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# The Potential Role for CapB in Pathogenesis of Francisella tularensis

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# THE POTENTIAL ROLE FOR *capB* IN THE PATHOGENESIS OF *FRANCISELLA TULARENSIS*

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

by

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| | |
|---|---|
| A260nm | absorbance at 260 nm |
| A595nm | absorbance at 595 nm |
| AGE | agarose gel electrophoresis |
| Amp | ampicillin |
| ATP | adenosine triphosphate |
| ATCC | American Type Culture Collection |
| *B. anthracis* | *Bacillus anthracis* |
| *B. licheniformis* | *Bacillus licheniformis* |
| *B. pumilus* | *Bacillus pumilus* |
| BSA | bovine serum albumin |
| BSL-3 | Biosafety level-3 |
| *B. subtilis* | *Bacillus subtilis* |
| bp | base pair |
| C | cytosine |
| $CaCl_2$ | calcium chloride |
| cAMP | cyclic adenosine monophosphate |
| CDC | Centers for Disease Control and Prevention |
| CDM | Chamberlain's defined medium |
| cDNA | complementary deoxyribonucleic acid |
| CFU | colony forming unit |
| cm | centimeter |
| $CO_2$ | carbon dioxide |
| DEPC | diethylpyrocarbonate |
| DMEM | Dulbecco's modified Eagle's medium |
| DNA | deoxyribonucleic acid |
| *E. coli* | *Escherichia coli* |
| EDTA | ethylenediaminetetraacetic acid |
| Erm | erthyromycin |
| ESI | electrospray ionization |
| FDA | Food and Drug Administration |
| *F. horactica* | *Francisella horactica* |
| *F. novicida* | *Francisella novicida* |
| FPI | *Francisella* pathogenicity island |
| *F. tularensis* | *Francisella tularensis* |
| G | guanine |
| GABA | γ-aminobutyric acid |
| GGT | γ-glutamyltranspeptidase |
| $H^+$ | hydrogen ion |
| HCl | hydrochloric acid |
| IFN-γ | interferon gamma |
| IL-1 | interleukin-1 |

| | |
|---|---|
| IPTG | isopropyl-β-D-1-thiogalactopyranoside |
| K | 1000 RPM |
| kb | kilobase |
| kDa | kilodalton |
| kg | kilogram |
| kV | kilovolts |
| LB | Luria Broth |
| LC/MS | liquid chromatography / mass spectrophotometry |
| LPS | lipopolysaccharide |
| LTC | Lieutenant Colonel |
| LVS | live vaccine strain |
| M | molar |
| MAC | membrane attack complex |
| MAJ | Major |
| MALDI-TOF | matrix-assisted laser desorption ionization-time of flight |
| MAPS | microtubule-associated proteins |
| Mbp | megabasepair |
| MDC | monodansylcadaverine |
| $Mg^{2+}$ | magnesium ion |
| mg | milligram |
| $MgCl_2$ | magnesium chloride |
| $MgSO_4$ | magnesium sulfate |
| ml | milliliter |
| mM | millimolar |
| mmol | millimole |
| $Mn^{2+}$ | manganese ion |
| $MnCl_2$ | manganese chloride |
| MOI | multiplicity of infection |
| mRNA | messenger ribonucleic acid |
| MTA | material transfer agreement |
| MW | molecular weight |
| m/z | mass/charge |
| NaCl | sodium chloride |
| $NaHCO_3$ | sodium bicarbonate |
| NBT/BCIP | p-nitro blue tetrazolium / 5-bromo-4-chloro-3-indoyl phosphate |
| NEB | New England Biolabs |
| nm | nanometers |
| nmol | nanomole |
| O-antigen | oligosaccharide antigen |
| $OD_{600}$ | optical density at 600nm |
| ORF | open reading frame |
| oriT | origin of transfer |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |

| | |
|---|---|
| PCR | polymerase chain reaction |
| PGA | polyglutamic acid |
| pKa | negative logarithm of an equilibrium constant |
| PMN | polymorphic nuclear cells |
| PTFE | polytetrafluoroethylene |
| RAW 264.7 | Mouse leukaemic monocyte macrophage cell line |
| RNA | ribonucleic acid |
| RPM | revolutions per minute |
| RT-PCR | reverse transcriptase-polymerase chain reaction |
| *S. epidermidis* | *Staphylococcus epidermidis* |
| *S. haemolyticus* | *Staphylococcus haemolyticus* |
| SOC | Super Optimal Broth with Catabolite repression |
| SOEing | Splicing by Overlapping Extension |
| SDS | sodium dodecyl sulfate |
| TE | 10 mM Tris-Cl, pH 7.5; 1 mM EDTA |
| TEM | transmission electron microscope |
| TEN | 25mM Tris-Cl; 10mM EDTA; 200mM $NaHPO_4$, pH 7.6 |
| TLR-4 | toll-like receptor #4 |
| TNF-α | tumor necrosis factor-alpha |
| tRNA | transfer ribonucleic acid |
| TSB-C | tryptic soy broth plus 1% cysteine hydrochloride |
| U112 | Utah strain 112 |
| V | volts |
| WHO | World Health Organization |
| X-gal | bromo-chloro-indolyl-galactopyranoside |
| α | alpha |
| β | beta |
| γ | gamma |
| μF | microFaraday |
| μg | microgram |
| μm | micromillimeter |
| μl | microliter |
| °C | degrees Celsius |

ABSTRACT

THE POTENTIAL ROLE FOR *capB* IN THE PATHOGENESIS OF *FRANCISELLA TULARENSIS*

By MAJ Eric R. Fleming, Ph.D.

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

Virginia Commonwealth University, 2009

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*Francisella tularensis* was a facultative intracellular pathogen and a gram-negative coccobacillus which has been categorized by the CDC as a potential class A select agent due to its highly infectious properties and high mortality rates. *Francisella tularensis* was also responsible for the zoonotic disease tularemia, which was usually transmitted by arthropod vectors or via contact with infected animals. *Francisella tularensis* subspecies *novicida* has been used by many researchers in genetic pathogenesis experiments to try to elucidate genes responsible for virulence factors. One of these virulence factors was a capsular material which has been thought to be involved in either increasing pathogenicity or infectivity of this organism upon engulfment by its principal host cell, the macrophage. There were many potential genetic loci which may be involved in this

biosynthetic process of encapsulation. One such locus has excellent homology to the capsule biosynthesis operon of *Bacillus anthracis*, which, under certain conditions, creates a polyglutamic acid capsule (PGA). A transposon mutation in the amide ligase (*capB*) in LVS has a reduced virulence in murine infection models. I wished to investigate whether *Francisella novicida* was capable of producing such a capsule and under which environmental conditions this capsule was made. I have created a site-directed mutant of the *capB* gene in *Francisella novicida* U112 using targeted mutagenesis via PCR SOEing and have introduced this mutation via electroporation of a suicide vector. I have tested our mutant against preimmune serum treatments and have shown reduced viability as well as a reduced capacity for replication inside RAW 264.7 murine macrophages. I assayed for production of a PGA capsule via immunodot blot and electron microscopy as well as analysis by mass spectrophotometry of capsular extracts. I also tested various media constituents and different environmental conditions to determine which external stimuli may contribute to PGA capsule biosynthesis as well as regulatory changes in transcript levels of this operon.

# CHAPTER ONE: INTRODUCTION

## Background

*Francisella tularensis* was a gram negative coccobacillus bacteria and was classified as a facultative intracellular pathogen, in which it can survive outside a host in the environment. It was highly infectious, with as few as ten organisms causing disease in compromised individuals, and can be easily disseminated. It was classified by the Center of Disease Control and Prevention as a category A select agent. The organisms, viruses, and toxins which comprise this list were known to have the potential to cause widespread disease, produce high levels of morbidity and mortality if not treated, and usually require specialized care and therapy for resolution. *Francisella tularensis* causes the zoonotic disease tularemia, which originally was discovered in Tulare County, California causing a plaque-like illness in local ground squirrels in 1911. The organism isolated from these animals was originally called *Bacterium tularense*, after the site of disease origin. In 1947, it was later renamed after the famous public health surgeon, Dr. Edward Francis, who dedicated his life to the study of this organism and the disease it causes. Tularemia was usually associated with rodents, rabbits, muskrats, and voles and has been nicknamed rabbit fever. In the 1930s and 40s, large waterborne outbreaks of tularemia occurred in regions of the Soviet Union and Europe. The largest outbreak on record occurred in Sweden in 1966 through 1967, in which over 600 farmers became infected due to the aerosolization of bacteria in rodent-infested hay (47, 111).

**Transmission**

Tularemia was usually transmitted in one of four ways: direct contact with infected animals, arthropod-borne, ingestion, or aerosolization. The severity of disease can be associated with the route of infection. There were many known reservoirs of *Francisella*, including 14 species of ticks, 6 species of flies, many known mosquito species, several hundred species of mammals, and several bird species. While most cases in the United States involve ticks or mammal exposure, there was a known rodent-mosquito cycle which takes place in the northern climes of Russia and Sweden. Direct contamination was usually due to the handling of skins or tissue of infected animals, and was mostly associated with the occupational hazards of animal handlers or farmers. Bites from infected animals have resulted in transmission, however this occurs rather infrequently. Ticks infected with *Francisella* were usually infective for life and there was evidence of transovarial transmission, in which female ticks pass the bacteria on to their offspring. Tick species known to transmit tularemia include *Dermacentor andersonii, Dermacentor variabilis*, and *Amblyomma americanum*. The mosquito species of *Aedes, Anopheles,* and *Culex* have been known to transmit tularemia as well. The deerfly *Chrysops discalis* was also known to transmit this disease, although infrequently. Humans were usually accidental hosts in this life cycle. Ingestion of relatively larger amounts of bacteria has been the source of waterborne outbreaks, and disease has resulted from the ingestion of contaminated meat. Some water borne cases have been affiliated with amoebae, such as *Acanthamoeba*, and it was known that these bacteria can persist in these single-celled organisms for some time. Aerosolization of

bacteria due to contaminated hay and soil as well as laboratory accidents have produced many instances of pneumonic tularemia. The fairly recent occurrence of exposure to laboratory workers at the clinical microbiology lab at Boston Medical Center clarifies the importance of established select agent protocols and laboratory notification of potentially dangerous organisms (152, 156). There have been no reported incidences of person to person transmission.

**Utilization as bioweapon**

Due to its infectivity and its ease of dissemination, *Francisella tularensis* has been an agent of interest to the military as a biological weapon and was developed and mass produced in both the United States and the Soviet Union in the early 1950s. One of the most virulent strains of *Francisella* was weaponized for use in the M143 bursting spherical bomblet. It was believed that in the Soviet Union, strains of *Francisella* were engineered to withstand various antibiotics and vaccine therapies as late as 1990. In 1972, the Biological and Toxic Weapons Convention was signed by the United States prohibiting the development, production and stockpiling of microbes or their poisonous products except in amounts necessary for protective and peaceful research. As of 1996, 137 different countries had ratified this treaty, however it was believed that several countries have ignored the specifics of this treaty and have continued production of biological weapons (111).

It has been suggested by Dr. Ken Alibek and a former Russian LTC that Russia used tularemia as a bioweapon at the onset of the Battle of Stalingrad in the fall of 1942.

It was recorded that Russia had already lost a least a million soldiers that preceding year, and when it become clear that the German forces (6th and 17th Armies) were pushing toward Stalingrad, Stalin ordered Order 227, in which "every granule of Soviet soil must be defended to the last drop of blood." That fall over one hundred thousand cases of tularemia (the majority of these were pulmonary infections) befell the soldiers, first the Germans, but then the Russians too were affected. Subsequent reports, however, have cast doubt on these suggestions, as tularemia was endemic to these regions of Russia, and that the severe conditions of the front promoted these infections. It has been reported that the regional crops had not been harvested and that by winter, the number of rodents cohabiting with soldiers was extreme. Soldiers from both sides were bedding in straw as well as eating rations frequently tainted with dead rodents (1).

In 1969, the World Health Organization (WHO) estimated that if 50 kg of virulent *F. tularensis* particles were aerosolized over a city with 5 million people, the result would be 250,000 illnesses and 19,000 deaths. Illness would be expected to persist for several weeks and disease relapses would occur during the following weeks or months. Recently, the Centers for Disease Control and Prevention (CDC) estimated the economic impact associated with an outbreak of tularemia to be $5.4 billion for every 100,000 people exposed (total base cost to society) (75, 187).

**Epidemiology, disease, and treatment**

Tularemia was usually found in the northern hemisphere in countries such as the United States, Russia, Europe, Japan, and Scandinavia. Cases have been reported as far

as south as Australia. In the U.S., about 200 cases were reported each year and were usually located in Midwestern states, such as Oklahoma, Missouri, and Arkansas. Human disease can usually be linked to increased prevalence of tularemia in local wildlife.

Tularemia as a disease usually manifests itself in one of six clinical syndromes: ulceroglandular (the most prevalent form), glandular, oropharyngeal, oculoglandular, typhoidal, and pulmonary (usually the most lethal). These manifestations depend on the route of infection, the particular strain, and the inoculum size. All of these forms, however, usually start with headache, sudden fever, myalgia, and chills after three to ten days of incubation. Bacteria were usually be found disseminated to the spleen, liver, lungs, kidneys, and other sites which harbor cells of the reticuloendothelial system. The glandular symptoms were the result of infected lymphatics and present as the classic enlarged buboes and lymphadenopathy similar to bubonic plaque. The most mortality (30 to 60%) was seen with typhoidal and pulmonary tularemia, which, if left untreated, may result in shock and pneumonia, respectively. Pulmonary tularemia was usually due to the inhalation of infectious organisms via aerosolization (47).

Treatment of this disease can be successful, however, history has shown that early and the correct antibiotic regimen against tularemia was paramount. Streptomycin and gentamicin were effective against *Francisella* and were bactericidal antibiotics. Ciprofloxacin has been a favorite choice of treatment due to its efficacy in pediatric cases as well as against relapses of diseases when initial therapy was not successful. Doxycycline was another favorite due to its low cost. Bacteriostatic drugs against

*Francisella* include tetracycline and chloramphenicol, which require longer treatment regimens. It has been shown that *Francisella* was resistant to most beta-lactams and azithromycin via in vitro suspectibility tests (43).

