PRODUCTION OF RECOMBINANT INJECTOSOME AND OUTER MEMBRANE PROTEINS FROM *YERSINIA PESTIS* KIM5

THESIS

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AFIT/GWM/ENP/09-S01

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THESIS

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Abstract

Molecular biology techniques and low cost reagents have lowered the barriers to entry for development of biological arsenals by non-state and state groups. Additionally, genetic engineering of epitope targets from such pathogens as *Y. pestis* would annul current detection methods and therapeutic treatments. Vaccines often have short shelf lives and are of minimal utility if not used prior to exposure to BW agents. *Camelidae,* including camels and llamas produce unique antibodies termed Nanobodies[®] (Nbs) or antigen specific fragments (V_{HH}) which are much smaller than traditional antibodies (15 vs. ~150 kDal) yet seem to attach with the same selectivity and affinity as full antibodies. V_{HH} are more stable than the fragile, more bulky antibodies and maintain their structure and function even at high temperature and humidity. It is thought that, due to these unique characteristics, V_{HH} could be reconstituted from a lyophilized pellet and used as a real time injectable immunotherapeutic to be used when warfighters have been exposed to BW. Two logical candidates for V_{HH} production are the low-calcium-response V protein (LcrV) and the needle-like Yop (Yersinia Outer-membrane Protein) Secretion Protein F (YscF). This effort successfully produced quantities greater than 1-mg purified native recombinant LcrV and YscF proteins as antigens for V_{HH} production.

Acknowledgments

To the Lord of Creation, Creator of the heavens and earth. I am everyday humbled and in amazement at the many blessings I have received. Thank you wife (your name is privacy act information so I can't use it), for your support (sometimes just tolerance) and many sacrifices for <u>our</u> career. I am very excited to see what lies ahead in our crazy adventure. Thank you Dr. Camilla Mauzy (you are DoD property and I can use your name) for your mentorship and friendship over the last few years, you will be a member of my family for many years to come. I am truly grateful for you always taking the time to teach me the ins and outs of fields ranging from polygenetic phenotype analysis to sushi.

Thank you Dr. John Schlager for letting me work in your branch and letting me make mistakes. Thank you Capt Sean Stevens for setting the bar high and teaching me what it means to be an officer. Thank you Dr. David Riddle and Mr. Jordan Williamson for your help with this project. Without your contributions this would not be possible.

To the team members of Bldg 837 (regardless of rank, education, or shoe size): my time spent working, laughing, and crying with you has enriched my life.

Jeremiah N. Betz

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PRODUCTION OF RECOMBINANT INJECTOSOME AND OUTER MEMBRANE

PROTEINS FROM *YERSINIA PESTIS* KIM5

1. Introduction

1.1 Chapter Overview

Yersinia pestis, formerly *Pasteurella pestis,* has long been a blight upon the human species.   As the causative agent of bubonic, septicemic, and pneumonic plagues, this bacterium has caused the deaths of hundreds of millions of people.   Due to its high mortality rate, potential for major public health impact, potential to cause public panic and social disruption, and special actions required by public health, the Centers for Disease Control has classified *Y. pestis* as a Category A Agent (CDC 2009).   This bacterial disease is endemic in a large portion of the globe including the continental United States, South America, Southeast Asia and parts of Africa (WHO 1970).   While transmission of this zoonotic disease is often through flea bites, indigenous populations of rats, mice, and other members of the order rodentia harbor the bacteria.   Aerosolized carrier byproducts or direct contact with an infected organism may also result in illness. The human infection type (bubonic, septicemic, and pneumonic) of *Y. pestis* may start in one or more systems in the body depending upon route of entry.   The WHO reports there are 1000-2000 naturally occurring reported cases each year (WHO 2009).   If untreated with antibiotics the fatality rate ranges from 50-60% for bubonic plague to nearly 100% for septicemic and pneumonic plague.   Taken

together, 1 in 7 cases are fatal among treated and untreated victims in the United States (WHO 2009).

1.2 Problem Statement

The barrier to entry in the fields of microbiology and molecular biology has never been lower. Literature is publically and easily available for culturing many hazardous agents, and several massive public electronic databases exist with the sequences of Marburg Virus, Variola Virus, and others. The medical community has several tools to combat these biological threats; however, genetic manipulation of critical genes and the long lag time for vaccine development may limit their effectiveness against purposeful targeted BW outbreaks.

1.3 Proposed Solution

One tool that may combat this threat is the use of immunotherapeutic administration of antigen specific fragments (V_{HH}). V_{HH} are produced by cleaving a variable, antigen-specific domain from naturally occurring single-domain Camelid antibodies.

The present study will overproduce and purify two *Yersinia pestis* KIM5 (Appendix C. Strains) proteins, LcrV and YscF, using molecular biology techniques, to be used as antigens for V_{HH} development. The DNA gene sequences coding for these proteins will be put into expression vectors and transformed into *E. coli.* The protein over-expression and purification will be performed without the aid of fused *N-* or *C-*terminal tags.

1.4 Research Questions

1. Can LcrV and YscF be over-expressed in *E. coli* and purified without tags?

2. Is the protein able to be over-expressed to a significant degree for purification?

3. Does the *E. coli* modify the proteins' primary structure post-translationally?

1.5 Significance of Results

The study of V_{HH} as immunotherapeutics for combating the deleterious effects from biological weapons of mass destruction is a credible and working pursuit for the DoD, as evident by their funding of immunotherapeutic research. Producing and purifying these two recombinant proteins is the initial step for production of the V_{HH}. In April 2009, 2 mg quantities of purified recombinant LcrV and YscF was shipped to Air Force Research Laboratory collaborators in Belgium for V_{HH} development. The efficacy of the V_{HH} to protect against *Y. pestis* infection will determine their future for *in vivo* DoD applications.

1.6 Research Focus

The methods and tools that will be applied answering the research questions are:

1. Clone genes for LcrV and YscF DNA and sequence verity constructs

2. Transfer genes into expression vectors

3. Transform constructs into expression strains and express the proteins.

4. Harvest and purify excess of 2 mg of both LcrV and YscF proteins.

1.7 Methodology

1.7.1 Plasmid Development

Genomic DNA extracted from *Y. pestis* KIM5 was used as a PCR template to clone gene sequences. Custom primers flanking the entire 5' to 3' region of

DNA coding for native LcrV and YscF proteins were employed to replicate the sequences. Amplification was followed by an initial round of ligation and transformation into pCR2.1 and TOP10F' (Appendix C. Strains). This construct was DNA sequence verified before restriction digest removal of the gene and the second round of ligation into pET24a+ and transformation into strain DH5α (Appendix C. Strains). Constructs were again sequence verified and pET24a+-LcrV and pET24a+-YscF were finally transformed into the *E. coli* expression strain BL21(DE3) (Appendix C. Strains).

1.7.2 Protein Expression

The LcrV and YscF constructs in the expression strain were initially tested for protein induction in small 3 mL volumes. The induced whole cell pellet was lysed and analyzed by SDS-PAGE. A very large induced band was observed at approximately the predicted MW of 10 and 32 kDal for YscF and LcrV, respectively. The LcrV protein production was successfully upscaled to 2 L without further modification; however, YscF production required multiple, smaller quantities in baffled flasks. After induction with IPTG, the cells were harvested, and resuspended for lysis via French Press. The lysed cell homogenates were centrifuged and the resultant pellet saved for further protein purification.

1.7.3 Protein Purification

The cell homogenates were further purified by ammonium sulfate precipitation. This step successively precipitates the suspended cellular constituents according to their solubility in increasing salt conditions. The fractions that contained the highest relative quantity of LcrV or YscF were used in

further purification steps. FPLC was the primary instrument for purification [GE AKTAexplorer™ system] utilizing HIC affinity, size exclusion, and anion exchange columns. LcrV and YscF had different purification schemes; however both yielded native protein in purities of about 99%.

1.8 Assumptions

Two assumptions were made when designing the experimental architecture for this research.

1. *E. coli* tertiary structure folding will be similar as in *Y. pestis* in that post-translational modifications and self-grouping (dimers, trimers, etc.) are the same in *Y. pestis* KIM5 and CO92 and *E. coli* BL21(DE3).

2. Purification in the manner described below will not significantly alter the structure, thus antigenic properties and epitope locations in the two proteins.

With respect to assumption 1, the primary structures of LcrV and YscF were sequenced and found 99% and 98% homologous to the NCBI published predicted sequences. The 1% and 2% difference is due to the post translational modification in which the methionine is cleaved from the N-terminus of both proteins. This irreversible cleavage is catalyzed by the enzyme methionine aminopeptidase (MAP) in *E. coli. Y. pestis* also has MAP and most likely also cleaves off this residue.

It has been assumed that due to the self-folding of most prokaryotic proteins *in vivo*, LcrV and YscF should be able to refold after being denatured. YscF in low salt (NaCl) conditions and high salt conditions rapidly precipitated out of solution, indicating that it is polymerizing as previously shown (Hoiczyk 2001).

1.9 Implications

This research completes the initial step by preparing the two recombinant proteins for V_{HH} production. By using native proteins as antigens, the V_{HH} produced may have a higher probability to recognize previously unknown epitopes found in protein clefts currently inaccessible by the much larger antibody molecule. Once the V_{HH} are produced and characterized, several *in vitro Y. pestis* neutralization and protein binding assays will quantitatively measure V_{HH} affinities. In addition, efficacy of V_{HH} protection will be examined by use of human primary macrophage model and an *in vivo* murine model.

1.10 Document Overview

This paper begins in Chapter 2 with the current threat assessment for WMD and leads to an understanding of the potential therapeutic uses of V_{HH} and the antigen choices. Chapter 3 describes the over-production of LcrV and YscF proteins, the first step to V_{HH} production. The results of the production are in Chapter 4 and conclusions in Chapter 5.

2. Literature Review

2.1 Background

This chapter lays out an argument for the DoD and other National Security Organizations to investigate the potential of V_{HH} as immunotherapeutics and the rationale and methods behind such an endeavor. This chapter also discusses the threat of biological agents past and present, the mechanisms of invasion and infection causing morbidity and mortality by many parasitic bacteria, and the use of biologically developed antibodies for combating disease.

2.2 Threat Assessment from WMD

2.2.1 Overview

Due to the heinous potential of biological weapons' effects on the human population, both direct (human morbidity and mortality) and indirect (economic, psychological, etc.), policy makers of the last century have made several global efforts to curtail the development, production, stockpiling, and acquisition of these agents (Inglesby 2000, Radosavljevic 2007). Policy creation or change has been in response to an exceptional event or events civilization deems morally reprehensible. The Geneva Protocol was the first modern (within last century) attempt to curb the use of biological weapons (Geneva 1925). This treaty opened for signature on June 17, 1925 and entered into force on February 8, 1928. The signatories called for the prohibition of both chemical and biological weapons, in response to the militaries' judicious use of chemical and, to lesser extent, biological weapons during WWI. The protocol stated that the use of such weapons "has been justly condemned by the general opinion of the civilized

world…" Much of the treaty was ignored and did little to prevent the subsequent biological arms race ensuing multilaterally between the US, the USSR, the UK, Japan, and several other states from the 1930s to the 1970s (Department of the Army 1997, Parlement of the United Kingdom 1974).

President Richard M. Nixon, on November 25, 1969, guided by his Secretary of Defense, Melvin Laird, and the National Security Council, issued the Statement on Chemical and Biological Defense Policies and Programs dramatically changing US policy on biological weaponry. This statement summarized 1) the US no-first strike policy with regards to chemical weapons; 2) the renouncement of use of lethal biological agents and weapons, and; 3) the continuance of very limited biological weapons research for defensive purposes. His political decision was motivated by the high cost of the US biological weapon program, low public opinion of the military during the Vietnam War era, and the strategic weapon redundancy due to our growing nuclear arsenal (Nixon 1969).

Nixon's pronouncement brought about the Biological Weapons Convention, a multilateral treaty, which, if completely enacted, would eliminate the development, stockpiling, and transferring of this entire class of weapons amongst the signatories. The treaty entered into force on March 26, 1975, and currently has 162 signatories who agree to disallow development, production, stockpiling, and transfer of biological weapons (CWTC 1972). Israel remains a non-member along with several other countries most of which are in continental Africa (United Nations 2009). Current BW threats vary in scope, scale, and target. They may range from large, highly funded state-sponsored bioweapons

programs to single-operatives. The latest threats have been towards the single-operative side of the spectrum (BBC-1 2001) (Hosenball 2008) (A Security Source 2009).

2.2.2 State Players (Since 1970s)

Despite being signatories of the 1972 Biological and Toxin Weapons Convention (BWTC) several states continued research in biological weaponry. The former Soviet Union was an early participant in biological weapon experimentation and believed the US' stance, voiced by Nixon, was a ploy to disarm all but itself of biological weapons (Alibek 1999). Between 1973 and 1974, as the US was disarming itself, the Soviet government developed a new organization, under the guise of civilian biotechnology research, named Biopreparat, to develop a host of cutting edge biological weapons. Several publically acknowledged incidences in the last 30 years indicate a robust, highly funded Soviet biological weapon machine (Alibek 1999, Davis 2006).

Georgi Markov, a Bulgarian critic of the Communist government, was assassinated in London on September 7, 1978, by use of a ricin-coated pellet. While ricin, a protein toxin found in the seeds of the castor bean plant, is not an infectious or living pathogen, its use is banned by the BWC of 1972. Recent evidence supports the argument that the assassination was made by the Bulgarian secret service aided by the Soviet KGB (The Economist 2009).

Another instance of an active Soviet post-BWTC development program was the accidental release of anthrax spores in April 1979 upon the town of Sverdlovsk (now Yekaterinburg, Russia). Sensationally called "the biological

Chernobyl," the accidental microbial release from a BW research facility caused the death of approximately 100 people (Meselson 1994, Alibek 1999).

As shown in the case of the former Soviet Union, signing the 1972 Biological Weapons Convention did not eliminate the aspiration, capability, or actions of a country to pursue the weaponization of biological organisms. Iraq signed the BWTC on 11 May 1972, but was not ratified until 1991. The Iraqi government, led by the former president Saddam Hussein, developed chemical and biological weapons programs in the mid 1980s to combat the Iranians during the Iran-Iraq war from 1980-1988 (Cordesman 1998). The strains investigated include *Bacillus anthracis, Yersinia pestis, Clostridium botulinum* and *perfringens,* species of afatoxin-producing fungi, and several others (Smith 1997). Acquisition of these strains, obtained through routing the orders to the American Type Culture Collection (ATCC) via the University of Baghdad, was met with very little opposition, as very little was required to prove the legitimacy and intent of such purchases. In a similar fashion, Libya signed the Geneva Protocol in 1925 and the BWTC in 1982 and developed limited biological weapon capabilities but failed to establish a significant program due to a lack of indigenous scientific and engineering infrastructure (NTI 2009). These three cases serve as examples of state/military funded biological weapons programs that were conducted in spite of the BWTC.

2.2.3 Non-state Players

While the quantities produced by large state funded research and development centers are orders of magnitude larger than can be produced by an

individual or small group, the most recent attacks have been from non-state sponsored organizations. Three intriguing cases are the *Salmonella* contaminated salad bar, the 2001 anthrax letters, and the recent mysterious deaths of forty Al-Qaeda terrorists in Algeria. These examples demonstrate the potential for small players to impact large populations.

As the only successful culture and release of a pathogen, the Rajneeshee cult, led by Bhagwan Shree Rajneesh, contaminated several restaurants with *Salmonella enterica* Typhimurium (Leitenberg 2005). The attack at The Dalles, Oregon, in 1984, sickened over 750 people, 45 of which were hospitalized (Grossman 2001). The event was the prototypical domestic terrorist event demonstrating the fragility and severe limitation of the public health community who protect such a large range of targets.

Another widely publicized terrorist/BW act was the 2001 anthrax letters, which targeted two US Senators and several media companies. The biological material contained in the letters, which caused five deaths and the infection of 17 others, was composed of dry *Bacillus anthracis* spores. While the media speculated wildly as to the grade of the spores, several subject matter experts found no evidence of significant weaponization (Alibeck 2002). The likely culprit was US scientist, Bruce Edward Ivins, Fort Detrick, Md, who had access to the strain (Hosenball 2008).

