach to purify and refold ESAT-6 results in a functional protein capable of liposomal membrane disruption as evidenced by the detection of the release of K^+ (Figure 3.8).

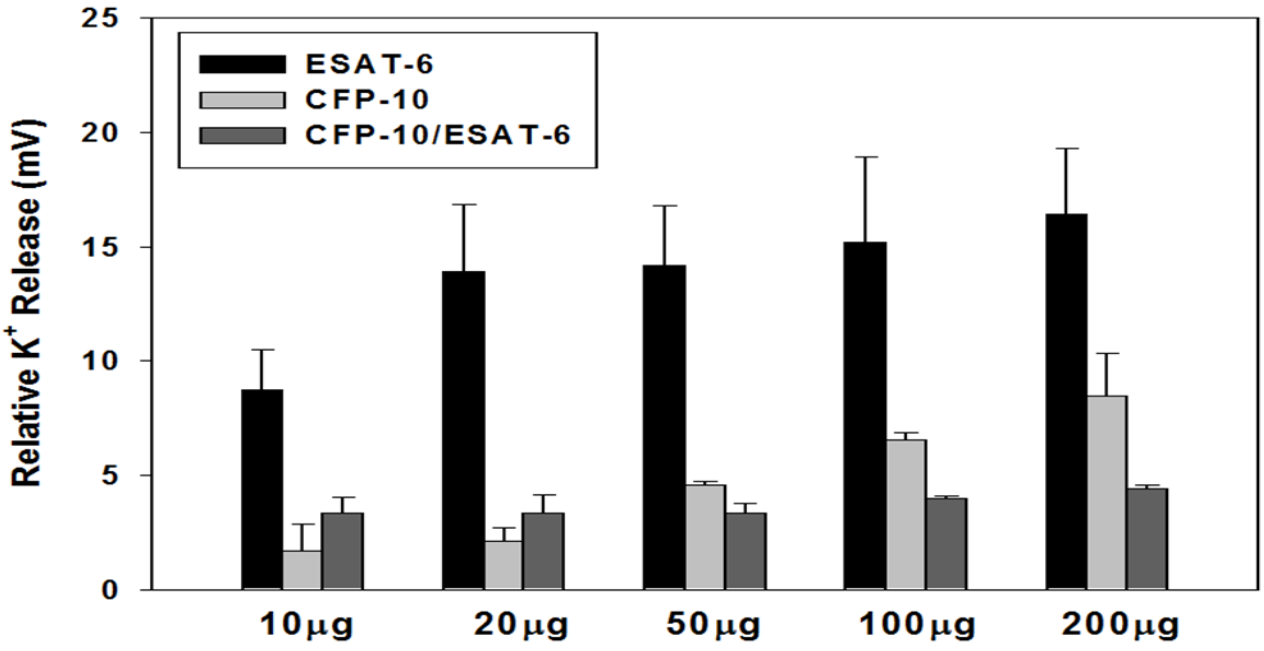

Figure 3.8: ESAT-6 disrupts liposomes by measuring the release of K^+. ESAT-6 is involved in a greater amount of K+ and while the heterocomplex appears to have no effect on disrupting liposomes.

CFP-10 appeared to disrupt the liposomal membrane in a dose dependent fashion albeit that the amount of K^+ release was substantially less than what was seen with ESAT-6. When the hetero-complex, ESAT-6/CFP-10, was tested it maintained a steady level of K^+ release despite the amount of protein introduced into the reaction mixture.