**Description of *Francisella* subspecies**

There were five main subspecies of *Francisella tularensis*: *tularensis, mediasiatica, holarctica, novicida,* and *japonica*. *Francisella tularensis tularensis* has been classified as type A, has the highest virulence of the subspecies, and requires manipulation in a BSL-3 laboratory. *Francisella tularensis holarctica* has less virulence and was classified as a type B organism. One strain of *holarctica*, LVS (live vaccine strain), has been used as a vaccine in the past against tularemic infections, however, the identification of the attenuation of this strain has never been uncovered which prevents its licensure with the FDA. *Francisella tularensis novicida* has been shown to be less virulent to humans and presents a tularemia like illness in animal models. *Francisella novicida* has nearly identical 16S ribosomal DNA to *Francisella tularensis*, but lacks a second copy of the *Francisella* pathogenicity island (FPI) which was essential to intracellular growth in macrophages (33, 115). It has also been established that *novicida* has a functioning restriction system, making transformation experiments more difficult. These characteristics have made this strain a choice of many researchers hoping to uncover the many various virulence factors responsible for its pathogenesis. This organism has many ORFs that do not match with known proteins of other bacteria, and

were deemed unique. In general, the *Francisella* genome has a relatively low G+C content (34%) and a small length of ~ 1.8Mbp (16).

**Bacterial capsule as a virulence factor**

Little was known regarding the virulence factors responsible for *Francisella's* pathogenesis. No information has been found regarding the presence of secreted toxins, although evidence of genes for pili formation and type I (ATP-binding cassette proteins) secretion exist (192). It was also known that the lipopolysaccharide (LPS) for *Francisella* does not elicit the same type of immune response characteristic of gram-negative bacteria. It does not cause the induction of IL-1 nor the strong induction of TNFα from macrophages.

Many bacteria produce a protective barrier around themselves traditionally comprised of polysaccharides and attached to either the outer membrane or the peptidoglycan layer called a capsule. This protective barrier can have many different properties depending on the bacteria and the nature of the capsule. Usually the capsule was made of a polymer of a weakly immunogenic material which can be used to aid the bacteria in avoiding aspects of both the innate and adaptive immune responses. Some capsules were able to inactivate complement by disrupting the formation of the MAC complex on the bacterial surface. Others mask pathogen-associated molecular patterns that may be recognized by instruments of the adaptive immune response. Some capsules give their bacteria adhesive properties so that may cluster and inhibit antibiotics or other bactericidal materials. Still others provide an ionic shell which aid certain bacteria from

desiccation as well as act as a hydrophilic barrier which can also protect the bacteria during stringent conditions. It can also act as an ionphilic barrier for important cations needed by the bacteria cell for metabolism. Historically, it has been shown that with many bacteria, if they were stripped of their capsular material, that the virulence potential for that organism in *in vitro* infection experiments or animal models was greatly reduced. A classic example of this would be the smooth:rough *Streptococcus* experiments performed by Avery. The bacterial capsule of *Francisella* has been an object of contention amongst many researchers, many of which, in years past, questioned its existence. However, Dr. Hood's analysis of the capsular material recovered from the virulent *F. tularensis* Schu S4 strain showed evidence of mannose, rhamnose, galactose, and two forms of fatty acid (68). It has been said however, that these samples may have been contaminated with LPS, and that additional analysis was necessary. Dr. Cherwonogrodsky published a seminal paper in 1994 in which he demonstrated an increase in both encapsulation and virulence with repeated growth of *Francisella* in the synthetic Chamberlain's media (32). Although there was no isolation or analysis of capsular material, the capsule was visualized by transmission electron microscopy and the reduction in viability of test animals was demonstrated in infection models. In recent years, Drs. Apicella and Inzana have elucidated the capsule of *Francisella* using immunoelectron microscopy with antibodies specific for individual sugars which comprise the capsule. Its relevance and role in pathogeneticity was still questioned, however, as Dr. Golovliov has presented data showing the loss of capsular material upon

phagolysosome entry by the bacteria in macrophage infection and that the capsular material may be involved in the actual escape of bacteria from the phagolysosome (57).

Genetically, the genes responsible for biosynthesis of this polysaccharide capsule have been elucidated and were in proximity with the genes responsible for O-antigen synthesis. Mutants have been made of several of these genes (*manC*, *manB*, and *GGT*) and the loss of capsular material has been observed (unpublished material). There were, however, other genes present in *Francisella* which could potentially encode for a protein capsule.

**Characteristics of polyglutamic acid**

Many of the *Bacillus* species encode for a capsule which consists of poly-γ-glutamic acid (PGA), a nonribosomal polyamino polymer that can be covalently attached or secreted from the bacteria. PGA was found usually in gram positive bacteria and when attached, was anchored to the peptidoglycan layer. Attachment of PGA to this layer was dependent on the expression of *capD*, which encodes a γ-glutamyltranspeptidase, which was also thought to be integral in PGA degradation. A recent *capD* mutation in *Francisella* (referred to as *GGT*) was found to have low intracellular viability not due to effects on polymerization of PGA, but due to its importance in the breakdown of glutathione for cysteine acquistion (2). PGA can be formed entirely of D-glutamate, as was the case in *Bacillus anthracis*, or consist of both isomer forms, D+L. Polymers consisting solely of L-glutamate were the rarest form of PGA. Isomers of glutamate were possible via glutamate racemase, which exists in *Francisella*. The potential for

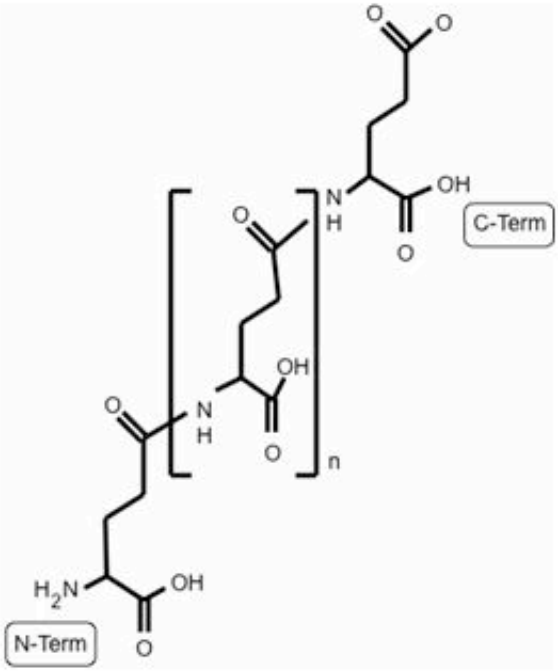
isomerization was important as polymers consisting of a singular form of glutamate will act as a thymus-independent type 2 antigen and will be incapable of inducing immunoglobulin class switching (183). It has been shown that PGA provides certain bacteria (ie. *Staphylococcus epidermidis*) salt tolerance and also gives *B. anthracis* antiphagocytic properties. PGA may also be a storage of both nitrogen and carbon in periods of starvation. PGA was polyanionic in nature and was highly water soluble, which aids in its hydrophilic properties (25, 63). Measurement of PGA from bacteria can be difficult as traditional protein assays such as Bradford and Lowery were not effective (1.25 mg/ml of L-glutamic acid solution measures as 0.42 μg/ml in the Bradford assay). PGA also does not stain with Coomassie blue in SDS-PAGE experiments (188). Polymer size usually varies due to the organism, but can measure from 160 kDa to 1500 kDa (> 1000 residues).

**Gene operon of polyglutamic acid**

In *B. anthracis*, this protein capsule operon was encoded by the plasmid OX2, which may have the propensity to transpose to other bacteria (12, 60). This operon consists of *capA, capB, capC, capD,* and *capE* genes (6). Some ambiguity still exists regarding whether *capE* was essential for polymerization to occur, as PGA has been created in reengineered tobacco plants with only the A, B, and C orthologs (169). The genes for *capA, capB,* and *capC* were located together in the *Francisella novicida* U112 genome at position 1272800, while it was believed that the analog for *capD* was located upstream at position 1229837. There were many lines of evidence that point to the

**Figure 1 A and B. The structure of polyglutamic acid, the organisms which produce PGA, and confirmation of known polymers.** A) PGA was made up as an anionic polymer of glutamic acid residues connected by gamma linkages, which were resistant to protease activity. These linkages provide PGA with specific properties. B) Many organisms were known to produce PGA and were capable of several different configurations of polymer, depending on the enantiomers of glutamate available.

**A**




**B**

| Organism | Conformation | Filament conformation | References |
|---|---|---|---|
| *Bacillus anthracis* | D | D | Hanby and Rydon (1946) |
| *Bacillus mesentericus* | D | D | Bruckner and Ivanovics (1937) |
| *Bacillus licheniformis* | D and L | D and L | Thorne and Leonard (1958) |
| *Bacillus megaterium* | D and L | D+L | Ashiuchi et al. (2003) |
| *Bacillus pumilus* | D and L | ND | Schneerson et al. (2003) |
| *Bacillus subtilis* | D and L | L and D+L | Tanaka et al. (1997) |
| *Planococcus halophilus* | D | D | Kandler et al. (1983) |
| *Sporosarcina halophila* | D | D | Kandler et al. (1983) |
| *Staphylococcus epidermidis* | D and L | ND | Kocianova et al. (2005) |
| *Natrialba aegyptiaca* | L | L | Hezayen et al. (2001) |
| *Hydra* | ND | ND | Weber et al. (1990) |

adapted from Candela T. and Fouet A. Molecular Microbiology 60: 1091-98

production of a PGA capsule by *Francisella*. First, several bacteria have been identified to contain the *cap* operon within their genome, with a few of these not being traditional gram-positive bacteria, such as *Leptospira interrogans* and *Fusobacterium nucleatum* (79). Second, *Francisella* has excellent homology (>50%) to most of these genes, and ablation of several of these genes has phenotypic effects (161). Third, it has been shown that gram-negative bacteria were capable of producing PGA. Dr. Ashiuchi reengineered *E. coli* bacteria with expression vectors to express the *cap* operon in an attempt to mass-produce PGA for industrial purposes. PGA has many uses such as a food preservative, a cryoprotectant, a cosmetic thickener, and as a biological adhesive. Dr. Ashiuchi found that the reengineered *E. coli* could produce PGA, however, at a much lesser yield (9). And finally, Dr. Su, in his transposon mutagenesis experiments of LVS, demonstrated a substantial decrease in virulence of the *capB* mutant in murine animal co-infection models. This data was supported by the generation of site-directed mutagenesis of this same gene to rule out the effects of potential transposon integration at other sites besides this gene of interest. Similar attenuation of virulence was seen (161).

The *capB* gene of this operon traditionally encodes an amide ligase, which was responsible for the gamma bond linkage between molecules of glutamic acid. This was the bonding of the gamma carboxyl group of glutamate to the amine group of the next glutamate. This linkage gives the polymer additional properties beyond the usual alpha peptide linkage. The gamma linkage was resistant to proteases unlike the alpha linkage. The polymerization of PGA requires ATP and the genetic sequence of *capB* has a consensus ATPase motif early in its sequence (GIRGKS). Unfortunately, the membrane

structure and organization of this enzyme and its protein-protein interactions have not been uncovered as isolation of this membrane protein complex was unstable. It was believed that CapB and CapC form a complex together due to the increase in ATPase activity and that these proteins were mainly involved in polymerization (25, 107).

**Mutagenesis of *Francisella***

Transposon mutagenesis of *Francisella* has been successful in elucidating many gene functions of unknown reading frames as well as genes required for intracellular growth, but for a long time, no confident allelic exchange system existed for site-directed mutagenesis of particular genes. Initial vectors designed for allelic exchange in *Francisella* lacked species-specific promoters driving various antibiotic cassettes. Similar transposon elements without *Francisella*-specific promoters also failed to be successful instruments of mutagenesis (54). The development of the pFNLTP series of plasmids by Dr. Tamara Maier opened the door for site-directed mutagenesis in *Francisella* (105). These series of plasmids were the result of integrating pTOPO with pFNL10, a native plasmid from *Francisella novicida* F6168 (120). This construct was electroporated in LVS and the resulting plasmids had deletions at the fusion point between these two original plasmids. However, these plasmids were functional shuttle vectors between *Francisella* and *E. coli* species and were relatively stable with repeated passage in these organisms. Dr. Karl Klose used these principles to design the pKEK887 plasmid which has a *Francisella novicida* FTN1451 promoter driving the erythromycin resistance gene (94). Unlike the pFNLTP series, however, this plasmid lacks the ability

to replicate in *Francisella*, making it an excellent candidate for use as a suicide vector in targeted insertion experiments for site-directed mutagenesis. The Klose plasmid also creates nonpolar mutations, depending on the selection of the flanking DNA around the desired target. Only certain antibiotic cassettes can be used in recombination experiments in *Francisella* due to the nature of this organism and as a member of the select agents of bioterrorism. The pKEK887 was used here to create a targeted insertion of the erythromycin cassette into the *capB* gene of *Francisella tularensis novicida* U112.

**Environmental factors involving encapsulation**

Many different environmental factors were known to induce the biosynthesis of encapsulation of bacteria. The PGA capsule of *Staphylococcus epidermidis* was induced by increases in NaCl concentration, and this capsule helps protect *S. epidermidis* from high salinity (79). In *Bacillus anthracis*, PGA encapsulation was induced with incubation of the bacteria in 5 to 10% carbon dioxide. The addition of sodium bicarbonate plus similar levels of carbon dioxide has been shown to increase PGA production in the reengineered *E. coli* (6, 9). In other *Bacillus* species, the presence of $Mg^{2+}$ or $Mn^{2+}$ increases capsule production. Various methods will be attempted to try to induce PGA capsule production in *Francisella novicida*.

**Research objectives**

The purpose of this dissertation project was to try to visualize the PGA capsule of *Francisella novicida* and to identify particular external stimuli that will facilitate its

biosynthesis. Its role in pathogenesis will also be investigated. My research has been separated into three specific aims:

**Isolate and characterize PGA mutants**

Site-directed mutagenesis of the *capB* gene in *Francisella novicida* was used to ablate the amide ligase activity of this gene's product. A suicide vector generated by PCR SOEing was introduced into *Francisella novicida* by electroporation. Loss of the transcript of *capB* will be confirmed by RT-PCR and characterization assays for the loss of encapsulation were performed.