The third case demonstrates the interest groups, such as al Qaeda, have acquiring CBW. Several newspapers have recently reported the accidental deaths of at least forty al Qaeda operatives. Initial reports from several UK

newspapers claimed the death may be attributed to the biological agent *Yersinia pestis*, while US newspapers have been much more conservative with their estimates and say the agent may be chemical or biological (Lake 2009, A Security Source 2009, The Daily Telegraph 2009). The three cases have and will continue to shape policy and funding regarding biological weapons.

2.3 Bacterial Based Biological Weapons (BBBW)

As with chemical or nuclear weapons, biological weapons require special materials to deliver an effective attack (9/11 Commission 2004). These special biological materials or "barriers to entry" can be easily acquired, when compared to nuclear and chemical weapons. This is in part due to dual use equipment, ease of culture methods, and easily available pathogen sequences published in articles and posted on the internet. The Australia Group (AG) is a cohort of over forty countries who meet annually to promote national export licensing which:

1. are effective in impeding the production of chemical and biological weapons;

2. are practical, and reasonably easy to implement; and

3. do not impede normal trade materials and equipment used for legitimate purposes (Australia Group 2008).

The domestic regulation of bacterial based biological weapons is difficulty due to dual-use of the processing vessels and equipment in legitimate public health, academia, commercial R&D, and medical establishments. Dual-use equipment, common at even small undergraduate research facilities and local public heath offices, include fermenters, autoclaves, centrifuges, and incubators (Shea 2004). These rudimentary culture devices are easily attained through

relatively anonymous sources such as ebay.com. Bacterial-based biological weapons are much easier to produce than spore-forming agents or viruses since they require less downstream handling.

The CDC classifies biological agents/diseases into three groups, based upon a metric which considers transmission, mortality rates, public perception, and public health resources (CDC 2009). Of the six Category A Agent/disease groups (Table 1) only two fall into the classification of BBBW, these include *Francisella tularensis* (the causative agent of Tularemia) and *Yersinia pestis*. Both these organisms are regularly transmitted by arthropod vectors and are naturally endemic in North America and other parts of the world. Both pathogens are harbored in small mammals as reservoir hosts (Morner 1992). As evident by centuries of morbidity and mortality, *Y. pestis* is transmissible from one human to another, making it unique among bacterial Category A Agents.

Table 1. CDC Category A Agents

Agents	Classification	Spread Human-to Human
Bacillus anthracis	Spore, Bacterial	No
Clostridium botulinum toxin	Toxin, Bacterial	No
Yersinia pestis	**Bacterial**	**Yes**
variola major	Viral	Yes
Francisella tularensis	Bacterial	No
filoviruses and arenaviruses	Viral	Yes

2.4 Yersinia pestis

2.4.1 Overview

With the exception of smallpox (with no known animal reservoir), and *C. botulinum* (a toxin producer found in food), all of the Category A Agents are zoonotic diseases, as they are shared by animals and humans (CDC 2009).

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Arguably, the genus *Yersinia* has impacted human kind more than any other pathogen due to its widespread and lasting effects. The causative agent of plague has been implicated in the death of over 200 million people in three pandemic waves in the last 1500 years. It was not until 1894, while investigating the Manchurian Pneumonic Plague epidemic in Hong Kong that bacteriologist Alexandre Emile Jean Yersin discovered the gram-negative bacillus causing the plague, satisfying Koch's Postulates.

2.4.2 Historical and Modern Classification Models

The study of relationships between living organisms is very important within the field of pathology. Edward Jenner's research and observations in the late 1700s, while pre-dating comparative genetics, uncovered the phenotypic and antigenic similarities between *Vaccinia virus* (the agent of cowpox) and *Variola major* (the agent of smallpox).

The genus *Yersinia* of the family Enterobacteriaceae contains over a dozen species including three which cause disease in humans (Table 2).

Table 2. *Yersina* Species and Diseases

Species	Disease
Y. enterocolitica	Yersiniosis
Y. pseudotuberculosis	Izumi-fever, gastroenteritis
Y. pestis	**Plague**

Y. pestis is hypothesized to have evolved from *Y. pseudotuberculosis* within the last 1,500 to 20,000 years (Achtman 1999). The large range in postulated dates is due to the large reproductive rates and extreme selection shown by pathogens. The level of divergence between species depends upon

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the strain and can be quantified using several different methods. Figure 1 shows the quantification of gene loss of function/inactivation in *Y. pestis* is compared to *Y. pseudotuberculosis.* The darker columns represent a relative loss of analogous gene clusters for *Y. pestis* strain CO92 and light blue (lighter) represent relative loss for *Y. pseudotuberculosis* strain IP32953 (Chain 2004)*.* Notably, large scale gene inactivation of *Y. pestis* may be the cause of its increased pathogenicity (Chain 2004).
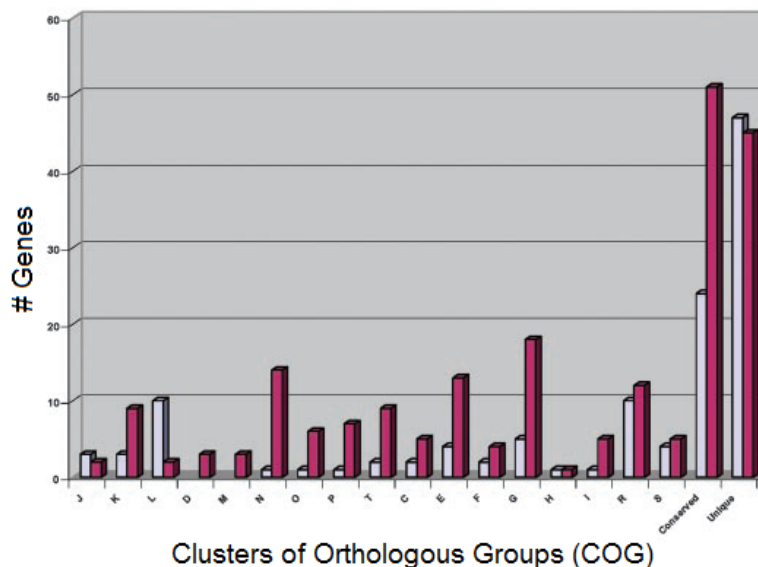
Figure 1. Active Y. pestis gene groups compared to Y. pseudotuberculosis

(Chain 2004)
Image used with permission of PNAS (copyright 2004 National Academy of Sciences, U.S.A) and Author

The historical (classical) subspecies classification subdivided *Y. pestis* strains into one of three biovars: antiqua, mediaevalis, and orientalis (closely related to Justinian's plague and the Black Death). Recently, microtus has been proposed as a possible fourth subspecies (Zhou 2004). These divisions were phenotypically differentiated using the strains' glycerol fermentation and nitrate

reduction abilities and geographic location. These phenotypic variations have been assumed to represent/model significant changes within the strain's genome and mark evolutionary divergences. Recent advances, chiefly the increased fidelity and affordability of sequence and mutation analysis (single nucleotide polymorphisms, variable number tandem repeats, and insertion deletions), have allowed researchers to challenge and build upon existing nomenclature paradigms (Touchman 2007). Proposed changes include addition of new biovars to complete reclassification based upon molecular signatures (Zhou 2004, Achtman 2004). The three classic biovars range in levels of morbidity and mortality ranging from the highly virulent CO92 (pronounced Colorado 92) to the avirulent KIM5 strain that can be handled at BSL2.

2.4.3 Forms of Infection

Another key factor in *Y. pestis* lethality is the form of infection within the host. The three common forms of infection include bubonic, septicemic, and pneumonic. Each has a different manifestation and mortality rate; however, patients are not limited to one form. The commonly referenced form is the bubonic plague. Within a less than a week of open skin contact to *Y. pestis* or a bite from an infected flea, symptoms of fever, headache, chills, and inflammation of the lymph nodes develop.
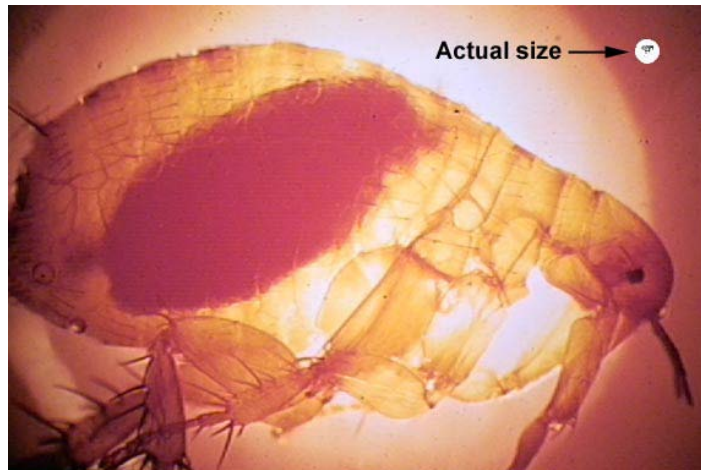
Figure 2. *Xenopsylla chepsis* **(oriental rat flee) engorged with blood (CDC 2009)**
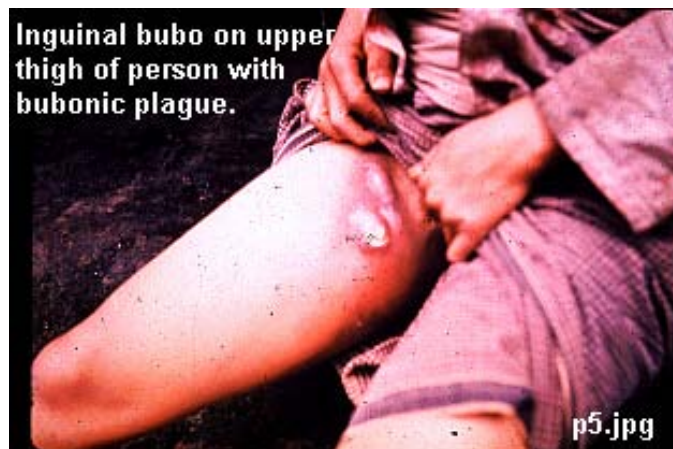
Figure 3. Inguinal Bubo (CDC 2009)

While this form is not always fatal, it often leads to the second more lethal form, septicemic plague. Septicemic diagnosis is given when an individual's blood has positive culture for the bacteria (Perry 1997). It has been reported that blood cultures range from 10 to 4 x 10^7 colonies/mL; patients with colony counts higher than 100/mL have higher fatality rates; however, survival with counts as high as 10^7/mL has been reported (Butler 1976).

Figure 4. Plague Bacteria in Blood (CDC 2009)

The third form, pneumonic plague, is transmitted when infectious droplets are successfully deposited deep within the respiratory system. In 1994 it was reported that only 12% of U.S. patients diagnosed with pneumonic plague developed it from primary bubonic or septicemic infections (Doll 1994). It should be noted that vaccines, whose efficacy has never been precisely calculated, reduce the incidence and morbidity of the disease in individuals bitten by infected fleas. A vaccine using attenuated live plague has been discontinued as it is still pathogenic and yields low protection from respiratory exposure (Titball 2001).

2.4.4 Human and Zoonotic Lifecycles

As previously mentioned, there are several routes of exposure to plague. The exposure methods to humans are:

1. Direct contact with infected wild or domestic animals (usually rodents)

2. Bite from infected flea

3. Person to person via infected bodily fluids (pneumonia plague)
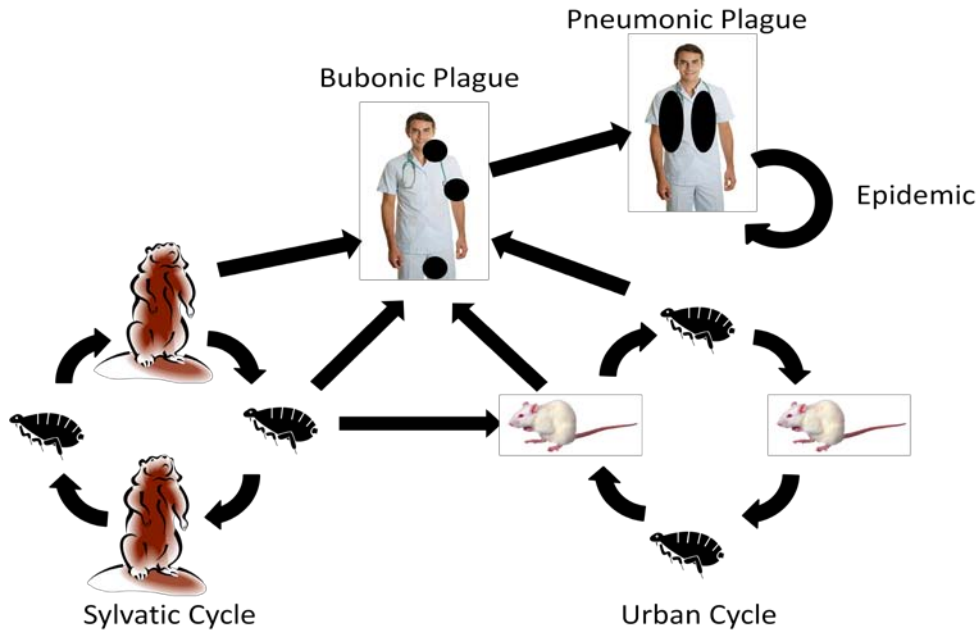
Figure 5. Plague Lifecycles

Adapted from Neal Chamberlain's Medical Microbiology: The Big Picture

All three of these routes of entry could be employed by a terrorist to infect an individual or population.

2.4.5 Genetic Components of Infectivity

In the same year (2001) the human genome was first published, researchers from the Wellcome Trust Sanger Centre released the sequence of *Y. pestis* CO92 (Parkhill 2001). CO92, the prototypal strain, has a genome of one chromosome with 4.65×10^6 base pair (bp) and three relatively smaller plasmids (circular DNA).
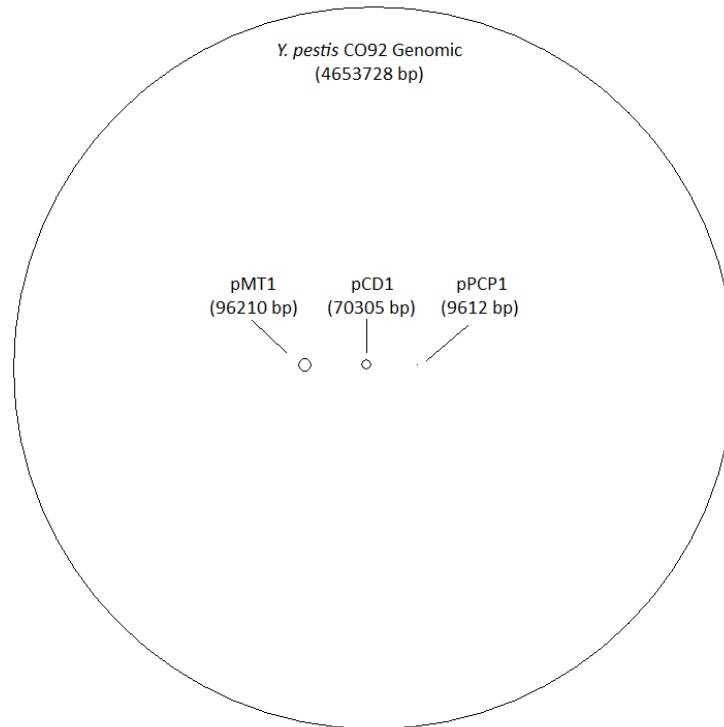
Figure 6. *Y. pestis* **CO92 Genetic Features (to scale)**

(Parkhill 2001)

The bacterium's chromosome encodes primarily the housekeeping genes but also includes several virulence factors (CEVF). CEVF are necessary for full virulence in the case of the *Y. pestis* model *Y. enterocolitica* (Heesemann 1984). While CEVF play a lesser role in pathogenicity, pCD1 the 70 kbp virulence plasmid, codes for many of the proteins primarily responsible for infection. The 70 kbp plasmid (pCD1 in *pestis*, pYVe in *enterocolitica*, and pYV or pIB1 in *pseudotuberculosis*) is common to all three pathogenic species within *Yersinia*. Two specific proteins of interest are coded in this plasmid: the low calcium response V protein (LcrV) and yop (*Yersinia* outer-membrane protein) secretion protein F (YscF). These two proteins, which are necessary for virulence, have

been used as target antigens for antibody production (Burrows 1956, Cornelis 1997).