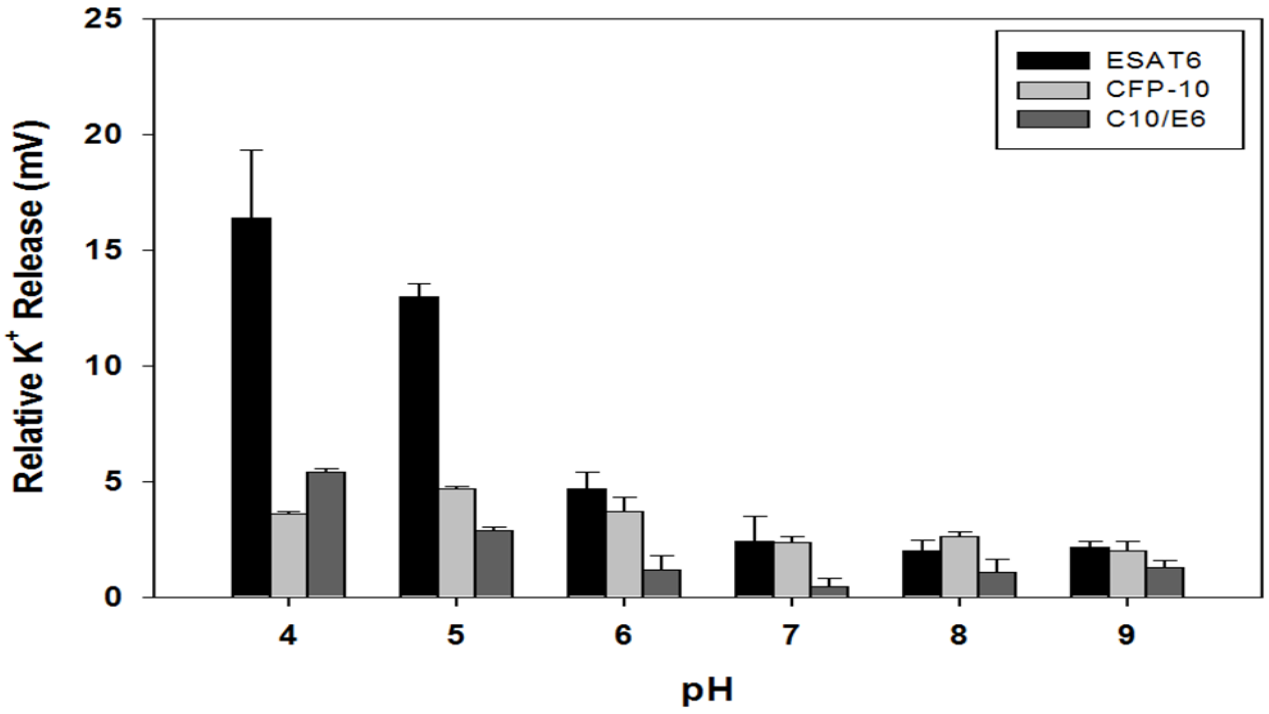

Figure 3.9: Comparison of purified proteins at various pH. As the pH is lowered it is shown that ESAT-6 (100 µg) has an increased effect on the disruption of liposomes. CFP-10 (100 µg) and the heterocomplex (200 µg) display activity at low pH but not to the extent as ESAT-6.

At a pH of greater than 6.5 ESAT-6 is unable to completely dissociate from CFP-10 [17] and here we have shown that at pH of 7.0 and greater there is no significant amount of K^+ detected and that pH 4.0 is the optimal pH to allow for a significant release

of K^+ (Figure 3.9). Here once again it is shown that the heterocomplex was unable to generate a K^+ reading similar to that of ESAT-6 alone. The results suggest that the heterocomplex did not dissociate at low pH.