**Demonstrate potential PGA production**

Immunoelectron micrographs using transmission electron microscopy was used with antibodies specific for PGA to try to visualize the PGA capsule of *Francisella novicida*. Extractions from the outer membrane were also be analyzed by mass spectrophotometry and amino acid analysis for presence of PGA.

**Determine environmental factors which may influence PGA production**

Various growth conditions were examined in order to determine which may promote PGA encapsulation of *Francisella novicida*. Outer membrane extractions from various growth conditions were blotted with the same antibodies used in the immunoelectron micrographs.

# CHAPTER TWO: MATERIALS AND METHODS

## Bacterial isolates, macrophage cell line, and cultivation conditions

*Francisella novicida* U112 was provided by Dr. Karl Klose from University of Texas, San Antonio. U112 was grown principally in three types of media: Trypticase Soy Broth with 1% cysteine hydrochloride (TSB-C), Chamberlain's defined media (CDM), or M9 minimal media plus 1% cysteine hydrochloride, all at 37°C. For molecular cloning, *E. coli* DH5α was used for all plasmid propagation and grown at 37°C in Luria Broth. *Klebsiella pneumoniae* was also grown at 37°C in Luria Broth. *Bacillus licheniformis* ATCC 9945 was provided by Dr. Thomas Kozel and was grown in PGA media at 30°C (177). Spectrophotometric measurements were made on a Bio-Rad SmartSpec Plus. RAW 264.7 murine macrophages were provided by Dr. Suzanne Barbour and were grown at 37°C with 5% $CO_2$ in DMEM with 10% heat-inactivated fetal bovine serum and 1mM nonessential amino acids (all media components from Gibco-BRL).

## Construction of suicide vector, pEF25

In order to create *Francisella* homologous DNA fragments needed for proper recombination with the U112 genome, genomic DNA was extracted from inocula of *Francisella novicida* U112 grown overnight in Trypticase Soy Broth with 1% cysteine hydrochloride using the Epicentre DNA extraction kit. Extracted genomic DNA (100 ng) was used as template in PCR reactions with primers listed in table 1. PCR was

performed using a MJ Research PTC-200 Thermocycler and using the Pfu polymerase (Stratagene). The erthyromycin cassette of pKEK887 was used as template for the antibiotic marker for this suicide vector. This vector has a *Francisella novicida* promoter driving the erthyromycin cassette, which was essential for expression of the cassette (94). Once all three PCR fragments were created and isolated from 1% agarose electrophoresis by the QIAGEN gel extraction kit, these DNAs were combined together into a single PCR SOEing reaction. The resulting fragment of ~3kb was subsequently restriction digested with SalI and ligated using NEB T4 ligase into pBlueScript SK+ plasmid using blue / white selection and selection for erthyromycin resistance at 100 µg/ml. Resultant colonies were initially verified for accuracy by MW via 1% agarose electrophoresis and additionally verified by QIAGEN plasmid prep DNA restriction with Sal I, liberating the DNA insert from the plasmid. One was designated pEF25.

**Electroporation of pEF25 into U112**

1 µg of pEF25 DNA resuspended in milliQ water was mixed together with 100 µl of thawed competent U112 in a 0.2 cm electroporation cuvette. These bacteria were made competent after overnight growth in TSB plus 1% cysteine to an $OD_{600}$ of 1.5, washed twice in 0.5M sucrose, and frozen to –80°C. The electroporation was performed as in Pavelka (97), at room temperature in a Bio-Rad Gene Pulser set at 2.5kV, 25 µF, and 600 ohms. The inoculum was then placed into 1ml of TSB plus 1% cysteine for a two hour recovery period in a shaking incubator at 37°C. The inoculum was then plated to TSB plates with 1% cysteine and 100 µg/ml erythromycin for two days at 37°C.

**RT-PCR / PCR for *capB* knockout confirmation**

RNA was isolated from wildtype and mutant bacteria using the RNAeasy QIAGEN extraction method and resuspended in DEPC-treated water. RNA was checked for quality by spectrophotometry and 260 / 280 ratio. 2 µg of RNA was used in an Invitrogen Thermoscript RT-PCR system to convert RNA to cDNA, utilizing the random hexamer protocol. Subsequent Taq PCR was performed using primers from table 1 under the following conditions: 1 cycle at 94°C for 2 min, followed by 30 cycles of 94°C for 30 seconds; 56°C for 30 seconds; 72°C for 1 min, 30 seconds; and an extension period at 72°C for 7 min. Presence of cDNA products was confirmed via 1% agarose gel electrophoresis.

**Growth curves and growth conditions**

Two hour timepoint measurements of $OD_{600}$ were made for logarithmic growth curves of bacterial cultures inoculated by normalized amounts from overnight cultures in TSB plus 1% cysteine or Chamberlain's defined medium (CDM). Various media were used including supplemented 0.5M, 1M, 1.5M, and 2M NaCl; 10mM, 25mM, 50mM, 100mM, 150mM, and 200mM $MgSO_4$; 50mM L-glutamic acid; and 10% carbon dioxide plus 0.5 to 1% $NaHCO_3$. Subtractive media were used as well, such as CDM without ferrous chloride; spermidine; and M9 minimal media plus 1% cysteine and without carbon sources. Congo Red was added to various agar media at a concentration of 100 µg/ml.

**TABLE 1**

| NAME | RS | PRIMER |
|---|---|---|
| UniUP | | TGCATTAGGAAGCAGCCCAGTAGT |
| UniDN | | TTCCTTTCGGGCTTTGTTAGCAGC |
| CapCF1 | Sal I | GATGCTGTCGACCCCATTGTCTTACATAGATAGC |
| U112CapR1 | | ACTACTGGGCTGCTTCCTAATGCAGGAGTTGGTA ATATTAAAGATATTGG |
| U112CapF2 | | GCTGCTAACAAAGCCCGAAAGGAACTACTGTTCT ATATCCTCCAGC |
| U112CapR2 | Sal I | GATGCTGTCGACCAATTGCTTTATCGGCTTATGC |
| CapB2F | | GCACAAAGCTTATCCCAAAGC |
| CapB2R | | GCAGCTACTGTCCCTGTTGGAG |
| SpoT2F | | TAACCATGCCGGATTAGGATCAGCA |
| SpoT2R | | CAGTTGGAAACTCTTGCGTTTGAG |
| PolA2F | | TGGAGGAAGTCCTAGCTTCTCGAA |
| PolA2R | | CAGCTTGATGATCTGATTGCGGA |
| CapC1F | | ATCCAGGTACAACCATACCAC |
| CapC1R | | TGGATCCGTTAACGCTCTCGA |
| | | |
| | | **PLASMIDS** |
| pFNL10 | | native plasmid from *F. novicida* F6168 |
| pFNLTP | | *F. tularensis* / *E. coli* shuttle plasmids derived from integration of pTOPO and pFNL10 |
| pFT7T | | pFNLTP7 shuttle plasmid with oriT addition |
| pKEK887 | | pET15b based plasmid with FTN_1451 promoter for *Erm* cassette |
| pEF25 | | pBluescript SK + plus flanking *capB* U112 genomic sequences and *Erm* cassette |

**RS = restriction sites**

**Immunoelectron microscopy**

Immunoelectron micrographs were created similar to the Sebastian protocol (150). 200-mesh Formvar carbon-coated copper grids were dropped onto 8 µl drops of bacteria for 1 min. The grids were then blocked in 0.5% fishskin gelatin in PBS with 0.1% Tween 20 for 5 min. Primary antibody diluted to 1:20 in PBS was placed onto these grids for a 20 min incubation, then washed for 1 min in 0.1 x PBS-Tween. Gold colloidal beads bound to Protein A were used as a secondary antibody diluted to 1:10 for another 20 min incubation. The grids were then washed in milliQ water twice for 1 min each. Grids were then dried overnight and visualized by a JEOL JEM-1230 TEM (70). Primary antibodies included a F26G3 monoclonal from Dr. Thomas Kozel (80) and a polyclonal from Dr. Rachel Schneerson (148), both against polyglutamic acid. Preimmune sera were used as a control.

**Sodium deoxycholate and serum sensitivity assays**

Both of these assays were modeled from assays performed by Cowley (39). Overnight cultures of bacteria were centrifuged down and washed twice in PBS. These bacteria were then diluted to $10^5$ CFU / ml and 100 µl were plated in duplicate into a 96-well culture plate. 100 µl of either sodium deoxycholate at concentrations ranging from 0.005% to 5% or preimmune sera at concentrations from 1% to 30% were incubated with the aliquoted bacteria for 45 min and 3 hours, respectively. These bacteria were then serially diluted and plated to TSB-C plates for incubation at 37°C. CFUs were counted and compared to controls.

**In vitro murine macrophage infection model**

Our in vitro infection model using RAW 264.7 murine macrophages was similar to the invasion protocol used by Tempel (170). 4 x $10^5$ RAW 264.7 macrophages were plated to a 24 well plate format and incubated overnight at 37°C in 5% $CO_2$. The next day, enough bacteria (determined by $OD_{600}$) were grown, washed several times and resuspended in PBS, so that infections of multiplicity of infections of 10 and 100 bacteria per cell were added to respective tissue culture wells for infection for 1 hour. DMEM culture media was then removed from each well and the macrophages were treated with DMEM plus 25 μg/ml of gentamicin. In the two hour experiment, wells were rinsed with PBS three times and then given DMEM plus 10 μg/ml of gentamicin for one hour. The wells were then washed three times in PBS before lysis with 1 ml of milliQ water and plating to TSB-C plates. In the 24-hour experiment, the 10 μg/ml gentamicin treatment was maintained overnight and lysis by water treatment occurs the next day.

**Outer membrane PGA extraction protocol**

A sodium acetate / glacial acetic acid extraction procedure was used on *Bacillus* species to isolate PGA (80). 10% sodium acetate and 1% glacial acetic acid was added to bacterial culture and shaken for at least two hours. This bacterial mixture was then centrifuged at 14K and passed through a .45 μm filter to separate the bacterial cells. The supernatant was mixed with two volumes of ethanol to precipitate the PGA overnight at 4°C. The ethanol mixture was centrifuged at 12K for 30 min at 4°C and the pellet was resolubilized in a 10% sodium acetate and 1% glacial acetic acid solution and then

reprecipitated in two volumes of ethanol overnight at 4°C. The resulting pellet was then dried for 10-15 min and resuspended in 1ml milliQ water.

**Anthrone carbohydrate and BSA protein assays**

The anthrone assay was a spectrophotometric tool to measure the monosaccharide concentration of a solution. The assay involves the hydrolysis of oligosaccharides and subsequent dehydration of monosaccharides (usually 400 µl of an organic solution) by 1 ml of sulfuric acid. This was mixed with 100 µl of 2% anthrone in ethyl acetate, which reacts to form a furfural compound. These solutions were then read at $A_{260}$nm after a 10 min incubation. Standards were made of increasing concentrations of glucose (84).

The BSA assay was based on the Bradford assay, which was used to measure solubilized protein. 200 µl of an acidic solution of Coomassie Brillant Blue G-250 dye was mixed with 800 µl of either the protein standard or sample and incubated at room temperature for at least 5 min. Similar spectrophotometric readings were made at $A_{595}$nm. Both of these assays were performed in duplicate.

**Dot blot protocol**

Three µl of extract were dropped onto strips of nitrocellulose and dried overnight. Strips were then blocked for at least two hours in 3% BSA in TEN buffer (25mM Tris-10mM EDTA-200mM $NaHPO_4$, pH7.6). Primary antibody was added to a final concentration of 50 µg/ml and was slowly shaken for two hours. The primary solution was removed and the strips were washed three times in wash buffer (Tris-buffered saline

or TBS) for 10 min each. Alkaline phosphatase-conjugated secondary antibody at a dilution of 1:10,000 was added and shaken for one hour. The strips were washed again three times in wash buffer (TBS) and were then incubated in Pierce 1-Step™ NBP/BCIT solution from Pierce for 30 min. The strips were dried overnight before interpretation and analysis.

**Desiccation protection model**

These experiments were modeled after similar experiments performed on *Azotobacter vinelandii* by Campos (23). *Francisella novicida* bacteria was grown overnight, washed and resuspended in M9 minimal media plus 1% cysteine without carbon sources. Cell density was then measured by $OD_{600}$, and normalized amounts were then aliquoted to separate microfuge tubes, left open, and stored at 30°C in a dry incubator. These samples were then resuspended each day in the same minimal media, diluted, and then plated to TSB-C plates for CFU counts. Experiments proceeded for three to four days of desiccation.

**Acid resistance assays**

These assays were modified from assays performed by Foster (52, 133). Overnight cultures were subjected to lower pH during stationary phase by the addition of drops of HCl. The pH of these cultures were measured by using pH strips and then incubated for two and four hours, shaking at 37°C. These bacteria were then diluted at

these timepoints and plated to TSB-C plates for CFU counting. Survival data were extrapolated from CFU of the wildtype bacteria compared to the mutant bacteria.

**Glutamate decarboxylase assay**

1.8 ml of overnight cultures were centrifuged at 12K for 3 min and the pellet washed and resuspended in 1ml PBS. The bacteria were pelleted again and vortexed vigorously. 500 µl of GAD reagent, defined as 1 g of L-glutamic acid, 0.05 g of bromocresol green, 90 g of NaCl, and 3 ml of Triton X-100 per liter, was added and vortexed. The solution was incubated at 37°C for four hours, checking for color change every hour. Assays using GAD reagent with Triton X-100 measures internal glutamate decarboxylation, thus the α-decarboxylation of glutamate to γ-aminobutyric acid (GABA) and carbon dioxide, a reduction reaction consuming an intercellular proton. Assays without Triton X-100 were a measure of the ability of the glutamate-GABA antiporter to function, thereby neutralizing the surrounding media (28, 130).

**Electrospray ionization mass spectrophotometry**

Dr. Connell Cunningham from Rohm and Haas, a subsidiary of Dow Chemical, performed ESI MS without chromatography on three PGA extraction samples: one PGA standard from Dr. Kozel of *B. licheniformis*, and two PGA extracts from U112 and *capB* mutant, respectively, grown at 1M NaCl in Chamberlain's defined media. Data forwarded was from mass/charge ratios from 100 to over 1000.