2.5 LcrV and YscF Proteins

2.5.1 LcrV

The low calcium response V or V-antigen was the first virulence determining factors of *Yersinia pestis* (Burrows 1956). Among the *Yersinia* species, the LcrV coding sequence demonstrates a 90-95% homology (Weeks 2002).

Figure 7. LcrV Amino Acid Comparison amongst *Yersinia* and Similar Species

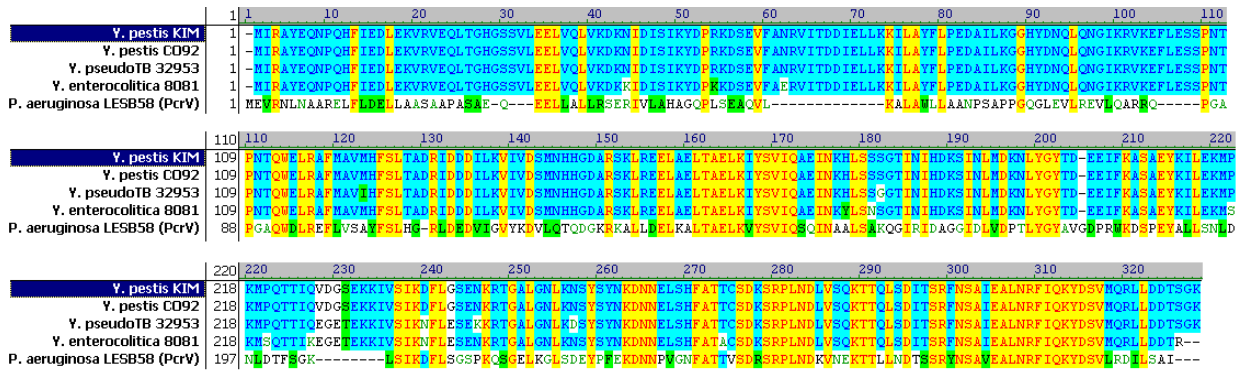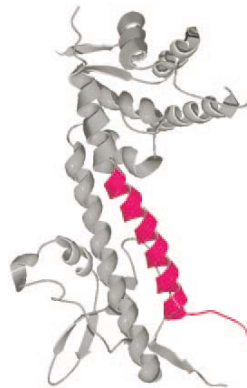**Figure 8. Structure of *Y. pestis* LcrV**

(Overheim 2005)
Image used with permission of American Society for Microbiology (license number 2170320732934) and Author

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The high level of homology is supportive evidence of the importance of this factor for the survival and infectivity of this species. The pCD1 (pYV) vector contains a 981 bp gene that codes for the 327 amino acid LcrV sequence. The role that LcrV plays has been somewhat illusive, most likely due to its multiple roles in pathogenicity. It has been shown that LcrV stimulates host expression of interleukin 10 (IL-10), suppressing the host's innate immune system (Nakajma 1995). Several other roles have been observed in animal models, many of which also involve suppression of the host response to infection.

2.5.2 YscF

Several gram-negative animal and plant pathogenic bacteria share a similar secretion mechanism for translocating proteins into a host's cytoplasm. These proteins, while species specific, are activated by contact with cell surface moieties and lead to suppression of host immune responses (Hueck 1998). Yesinia species, *Salmonella typherim, Shigella flexneri*, and enteropathogenic *E. coli* all share this type three secretion system (T3SS or TTSS). A key player in the T3SS is a small surface expressed protein named YscF. YscF also shares a high level of homology amongst *Yersinia* species as demonstrated in Figure 9.
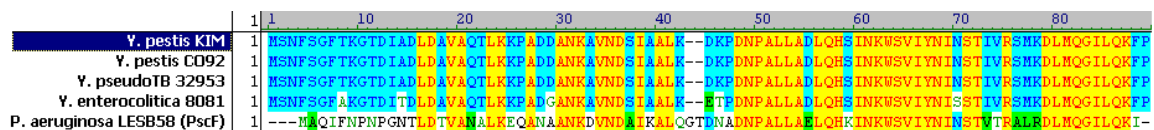
Figure 9. YscF Amino Acid Comparison amongst *Yersinia* and Similar Species
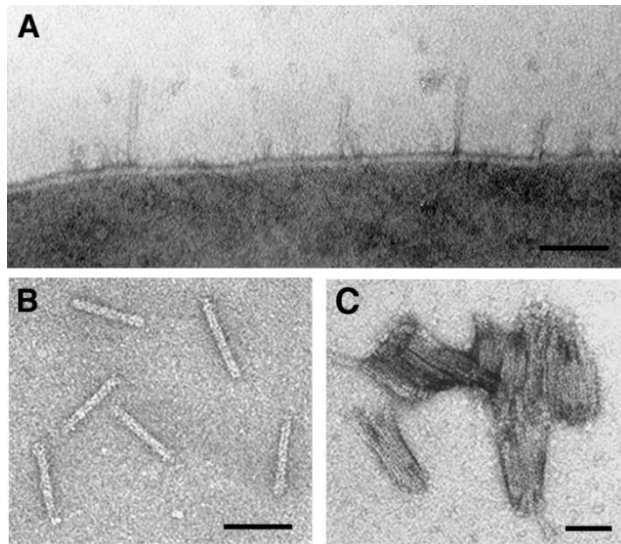
Figure 10. YscF Images

(A) in vivo cross section analysis of needles of *Y. enterocolitica*; (B) Isolated needles; (C) Aggregated upon Ca+2 addition (bars are 50 nm) (Hoiczyk 2001)
Image used with permission of PNAS (copyright 2001 National Academy of Sciences, U.S.A) and Author

The pCD1 (pYV) vector also contains the 264 bp coding for the 88 amino acid sequence of YscF weighing 7 kDal. This small protein is a monomer creating relatively large hollow needle-like polymers. YscF has also been demonstrated to serve as protective antigen as it issues a robust immune response (Swietnicki 2005). This needle (injectosome) provides the channel for the Yops to move from *Yersinia* into the host cell as depicted in Figure 10.
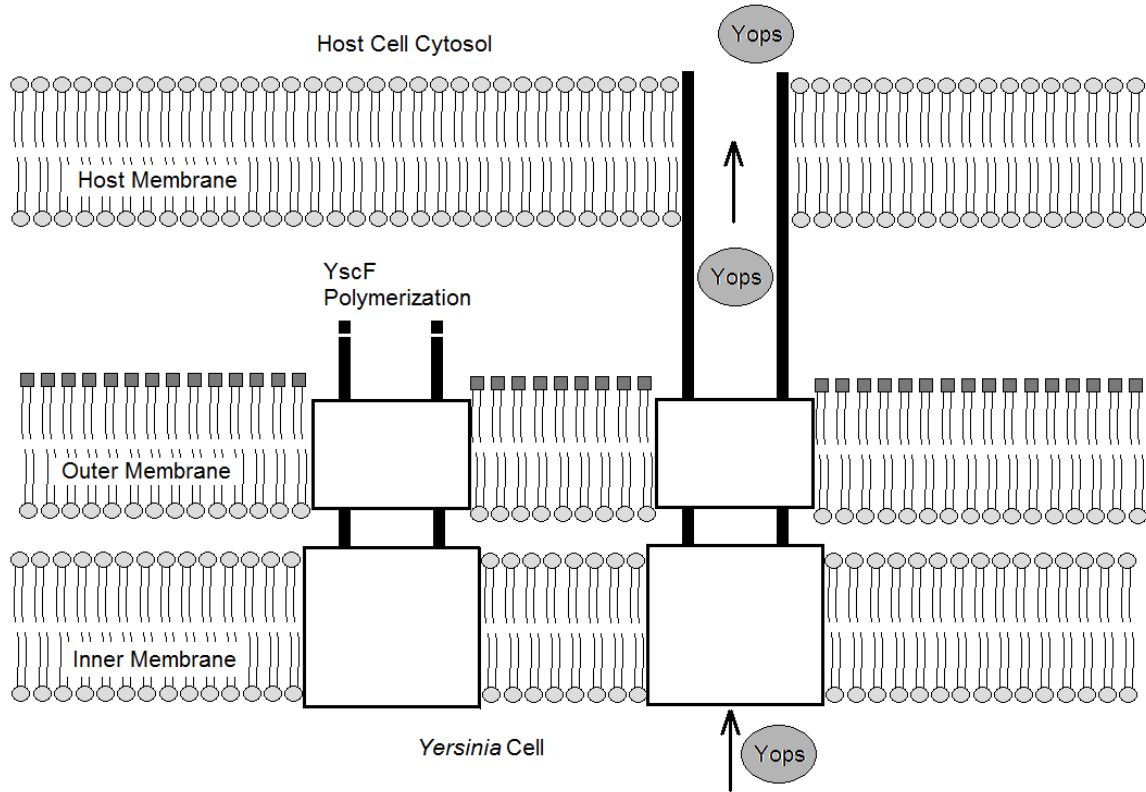
Figure 11. YscF Injectosome Penetrating Host Cell

As both LcrV and YscF are located on the exterior of *Yersinia*'s outer membrane and are necessary for pathogenicity, they are ideal candidates for antibody production to target *Y. pestis*.

2.6 Antibodies and Antigen Specific Camelidae Fragments

2.6.1 Overview

Antibodies or immunoglobulins (Ig) are proteins produced by living organisms in response to the presence of an antigen. The innate immune systems of vertebrates quickly and non-specifically respond to foreign objects with increased circulation, elevation of temperature, and recruitment of cellular and non-cellular helpers. However, the adaptive immune system develops a targeted, more lethal attack. Previously produced antibodies are constitutively

expressed to some degree after exposure, providing a faster response to future attacks.

2.6.2 Structure of Antibodies

Antibody responses exist in all vertebrates. The most common immunoglobulin species (up to 75% of immunoglobulins in human serum) is immunoglobulin G (IgG). This Y-shaped molecule is structurally composed of two heavy chains and two light chains. The heavy chain can be broken down further to smaller domains consisting of a variable region (V_H) and a larger constant region (C_H). Similarly, the light chain can be broken into a small variable region (V_L) and only slightly larger constant region (C_L). As displayed in Figure 12 the four regions come together to form the quaternary structure of the protein.
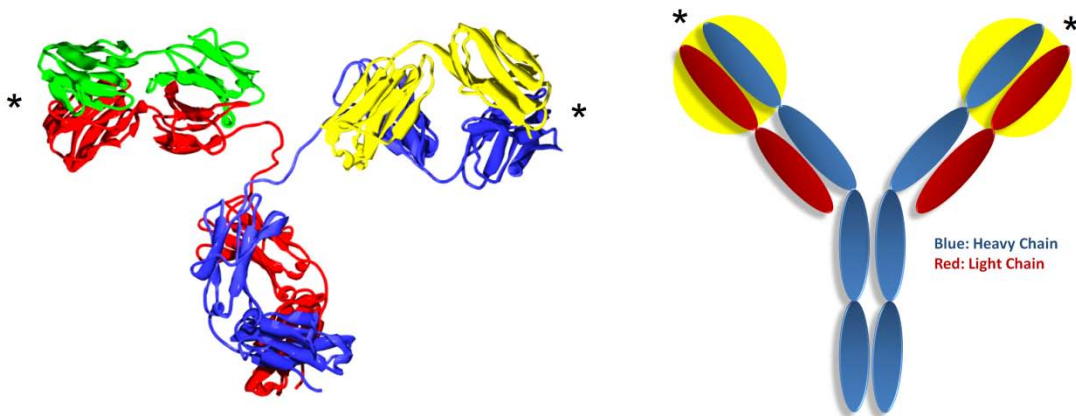
Figure 12. Immunoglobulin G Structure

*denotes variable region conferring variability and specificity
(Ribbon Structure from Wikipedia)

2.6.3 In vivo and in vitro Antibody Production

Predating the discovery of antibodies in the late 19th century, scientist recognized the ability for the human body to respond to a pathogen and acquire immunity to future exposures. Edward Jenner's model of using a surrogate, or later attenuated and killed pathogens, demonstrated the body's ability to generate antibodies without exposure to full virulent pathogens. The polyclonal antibodies produced in response to the pathogen have affinities for many areas of the antigen, called epitopes. This antibody fraction or titer can be isolated and purified from whole blood.

Often pathogens possess surface proteins or features that are very antigenic, that is, they stimulate strong antibody producing activity by the adaptive immune system. To produce such antigen-specific IgG's researchers can isolate these antigenic proteins from the pathogen *in vivo* or, by gene cloning, make recombinant proteins in cell culture. These purified proteins can be directly injected into an organism with an immune system and the resultant polyclonal antibodies may be harvested, purified, and used. In addition, an outstanding IgG may be followed up with monoclonal antibody production which allows for perpetual *in vivo* production. Additionally, while polyclonal antibodies target many different epitopes, monoclonal antibodies are produced as a single IgG species and target a single epitope. To accomplish this, an *in vitro* method was developed in the 1970s to combine functional B-cells from an exposed organism's spleen or blood and fuse them with a myeloma cell line that no longer

is able to secrete antibodies. These hybridomas (hybrid + myeloma) can be grown with simple cell culture methods to produce monoclonal antibodies.

While the above methods of antibody/vaccine production have been commonly used for many years, a new technique of vaccine/antibody production has emerged. Scientists have developed plasmids that, when directly injected into organisms, express antigenic proteins (Tang 1992). As of early 2005 over 170 DNA vaccine production and related technology patents have been filed (DNAvaccine.com 2004). While the possibility of converting the production of our nation's expensive and delicate vaccines to this new process has potential benefits, the antibody response is still insufficient for many disease vaccines. In 2006 a firm from the UK described the first positive results for a DNA-based flu vaccine which has been moved to Stage II clinical trials (Barnes 2006).

2.6.4 Problems with Antibody-based Therapeutics

While, antibodies from most vertebrate species act with the same mechanism, foreign antibodies are themselves very immunogenic. With a properly functioning immune system, humans produce an antibody response in 1-2 weeks after exposure to antibodies produced in a foreign organism (Isaacs 1990). The immunogenicity of antibodies poses two different problems. First, patients previously treated or exposed to a non-human antibody, perhaps from a mouse, would be less likely to benefit from further antibody treatments produced by the mouse. Second, diagnostic tests using antibodies derived from mice, namely enzyme-linked immunosorbant assays, can produce erroneous results due to interferences of the human anti-mouse antibodies or HAMA. In 1990

research found that 2 of 50 surveyed hospital patients produced a positive titer for human anti-mouse antibodies (Kricka 1990). In an attempt to trick the human immune system to accepting non-human antibody therapies, researchers have "humanized" antibodies by various means. Humanized antibodies or chimeric antibodies can be produced by fusing the non-human derived variable domain coding DNA with human constant coding DNA (Wu 2005). This fused DNA sequence can be used to produce monoclonal antibodies without using humans as laboratories for antibody production. Another approach to avoid immunogenicity of the antibody therapeutic involves antibody truncation, eliminating the common targets for the human anti-mouse antibody response for example. Truncated antigen-binding fragments (Fab) include one light chain and the top half of the heavy chain that included the binding site.

Blue: Heavy Chain
Red: Light Chain
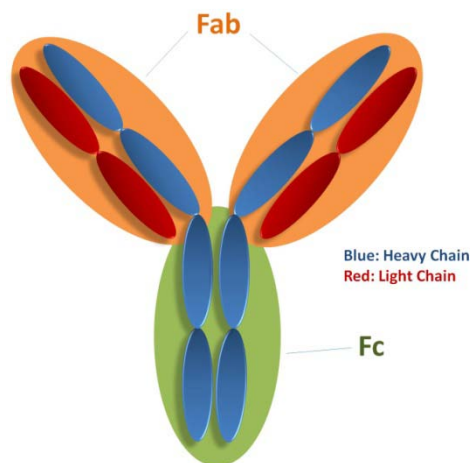
Figure 13. Fab

Besides immunogenicity to therapeutics, other difficulties arise with storage and stability of therapeutic and diagnostic antibodies. Antibodies tend to degrade at room temperature; storage at 4°C (typical refrigerator temperature)

should not exceed two weeks with the optimal temperature of -20°C (abcam 2009). V_{HH} have been found to be show affinities at temperature as high as 90°C due to their ability to refold after denaturation (Linden 1999).