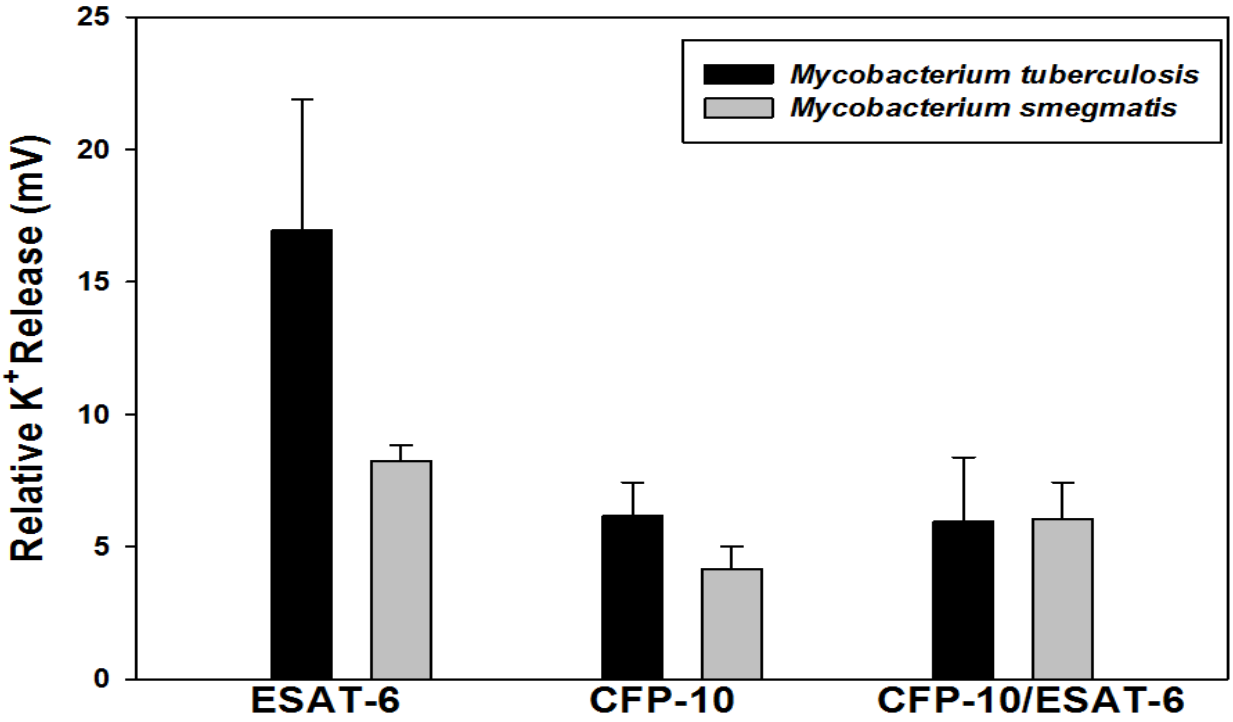

Figure 3.10: K^+ release from virulent and avirulent *Mycobacteria* at pH 4.0. *Mycobacterium tuberculosis* ESAT-6 shows a greater release of K^+.

To this point we have shown that using K^+ release we are able to distinguish the various activities of recombinant *Mycobacterium* tuberculosis secreted factors ESAT-6, CFP-10, and heterocomplex on liposomal membranes. We next compared those activities to that of *Mycobacterium smegmatis*. We used

recombinant *Mycobacterium smegmatis* ESAT-6, CFP-10, and heterocomplex which had previously been purified by the same approach we have shown here for ESAT-6 (data not shown). When comparing all proteins of both species at pH 4.0, we see that the activity of CFP-10 and heterocomplex of both species are similar in liposomal disruption. On the other hand ESAT-6 of *Mycobacterium tuberculosis* shows a greater amount of K^+ being released when compared to that of *Mycobacterium smegmatis* (Figure 3.10).

3.6 COMPLEX DOES NOT DISSOCIATE AT LOW PH

In order to confirm that ESAT-6 was not dissociating from CFP-10 at pH 4.0 we carried out an aggregation assay. Here we showed that at low pHs ESAT-6 formed aggregation in solution while little aggregation was observed at pH 7.0. In contrast, CFP-10 alone and the heterocomplex showed no signs of aggregation, even at low pHs (Figure 3.11). Thus the data suggests that at the low pH ESAT-6 forms aggregation in solution, but in heterocomplex ESAT-6 is not dissociating from CP-10, thus prevents ESAT-6 from interacting with membranes.