**Amino acid analysis**

Amino acid analysis was performed by the Amino Acid Laboratory at the University of California at Davis, Davis, CA. Bacterial extracts or samples were mixed 1:1 with 6% sulfosalicylic acid, which acts to deproteinize the sample, then centrifuged at 14K and filtered through a .45μm PTFE filter, adjusted to pH 2.2, and then loaded into a Biochrom 30 amino acid analyzer for free amino acid analysis. To examine the total amino acid profiles, the pellet and supernatant samples were dried slightly in a SpeedVac, then transferred to 10ml ampules for hydrolyzation. Two mls of 6N HCl (containing 200 nmol/ml norleucine as an internal standard) was added, then purged with nitrogen gas for 5 min, and sealed using an open flame. The ampules were then heated at 110°C for 24 hours, cooled, and then purged again with nitrogen gas to dry. 10 ml of 0.2M lithium citrate loading buffer was used to dissolve the samples, filtered through a 0.2 μm filter, and loaded into the Biochrom 30 Amino acid analyzer.

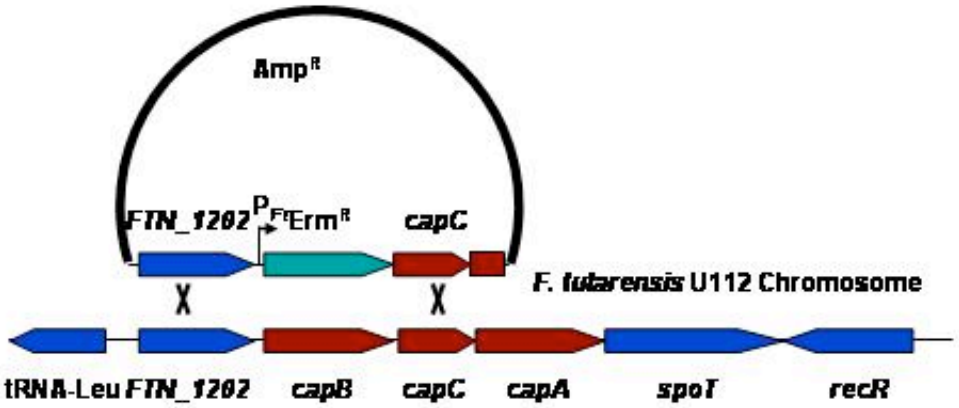
CHAPTER THREE: RESULTS OF EXPERIMENTS

**Creation of the *capB* mutant in *F. novicida* U112**

The flanking DNA sequences of *capB* used as the U112 homologous DNA sequences for the suicide vector were approximately 980bp and 1050bp, respectively and were PCR amplified from U112 genomic DNA using the primers CapCF1, U112 cap R1 and U112 cap F2, U112 cap R2, respectively. The erythromycin cassette from pKEK898 (approximately 1550bp) was amplified from a dilution of this plasmid using the universal primers UniUP and UniDN as described by Liu et al (94). All three PCR fragments were amplified using the following Pfu PCR conditions: 1 cycle at 95°C for 10 min, followed by 30 cycles of 95°C for 60 seconds; 55°C for 80 seconds; 72°C for 3 min; and an extension period at 72°C for 10 min. These fragments were then electrophoresed using 1% agarose at 95V and gel purified using the QIAGEN gel extraction kit, resuspending in 20 ul TE. All three fragments were placed together as template in a single Pfu PCR SOEing reaction using the outside primers CapCF1 and U112 cap R2. The universal sequences from the erythromycin cassette were embedded into the primers U112 cap R1 and U112 cap F2, which acted as complimentary sequences for the PCR SOEing. At least 25ng of each PCR fragment was needed for the PCR SOEing reaction, using the following parameters: 1 cycle at 95°C for 5 min, followed by 30 cycles of 95°C for 60 seconds; 55°C for 40 seconds; 72°C for 6 min, 30 seconds; and an extension period at 72°C for 10 min. The resulting fragment was approximately 3.2kb and once gel purified as stated before, it was used as template in a subsequent PCR reaction with the same

parameters as the PCR SOEing reaction. This amplified product was then gel purified and 500 ng of this product was used in a 25 µl Sal I restriction digest to cleave off the ends of the primer sequences. At the same time, pBluescript SK+ was propagated in a DH5α overnight culture and plasmid DNA was acquired using the QIAGEN miniprep kit. This plasmid was capable of blue / white selection of recombinant DNA colonies due its genotype. Approximately 5 µg of plasmid DNA was used in a 50 µl Sal I restriction digest and after 1% agarose electrophoresis, was gel purified and resuspended in 50 µl TE. These two Sal I digested DNAs were mixed together in a 20 µl NEB T4 ligation reaction using appropriate controls to determine plasmid religation efficiency and amount of uncut plasmid in our plasmid restriction digest. Approximately 8 µl of these ligation reactions were used in 100 µl *E. coli* transformation reactions, using DH5α made competent after $CaCl_2$ washes. The bacteria-DNA mixtures were heat shocked for 40 seconds at 42°C and then incubated at 37°C in rich SOC media for 40 minutes before plating aliquots to LB agar Ampicillin plates at 100 µg/ml and with both X-gal (80 µg/ml) and IPTG (20mM). After several attempts using increased amounts of insert DNA, two to three white colonies were inoculated in 2ml of LB Amp100 broth and checked the next day via QIAGEN miniprep and 1% agarose electrophoresis. Two clones had MW of approximately 6.2 kb. These clones were confirmed as correct recombinants by Sal I restriction of plasmid DNA and 1% AGE, resulting in excision of the DNA insert from the plasmid DNA. They were also confirmed by PCR of the diluted plasmid DNA using the Liu et al. (94). universal primers to amplify the erthyromycin

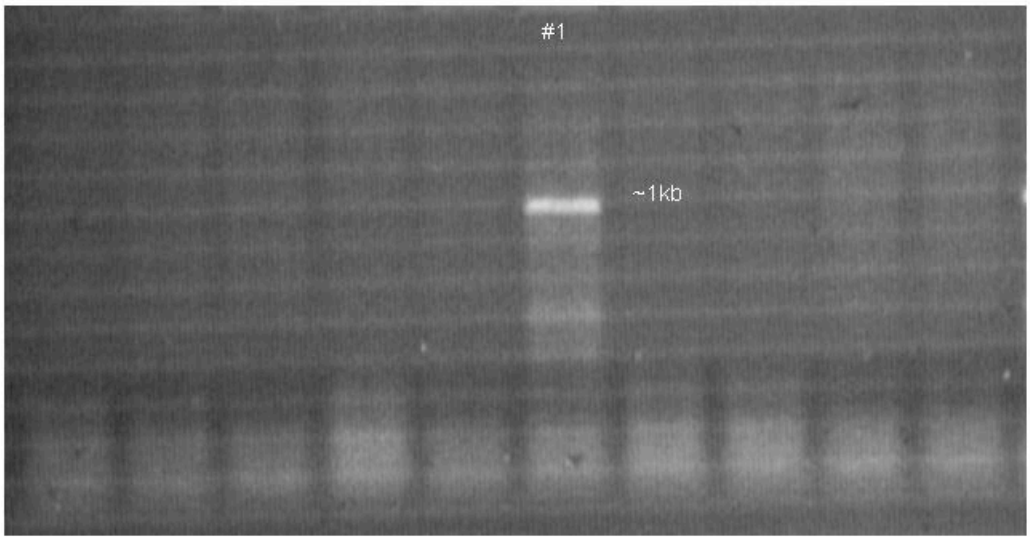
**Figure 2. Method for targeted mutagenesis of the Fn *capB* gene, which was predicted to encode an amide ligase.** PCR SOEing was used to construct a nonpolar *ΔcapB*::Erm allele, which was cloned into pBS SK+ and transformed into *F. novicida* U112 with selection for erythromycin-resistant (100 mg/ml) recombinants according to Liu et al. (94). A double recombination event was necessary for the creation of the *capB* mutant, which was introduced into *F. novicida* U112 by electroporation.



cassette. The bacteria were inoculated in 4 ml of LB Amp100 broth for plasmid DNA from a miniprep, resuspending in 50 µl of milliQ water instead of TE. 1 µg of pEF25 was electroporated in *F. novicida* U112 made competent by washes in 0.5M sucrose. As a positive control for electroporation, pFT7T, a derivative of the Maier shuttle plasmid pFLNTP7 (105), was used. This plasmid has a kanamycin resistance cassette and was known to create kanamycin resistant *Francisella* at 15 µg/ml. TSB-C plates with 100 µg/ml of erthyromycin were used to isolate recombinant *Francisella* having a double recombination event with pEF25 (Fig. 2). Several methods were used to ensure that the picked, erthyromycin resistant *Francisella* colonies were targeted insertions into our gene of interest.

First, approximately 23 *Francisella* colonies grew on the experimental electroporation plates. These colonies were patched to another TSB-C plus Erm 100 µg/ml plate as well as used to inoculate 2 ml of fresh TSB-C media at 37°C overnight. These cultures were then centrifuged, washed with PBS twice, and resuspended in 200 µl of PBS. 2 µl of these cultures were used as template in a Taq PCR reaction using the Liu et al. (94) universal primers (most culture media contains ingredients which may inhibit PCR reactions) to amplify the embedded erthyromycin cassette. Genomic U112 DNA was used as a negative control and a dilution of pKEK887 was used as a positive PCR control. The parameters of the PCR reaction were as follows: 1 cycle at 95°C for 7 min, followed by 30 cycles of 95°C for 30 seconds; 55°C for 30 seconds; 72°C for 1 min, 30 seconds; and an extension period at 72°C for 7 min. Upon 1% AGE (Fig. 3), two of the

**Figure 3. Screening of the potential recombinant clones via PCR of the erythromycin cassette.** I initially screened our Erm resistant *Francisella* colonies by performing PCR of the erythromycin cassette on bacterial inocula using the Uni primers shown in table 1. This PCR product was about 1.1 kb in length. This product demonstrated the existence of the Erm cassette in the genome of our potential mutant. Our transformation efficiency was approximately 10%.



23 colonies presented a ~1.5kb band which ran at the same MW as the positive control. These colonies were termed EF98 and one was selected for the remaining experiments.

Second, genomic DNA was isolated from *capB* mutant EF98 using the Epicentre DNA purification kit and this DNA was used, along with genomic DNA from U112, as template in another Taq PCR reaction using primers CapCF1 and U112 Cap R2, with the following parameters: 1 cycle at 95°C for 7 min, followed by 30 cycles of 95°C for 30 seconds; 60°C for 30 seconds; 72°C for 3 min, 15 seconds; and an extension period at 72°C for 7 min. When compared by 1% AGE, the PCR product for EF98 had a slightly higher MW than the PCR product from U112 (data not shown). The difference in MW was due to the incorporation of the larger erthyromycin cassette into the genome of our recombinant. Additionally, these PCR products were subjected to Bgl II restriction digests and were run on 1% AGE (Fig. 4). The FTN1451 promoter of the erthyromycin cassette has a Bgl II site, which was lacking in the U112 sequence (94). Two bands at 1.1 and 1.8 kb were seen for *capB* mutant EF98, while the wildtype U112 PCR product was uncut. This demonstrates the incorporation of this antibiotic cassette into our gene of interest.

Third, I wanted to determine whether the transcript for *capB* was lost in our recombinant clone. After overnight cultures were made, RNA was procured from *capB* mutant EF98 and U112 using the QIAGEN RNAEasy kit. The 260 / 280 ratio for EF98 and U112 was 1.825 and 1.7713, respectively. Both RNAs were measured to be approximately 2 µg/µl and 1 µl of each was used to create cDNA utilizing the Invitrogen RT-PCR System with the random hexamer primer protocol. A reaction for each was run

**Figure 4. Bgl II restriction of *capB* PCR product.** Our *capB* mutant EF98 was confirmed via PCR using our outermost primer set and restricting with Bgl II. The only Bgl II site present should be the site located inside the promoter sequence of the erythromycin cassette (94). This restriction provides evidence that our antibiotic cassette incorporated itself at the *capB* locus. The product from wildtype bacteria remains uncut at ~3kb.

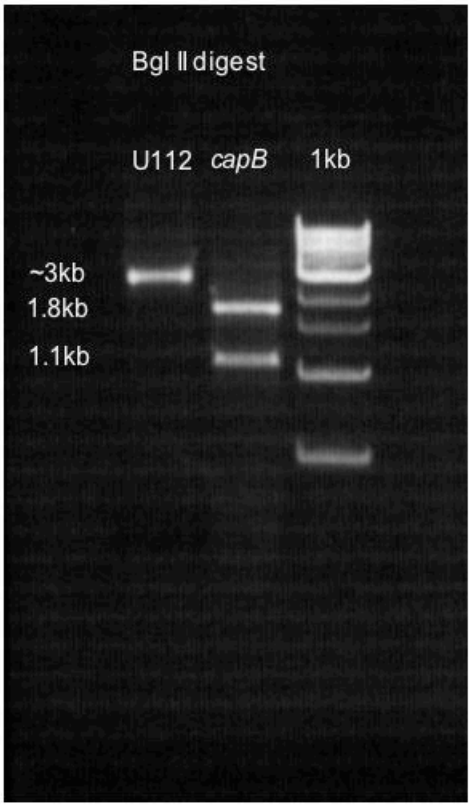
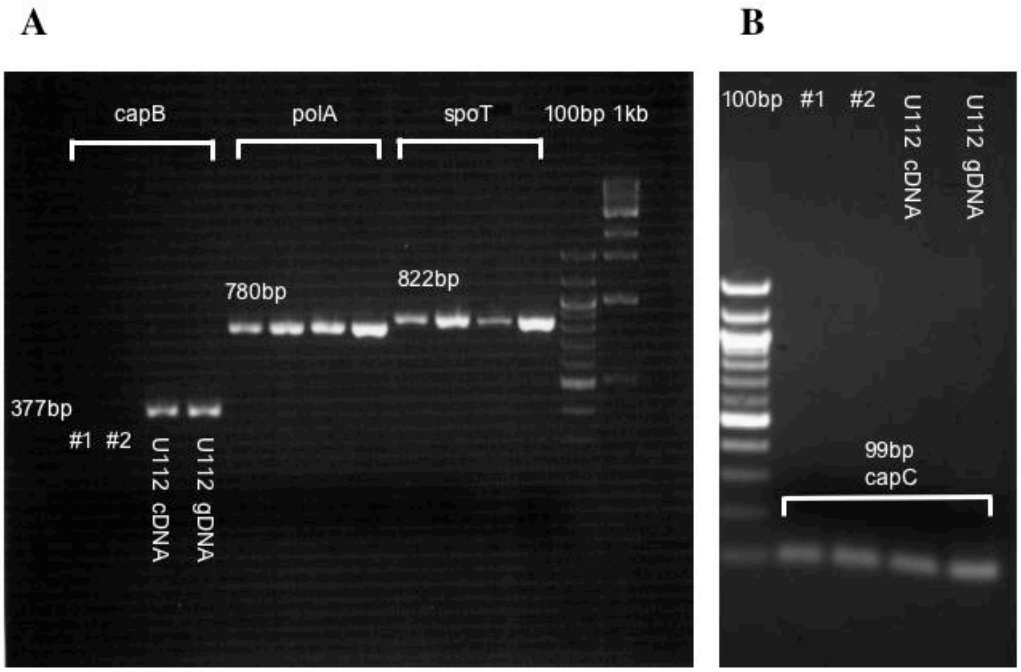


**Figure 5 A and B. RT-PCR for transcriptional analyses.** A) Loss of expression of the *capB* transcript was shown by electrophoresis after RT-PCR using random hexamer primers and subsequent PCR with gene specific primers (table 1). The two clones of EF98 do not amplify the 377bp PCR fragment for *capB*, but do amplify transcripts for the genes *polA* and *spoT*. A genomic DNA amplification was also shown as a positive control. B) RT-PCR and subsequent PCR was also performed on the immediately downstream transcript of *capC*, presenting our site-directed mutagenesis of *capB* as nonpolar, thus attributing any changes in phenotype to the loss of *capB* gene function. Negative RT controls demonstrated no DNA contamination of our RNA preparations.