In 1993 a research group in Belgium published their investigations into the unique antibody structure produced by camelids, including camels and llamas (Hamers-Casterman 1993). It was later found that, due to a G to A mutation, the splice site was destroyed after the C_H1 exon. Further mutations likely increased the hinge exon length by transposon(s) insertion (Nguyen 2000). Due to these mutations, the *Camelidae* family naturally produces both fully functional single chain antibodies (SCAB) and normally structured IgG. Further work with SCAB demonstrated an ability to further reduce the size and change the physical properties. The biotech company, Ablynx, has commercialized the truncation of the variable region and named these molecules "Nanobodies[®]" also known as V_{HH}. These small structures (15 kDal) are, relative to full antibodies, more heat and pH resistant and stimulate a low immunogenic response when injected into primates (Gibbs 2005, Ablynx 2007). Figure 14 is a representation of the unique structure of single domain *Camelid* antibodies when compared with Figure 12. They also can be produced using lower eukaryotic microorganisms such as yeast and prokaryotes such as *E. coli* in quantities of grams per liter (Frenken 1998).
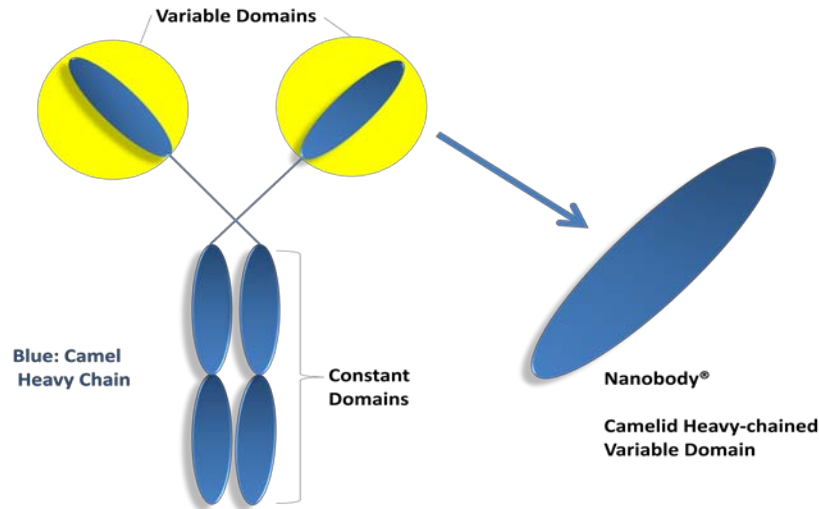
Figure 14. Single Domain *Camelidae* Antibodies

2.6.5 Medical and Military Applications

The environment in theater with which the warfighter must endure presents a unique set of problems in diagnosis and detection of pathogens. The traditional antibody detection method, Enzyme-Linked ImmunoSorbent Assay (ELISA), has limitations for field use due to the instability of the antibody in the wide temperature range found in theater. This new subclass of antibodies, V_{HH}, may solve several of these durability issues for use as both a sensor capture element and as a immunotherapeutic. In sensors, such stability is critical for the ng/mL to pg/mL sensitivities for marker detection and quantification. V_{HH} stability may enable stockpiling of immunotherapeutics stored at room temperature. This not only has beneficial applications for remote field hospitals, but for individual warfighter kits.

The use of antibody based passive immunotherapeutics is well known, but inherent difficulties have limited their use and approval. Only twenty-five, as of 2007, have been approved since the inception of the first therapeutic monoclonal

antibody FDA-approved in 1986. The majority have applications in cancer treatment and other civilian life-threatening diseases; however, only one, Palivizumab (Respiratory Syncytial Virus), is designed to help prevent (not treat) a pathogenic organism. Most of the difficulties in acquiring FDA approval revolve around the body's natural immune response. V_{HH} inherent physical characteristics may lend themselves to use as on-site, real-time protection post-BW attack by blocking the ability of bacteria in the initial infectivity pathway. By reducing the size of the therapeutic, there is a reduction in the number of antigenic sites. As of 2006, multiple injections of V_{HH} have not triggered an immunogenic response in the mouse model (Coppieters 2006). Oral immunotherapy with V_{HH}, engineered for proteolytic stability, prevented diarrhea caused by the rotavirus (Vaart 2006). For these reasons V_{HH} may serve as a battlefield-ready, stable immunotherapeutic.

3. Methodology

3.1 Introduction

To produce V_{HH} in *Camelidae* (llama), a total of 1 to 2 mgs of purified protein must be prepared to use as an antigen. The protein, once purified, should be >95% pure to lower nonspecific antigenic response due to impurities. Therefore, an excess of 2 mg of LcrV and YscF protein need to be purified to accomplish this level. Overexpression of these proteins in a bacterial host required a protein expression vector, containing the appropriate gene, transformed into an *E. coli* expression strain. The construct, once built, was verified, induced, and the induced protein purified. Protein productions with N- and C-terminal tags, such as poly-histidine tails, have become popular due to the ease of purification post-induction. Affinity columns have been developed that bind the poly-histidine tail, selectively removing the fused protein from cell lysate, aiding in purification. However, production of the native protein without non-native protein fusions is advantageous to antibody production, as native proteins contain the correct secondary and tertiary structures. However, by expressing proteins without the His tags, production and purification of LcrV and YscF was much more difficult and time consuming. By doing so, new and perhaps unalterable cleft epitopes will be presented for *in vivo* V_{HH} production. The methodology is divided into three parts as is described in the objectives: 1) construction of LcrV and YscF expression plasmids; 2) expression of native proteins in *E. coli* and; 3) purification of these proteins using fast protein liquid chromatography (FPLC).

3.2 Assumptions

There are several key assumptions that were made prior to the experimental process.

1. *E. coli* 2^0 and 3^0 structure folding of the produced proteins will be similar as in *Y. pestis.*

2. Purification in the manner describe below will not significantly alter the structure thus antigenic properties of the two proteins.

3. The LcrV and YscF proteins will not be toxic to the *E. coli* cell upon induction.

3.3 Development of LcrV and YscF expression plasmids

The goal of this objective was construction of two *E. coli* vectors, one an expression plasmid coding for the native LcrV protein and the other YscF protein. These steps are graphically represented in Figure 15.

3.3.1 Design NdeI and XhoI primers for both LcrV and YscF coding DNA from *Y. pestis* strain KIM5 as a target for PCR amplification.

3.3.2 Develop PCR amplification protocols for creating NdeI/XhoI sites 5' and 3', respectively, to the LcrV and YscF coding PCR product from *Y. pestis* KIM5 genomic DNA.

3.3.3 Ligate the LcrV and YscF PCR products into pCR2.1 vector plasmids.

3.3.4 Transform pCR2.1-LcrV and pCR2.1-YscF vectors into the non-expression TOP10F' *E. coli* strain (see Appendix C for genotypes).

3.3.5 Isolate each plasmid construct and verify the gene sequences using dideoxy sequencing. Once verified, batch purify plasmid construct for stocks.

3.3.6 Digest pCR2.1-LcrV, pCR2.1-YscF, and pET24a+ plasmids and isolate fragments for ligation.

3.3.7 Ligate gel-isolated LcrV and YscF DNA gene fragments into the pET24a+ plasmid.

3.3.8 Transform plasmids into the non-selective, non-expression *E. coli* strain DH5α and verify with restriction digestions.

3.3.9 Purify verified plasmid constructs and transform into *E. coli* expression strain BL21(DE3).
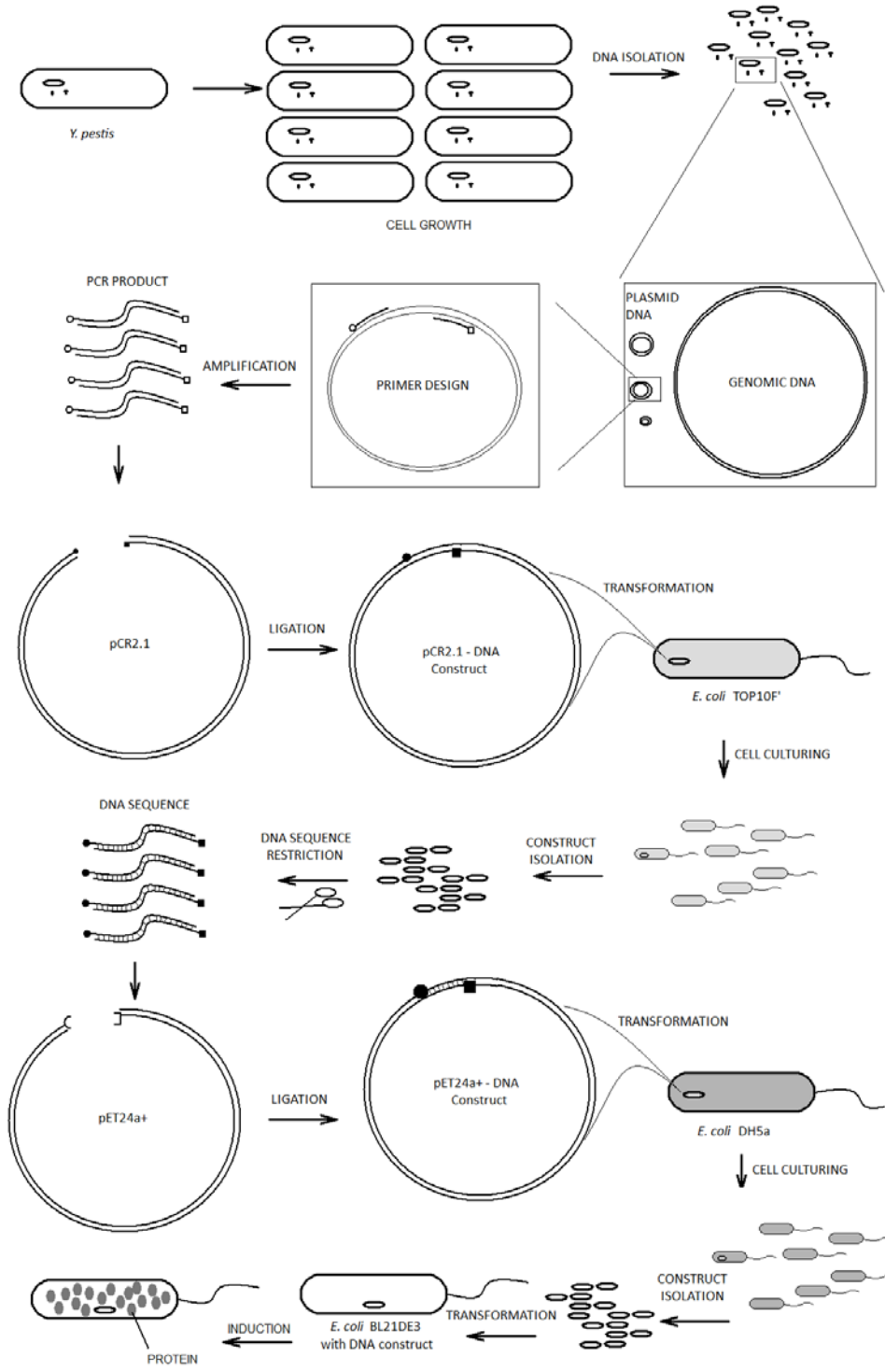

Figure 15. Development of Expression Plasmids

3.3.1 Design NdeI and XhoI primers for both LcrV and YscF coding DNA from *Y. pestis* strain KIM5 as a target for PCR amplification

The DNA sequences from which LcrV (Table 3Table 3) and YscF (Table 4) proteins are encoded were taken from http://www.ncbi.nlm.nih.gov/. The sequences coding for both proteins are identical in both CO92 (virulent) and KIM5 (avirulent) strains of *Y. pestis*. The LcrV coding DNA contains 981 bp coding for a 37.2 kDal protein. The YscF coding DNA is much smaller, with 264 bp producing a 9.5 kDal protein.

Table 3. LcrV DNA Sequence

(Start and Stop Codons Underlined and Bolded)

```
atttaat atg att aga gcc tac gaa caa aac cca caa cat ttt att
gag gat cta gaa aaa gtt agg gtg gaa caa ctt act ggt cat ggt
tct tca gtt tta gaa gaa ttg gtt cag tta gtc aaa gat aaa aat
ata gat att tcc att aaa tat gat ccc aga aaa gat tcg gag gtt
ttt gcc aat aga gta att act gat gat atc gaa ttg ctc aag aaa
atc cta gct tat ttt cta ccc gag gat gcc att ctt aaa ggc ggt
cat tat gac aac caa ctg caa aat ggc atc aag cga gta aaa gag
ttc ctt gaa tca tcg ccg aat aca caa tgg gaa ttg cgg gcg ttc
atg gca gta atg cat ttc tct tta acc gcc gat cgt atc gat gat
gat att ttg aaa gtg att gtt gat tca atg aat cat cat ggt gat
gcc cgt agc aag ttg cgt gaa gaa tta gct gag ctt acc gcc gaa
tta aag att tat tca gtt att caa gcc gaa att aat aag cat ctg
tct agt agt ggc acc ata aat atc cat gat aaa tcc att aat ctc
atg gat aaa aat tta tat ggt tat aca gat gaa gag att ttt aaa
gcc agc gca gag tac aaa att ctc gag aaa atg cct caa acc acc
att cag gtg gat ggg agc gag aaa aaa ata gtc tcg ata aag gac
ttt ctt gga agt gag aat aaa aga acc ggg gcg ttg ggt aat ctg
aaa aac tca tac tct tat aat aaa gat aat aat gaa tta tct cac
ttt gcc acc acc tgc tcg gat aag tcc agg ccg ctc aac gac ttg
gtt agc caa aaa aca act cag ctg tct gat att aca tca cgt ttt
aat tca gct att gaa gca ctg aac cgt ttc att cag aaa tat gat
tca gtg atg caa cgt ctg cta gat gac acg tct ggt aaa tga
cacgagg
```

Table 4. YscF DNA Sequence

(Start and Stop Codons Underlined and Bolded)

```
    aaaataa atg agt aac ttc tct gga ttt acg aaa gga acc gat
atc gca gac tta gat gcg gtg gct caa acg ctc aag aag cca gca
gac gat gca aac aaa gcg gtt aat gac tcg ata gca gca ttg aaa
gat aag cct gac aac ccg gcg cta ctt gct gac tta caa cat tca
att aat aaa tgg tcg gta att tac aat ata aac tca acc ata gtt
cgt agc atg aaa gac tta atg caa ggc atc cta cag aag ttc cca
taa tatgaaa
```

The pET24a+ vector (Appendix D. pET24a+ Vector Map) is designed to enhance expression driven by production of mRNA using T7 RNA polymerase targeting the T7 promoter (T7P) upstream of the inserted DNA sequence. Expression is controlled by *lacUV5* promoter and operator regions. The operator region is normally bound with the repressor protein blocking transcription. Lactose or an analogue such as isopropyl-β-D-thio-galactoside (IPTG), bind to the repressor, releasing operation region, greatly increasing transcriptional expression. For ligation with correct orientation (T7P 5' of translated sequence) in the pET24a+ expression vector the primer sets were designed with NdeI and XhoI 3' overhangs. The restriction enzyme NdeI selectively digests (cleaves DNA backbone) the DNA recognition sequence CATATG. This sequence is cut asymmetrically, leaving an overhang (Figure 16).
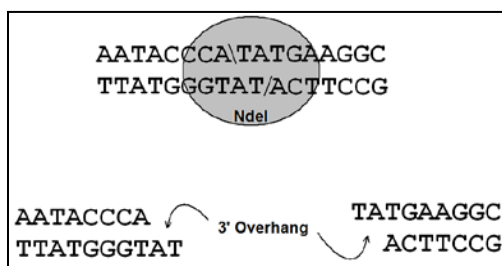

Figure 16. NdeI Restriction Enzyme Leaving 3' Overhang

Since the start codon common to most living organism is ATG, the NdeI restriction site is easily constructed by adding CAT (the complimentary three bases) upstream to the start codon. The complimentary strand's primer was tagged with an XhoI site (C/TCGAG). The final primer design for amplification of LcrV and YscF DNA amplicons are presented below in Table 5.