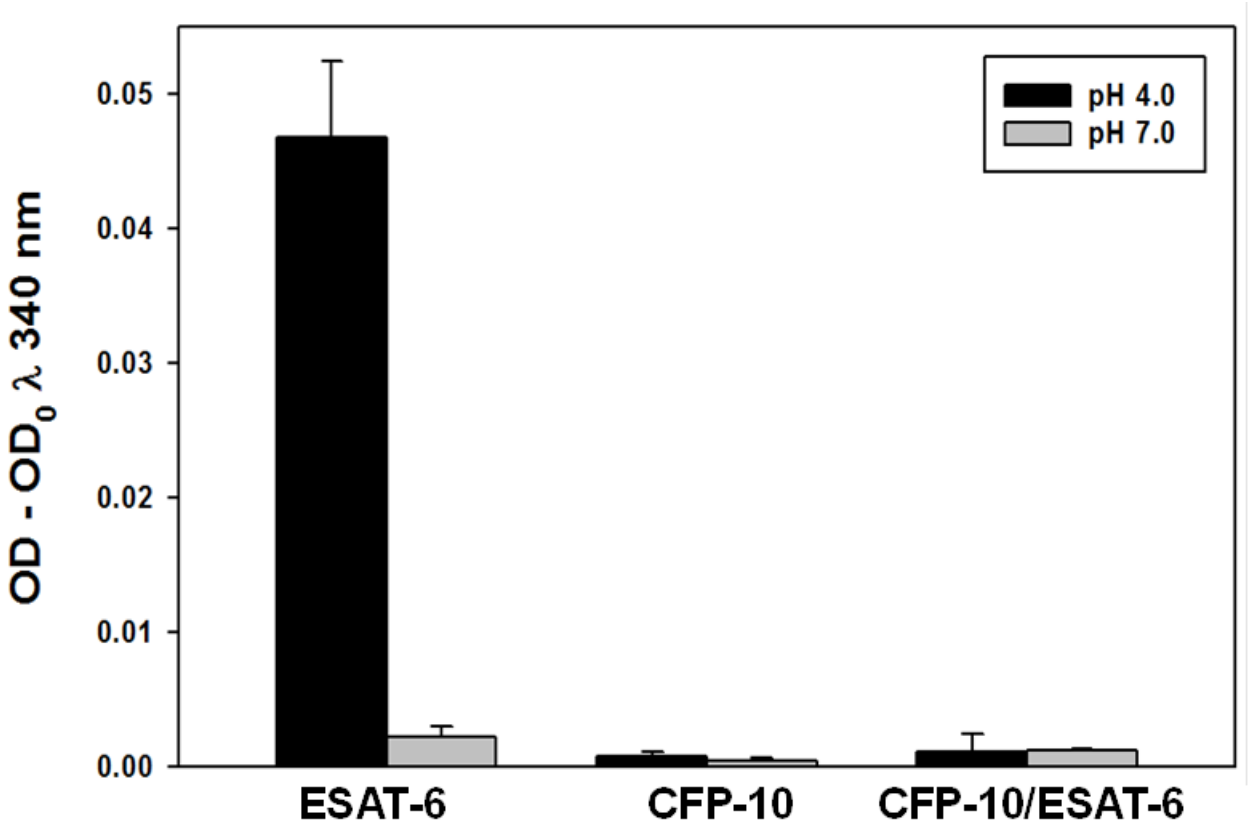

Figure 3.11: ESAT-6 forms aggregates at pH 4.0. While CFP-10 and the hetero-complex forms no aggregates at both pH 4.0 and pH 7.0.

CHAPTER 4: DISCUSSION

Our approach of purification resulted in a substantial amount of protein yields for *Mycobacterium tuberculosis* ESAT-6, CFP-10 and heterodimer. It is about 20-30 mg on average from each preparation, and achieved purity upwards of 90%. Other groups working with such proteins purify these two proteins from the culture filtrate of *Mycobacterium tuberculosis*. *Mycobacteria* tuberculosis has a doubling time of 12-24 hours. With such a long doubling time it could take several weeks to culture the bacteria, which results in limited yields of proteins. When one compares the doubling rate of *Escherichia* coli, which is about twenty minutes, a large culture could be generated, allowing for the potential to isolate a large of amount of proteins of interest. Groups have utilized an expression system for the expression of ESAT-6 and CFP-10 in *Mycobacterium smegmatis*, although this particular species grows at a much faster rate, two to three days for colony appearance. Still quite slow when compared to the potential yield one could generate using *Escherichia coli*. An additional advantage of having *Escherichia coli* is that when the proper facilities required for work with *mycobacteria* species is not available, such as a biosafety level III lab, one could study *mycobacteria* utilizing the fast growing *Escherichia coli*. This approach has