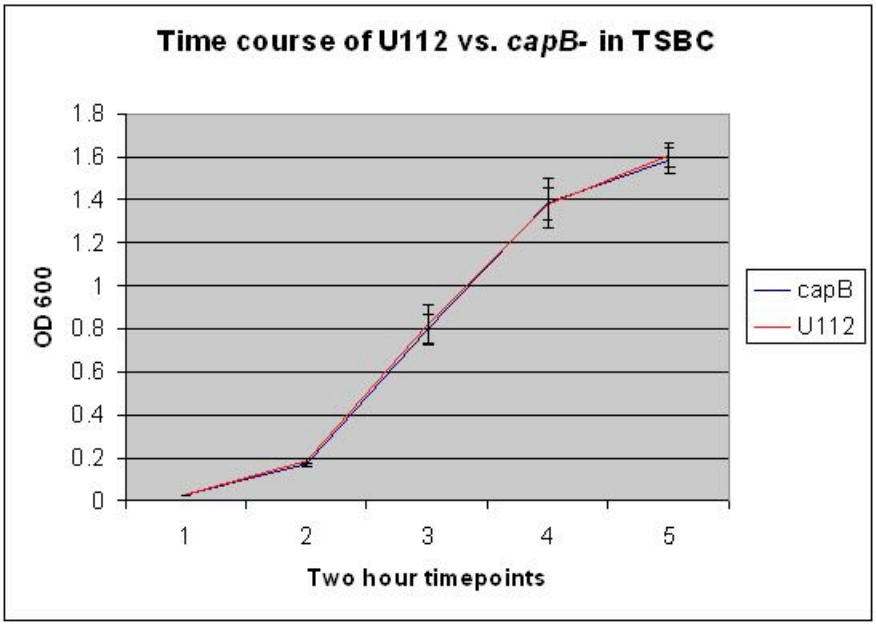


without RT as negative control for cDNA (data not shown). Specific primers for various transcripts were used in the subsequent Taq PCR reaction, including *capB, polA, spoT*, and *capC* (see table 1). The reaction parameters were as follows: 1 cycle at 94°C for 2 min, followed by 29 cycles of 94°C for 30 seconds; 56°C for 30 seconds; 72°C for 1 min, 30 seconds; and an extension period at 72C for 7 min. The original RNAs were run as template as controls for potential DNA contamination. These samples were analyzed by 1.5% AGE at 95V (Fig. 5). The 822bp and 780bp PCR products for *spoT* and *polA* were seen for both cDNAs of EF98 and U112. However, the 377bp PCR product for *capB* was not seen for EF98 cDNA, but was present for U112 cDNA. Just as important, both cDNAs amplified the downstream 99bp *capC* PCR product, demonstrating that the targeted disruption of *capB* was nonpolar and did not affect the transcripts downstream. No amplification was seen with the RNA samples (data not shown).

**Initial phenotypic characterization of *capB* mutant**

Initial investigation into differences between *capB* mutant and U112 demonstrated no differences. Both bacteria grew similarly in two hour timepoints growth experiments to stationary phase in both TSB-C (Fig. 6) and CDM. Final overnight $OD_{600}$ for TSB-C and CDM averaged 2.1 and 1.8 for both bacteria, respectively. Colony morphology on Cysteine Heart Agar plus defibrinated blood and 1% IsoVitalex, TSB-C, and CDM plates was the same as well, presenting as a smooth, small, opaque colony with no change to its edges. Several experiments were performed to determine if changes to the outer membrane by our mutant could be ascertained. Congo Red was added to 100

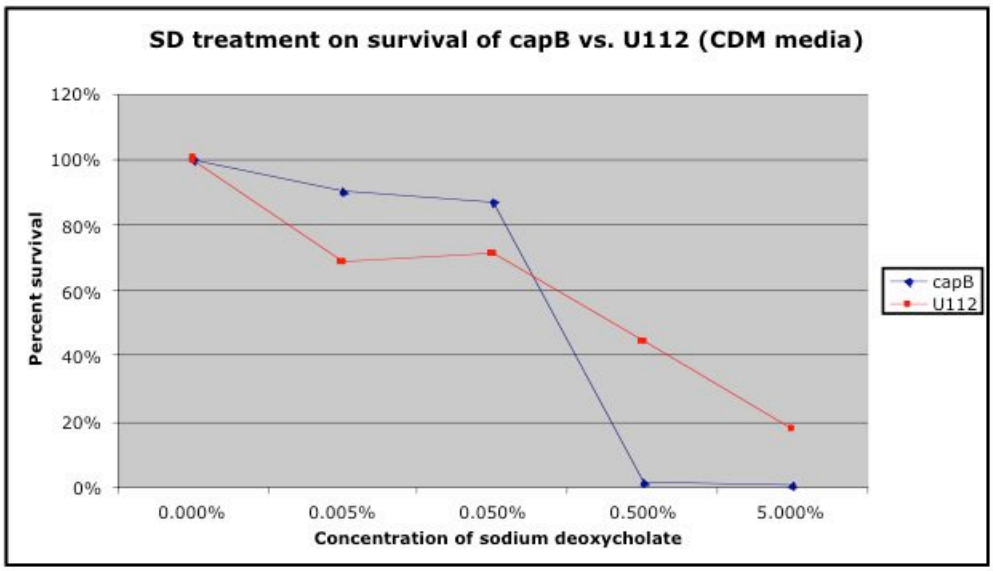
**Figure 6. Time course experiment of U112 vs. *capB* mutant EF98 in TSB-C.** There was no growth disadvantage for the *capB* mutant when compared to the wildtype in TSB-C. Similar data were obtained for growth in CDM (data not shown). Overnight cultures were also similar in final $OD_{600}$.



μg/ml to TSB-C plates and both bacteria were plated this agar. This dye binds to lipoproteins on the surface of bacteria and turns bacteria various shades of red depending on the amount of dye incorporated. This dye has been used to characterize many O-antigen mutants in gram-negative bacteria, including *Francisella holarctica* LVS, in which dye was able to bind to lipoproteins normally protected by the polysaccharides of the O-antigen. LVS colonies that normally appear pink in color contrasted with the dark red color seen in galactosyl transferase mutants or *wbtA* mutants. If a PGA capsule were providing any protection to lipoproteins on the surface, there may be a color differential seen on these plates (91, 128). However, no change in color was seen between the mutant and wildtype U112 (data not shown).

Variable results have been seen in experiments on *F. novicida* O-antigen mutants and their sensitivity to sodium deoxycholate, a water-soluble ionic detergent commonly used in cell lysis, liposome preparation, and isolation of membrane proteins and lipids. Cowley et al. (39) has stated that the activity of hydrophobic antibiotics and certain detergents against the cell membrane of gram-negative bacteria was largely influenced by the association between LPS and other molecules of the outer membrane. If the equilibrium of divalent cations was upset by the loss of largely negative components of the outer membrane, increased susceptibility to these type of detergents may result. Cowley et al. (39) has shown that an *wbtA* mutant has a increased sensitivity to increasing concentrations of sodium deoxycholate. However, Li et al. (94) has reported an increase in resistance of an LVS galactosyl transferase mutant to sodium deoxycholate. Raynaud

**Figure 7. Treatment of bacteria with variable concentrations of sodium deoxycholate.** Equal numbers of bacteria were subjected to treatment with 0 to 5% sodium deoxycholate (an ionic detergent) for 45 min to disrupt the cell envelope. At 0.5%, the *capB* mutant was highly sensitive compared to wild-type. Sodium deoxycholate was a water soluble ionic detergent/bile salt commonly used in applications ranging from cell lysis (RIPA Buffer), liposome preparation, and isolation of membrane proteins and lipids. Changes to outer membrane architecture could cause increased sensitivity to sodium deoxycholate.



et al. (128) has reported no difference in sodium deoxycholate sensitivity between a *wbtA* mutant and wildtype LVS (39, 91, 128).

I performed similar experiments with our *capB* mutant to determine whether a difference in sodium deoxycholate sensitivity could be seen. At concentrations less than 0.05%, no statistical difference could be seen between the *capB* mutant and wildtype U112 (Fig. 7). However, at 0.5% and 5%, a statisticaldifference was seen in viability between these two bacteria. At 0.5% sodium deoxycholate, up to 44% of the wildtype U112 bacteria survived treatment while only 1% of the *capB* mutant bacteria survived ($p=.0002$). At 5% sodium deoxycholate, 18% of wildtype survived compared to 1% of the *capB* mutant ($p<.0001$). These results suggest an ionic difference at the outer membrane of the *capB* mutant, which may be the loss of a polyanionic barrier helping to stabilize the integrity of the outer membrane.

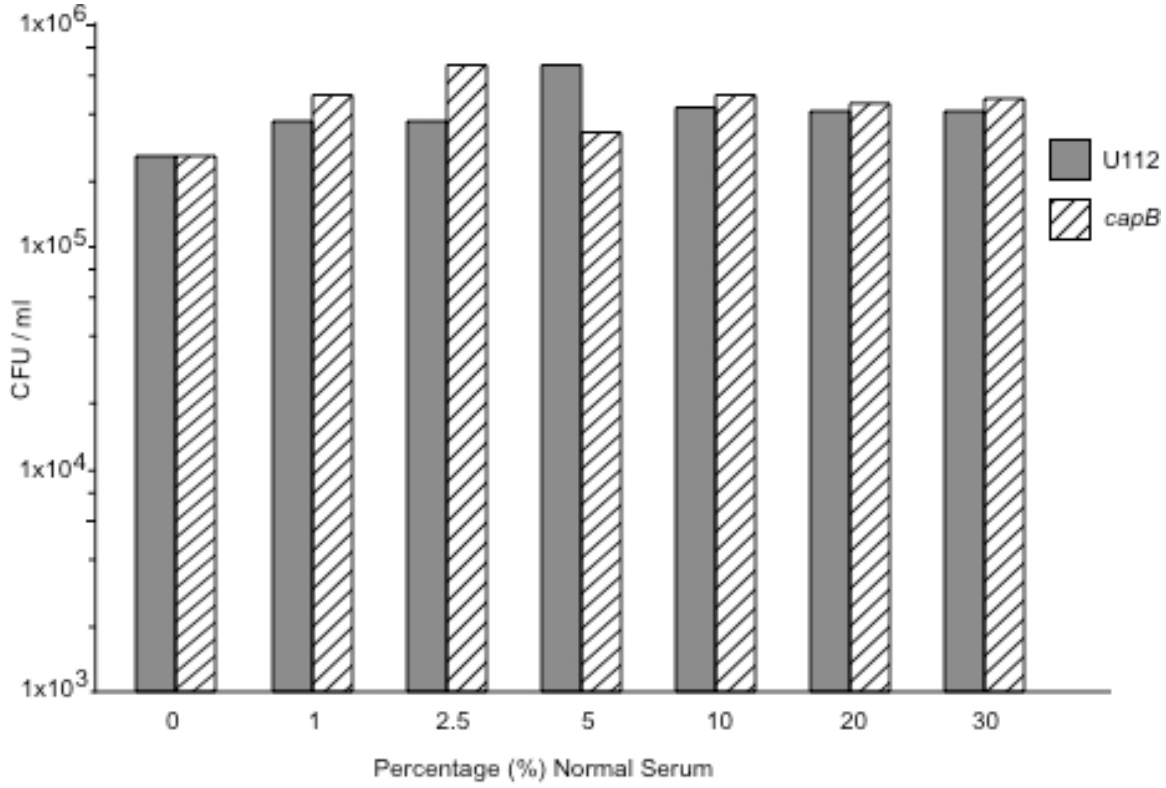
**Serum sensitivity assays and in vitro macrophage infection studies**

Another set of experiments performed to determine changes made to the outer membrane include preimmune serum sensitivity experiments. Preimmune serum has bactericidal properties due to the complement proteins it contains, which ultimately leads to formation of the MAC complex and cell lysis. The formation and deposition of complement proteins has been shown to occur more readily in the absence of O-antigen chains, which cover the lipid A determinants recognized by complement. This phenomenon involves both the classic and alternative complement pathways (158). Sandstrom et al. (140) described a capsule-deficient *Francisella* LVS and its

susceptibility to serum treatment. This killing by serum was ablated by heat-inactivation of the serum, demonstrating that complement was directly involved. These mutants, however, were created via chemical mutagenesis (acridine orange), were not genetically verified, and were termed capsule mutants due to their rough morphology (140). It is likely that these capsule mutants were actually LPS mutants, due to the familiar phenotype seen in LPS mutant serum experiments. It was believed that in the case of several PGA producing bacteria that loss of encapsulation may allow increased deposition of complement proteins as well. To test whether our mutant would have limited viability in preimmune serum, I incubated normalized amounts of bacteria, after being washed in PBS, with increasing amounts of preimmune rabbit serum, to final concentrations of 1, 2.5, 5, 10, 20, and 30%. After treating cells for three hours in a 96-well format in duplicate, 100 µl aliquots were diluted in PBS and plated to TSB-C plates for measurement of CFUs. Mean counts for CFUs for these experiments ranged from 16.5 to 63, and although there were experiments at median serum concentrations which demonstrated a statistical difference between U112 and *capB*, our counts for *capB* were lower than wildtype and there was no significant decrease in CFU as serum concentrations increased (Fig. 8). One would expect reduced viability of both bacteria with increased serum, yet this was not seen.