Table 5. LcrV and YscF Primers

LcrV	Forward Primer	LcrVNde-I	5'-TAGCATATGATTAGAGCCTACGAAC-3'
	Reverse Primer	LcrVXho-I	5'-CTCGAGTCATTTACCAGACGTGTC-3'
YscF	Forward Primer	YscFNde-I	5'-TAGCATATGAGTAACTTCTCTGG-3'
	Reverse Primer	YscFXho-I	5'-CTCGAGTTATGGGAACTTCTGTAG-3'

3.3.2 Develop PCR amplification protocols for creating NdeI/XhoI sites 5' and 3', respectively, to the LcrV and YscF coding PCR product from Y. pestis KIM5 genomic DNA

The commercially synthesized primers were resuspended with DNAse/RNAse Free PCR grade water; and aliquots of the resuspended primers were diluted to a concentration of 1 ug/uL with water. The KIM5 genomic DNA was diluted to 3 ng/uL prior to amplification. AccuPrime™ *Taq* DNA Polymerase (Invitrogen™) and 10x buffer II were selected based on ease of use and sufficient amplification fidelity. The reaction volume (30 uL):

- 3 uL 10x PCR Buffer II (includes $MgCl_2$ and dNTPs)
- 0.5 uL AccuPrime™ *Taq* Polymerase
- 3 uL (0.1 ug/uL) forward primer (LcrVNde-I or YscFNde-I)
- 3 uL (0.1 ug/uL) reverse primer (LcrVXho-I or YscFXho-I)
- 19.5 uL DNAse/RNAse Free PCR grade water
- 1 uL (3 ng/uL) purified Genomic KIM5 DNA

The PCR thermocycling conditions differed for LcrV and YscF due to the lengths of amplicons. The longer the amplified region the longer the time needed during the elongation stage (secondary cycles). The PCR reactions were separated into two stages: an initial ten cycles were performed using less restrictive parameters (lower annealing temperatures and longer elongation time), followed by 25 cycles at more stringent hybridization parameters (Table 6).

Table 6. PCR Conditions

LcrV				**YscF**			
Stage	Quantity	Temp (°C)	Time (sec)	Stage	Quantity	Temp (°C)	Time (sec)
Initial Denaturation	1	94	90	Initial Denaturation	1	94	90
Primary Cycles	10	94	60	Primary Cycles	10	94	60
		55	60			55	60
		72	80			72	45
Secondary Cycles	25	94	60	Secondary Cycles	25	94	30
		60	45			62	30
		72	60			72	45
Final Hold	1	4	variable	Final Hold	1	4	variable

The above reactions were run in duplicate with negative controls (water in place of template DNA). The reactions were run on 0.75% agarose gels with ethidium bromide staining. The appropriate bands (LcrV ~985 bp and YscF ~270 bp) were identified, excised, and isolated using a DNA Extraction Kit (QIAGEN™) according to the manufacturer's suggested protocol. The purified DNA fragments were stored at -20°C prior to ligation.

3.3.3 Ligate the LcrV and YscF PCR products into pCR2.1 vector plasmids

As an artifact of *Taq* polymerase activity, adenine bases are usually added to the 3' ends of the amplicons. The pCR2.1 vector supplied by Invitrogen™ is

provided linearized with 3' thymine overhang ends, which take advantage of this inherent property of *Taq* polymerase by providing a nonspecific overhang for any PCR product. These adenine and thymine overhang ends hybridize, covalently joining the pCR2.1 vector with the isolated PCR DNA fragments. Ligation was completed using "The Original TA Cloning Kit" by Invitrogen™". The ligation reactions (see below) totaling 15 uL were incubated at 14°C for 26 hours. The resultant constructs ligating the pCR2.1 vector with either LcrV and YscF were named pCR2.1-LcrV and pCR2.1-YscF, respectively.

Ligation Mix

- 0.5 uL (0.025 ug/uL) pCR2.1 vector
- 1.5 uL 10x ligation buffer
- 6 uL Water
- 1 uL T4 DNA ligase
- 6 uL purified PCR product

3.3.4 Transform pCR2.1-LcrV and pCR2.1-YscF vectors into the non-expression TOP10F' E. coli strain

The *E. coli* strain TOP10F' (see Appendix C. Strains for genotype) was selected due to its very low background expression of the protein in the expression vector in the absence of inducing conditions. The constructs pCR2.1-LcrV and pCR2.1-YscF were transformed into One Shot™ TOP10F' chemically competent cells (Invitrogen™). Once the purified plasmid was placed into cold competent cells, the mixture was incubated on ice for 30 min then heat shocked at 42°C 30 s. To both cultures, 250 uL of room temperature S.O.C. media (Invitrogen™) was added, and then incubated in a shaking incubator for 1 hr at 37°C at 225 RPM. 10 and 100 uL aliquots of the transformation solution were

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plated on S-Gal/KAN/LB agarose (Sigma-Aldrich Co.[©]) and incubated at 37°C overnight. White colonies, representing clones with inserts, were picked and used to inoculate 3 mL LB broth [50 ug/mL KAN] at 37°C at 225 RPM overnight.

3.3.5 Isolate each plasmid construct and verify the gene sequences using dideoxy sequencing

The inoculates (1 mL) were spun (12.5 RCF, 5 min) and the DNA purified from the cell pellet using S.N.A.P. mini prep kit (Invitrogen™) according to the manufacturer instructions. The purified plasmid containing the gene inserts were double digested with NdeI and XhoI restriction enzymes overnight.

Purified LcrV-pCR2.1 and YscF-pCR2.1 vectors (200 ng) were sent to the Ohio State University's Plant Microbe Genome Facility in Columbus, OH for DNA sequencing to verify the fidelity of the amplification, ligation, and transformation. By pair-wise alignment analysis, the two vector inserts were identified as containing the correct LcrV and YscF DNA sequences without any mutations. Unreadable base calls were analyzed using the software package ABI Sequencing Analysis 5.2 Patch 2.
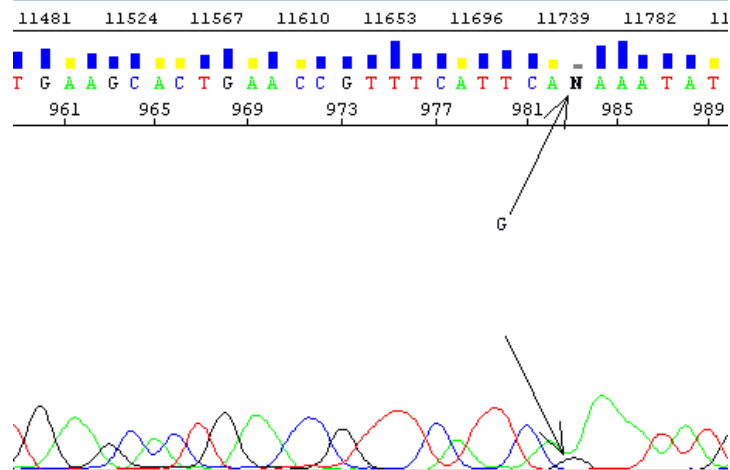
Figure 17. Sequencing Chromatographs

In order to ensure sufficient plamid for further manipulations, two 1 L flasks containing 250 mL of LB broth [50 ug/mL kanamycin (KAN)] were inoculated with 1 mL of the verified LcrV/YscF-pCR2.1 inoculums. These were grown overnight at 37°C at 225 RPM and harvested by centrifugation with a Sorvall RC-5B Refrigerated Superspeed Centrifuge at 6000 g for 15 min at 4°C in a SLA-3000 rotor. The pellets were purified with QIAGEN Endofree Plasmid Maxi Kits according to the manufacturer's directions. The preps produced 1.2 ug and 1.7 ug high purity pCR2.1-LcrV and pCR2.1-YscF plasmids, respectively, which were stored at -20°C.

3.3.6 Digest pCR2.1-LcrV, pCR2.1-YscF, and pET24a+ plasmids and isolate fragments for ligation

The screening vector pCR2.1 is not designed as an expression vector and thus is not used for large scale protein production. After sequence verification and large scale production, pCR2.1-LcrV, pCR2.1-YscF, and pET24a+ vector were digested with the restriction enzymes NdeI and XhoI. The LcrV gene

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sequence contained a naturally occurring XhoI site (CTCGAG italicized in Table 3), which could fragment the gene into two pieces upon XhoI digestion. Fortunately, neither XhoI restriction site was preferentially cut, and, a partial XhoI digestion was conducted by stopping the digest after 1-hr. Sufficient full size product could be recovered at this point (Figure 18). The LcrV and YscF DNA containing bands, as well as the linearized pET24a+ vector, were separated by agarose gel and the DNA fragments isolated using DNA Extraction Kit (QIAGEN™) according to the manufacturer's suggested protocol. The purified DNA was stored at -20°C prior to ligation.
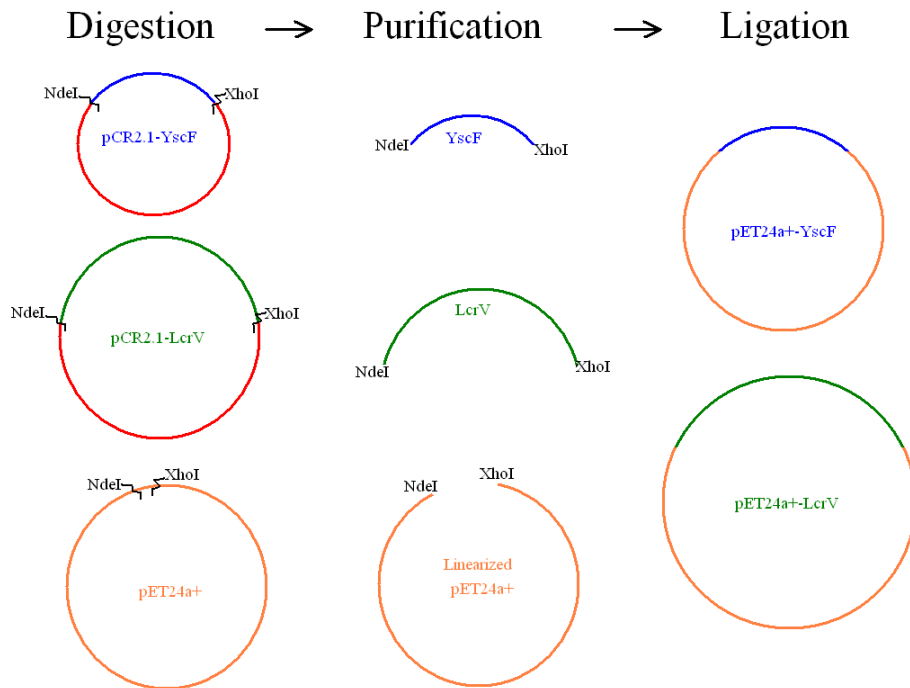

Figure 18. Depiction of Plasmid Switching

3.3.7 Ligate gel-isolated LcrV and YscF DNA gene fragments into the pET24a+ plasmid

Following digestion and purification of the expression vector components (LcrV, YscF, and linearized pET24a+ vector), the complementary overhang ends were ligated. The ligation reactions (see below) totaling 20 uL were incubated at 23°C for 4 hours and stored at -20°C until transformation.

Ligation Reaction

- 4 uL linearized pET24a+ (~10-ng/uL)
- 8 uL purified LcrV digest product or 5 uL YscF digest product
- 4 uL 5x ligation buffer
- 1 uL water (LcrV) or 4 uL water (YscF)
- 3 uL T4 DNA ligase diluted 1/10

3.3.8 Transform plasmids into the non-selective, non-expression *E. coli* strain DH5α and verify with restriction digestions

Ideally, the expression vector pET24a+ vector will not express (produce transcripts) the protein encoded in the vector; however, low level background transcription often does occur from minor promoter/operator regions in non-inducive conditions, producing very small quantities of protein. The energy required for protein production or the toxicity of the protein lowers the cell's reproductive rate thus negatively affecting the cell's competitiveness within a heterogeneous culture. If this occurs, this effect selects against the cells containing the correct gene insert. DH5α (Appendix C. Strains) does not promote this background expression and also carries the *recA1* and *endA1* mutations which help stabilize the plasmids and increase plasmid isolation yields. The same protocol from section **3.3.4** was used for this transformation and

culture. Blue/White screening capability have not been constructed within the pET24a+ vector; therefore, additional screening was necessary.

3.3.9 Purify verified plasmid constructs and transform into *E. coli* expression strain BL21(DE3)

The *E. coli* strain BL21(DE3) was selected as it is able to overexpress the transcripts, thus the proteins, using the T7 RNA promoter induced by the synthetic lactose analog, IPTG. The expression vector containing the LcrV and YscF genes were purified from the DH5α isolated constructs (similar to protocols sections **3.3.5** and **3.3.8**) and transformed into Novagen Bl21(DE3) Chemical Competent Singles™ as in section **3.3.4**. Colonies were picked and selected using restriction digestion.

3.4 Expression of recombinant LcrV and YscF proteins

3.4.1 Overview

With the expression vectors constructed and verified, the next step was to express recombinant LcrV and YscF. The process of producing the protein was broken into three tasks. First, small test protein expressions were conducted to verify for the correct protein size. Second, large scale test expressions were conducted and the protein gel isolated and sequenced to verify the correct amino acid sequence. Lastly, if insufficient expression was observed, conduct growth/induction optimization tests and repeat task two. Figure 19 represents the steps used in expressing LcrV and YscF. The optimized LcrV and YscF cultures were lysed and the cellular debris pelleted by centrifugation. The LcrV

expression did not require optimization, whereas YscF did require additional testing.
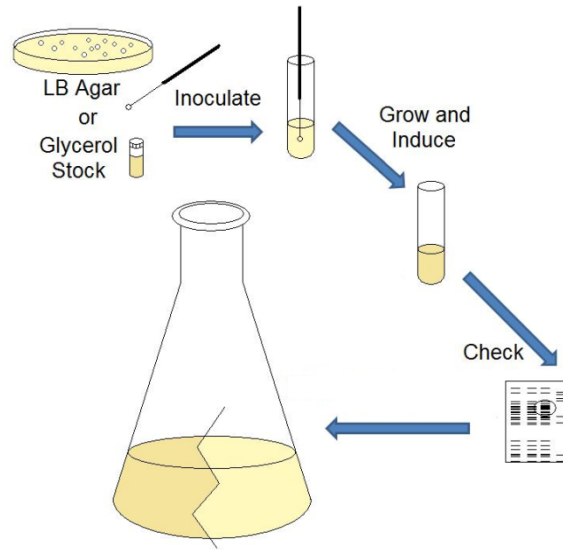
Figure 19. Protein Expression Overview

3.4.2 Protein Test Expressions

In the previous section **3.3** the methods for plasmid construction was described. For test expressions, five colonies for both pET-LcrV and pET-YscF were picked and tested in parallel. A loop of ~10 uL overnight saturated culture was used to inoculate 3 mL of LB broth [50 ug/mL KAN]. The culture was grown to an OD_{600} ~0.5 before induction with IPTG [0.1 M]. After induction, the cultures were allowed to grow at 37ºC overnight. The cultures were then spun at 10K RCF for 10 minutes, resuspended in SDS loading dye, and visualized by electrophoresis with Coomassie staining.

The construct which produced the highest level of protein was selected for each LcrV and YscF. The protein products for both LcrV and YscF constructs

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were submitted for analysis using liquid chromatography coupled with mass spectroscopy (LC/MS) of tryptic peptides.

3.4.3 Large Scale Production

In order to produce sufficient protein the production was scaled up ~1000 fold from 3 mL to 2 L. For LcrV, a 100 mL overnight seed culture grown to maximal density was transferred *en masse* into 4 L Erlenmeyer flask with 2 L LB broth [50 ug/mL KAN]. The culture was grown to OD_{600} ~0.5, induced with 0.1 M IPTG, and incubated overnight at 37°C at 225 RPM.