also been used in the purification of *Mycobacterium smegmatis* ESAT-6 and CFP-10 expressed in *Escherichia coli*, with success.

Many groups when purifying ESAT-6 and CFP-10 resulted in generating an insoluble product, especially with ESAT-6. Groups who have expressed ESAT-6 in *Escherichia coli* has utilized dialysis for refolding, which has a time disadvantage compared to the on-column refolding developed by us. This method does save time as it can be done in a single day. In addition to saving time the volume of buffers needed is reduced when folding ESAT-6 through an on column method. With that being said, our on-column approach using affinity chromatography is accomplished using one single column, in which we can use this system to purify protein and fold the protein of interest without changing columns. Through several column changes one can lose product, so using a single column for multiple steps prevents the loss of product. An additional advantage is that as the urea is being diluted one could get proper folding, but the potential to form aggregates may also occur. Allowing protein to be bound to resin can reduce aggregation during the refolding process, since the protein are immobilized on the matrix until complete folding has occurred. Once the protein has properly folded in the majority of cases aggregation is prevented.

Following purification we had to verify that purified ESAT-6 and CFP-10 were able to bind each other. We accomplished this

by way of native gel binding assay. In this assay we maintained the concentration of CFP-10 while increasing the concentration of ESAT-6. We were able to visualize complex formation by the disappearance of CFP-10 band and the appearance of heterocomplex. To further show that our approach to refolding ESAT-6 while bound to resin is advantageous. After refolding and having previously purified CFP-10 one can load, by injection, the purified CFP-10 onto the column having harboring refolded ESAT-6. After incubation the column is washed with buffer allowing any unbound CFP-10 to flow through. The heterocomplex was eluted from the column and it is from our experience that the complex has been isolated to 100% purity in a 1:1 molar ratio.

Having purified CFP-10, ESAT-6, and ESAT-6/CFP-10 we wanted to determine if using K^+ release assay would be a suitable approach to studying these proteins. With the understanding that ESAT-6 displays activity at a low pH of about 4.5, we looked to see at which amounts of proteins give an appreciable signal and if that signal reflects any differences between all three proteins being studied. Our results had indicated that at a pH of 4.0 ESAT-6 activity reached a saturation point at about 20 µg. CFP-10 showed a steady increase in the release of potassium, lower than ESAT-6, in which it can be assumed that membrane disruption may be is dose dependent. The hetercomplex

showed very little activity when compared to the ESAT-6 and CFP-10 alone. This result was surprising as we had expected the heterocomplex to give similar results as ESAT-6. Previous work done by other groups has identified two forms of ESAT-6 using 2-D electrophoresis. They had distinguished the two forms by mass spectrometry and found that one form of ESAT-6 was N-terminally acetylated [18]. They give speculation that this post translational modification may play a role on how ESAT-6 and CFP-10 associate and dissociate with each other specifically ESAT-6 N-terminal acetylation may prompt the dissociation of the complex.

In order to see why activity was low with the complex when compared to ESAT-6 and using the information provided in the literature, we wanted to investigate the notion that the low activity may be attributed to the inability of ESAT-6 to dissociate from CFP-10. Our approach to investigate dissociation was to use an aggregation assay. During this assay we compared all three proteins at two different pHs, one at pH 4.0 and the other at pH 7.0. From the data it seems that the heterocomplex is not dissociated. At a low pH we see that ESAT-6 has a tendency to form aggregates, which seen through the spectrophotometer readings. Our thought is that since ESAT-6 displays aggregation the heterocomplex should display similar results at pH 4.0. We did not see the expected aggregation,

suggesting that ESAT-6 and CFP-10 maintain interaction despite the drop of pH, which is consistent with the result of our K^+ release assay where we were not able to record a strong signal with the heterocomplex. This data may further give insight as to the role that CFP-10 may have as well. We were not able to detect aggregation with CFP-10 indicating that it maintains stability at low pH. This stability may further implicate that the role of CFP-10 may aid in the stabilization of ESAT-6 in a low pH environment.