Several characterizations of *capB* mutants in *Francisella* have shown a decrease in virulence in animal infection models, which has been attributed to attenuated pathogenesis due to reduced bacterial burden in macrophages and other target cells and reduced dissemination. Su et al. (161) presented a substantial reduction in virulence of

**Figure 8. No statistical significant difference between bacteria treated with preimmune serum.** Our studies seem to indicate that our *capB* do not fare as well as their wildtype counterpart against blocking the effects of complement. However, no statistical significant difference was seen at any concentration of serum, as increases in serum treatment did not demonstrate reduction in number of CFUs.



both transposon and direct mutagenesis of *capB* compared to wildtype in intranasal LVS-infected BALB/c mice. In these co-infection experiments, only the wildtype bacteria were isolated from the organs of infected mice in these 1:1 ratio infections. The attenuation was corrected via complementation with the *capB* gene. Singular murine infection experiments with the mutant only were performed as well, compared to mice infected only with LVS. These mutant infections presented a 1,000 fold reduction in bacterial burden of the lungs (161). A negative selection screen by Weiss et al. (186) of *F. novicida* transposon mutants also elucidated *capB* as encoding a potential virulence factor for *Francisella*. Genomic DNA from the transposon input bacteria was collected as well as DNA collected from the bacteria in the spleens after 48 hours of intraperitoneal infection. These DNAs were used to probe a *Francisella* microarray, which demonstrated an absence of the *capB* mutant bacteria DNA in the infected spleens (186). Several other discovered infection-attenuated mutants were subjected to in vitro macrophage infection models, in which bone-marrow derived macrophages were used. Two groups of mutants were determined, one having reduced intracellular growth capability and the other which replicated just as the wildtype. The *capB* mutant was not one of the bacteria selected for these experiments.

I infected RAW 264.7 murine macrophages in a 24-well format with both our U112 wildtype bacteria and the *capB* mutant. $4 \times 10^5$ macrophages were plated per well and enough bacteria to provide both a multiplicity of infection (MOI) of 10 and 100 were used. The bacteria were added for an infection period of one hour, then were either incubated for an additional hour or 24 hours. Extracellular bacteria were then eliminated

**Figure 9. Macrophage infection model (2hr) demonstrates no potential entry differences between U112 and *capB*.** Murine macrophage cell line RAW 264.7 were plated and infected with *F. novicida* for 1 hr, after which the cells were washed in gentamicin (25 mg/ml) for an additional hour, washed with PBS, then lysed with milliQ water before plating onto TSB-C plates. At both MOIs of infection, there were no significant differences in the number of the *capB* bacteria compared to WT.

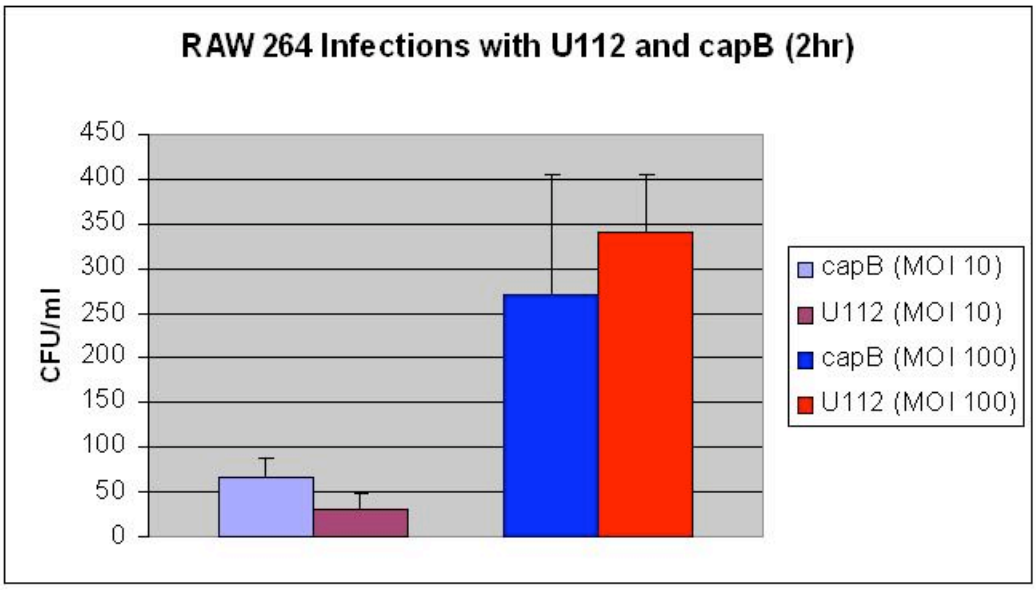
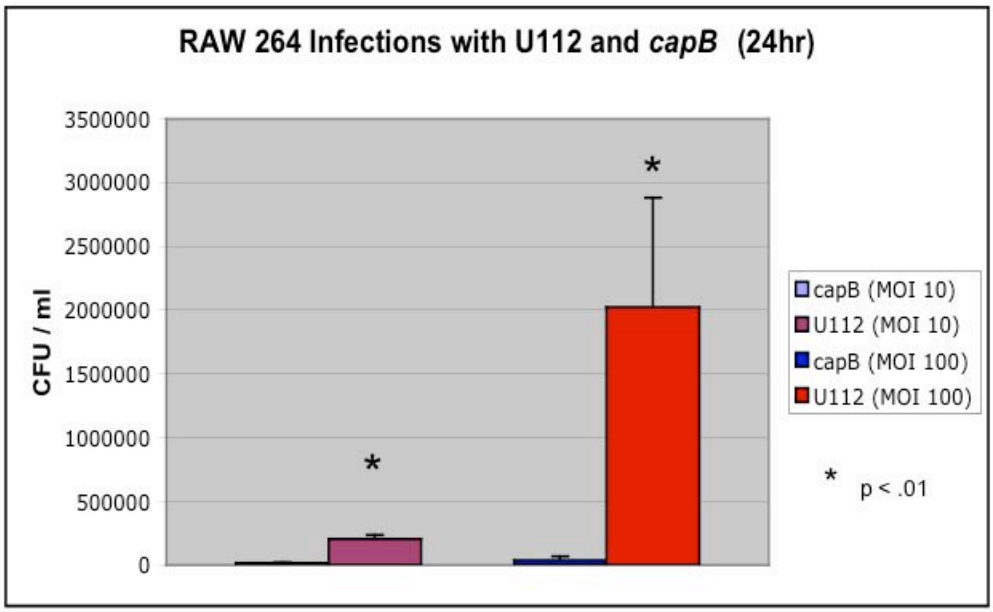


**Figure 10. Macrophage infection model (24hr) demonstrates replicative potential differences between U112 and *capB*.** As for the replicative capacity data, at both MOIs, *capB* show a decrease in replication inside the RAW 264.7 macrophages when compared to the wildtype U112.



with gentamicin washes. Infected cells were lysed with water and plated to TSB-C plates for CFU counts. No difference was seen at the lower MOI at two hours nor at the higher MOI at two hours, indicating no statistical difference in entry between the two bacteria types (Fig. 9, 10). At 24 hours, both MOIs demonstrated approximately a 1.5 log increase in CFUs between the wildtype U112 and *capB* ($p<.01$). This data suggests that the *capB* mutant has a reduced intracellular growth capability in macrophages.

**Attempted visualization of the PGA capsule of *Francisella***

There were several methods which can used to visualize the capsule of encapsulated bacteria. In certain bacteria which elicit a relatively large capsule, such as *Streptococcus pneumoniae* or *Kiebsiella pneumoniae*, the capsule can be visualized using a light microscope and particular stains. The PGA capsule of *Bacillus*, for instance, can be visualized using nigrosin in wet or dry mount preparations. This was due to two important factors: the size of the bacterium and the size of its capsule. Most *Bacillus* species were rather large (1-2 micron), and the capsule appears as a halo around the bacteria. Due to the relatively small size of *Francisella* (.2 to .4 micron), I had a difficult time using these types of techniques to even visualize the polysaccharide capsule. Although there appeared to be a halo around our wet and dry preparations of *Francisella* which were grown in repeated passages of CDM, these images were not conclusive of encapsulated bacteria (data not shown). These images also were unable to ascertain the difference between a polysaccharide capsule and one made of PGA. In order to specifically visualize a PGA capsule in *Francisella*, antibodies specific to PGA were

needed for use in immunoelectron microscopy studies. F26G3 was a monoclonal antibody against the PGA of *B. licheniformis*, which has been shown to cross-react with PGA from *B. anthracis* (81). I also obtained a polyclonal antibody against *B. pumilus*, which reacts with PGA made by *Staphylococcus epidermidis* (79, 80, 81). One problem, however, was that no experimentation has been performed to determine the limit of crossreactivity amongst antibodies to PGA and different polymers of PGA (see Fig. 1).

Initially, I used a mounted immunoelectron technique, which has been used to visualize *Kiebsiella pneumoniae* and its capsule. After both wildtype U112 and *capB* mutant were grown in CDM, these bacteria were washed in PBS and resuspended in antisera. These samples were then fixed in 5% glutaraldehyde-0.1 M sodium cacodylate buffer (pH 7.3) with 0.15% ruthenium red. Following several washes with this same buffer but with 0.05% ruthenium red, cells were fixed for one hour in 2% osmium tetroxide with 0.05% ruthenium red. The bacteria were again washed in this buffer with 0.05% ruthenium red and embedded in 2% agar before dehydration with increasing acetone solutions and embedding in Spurr's plastic (116). Ultrathin sections were cut and placed upon Formvar grids for inspection on a JEOL JEM 1230. Although this process was able to provide fair images of the embedded bacteria, the blade of the ultratome would push the bacteria in and out of the matrix, not slicing the bacteria as cross sections. Although other bacterial capsules have been seen using this technique, it seemed that the capsule of *Francisella* was fairly loosely associated and would not withstand such harsh treatment. It was determined that using a whole mount technique would enhance our chances for this visualization.

I then attempted to use a 1% phosphotungstic acid to negatively stain whole mounts of bacteria after incubating in CDM. While this process provided nice images of the bacteria capsule, it was not an immunoprocedure in which I could use antibodies to differentiate between types of capsule being produced. Sebastian et al. (150), in his immunoelectron micrographs of LVS O-antigen mutants, cited the procedure performed by Huebner et al. (70) in their characterization of capsular polysaccharide antigen of *Enterococcus faecalis* and *E. faecium*. This procedure did not require any negative staining of the whole mounted bacteria, and utilizes antibodies so that specific identification of components of the bacterial capsule can be made. Empiric determination of the primary antibody dilution was made and a 1:20 dilution in PBS proved effective. Visualization of reacting antibodies was made with Protein A gold colloidal particles at 10nm. Several milliQ water washes were necessary at the end of the procedure to avoid "salting out" on the grids, which occurs when the PBS from the antisera was not adequately removed and dries into a salt film with covers the grids, making analysis much more difficult (70).

Immunoelectron micrographs were obtained of the wildtype U112 and the *capB* mutant when grown in standard media of TBS-C or CDM following treatment with the F2G63 monoclonal antibody, and this demonstrated gold particles either at the periphery of both bacterial strains or in diffuse material outside attached to the Formvar grids (data not shown). The experiments with the polyclonal antibody also did not seem specific to any material on the grids (data not shown). Capsular material was seen, primarily in the CDM preparations, but there was no difference in specificity observed with the

antibodies when comparing the U112 and *capB* samples. Images of bacteria subjected to various environmental conditions will be discussed later.

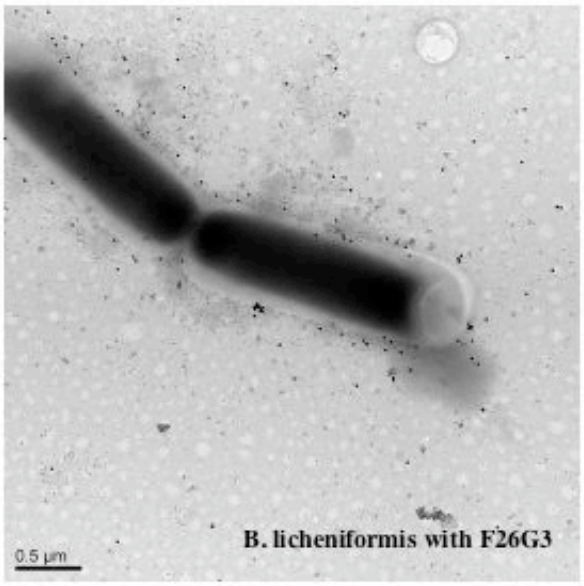
Positive control immunoelectron micrographs were performed on *B. licheniformis* grown in PGA medium at 30°C with shaking at a lower RPM of 200. These bacteria were treated with both the F26G3 monoclonal and the *B. pumilus* polyclonal. These images revealed diffuse material surrounding most of the gram-positive bacteria seen, and the secondary gold particles demonstrated association with this diffuse material in equal density as that seen in our experimental grids (Fig. 11). This association was more prominent with the monoclonal than with the polyclonal, as with the experimental grids.