In order to express the protein YscF in BL21(DE3), smaller volume Erlenmeyer flasks with baffles were required. The 2 L volume was split between eight 1 L baffled flasks containing 250 mL of LB with KAN. The large production cultures were grown to OD_{600} ~1.2, induced with 0.1 M IPTG, and incubated overnight at 37°C at 225 RPM.

3.4.4 Harvesting

The cells were harvested by centrifugation with a Sorvall RC-5B Refrigerated Superspeed Centrifuge and SLA-3000 Fixed Angle Rotor at 6K g for 15 min at 4°C. The supernatant was removed and the pellet was resuspended with 6 mL of 20 mM PB (pH 6.6) per gram of cells with 1 mL of 10x protease inhibitor cocktail added to limit proteolytic degradation. The cell slurry mix was kept on ice for the entire resuspension process. Following resuspension the cell slurry was lysed by two passes through a Thermo Electron French Press Cell Disrupter and a 40K French Pressure Cell. The YscF cell resuspension mix, in addition to the protease inhibitor, contained 1% Triton and 100 ug/250 mL

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lysozyme. The lysed cell slurries were then centrifuged at 15K x g 20 min at 4°C to pellet cellular debris. The supernatant was removed and stored at -20°C.

3.5 Protein Purifications

3.5.1 Overview

Due to physical differences (size, hydrophobicity, and solubility), purification of LcrV and YscF required different protein purification procedures. The primary instrument used in purification was an Amersham Biosciences AKTAexplorer Fast Protein Liquid Chromatography (FPLC). FPLC is very similar to high performance liquid chromatography (HPLC) but the FPLC columns tolerate larger quantities of crude protein extract at lower pressures without clogging the column or overpressure damage. Columns of varying properties were employed in series to achieve the final LcrV and YscF purity levels.

3.5.2 LcrV Protein Purification Protocol

3.5.2.1 Ammonium Sulfate Precipitation

A quick, cheap, and simple method for removing many unwanted proteins is precipitation using ammonium sulfate (ASP). Ammonium sulfate is a highly soluble salt which effectively dehydrates the area surrounding the proteins in solution. As the proteins become less soluble they precipitate out of solution and are easily removed via centrifugation.

The LcrV pellet from **3.4.4** was resuspended in 50mM phosphate, 150mM NaCl (pH 7.2 buffer). Several ammonium sulfate precipitations, or "cuts," were made by progressively adding quantities of ammonium sulfate salt to the LcrV solution. After each cut the solution was spun down, and the precipitate and

supernatant was analyzed for LcrV. The 1.5 M ammonium sulfate fraction (tube 2 in Figure 20) precipitated most of the unwanted proteins leaving LcrV in solution.
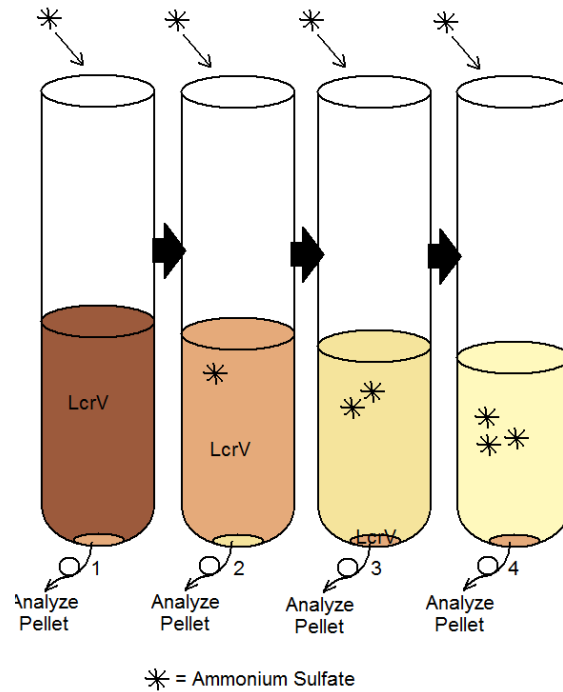
Figure 20. Test Ammonium Sulfate Precipitation Overview

3.5.2.2 FPLC Purification Step 1

The 1.5 M ammonium sulfate solution containing LcrV was loaded onto a Source 15PHE 4.6/100 PE column using a protocol established by Mr. Jordan Williamson with conditions described in Appendix A. The column fractions were stored on ice, pooled, and concentrated using a 10 kDal spin column concentrator.

3.5.2.3 FPLC Purification Step 2

The pooled and concentrated LcrV solution was loaded onto a HiTrap column to exchange it from high salt buffer (50 mM sodium phosphate and

residual ammonium sulfate, pH 7.2) to a lower salt buffer (20 mM Tris, pH 8). The lower salt LcrV solution was further purified using a MiniQ ion-exchange column from GE Healthcare Life Sciences using the method in Appendix B. Fractions 12-14 and 16-18 were pooled (most likely different quaternary structures of LcrV). This pooled sample was treated with 100 ug of lysozyme per 250 mL total solution for 5 min. The precipitant was pelleted and the supernatant was passed though a size exclusion column to yield the final solution containing ~99% purified LcrV.

3.5.3 YscF Protein Purification Protocol

3.5.3.1 Ammonium Sulfate Precipitation

A 20% ammonium sulfate concentration with lysozyme precipitated YscF from solution. The pelleted YscF precipitate was used for further purification, unlike LcrV, which used the fraction that remained in solution. YscF (9.5 kDal) is a relatively small protein and to remove higher molecular weight contaminants, the pellet was twice washed with 20 mM Tris, 1% (v/v) Triton X-100, 150 mM NaCl (pH 8), as we have seen that YscF is not soluble in NaCl solutions. After washings, the YscF containing pellet was resuspended in 20 mM Tris, 0.05% (v/v) Tween 20 (pH 8).

3.5.3.2 FPLC Purification

The resolubilized YscF solution was loaded onto a size exclusion column yielding the final solution containing purified YscF.

4. Results and Discussion

4.1 Overview

Much of the early LcrV and YscF cloning steps were conducted in parallel whereas the expression and purification steps were conducted separately. The results of the LcrV and YscF production will be described separately.

4.2 LcrV

Figure 21 depicts each of the steps required to build an LcrV recombinant protein producing construct from amplification of genomic *Y. pestis* DNA. Lanes C and D from Figure 21 are from the amplification of genomic *Y. pestis* KIM5 and CO92 DNA, respectively. CO92 DNA was not used for the building the construct; however, sequences are completely homologous.
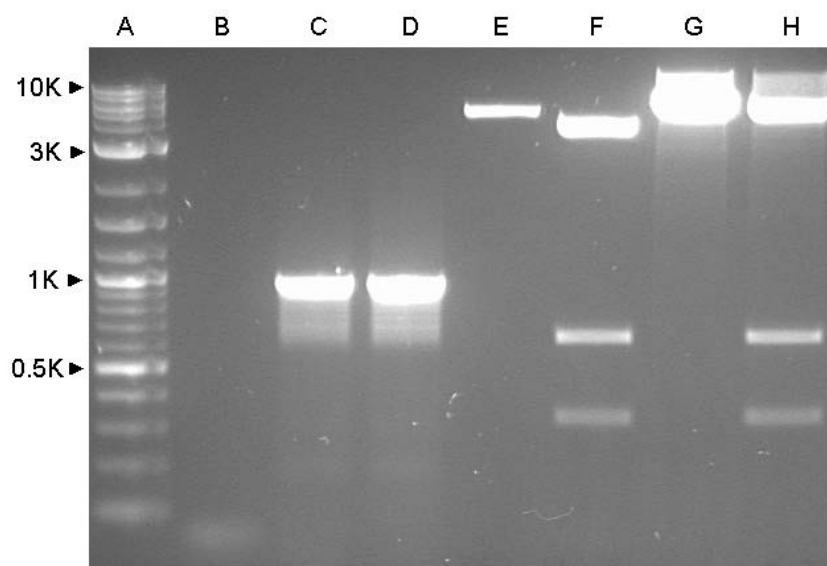

Figure 21. LcrV DNA

The KIM5 DNA amplicon from lane C was ligated to pCR2.1 vector, transformed into TOP10F', and plated onto LB agar plates with KAN. When the pCR2.1 vector is transformed into TOP10F' without the insertion of a piece of

DNA, the enzyme β-galactosidase is produced. β-galactosidase metabolizes S-gal® (a modified galactose sugar) producing an insoluble blue product. Therefore, white colonies (Figure 22) have a DNA sequence inserted in the pCR2.1 plasmid disrupting the βgal gene sequence and preventing the S-gal® metabolism.
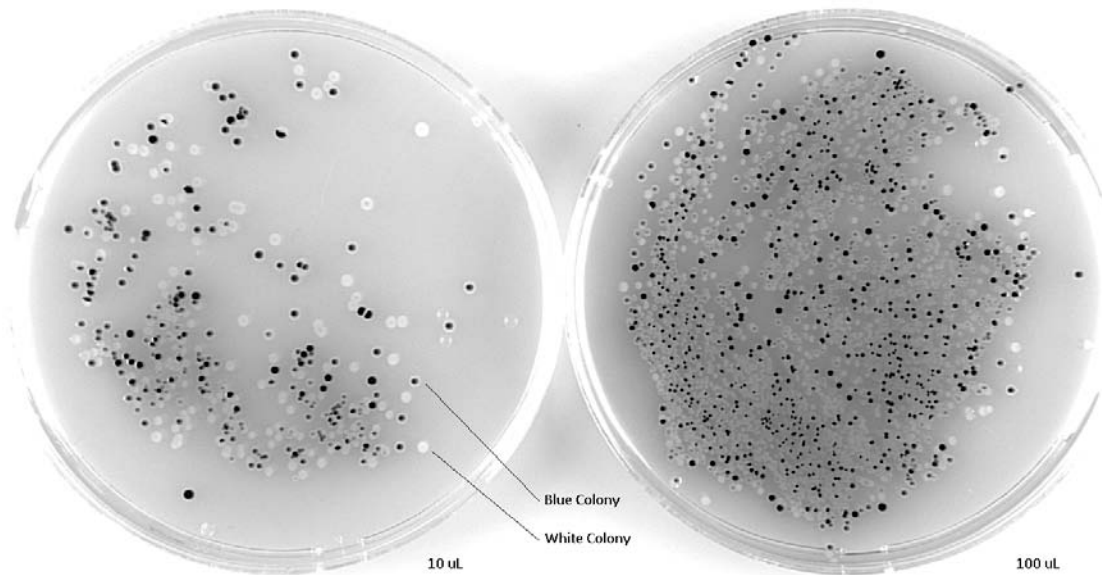
Figure 22. Blue/White Transformation Screening

Several clones were selected, plasmid DNA isolated, digested with the restriction enzymes NdeI and XhoI and the fragments analyzed via agarose gel. This step screened out plasmid inserts that were not the correct size as predicted by published LcrV DNA sequence. Lanes E (NdeI) and F (NdeI and XhoI) are pCR2.1-LcrV digests. The LcrV DNA bands in lanes F and H are cut with XhoI (CTCGAG) twice, as there is an additional XhoI site internal to the sequence (Table 3). The colonies with correctly sized inserts (not shown) were DNA sequenced using the primers T7P and R which hybridize just 5' and 3' to the

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insert sequence. Using this vector, the PCR fragment is not directionally aligned into the vector. Sequencing of both 5' and 3' directions aids in obtaining complete sequence from long inserts, as DNA sequences by standard methods often become less reliable after 1000 bp. Also, difficult regions to sequence, such as repeat strings of >6 nucleotides, are often miscalled by the software that translates the chromatogram to a sequence. The sequence from colony 1 is given in Appendix G.

The pCR2.1-LcrV vector was digested, the DNA insert ligated into the expression vector pET24a+, and the construct transformed into the non-expression strain DH5α. The following picture is an example of a successful transformation into *E. coli.*
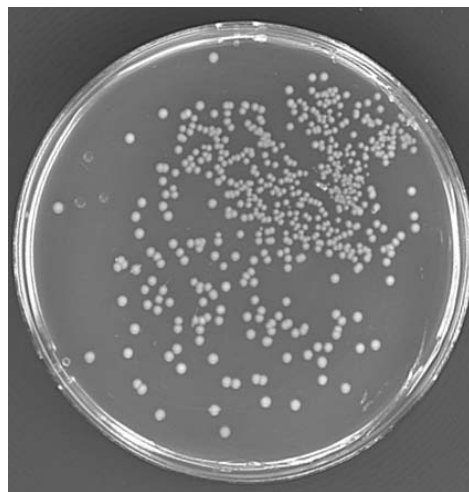

Figure 23. Transformation Plate

The correct insertion of the LcrV DNA into pET24a+ was initially screened with NdeI/XhoI digest and analyzed with gel electrophresis. Figure 21 lane G is the singly cut (NdeI) and Lane H is the double cut construct. pET24a+-LcrV was

tranformed into BL21(DE3), colonies picked, and test expressions were conducted to screen the colony producing the highest levels of LcrV. Figure 25 (D induced) has a band that is much larger then the uninduced control lane. The predicted size of LcrV (37.24 kDal) was predicted using ExPASy, a protein identification and analysis software set available at (http://ca.expasy.org/). The protein from the test inductions ran at 35 kDal (Figure 24). Following the successful induction, the constructs were archived in 15% glycerol and stored at -80ºC. This allows for future production of the protein without transformation. The vector map for pET24a+-LcrV is depicted in Appendix H.
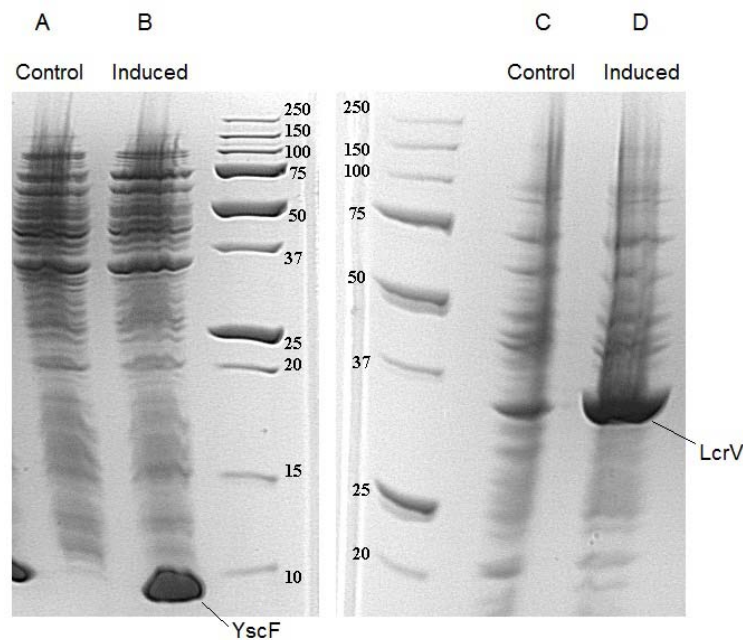

Figure 24. LcrV and YscF Test Expressions

The identity of the band was further confirmed by protein sequencing using LC/MS by the proteomics facility in the Applied Biotechnology Branch of the Air Force Reseach Laboratory run by Dr. Pavel Shiyanov. The results can be

seen in Appendices E and F. The N-terminal methionine on both LcrV and YscF were not detected, resulting in 99% and 98% coverage to the predicted sequences, respectively. This 1-2% difference is due to methionine processing which occurs in the cytoplasm of *E. coli* by methionine aminopeptidase (MAP or ampM) (Gellissen 2005). This results in the cleavage of the *N*-terminal methionine residue and accounts for the less than perfect homology. The enzyme MAP is also produced by *Yersinia* species; therefore the post translational cleavage of *N*-terminal methionines by produced *E. coli* does not significantly alter the recombinant protein from its native form (NCBI 2009).

Large scale growth of recombinant LcrV was easily upscaled from 2 mL to 2 L without modifying the procedure. Cell lysis (in conjunction with Mr. Jordan Williamson and Dr. David Riddle) was greatly aided by the addition of lysozyme, which cleaves 1,4-beta-linkages in *E.* coli's cell wall. These cell wall constituents, if untreated, may stick to isolated proteins and hinder purification. Figure 27 (Appendix B) would be much purer had lysozyme treatment been used, as with YscF, before purification with the FPLC.