4.1 Future Direction

The issue of dissociation and association of the heterocomplex should be resolved. One step towards such resolution would be to subject purified ESAT-6 to mass spectrometry [18, 19]. Using this approach one could determine the various forms of the ESAT-6, especially acetylated or non-acetylated form. It was reviewed that N-acetylation more commonly occurs with eukaryotic proteins, yet although rare can occur with prokaryotic proteins being that they do harbor a N-terminal acetyltransferase [20]. Acetylation occurs on amino acids threonine and serine. Okkels et al had shown that the acetylated form of ESAT-6 was found on a threonine residue [18]. Many groups have utilized mutations which can mimic acetylation [21, 22, 23, and 24].

Through the works presented here it could possible to further characterize the interaction of these proteins on an individual basis and a complex. Deletions of either N- or C-terminus of the proteins could aid in the identification of the initial interactions of protein with lipid membranes. Additionally identifying the major contributor to the interaction it could also be possible to map the interaction. One advantage is that both CFP-10 and ESAT-6 have no cysteine. Ideally one can introduce a single point cysteine mutation into the proteins. An environmental sensitive fluorophore, 7-Nitrobenz-2-oxa-1,3-diazole (NBD), can bind the cysteine and emit fluorescence when this dy is inserted into a non-polar environment. Thus, one could identify where that amino acid in relation to exposure to an aqueous or nonaqueous environment [25, 26].

An additional direction is on further investigation of interaction of the potential pore forming toxin with lipid membrane. As reviewed in Biological Cell, cholesterol is an important contributing factor to activity of toxin, such as serving a receptor or providing an ideal environment for the formation of pores on the host cell (27). We currently used DOPC as lipid membrane for K^+ release assay. It could be possible to compare these results with the results of future

experiments in which one would generate liposomes containing various concentrations of cholesterol [17].

Another approach to support the idea that ESAT-6, CFP-10, or CFP-10 are forming pores we could make use of molecular rulers to measure the size of these pores. Using various sizes of ethylene glycol's and/or polyethyelene glyocols is could be possible to determine the size of pores generated by these proteins (28). Determination of pore sizes could give a good indication of the composition of the pore itself as it relates to recombinant proteins interacting with prepared liposomes.

REFERENCES

1. **World Health Organization**. (2011) *Global Tuberculosis Control: WHO Report 2011.*
2. **Centers for Disease Control**. (2010) *Reported tuberculosis in the United States, 2010.*
3. **Styblo K.** (2008) Recent advances in epidemiological research in tuberculosis. *Advance Tuberculosis Research* 20: 1-63
4. **Mahairas, G.G, P.J. Sabo, M.J. Hickey, D.C. Singh, and C.K. Stover.** (1996) Molecular Analysis of Genetic Differences between *Mycobacterium bovis* BCG and Virulent *M. bovis*. *Journal of Bacteriology* 178: 1274-1282.
5. **Champion, P.A, S.A. Stanley, M.M. Champion, E.J. Brown, and J.S. Cox.** (2006) C-terminal signal sequence promotes virulence factor secretion in Mycobacterium tuberculosis. *Science* 313: 1632-1636.
6. **Abdallah, M.A., N.C. van Pittius, P.A. Digiuseppe Champion, J. Cox, J. Luirink, C.M.J.E. Vandenbroucke-Grauls, B. J. Appelmelk, and W. Bitter**. (2007) Type VII secretion-mycobacteria show the way. *Nature Reviews* 5: 883-891.
7. **Gao, L.Y, S. Guo, B. McLaughlin, H. Morisaki, J.N. Engel, and E.J. Brown**. (2004) A mycobacterial virulence gene cluster extending RD1 is required for cytolysis, bacterial spreading and ESAT-6 secretion. *Molecular Microbiology* 53: 1677-1693.