**Outer membrane extraction for PGA**

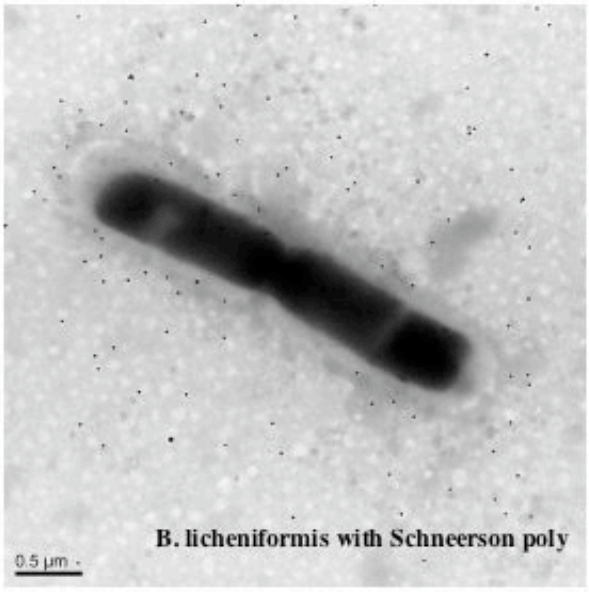
For more definitive analyses to be performed on the capsule of *Francisella*, an appropriate capsule extraction method was needed in order to perform both biochemical and immunological analyses. In earlier times, capsule extraction for the polysaccharide component of *Francisella* required medium concentration NaCl washes from cultures, as was performed by Hood (68). Detergents, such as dilutions of cetavalon or Zwittergent 3-14, have been used to strip off the outer layer of bacteria for analysis of the capsule (45). Many other methods have been described as well, ranging from Waring blender use to sonication. To complicate matters, I was looking for a PGA extraction procedure for a gram-negative bacteria, as most of the gram-positive bacterial extraction procedures I researched included harsher techniques. Initially, I tried extracting the capsule from bacterial cultures using both the agitation of autoclaved glass beads and 10mM EDTA,

**Figure 11 A and B. Positive control immunoelectron micrographs of *B. licheniformis* with antibody against PGA.** *B. licheniformis* was grown with reduced agitation at 30°C for several days until turbid cultures were seen. Cultures were placed onto Formvar grids and treated with A) F26G3 monoclonal against *B. licheniformis* or B) polyclonal against *B. pumilus* from Dr. Rachel Schneerson. In both cases, the antibodies were visualized by secondary gold colloidal particles (10nm) bound to Protein A.

**A**




**B**



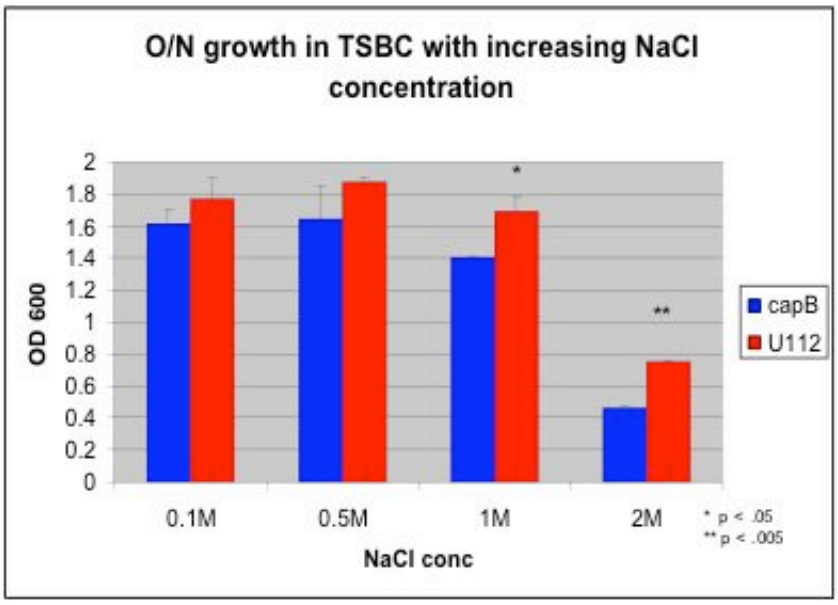
respectively. These procedures were very harsh and resulted in crude extracts which required additional enzymatic treatments and had inconsistent initial results with both the Bradford assay and the anthrone assay, which measure protein and simple sugar concentrations, respectively. Several protocols were then attempted, including use of detergents, such as cetavalon and zwittergent 3-14; and PGA extraction procedures against *Bacillus anthracis* from both Chabot et al. (29) and Kozel et al. (80). Most of these included two to three ethanol precipitations, as both polysaccharide and PGA polymers can be precipitated similarly. The Chabot et al. protocol (29) called for treatment of the bacterial supernatant with a nuclease in buffer containing 0.1M Tris, 10mM $MgCl_2$, 0.1M NaCl, and 0.02% sodium azide. It also requires a 5% trichloroacetic acid precipitation, which can be used in both protein and nucleic acid precipitations, depending on the concentration. I, however, proceeded to use the Kozel et al. (80) technique as this procedure incorporates the supernatant of the culture as well as washes from the bacteria. It requires washes with 10% sodium acetate and 1% glacial acetic acid, as well as two ethanol precipitations. These extractions were used in many different assays to determine potential PGA levels, but initially, anthrone and BSA assays were performed to uncover their biochemical nature. Extract from U112 grown in CDM produces approximately 8 μg/ml and 2.46 μg/ml of glucose equivalent and BSA equivalent, respectively. CDM media itself has at least 3.9 mg/ml of glucose and BSA results of fresh media usually were <0.2 μg/ml.

**Differences in bacterial growth**

It has been shown that PGA was capable of protecting several bacteria during growth in high salt conditions (71). Most neutralophiles can tolerate salt concentrations up to 2M NaCl, whereas at higher concentrations, the loss of intracellular water, thus turgor pressure, causes plasmolysis (21). *Staphylococcus epidermidis* was one of the best known bacteria in which PGA protects this organism due to its high salt environment, the human skin. It has been shown that *capB* mutants in *S. epidermidis* not only downregulate *capB* expression in periods of high salt (2M NaCl), but also have a growth disadvantage when compared to wildtype (79). Similar growth experiments were performed with our *capB* mutant in comparison with its wildtype control. Overnight growth in both TSB-C and CDM with incremental increases in NaCl concentration (0.1M, 0.5M, 1M, and 2M) were made. These cultures were started from aliquots of overnight cultures in their respective media into test tubes with 3ml of fresh media plus the NaCl addition. There were statistical differences in final $OD_{600}$ readings in our TSB-C experiments at 1M and 2M concentrations ($p<0.05$ and $p<0.001$, respectively), in which the wildtype U112 outgrew the *capB* mutant (Fig. 12, 13). Similar data was obtained in our CDM experiments, in which the U112 growth advantage was more apparent at 1M ($p<0.05$). It also seemed that both strains better tolerated with the higher salt concentrations in TSB-C than in CDM (at 1M NaCl, $OD_{600}$ range of 1.4 to 1.7 vs. 0.5 to 0.7). This was probably due to the fact that CDM has approximately double the amount of NaCl as TSB-C (9.9g per liter in CDM vs. 5g per liter in TSB-C).

**Figure 12 A and B. Differences seen in growth cultures in TSB-C when grown at NaCl concentrations greater than 1M.** A) When grown in TSB-C, the *capB* mutant showed significant reduction in growth when compared to wildtype U112 at both 1M and 2M NaCl concentrations for overnight inoculations. B) The time course experiment performed at 1M NaCl in TSB-C demonstrated a significant difference in growth at late log phase between the *capB* mutant and the wildtype U112.

**A**




**B**

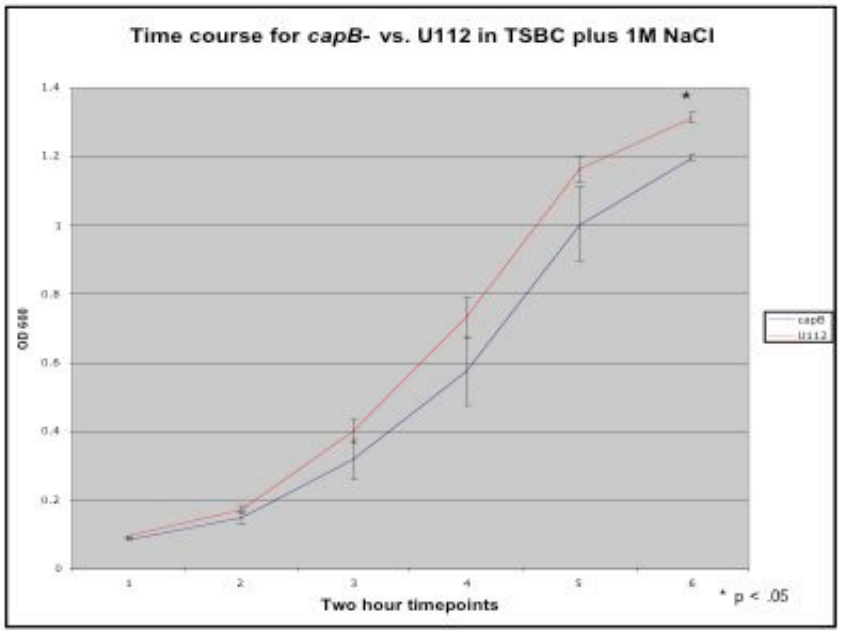
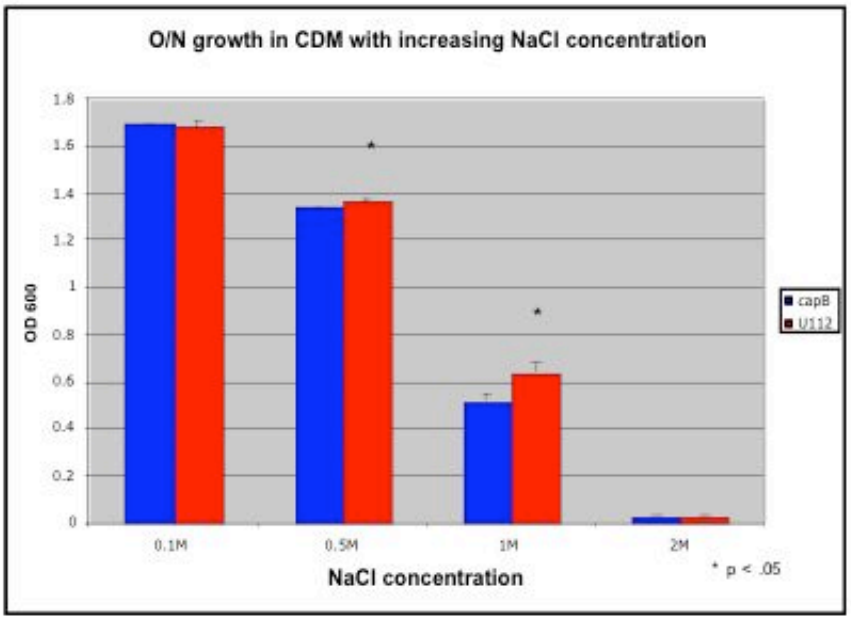
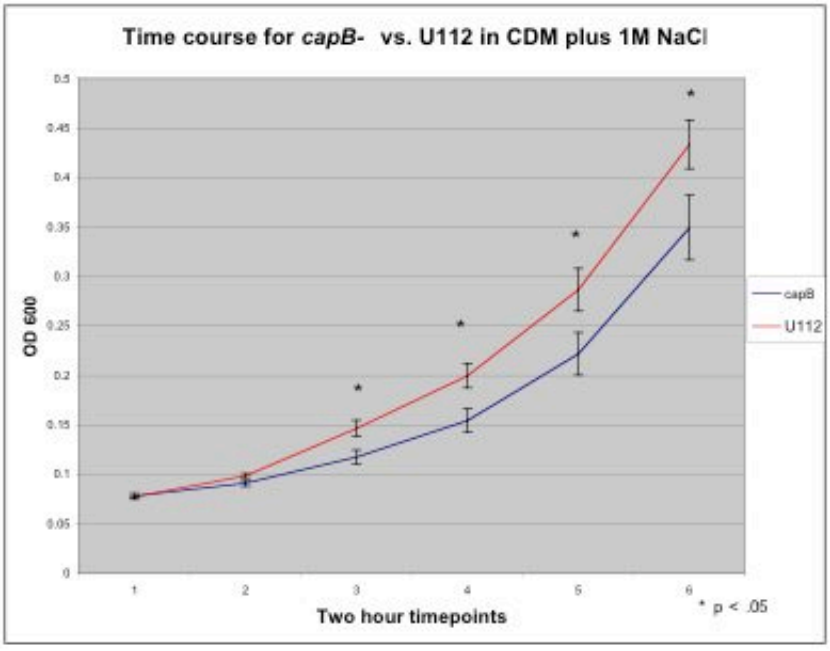


**Figure 13 A and B. Differences seen in growth cultures when grown in CDM at NaCl concentrations at 1M.** A) In the synthetic media CDM, the *capB* mutant also showed significant reduction in growth when compared to wildtype at both 0.5M and 1M NaCl concentrations for overnight inoculations. B) The time course demonstrates a significant difference in $OD_{600}$ at growth points during log phase when grown in CDM plus 1M NaCl.

**A**




**B**

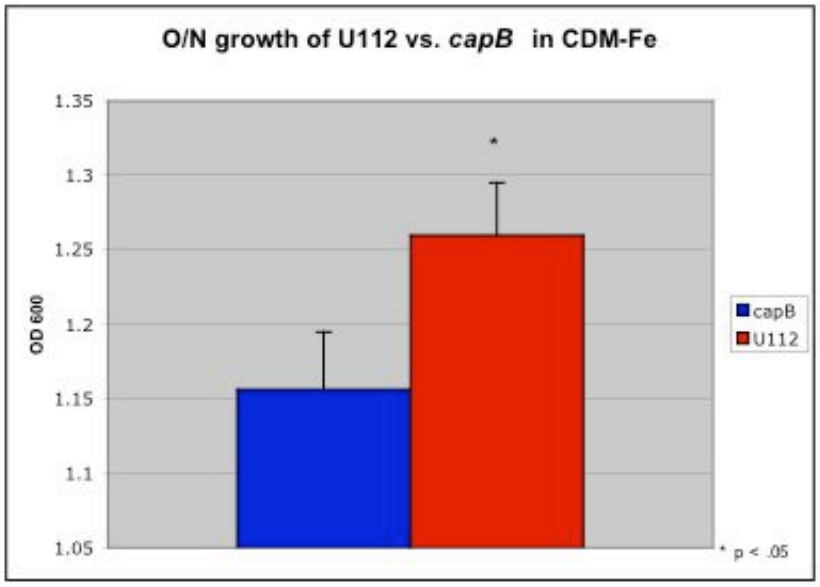


These growth advantages of U112 over the *capB* mutant were also demonstrated in time course experiments, which demonstrated the growth differential between U112 and *capB*. $OD_{600}$ readings between the two bacteria, grown in TSB-C plus 1M NaCl, did not show a statistical difference in growth until twelve hours after inoculation, whereas there was a statistical difference seen at an earlier timepoint (six hours) with growth in CDM plus 1M NaCl. Again, this difference attributed to the higher NaCl concentration in CDM. All of this data suggest that this growth advantage imparted by functional *capB* may aid in *Francisella*'s persistence in harsh, external environments, such as the skin of animals.