The series of FPLC purification steps performed by Riddle and Williamson resulted in a product of 99% purity.

4.3 YscF

Figure 25 depicts each of the steps required to build an YscF recombinant protein producing construct from amplification of genomic *Y. pestis* DNA. Figure 25 lanes C and D show the amplification of genomic *Y. pestis* KIM5 and CO92 DNA, respectively.
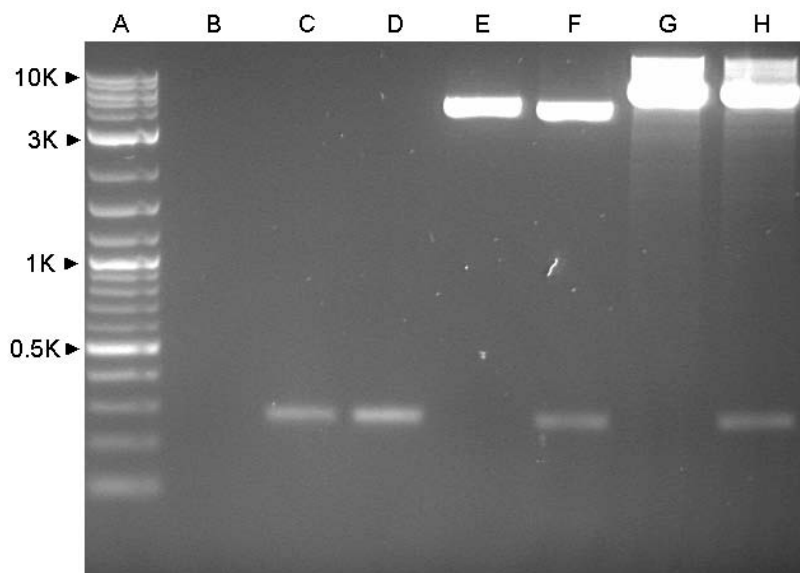
Figure 25. YscF DNA

The same process was used for YscF as was described in 4.2. The lanes represent (A) Ladder, (B) Negative Control, (C) YscF DNA from KIM5 Genomic, (D) YscF DNA from CO92 (for reference only), (E) pCR2.1-YscF NdeI digest, (F) pCR2.1-YscF NdeI/XhoI digest, (G) pET24a+-YscF NdeI digest, and (H) pET24a+-YscF NdeI/XhoI digest. The sequence for pCR2.1-YscF using the T7P primer is given in Appendix G. The vector map for pET24a+-YscF is depicted in Appendix I. A whole cell crude protein sample from the 3 mL test induction was run on a SDS-PAGE gel with Coomassie staining (Figure 24). The colony producing the highest level of protein was archived in glycerol and stored at -80ºC.

Large scale production of recombinant YscF required using multiple flasks of smaller volumes. Multiple attempts using a standard 4 L flask without baffles with 2 L of culture did not produce a noticeable YscF band. Spitting the 2 L

culture between eight 1 L flasks with baffles each with 250 mL of culture produced a very noticeable band in the range of YscF's predicted size. One explanation is the YscF producing culture strictly requires high levels of oxygenation. The large 2 L culture, grown in a smooth bottom flask, did not produce a large froth compared to the flasks with baffles. Also, perhaps without the baffles agitating the *E. coli*, a film could develop that repressed YscF expression.

5. Conclusions and Future Research Considerations

5.1 Overview

The production and purification of the two recombinant proteins LcrV and YscF is an initial step and an important milestone in accomplishing V_{HH} production as described in the DTRA proposal titled "Use of Epitope-directed Nanobodies® as Passive Immunotherapeutic Agents Against *Yersinia pestis.*" Several choke points and setbacks had to be dealt with during this project. Initially the *E. coli* strain NOVABLUE (Novagen) was used as the non-expression host for pET24a+-LcrV and YscF. After several transformation attempts, we came to the assumption that perhaps even basal expression, albeit low, killed the bacteria with the successful construct insert. The LcrV protein was suspected of being cytotoxic as it was thought to be a transmembrane protein with both hydrophobic and hydrophilic moieties. However, upon closer analysis with protein sequence analysis software (http://ca.expasy.org/tools/protparam-doc.html), the primary structure is largely charged with 52 positively and 43 negatively charge amino acids. LcrV's total average hydropathicity score was found to be -0.549 (charge amino acids are given negative values and uncharged positive). Since we believed the protein to be toxic we decided to try another non-expression *E. coli* strain DH5α. After multiple attempts with a two different lots of DH5α, the construct was successfully transformed. The LcrV purification was relatively straight forward. The protein stayed in solution during concentration and purification.

YscF, after production and during purification, indicated a high propensity to self-aggregation. The aggregation caused the small protein to behave like a much larger protein making purification and recovery difficult and unpredictable. Unsuccessfully, several detergents added to the YscF precipitant to resolubilize the protein. The process was unnecessary due to the discovery of the solubility properties of YscF in high and low salt (NaCl) conditions. YscF even in low concentrations self-agglomerates in even low salt conditions; however, it stays in solution and can be easily purified if salt-free buffers are used. This finding is not surprising as YscF is a small protein that forms needles by agglomeration. While significant effort was put into developing and optimizing the purification protocols, the entire process is evidence that fusion tags are not necessary for production of recombinant proteins. The proteins produced as described in section **5** using the methods from section **4** were shipped to AF collaborator Dr. Serge Muyldermans (University of Belgium) for V_{HH} production.

5.2 Continuation of Research

The proteins produced will be used as antigens in the *Camelidae* species *L. glama* to produce a natural immune response leading to the production in the host of antibodies and V_{HH}. Post-injection blood will be drawn and the peripheral blood lymphocytes isolated. cDNA will be produced from lymphocyte mRNA coding for the VHH domains from the Camelid antibodies, and these will be used as a phage display to screen for the highest binding affinity to the protein antigens used. Once panning is completed, the final DNA sequence coding for the peptide with the highest binding affinity will be ligated into a vector and

transformed into a bacterial cell line to produce unlimited supplies of the LcrV and YscF V_{HH}.

The developed LcrV and YscF V_{HH} will be tested using an *in vitro* model for the attenuation of infection using the avirulent *Y. pestis* KIM5 strain in human primary macrophage cells. Following characterization in the *in vitro* model, the V_{HH} immunotherapeutic will be tested in an *in vivo* murine model for efficacy against *Y. pestis* KIM5 exposure. In addition, the use of multiple V_{HH} injections will be examined to see if the period of protection can be extended and, if so, for how long.

If V_{HH} are successful in producing protection from *Y. pestis* <u>post</u>-exposure, the immunotherapeutic will be tested in an *in vivo* model using a highly virulent *Y. pestis* strain such as CO92. If such data supports the use of *Y. pestis* immunothepeutics, development of V_{HH} to other agents (Botulinum toxin, *F. tularensis*, etc.) will proceed, with hopes of developing a V_{HH} "cocktail" capable of providing a period of protection post-exposure, which will allow for the completion of mission requirements in the event of a BW attack.

Appendix A. LcrV Protein Purification with Source 15PHE 4.6/100 PE

Starting Buffer: 50 mM phosphate, 1.5 M ammonium sulfate, pH 7.0

Elution Buffer: 50 mM phosphate, pH 7.0

Flow Rate: 1.0 mL/min

Buffer Gradient:
        Step 1: 0% elution buffer for 10 min
        Step 2: 70% elution buffer for 15 min
        Step 3: 80 % elution buffer for 10 min
        Step 4: 100% elution buffer for 15 min

Sample Volume: 500 uL

The LcrV eluted out at approximately fraction 21 as shown in

Figure 26.

Figure 26. Output from FPLC for LcrV 15PHE
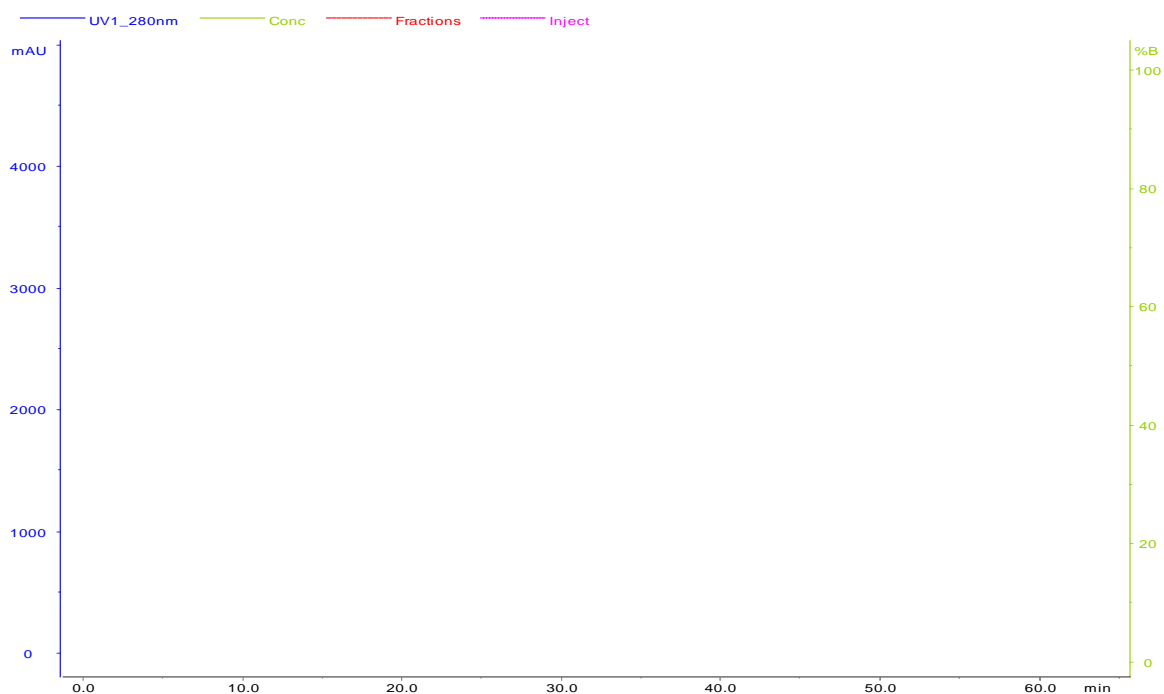
Appendix B. LcrV Protein Purification with MiniQ 4.6/50 PE

Starting Buffer: 50 mM phosphate, 1.5 M ammonium sulfate, pH 7.0

Elution Buffer (B): 1 M NaCl, 20 mM Tris, pH 8

Flow Rate: 0.5 mL/min

Buffer Gradient: 0 to 40% Elution Buffer for 24 minutes

The LcrV eluted out at fractions 12-14 and 16-18 as shown in Figure 27.
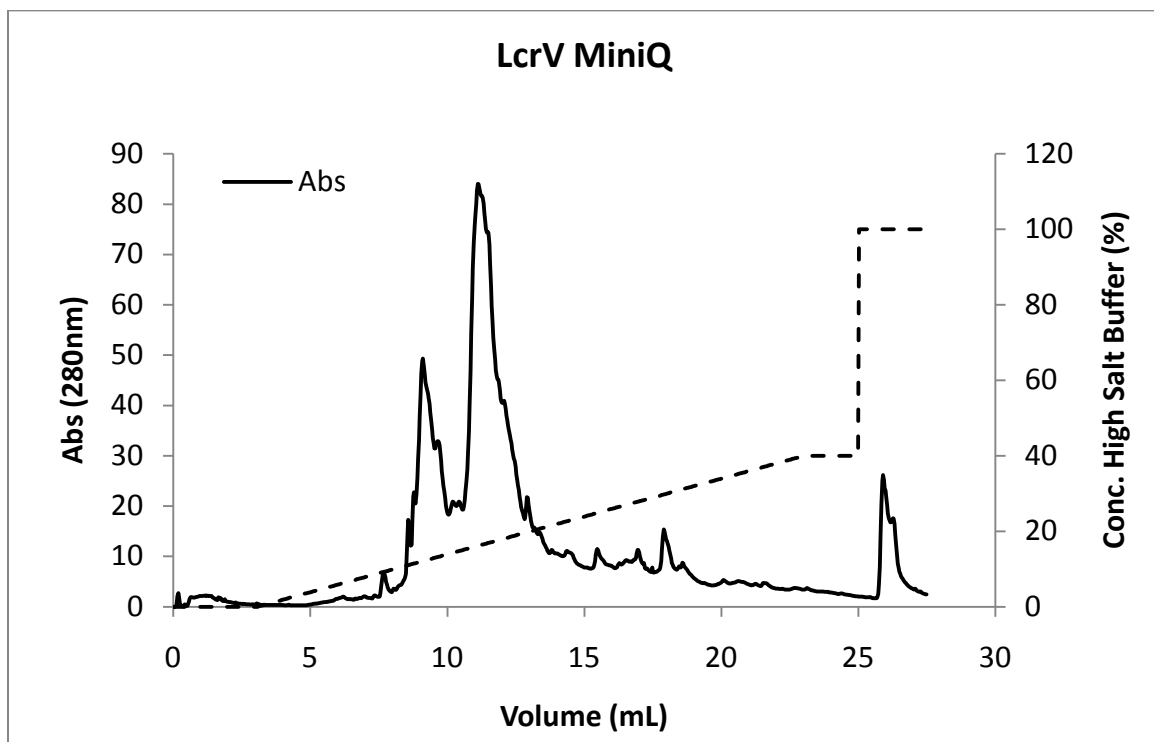
Figure 27. Output from FPLC for LcrV MiniQ

Appendix C. Strains

E. coli Strains (in order of use)

TOP10F' (Invitrogen™): F'[lacI^q Tn10(tet^R)] mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 deoR nupG recA1 araD139 Δ(ara-leu)7697 galU galK rpsL(Str^R) endA1 λ$^-$

DH5α (Invitrogen™): F$^-$ endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r$_K^-$ m$_K^+$), λ–

BL21(DE3) (Novagen™): F$^-$ ompT gal dcm lon hsdS$_B$(r$_B^-$ m$_B^-$) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])

Y. pestis Strains (CO92 DNA for reference only)

KIM5 (Matt Niles at North Dakota): biovar Mediaevalis, pgm-

[DNA ONLY] CO92 (BEI Resourses): biovar Orientalis, isolated from a fatal human case of primary pneumonic plague presumably contracted from an infected cat, Colorado, U.S.A., 1992

Appendix D. pET24a+ Vector Map

XhoI

NdeI

Ribosome
Binding Site

T7 Promoter
(T7P)

f1 origin

Kanamycin
Resistance
Gene

pET24a+ Map

(not to scale)
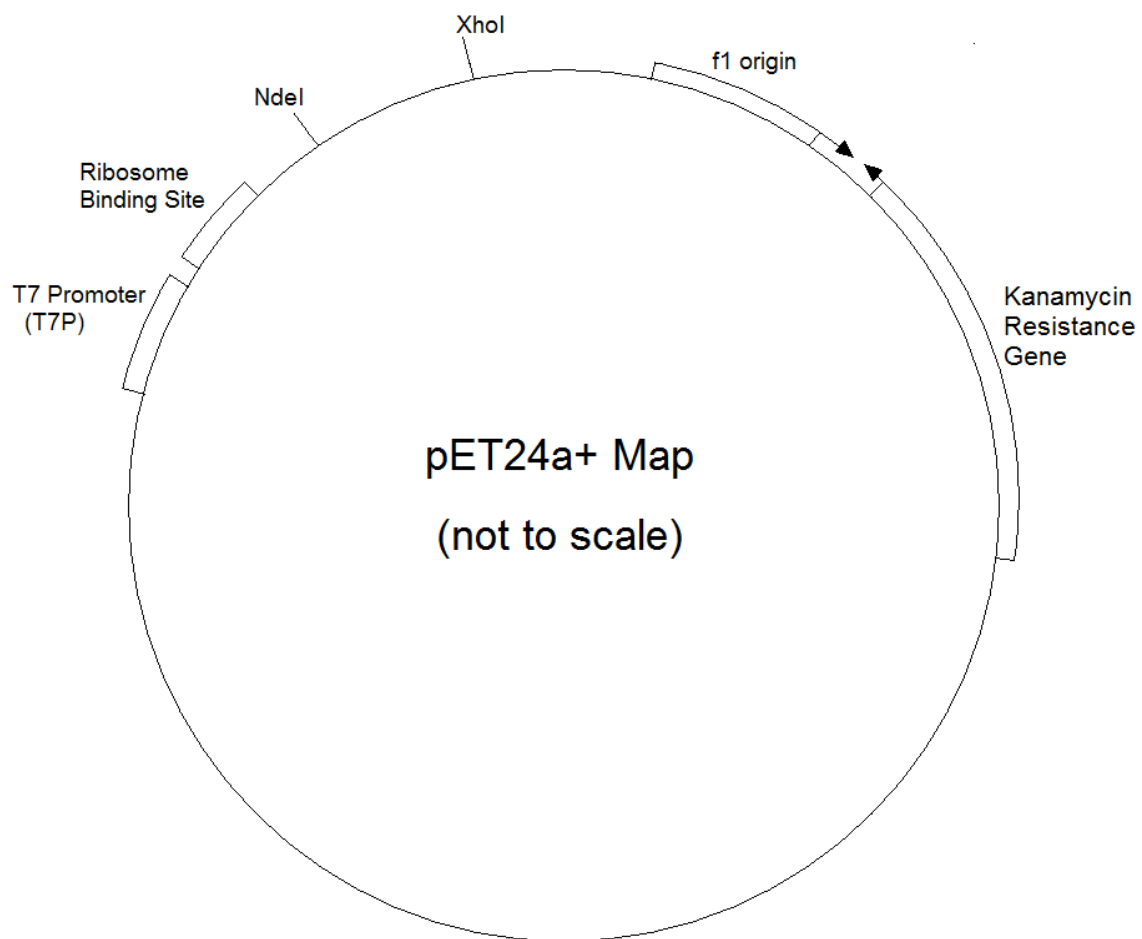
Appendix E. LcrV Protein Sequencing Results