8. **Kinhikar, A.G., I. Verma, D. Chandra, K.K. Singh, K. Weldingh, P. Andersen, T. Hsu, W.R. Jacobs Jr., S. Laal.** (2010) Potential role for ESAT6 in dissemination of *M. tuberculosis* via human lung epithelial cells. *Molecular Microbiology* 75:92-106.
9. **Smith, J., J. Manoranjan, M. Pan, A. Bohsali, J. Xu, J. Liu, K.L. McDonald, A. Szyk, N. LaRonde-LeBlanc, and L.Y. Gao.** (2008) Evidence for Pore formation in Host Cell Membranes by ESX-1 –Secreted ESAT-6 and Its Role in *Mycobacterium marinum* Escape from the Vacuole. *Infection and Immunity* 76: 5478-5487.
10. **Pallen, M.J.** (2002) The ESAT-6/WXG100 superfamily-and a new Gram Positive secretion system. *Trends in Microbiology* 10: 209-212.
11. **Renshaw, P.S., P. Panagiotidou, A. Whelan, S.V. Gordon, R.G. Hewinson, R.A. Williamson, and M.D. Carr.** (2002) Conclusive Evidence That the Major T-cell Antigens of the *Mycobacterium tuberculosis* Complex ESAT-6 and CFP-10 Form a Tight, 1:1 Complex and Characterization of the Structural Properties of ESAT-6, CFP-10, and the ESAT-6/CFP-10 Complex. *The Journal of Biological Chemistry* 277: 21598-21603.
12. **Renshaw, P.S., K.L. Lightbody, V. Veverka, F.W. Muskett, G. Kelly, T.A. Frenkiel, S.V. Gordon, R.G. Hewinson, B. Burke, J. Norman, R.A. Williamson, and M.D. Carr.** (2005) Structure

and function of the complex formed by the tuberculosis virulence factors CFP-10 and ESAT-6. *European Molecular Biology Organization* 24: 2491-2498.

13. **Silberstein, A., T. Mirzabekov, W.F. Anderson, and Y. Rozenberg.** (1999) Membrane destabilization assay based on potassium release from liposomes. *Biochemica Et Biophysica Acta* 1461: 103-112.
14. **Sun, J. G. Vernier, D.J. Wigelsworth, and R.J. Collier.** (2007) Insertion of Anthrax Protective Antigen into Liposomal Membranes, Effects of a Receptor. *The Journal of Biological Chemistry* 282: 1059-1065.
15. **Berthet, F.X., P.B. Rasmussen, I. Rosenkrands, P. Andersen, and B. Gicquel.** (1998) A *Mycobacterium tuberculosis* operon encoding ESAT-6 and a novel low-molecular mass culture filtrate protein (CFP-10). *Microbiology* 144: 3195-3203.
16. **Stanley, S.A., S. Raghavan, W.W. Hwang, and J.S. Cox.** (2003) Acute infection and macrophage subversion by *Mycobacterium tuberculosis* require a specialized secretion system. *PNAS* 100: 13001-13006.
17. **De Jonge, M.I., G. Pehau-Arnaudet, M.M. Fretz, F. Romain, D. Bottai, P. Brodin, N. Honore, G. Marchal, W. Jiskoot, P. England, S.T. Cole, and R. Brosch.** (2007) ESAT-6 from *Mycobacterium tuberculosis* Dissociates from Its Putative Chaperone CFP-10 under Acidic Conditions and Exhibits

Membrane-Lysing Activity. *The Journal of Bacteriology* 189: 6028-6034.

18. **Okkels L.M., E.C. Muller, M. Schmid, I. Rosenkrands, S.H.E. Kaufmann, P. Andersen, and P.R. Junblut.** (2004) CFP-10 discriminates between nonacetylated ESAT-6 of *Mycobacterium tuberculosis* by differential interaction. *Proteomics* 4: 2954-2960.
19. **Yang, Y, H.C. Hang.** (2011) Chemical Approaches for the Detection and Synthesis of Acetylated Proteins. *ChemBioChem* 12: 314-322.
20. **Polevoda, B. and F. Sherman.** (2003) N-terminal Acetyltransferases and Sequence Requirements for N-terminal Acetylation of Eukaryotic Proteins. *The Journal of Molecular Biology* 325: 595-622.
21. **Li, M., J. luo, C.L. Brooks, and W. Gu.** (2002) Acetylation of p53 Inhibits Its Ubiquitination by Mdm2. *The Journal of Biological Chemistry* 277: 50607-50611.
22. **Luo, J., M. Li, Y. Tang, M. Laszkowska, R.G. Roeder, and W. Gu.** (2003) Acetylation of p53 augments its site-specific binding both in vitro and in vivo. *PNAS* 101: 2259-2264.
23. **Matsuzaki, H. H. Daitoku, M. Hatta, H. Aoyama, K. Yoshimochi, and A. Fukamizu.** (2005) Acetylation of Foxo1 alters its DNA-binding ability and sensitivity to phosphorylation. *PNAS* 102: 11278-11283.

24. **Hatta, M., F. Liu, and L.A. Cirillo.** (2009) Acetylation curtails nucleosome binding, not stable nucleosome remodeling, by FoxO1. *Biochemical and Biophysical Research Communications* 379: 1005-1008.

25. **Johnson, A.E.** (2005) Fluorescence Approaches for Determining Protein Conformations, Interactions and Mechanisms at Membranes. *Traffic* 6: 1078-1092.

26. **Shepard, L.A., A.P. Heuck, B.D. Hamman, J. Rossjohn, M.W. Parker, K.R. Ryan, A.E. Johnson, R.K. Tweten.** (1998) Identification of Membrane-Spanning Domain of the Thiol-Activated Pore-Forming Toxin *Clostridim perfringens* Perfringolysin O: An α-Helical to β-Sheet Transition Identified by Fluorescence Spectroscopy. *Biochemistry* 37: 14563-14574.

27. **Geny, B., M.R. Popoff.** (2006) Bacterial protein toxins and lipids: role in toxin targeting and activity. *Biological Cell* 98: 633-651.

28. **Nablo, B.J., K.M. Halverson, J.W.F. Robertson, T. L. Nguyen, R.G. Panchal, R.Gussio, S.Bavari, O.V. Krasilnikov, and J.J. Kasianowicz.** (2008) Sizing the *Bacillus anthracis* PA63 Channel with Nonelectrolyte Poly(Ethylene Glycols). *Biophysical Journal* 95: 1157-1164.

CURRICULUM VITA

Joaquin A. De Leon III was born in Muleshoe, Texas. He is the eldest son of Joaquin and Virginia De Leon. Joaquin graduated from Clovis High School, Clovis, New Mexico, in 1998 and entered Eastern New Mexico University to pursue a Bachelors of Science in Biology. He worked as a pharmacy technician during his undergraduate work until graduating with a B.S. in Biology in the fall of 2005. He began his graduate work at ENMU as a Bridges Scholar in the spring of 2006 where he worked with the acid tolerant bacteria, *Halothiobacillus neapolitanus*. His work on *H. neapolitanus* resulted in a small publication in 2007. In 2008 transferred to the University of Texas at El Paso to further pursue his graduate studies. During this time he taught many undergraduate lab courses in microbiology, anatomy and physiology, and general biology. Joaquin's research focused on *Mycobacterium tuberculosis* where under the mentorship of Dr. Jianjun Sun. he gained valuable experience and appreciation for the art of protein purification.

Permanent Address: 2901 Duckworth Ave.
Clovis, New Mexico 88101

This thesis was typed by Joaquin A. De Leon III.