Mascot Search Results

Protein View

Match to: Q1BZU5|Q1BZU5_YERPA Score: 4415
V antigen, antihost protein/regulator - Yersinia pestis (biovar Antiqua strain Antiq
Found in search of C:\Xcalibur\rlp\20080716a03.RAW

Nominal mass (M_r): 37217; Calculated pI value: 5.57
NCBI BLAST search of Q1BZU5|Q1BZU5_YERPA against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Yersinia pestis Antiqua

Variable modifications: Carbamidomethyl (C),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: 99%

Matched peptides shown in Bold Red

```
  1 MIRAYEQNPQ HFIEDLEKVR VEQLTGHGSS VLEELVQLVK DKNIDISIKY
 51 DPRKDSEVFA NRVITDDIEL LKKILAYFLP EDAILKGGHY DNQLQNGIKR
101 VKEFLESSPN TQWELRAFMA VMHFSLTADR IDDDILKVIV DSMNHHGDAR
151 SKLREELAEL TAELKIYSVI QAEINKHLSS SGTINIHDKS INLMDKNLYG
201 YTDEEIFKAS AEYKILEKMP QTTIQVDGSE KKIVSIKDFL GSENKRTGAL
251 GNLKNSYSYN KDNNELSHFA TTCSDKSRPL NDLVSQKTTQ LSDITSRFNS
301 AIEALNRFIQ KYDSVMQRLL DDTSGK
```

Appendix F. YscF Protein Sequencing Results

{MATRIX SCIENCE} Mascot Search Results

Protein View

Match to: Q1BZW7|Q1BZW7_YERPA Score: 2361
Type III secretion protein - Yersinia pestis (biovar Antiqua strain Antiqua)
Found in search of C:\Xcalibur\rlp\20080716a01.RAW

Nominal mass (M_r): **9484**; Calculated pI value: **6.54**
NCBI BLAST search of Q1BZW7|Q1BZW7_YERPA against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Yersinia pestis Antiqua

Variable modifications: Carbamidomethyl (C),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **98%**

Matched peptides shown in Bold Red

```
    1 MSNFSGFTKG TDIADLDAVA QTLKKPADDA NKAVNDSIAA LKDKPDNPAL
   51 LADLQHSINK WSVIYNINST IVRSMKDLMQ GILQKFP
```

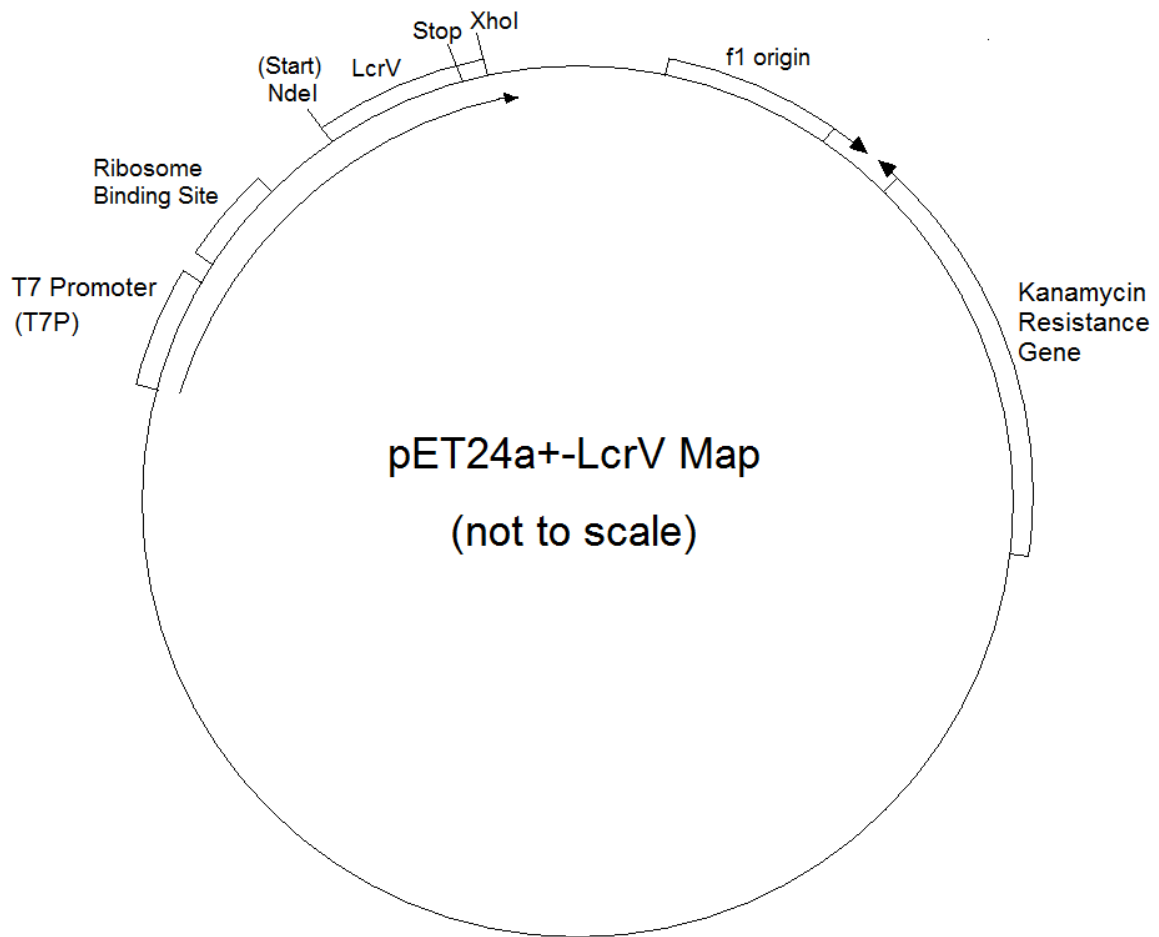# Appendix G. LcrV and YscF 5' DNA Sequencing Results

LcrV

```
>jr-YpL1T7P-T7P_039_B09.ab1
NNNNGNNNNNNCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGGCTTAGCATATGAT
TAGAGCCTACGAACAAAACCCACAACATTTTATTGAGGATCTAGAAAAAGTTAGGGTGG
AACAACTTACTGGTCATGGTTCTTCAGTTTTAGAAGAATTGGTTCAGTTAGTCAAAGAT
AAAAATATAGATATTTCCATTAAATATGATCCCAGAAAAGATTCGGAGGTTTTTGCCAA
TAGAGTAATTACTGATGATATCGAATTGCTCAAGAAAATCCTAGCTTATTTTCTACCCG
AGGATGCCATTCTTAAAGGCGGTCATTATGACAACCAACTGCAAAATGGCATCAAGCGA
GTAAAGAGTTCCTTGAATCATCGCCGAATACACAATGGGAATTGCGGGCGTTCATGGC
AGTAATGCATTTCTCTTTAACCGCCGATCGTATCGATGATGATATTTTGAAAGTGATTG
TTGATTCAATGAATCATCATGGTGATGCCCGTAGCAAGTTGCGTGAAGAATTAGCTGAG
CTTACCGCCGAATTAAAGATTTATTCAGTTATTCAAGCCGAAATTAATAAGCATCTGTC
TAGTAGTGGCACCATAAATATCCATGATAAATCCATTAATCTCATGGATAAAAATTTAT
ATGGTTATACAGATGAAGAGATTTTTAAAGCCAGCGCAGAGTACAAAATTCTCGAGAAA
ATGCCTCAAACCACCATTCAGGTGGATGGGAGCGAGAAAAAAATAGTCTCGATAAAGGA
CTTTCTTGGAAGTGAGAATAAAAGAACCGGGGCGTTGGGTAATCTGAAAAACTCATACT
CTTATAATAAAGATAATAATGAATTATCTCACTTTGCCACCACCTGCTCGGATAAGTCC
AGGCCGCTCAACGACTTGGTTAGCCAAAAACAACTCAGCTGTCTGATATTACATCACGT
TTCAATTCAGCTATTGAAGCACTGAACCGTTTCATTCANAAATATGATTCAGTGATGCA
ACGTCTGCTAGATGACACGTCTGGTAAATGACTCGAG
```

YscF

```
>jr-YpY1T7P-T7P_037_D09.ab1
NNNNNNNNNNGCTCGAGCGGCCGCCAGTGTGATGGATATCTGCAGAATTCGGCTTTAGC
ATATGAGTAACTTCTCTGGATTTACGAAAGGAACCGATATCGCAGACTTAGATGCGGTG
GCTCAAACGCTCAAGAAGCCAGCAGACGATGCAAACAAAGCGGTTAATGACTCGATAGC
AGCATTGAAAGATAAGCCTGACAACCCGGCGCTACTTGCTGACTTACAACATTCAATTA
ATAAATGGTCGGTAATTTACAATATAAACTCAACCATAGTTCGTAGCATGAAAGACTTA
ATGCAAGGCATCCTACAGAAGTTCCCATAACTCGAG
```

Appendix H. pET24a+-LcrV Construct

pET24a+-LcrV Map

(not to scale)

Appendix I. pET24a+-YscF Construct

pET24a+-YscF Map

(not to scale)

Labels on the plasmid map:
- Stop
- XhoI
- (Start) NdeI
- YscF
- f1 origin
- Ribosome Binding Site
- T7 Promoter (T7P)
- Kanamycin Resistance Gene
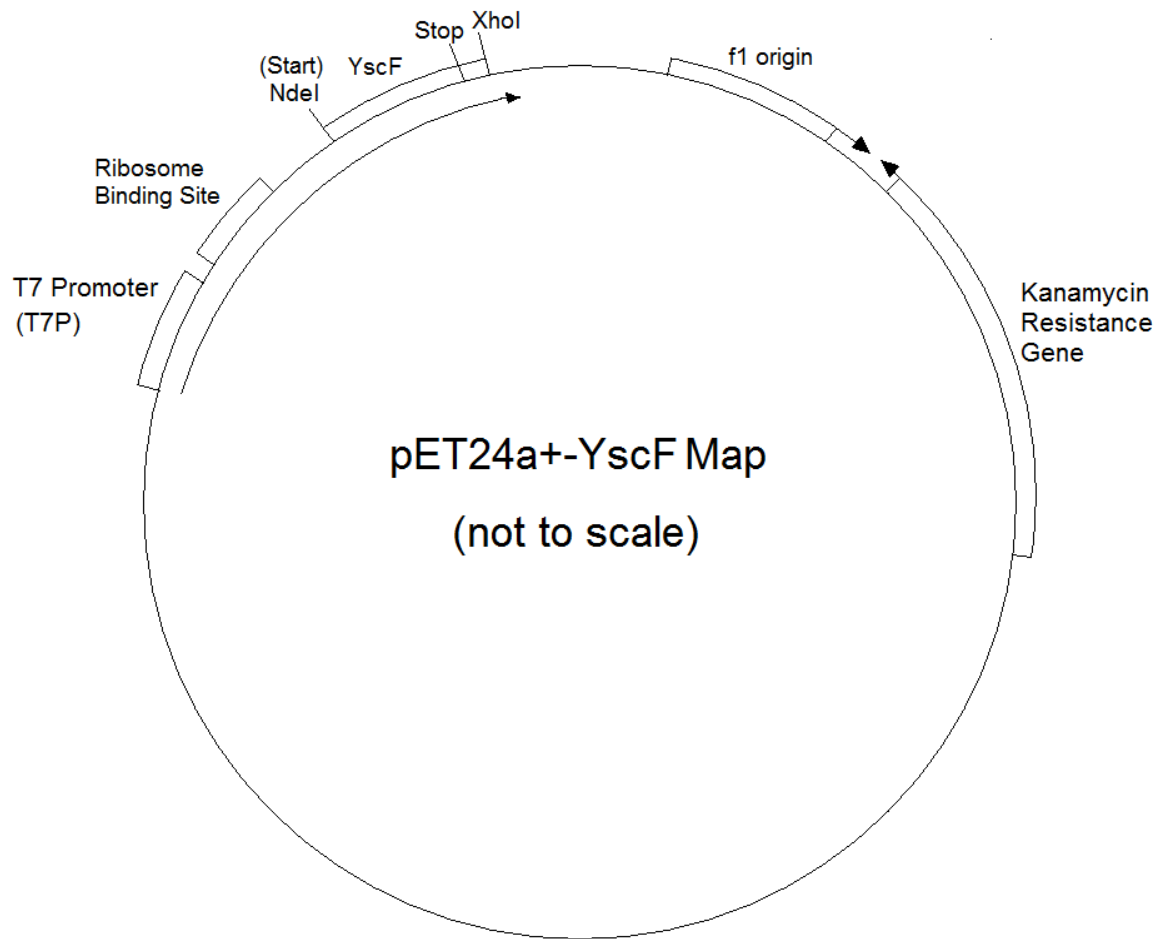
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A Security Source. <u>Home>News>World News>Africa and Indian Ocean>Algeria.</u> 19 January 2009. 16 February 2009 <http://www.telegraph.co.uk/news/worldnews/africaandindianocean/algeria/4287469/Black-Death-kills-Jews-operatives-in-Algeria.html>.

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<u>Bin Laden's Biological Threat.</u> Dir. BBC-1. 2001.

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14. ABSTRACT

Molecular biology techniques and low cost reagents have lowered the barriers to entry for development of biological arsenals by non-state and state groups. Additionally, genetic engineering of epitope targets from such pathogens as *Y. pestis* would annul current detection methods and therapeutic treatments. Vaccines often have short shelf lives and are of minimal utility if not used prior to exposure to BW agents. *Camelidae,* including camels and llamas produce unique antibodies termed Nanobodies® (Nbs) or antigen specific fragments (V_{HH}) which are much smaller than traditional antibodies (15 vs. ~150 kDal) yet seem to attach with the same selectivity and affinity as full antibodies. V_{HH} are more stable than the fragile, more bulky antibodies and maintain their structure and function even at high temperature and humidity. It is thought that, due to these unique characteristics, V_{HH} could be reconstituted from a lyophilized pellet and used as a real time injectable immunotherapeutic to be used when warfighters have been exposed to BW. Two logical candidates for V_{HH} production are the low-calcium-response V protein (LcrV) and the needle-like Yop (Yersinia Outer-membrane Protein) Secretion Protein F (YscF). This effort successfully produced quantities greater than 1-mg purified native recombinant LcrV and YscF proteins as antigens for V_{HH} production.

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Yersinia pestis, immunotherapeutic, nanobody, protein antigen

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