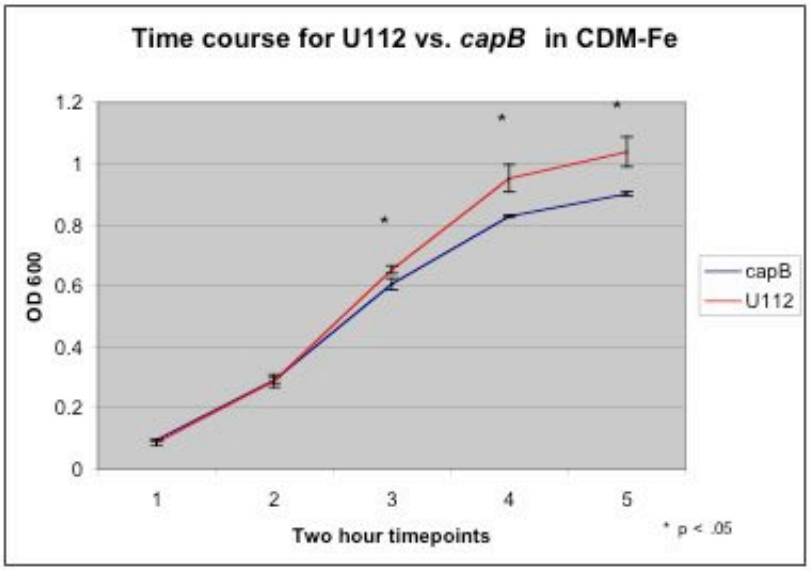
Another growth condition tested was the absence of ferrous iron in CDM. There have been many experiments performed which show that iron was essential for growth as well as intracellular growth and that many bacteria have evolved various tools to confront this problem. Sullivan et al. (162) has described how siderophores are used by *Francisella* in its acquistion of inorganic iron. These iron-siderophore complexes were then bound to receptors at the cell surface, and taken up by the organism. It has been suggested that in other species, PGA, like other anionic polymers, acts to sequester cations as a protective mechanism against certain metal toxins (110). It was also well known that under iron-restricted conditions, *Francisella* tends to upregulate genes needed for intracellular survival, such as the genes of the pathogenicity islands as well as regulators (86, 115). As far as the *cap* operon was concerned, *capD*, which encodes a γ-glutamyltranspeptidase, has been shown to have a decreased protein level in a LVS iron-deficient model (90). However, Deng et al. (42) have shown via microarray experiments

**Figure 14 A and B. Reduction in growth of the *capB* mutant when grown without ferrous iron.** A) Shown was a growth reduction of *capB* compared to wildtype when grown in CDM without iron for overnight culture. B) A significant difference was seen in the mid to late log phase of growth. Therefore iron restriction has an effect on a *capB* mutant. It has been suggested that low $Fe^{2+}$ containing environments may act as a signal to increase virulence factors needed for intracellular survival (known increase in expression of pathogenicity island genes). The reverse being that high $Fe^{2+}$ environments may signal adaptation to extracellular environments ie.(potentially encapsulating).

**A**




**B**



of LVS in iron-restricted media, that *capD* undergoes an increase in expression (1.595 mean comparative expression level of iron-replete / iron-deplete conditions, n=7). Their data also demonstrates reduced expression of *capB* in LVS in iron-restricted media (.749 mean comparative expression level of iron-replete / iron-depleted conditions, n=7). Deng et al. (42) also showed similar expression levels of genes thought to belong to this operon, suggesting a common mode of regulation. Our iron-depleted CDM media experiments demonstrated a slight growth advantage by U112 when compared to our *capB* mutant in our overnight growth curve (mean $OD_{600}$ of 1.260 for U112 vs. 1.156 for *capB*, $p<0.05$) (Fig. 14). This statistical difference was seen at hour six during the logarithmic phase of growth and throughout until stationary phase was achieved, $p<0.05$). These data suggest that *Francisella* may, amongst other external stimuli, use free iron concentrations as a molecular signal for intracellular vs. environmental growth.

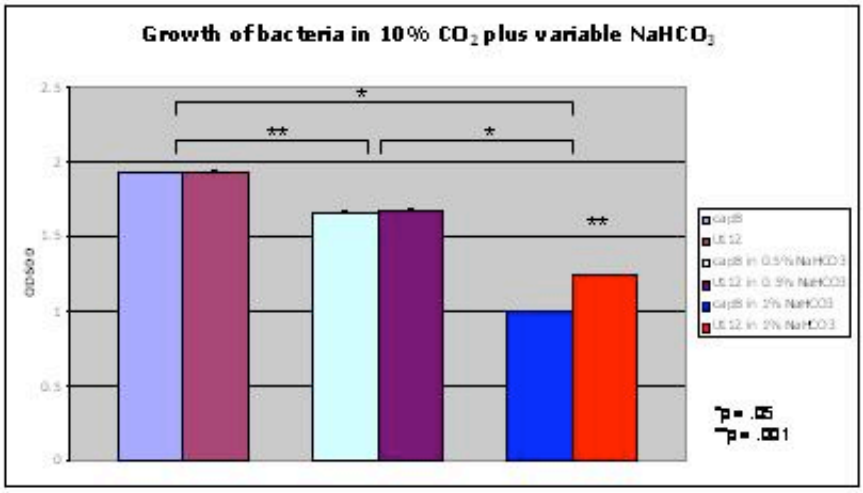
Magnesium concentration has been shown to affect PGA production in several of the *Bacillus* species. It was thought that magnesium was required by the ATPase activity in the amide ligase reaction (6, 25). The usual range of magnesium seen to increase PGA production in *B. anthracis* was 0.05% to 1%. Overnight cultures from both U112 and the *capB* mutant were used as inocula to initiate new overnight cultures with increasing concentrations of $MgCl_2$ as well as $MgSO_4$, respectively. One µl of culture was used to inoculate 2 ml of CDM plus additions of the Mg salt solutions. The $MgCl_2$ and $MgSO_4$ concentrations tested were .5, 1, 5, 10, 25, 50, 100, 150, and 200 mM. There was no inhibition of growth seen with either strain at the high concentrations (data not shown).

There also was no statistical difference in stationary growth measured by final $OD_{600}$ between the wildtype U112 and the *capB* mutant at any of these concentrations.

Another factor that has been demonstrated to influence PGA production in *B. anthracis* was aerobic growth at 37°C with 5% $CO_2$ and 0.8% $NaHCO_3$. It was believed that the $CO_2$ levels indirectly influence the promoter region for transcription of the *cap* operon in *B.anthracis* (6, 106). The genetic element recognized as well as the proteomic entities involved were not known. Sodium bicarbonate, however, acts as a buffer to the increase in pH due to $CO_2$. $CO_2$ reacts with water to become bicarbonate and a proton, which acts to lower the pH of the media. However, increasing the bicarbonate concentration forces the reaction in the opposite direction, increasing the pH of the media until equilibria was reached (106). Overnight cultures of both U112 and the *capB* mutant in CDM were again used as inocula to prepare cultures grown in 10% $CO_2$ with 0.5 and 1% additions of sodium bicarbonate for incubation overnight. There was a decrease in overall growth of both bacteria as the concentration of sodium bicarbonate increased (1.938, 1.670, and 1.119: mean growth of both bacteria at $NaHCO_3$ of 0%, 0.5%, and 1%). However, there was no statistical difference seen in the final $OD_{600}$ of these two strains. As expected, I observed an increase in pH in both bacterial cultures as level of bicarbonate increased, with a slight difference in pH between U112 and the *capB* mutant. The wildtype U112 consistently had a 0.5 pH increase when compared to its *capB* mutant, at all levels of sodium bicarbonate (table 2), using pH strips with a range from 2 to 9.

**Figure 15 A and B. The effects of 10% $CO_2$ and variable concentrations of $NaHCO_3$ on wildtype U112 and *capB* mutant.** A) As concentrations of $NaHCO_3$ increased in these overnight cultures in CDM at 10% $CO_2$, a reduction in growth of both strains was observed. No significant difference between wildtype U112 and *capB* mutant was seen until 1% $NaHCO_3$. B) The pH readings in the culture after overnight growth under these conditions are shown. It was consistently observed that the *capB* mutant had a reduced pH when compared to U112. Both data indicate that optimal growth of these bacteria takes place at pH < 7.

**A**




**B**

| Strain and condition | pH reading |
|---|---|
| | |
| capB | 6 |
| U112 | 6.5 |
| | |
| capB in 0.5% NaHCO3 | 7.5 |
| U112 in 0.5% NaHCO3 | 8 |
| | |
| capB in 1% NaHCO3 | 8.5 |
| U112 in 1% NaHCO3 | 9 |

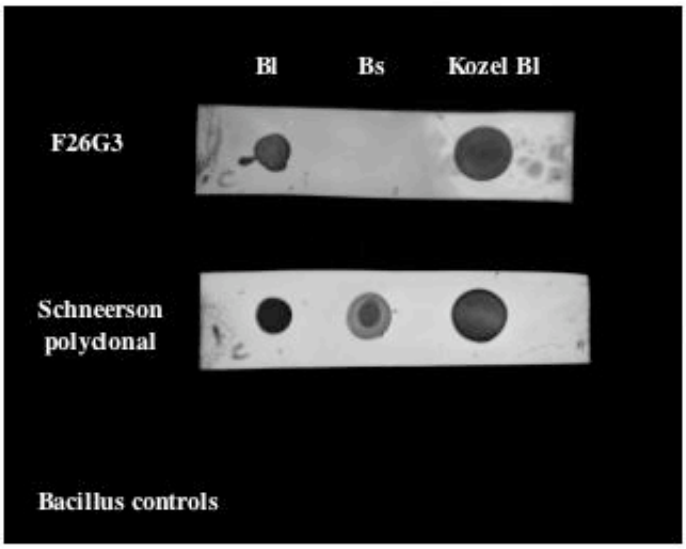
**Immunodot blot analysis for PGA**

Dot blots were screened using both the monoclonal F26G3 and the polyclonal antibody against PGA from *B. pumilus*, which were obtained as gifts. Extracts from both wildtype U112 and the *capB* mutant grown in various environmental conditions were placed onto nitrocellulose and dried overnight. These blots were blocked with 3% BSA and incubated with each antibody separately in TEN buffer at 50 µg/ml and then incubated with secondary alkaline phosphatase-conjugated antibody for visualization via NBT/BCIP substrate. It has been shown that the F26G3 antibody reacts with the PGA polymer from *B. anthracis*, which was in the D-form, as well as the D+L form of *B. licheniformis*. The polyclonal against *B. pumilus* has been shown to react with PGA made from *B. pumilus* (D+L) and the PGA made from *S. epidermidis* (D+L) (79). Control blots were performed in which extractions on *B. licheniformis* and *B. subtilis* cultures grown in PGA expression medium overnight. PGA provided by Dr. Kozel acted as a positive control, as this PGA was extracted using the same procedure. The F26G3 antibody reacted with our *B. licheniformis* extraction and the positive control, but did not react with our *B. subtilis* extraction (Fig. 16). The polyclonal had strong reactions with both the positive control and the *B. licheniformis* extraction, and had a substantial reaction with our *B. subtilis* extract. To demonstrate specificity of these antibodies to the polymer, an additional control was performed in which aliquots of 25mM and 250mM L-glutamic acid were blotted and screened. There was no reaction seen at either concentration with the monoclonal.

U112 and the *capB* mutant were grown in CDM at 1M NaCl and 50 mM $MgCl_2$,

**Figure 16 A and B. Control immunodot blots with PGA extracts from *Bacillus* species and increasing concentrations of L-glutamic acid.** A) To test whether the OM extraction procedure was efficient, extractions were performed on several *Bacillus* species (Bl = *B. licheniformis*, Bs = *B. subtilis*, Kozel Bl = PGA of *B. licheniformis* from Dr. Kozel). These extracts were probed with both F26G3 and the Schneerson polyclonal. The F26G3 recognized our Bl extract but not our Bs extract. The polyclonal recognized both Bl and Bs extracts. Both antibodies recognized the positive control from Dr. Kozel. B) To further test the specificity of these antibodies for PGA and not singular L-glutamic acid, I tested both 25 and 250mM L-glutamic acid solutions against both F26G3 and the polyclonal. No significant signal was seen for these concentrations.

**A**




**B**

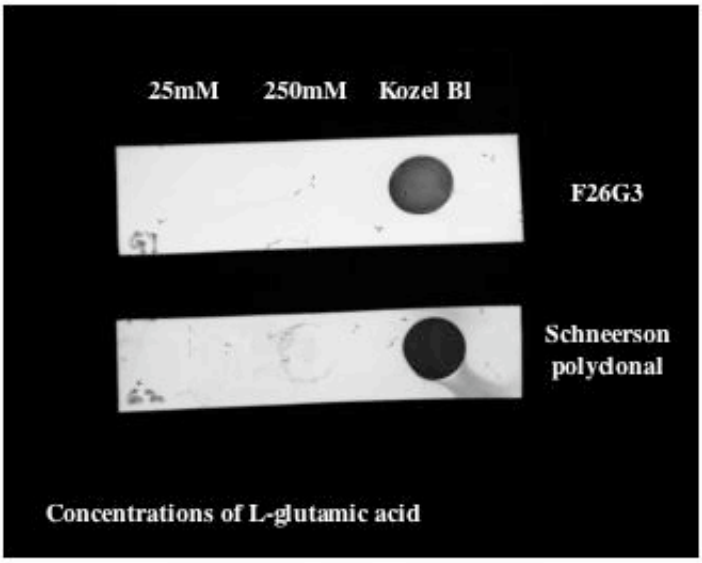
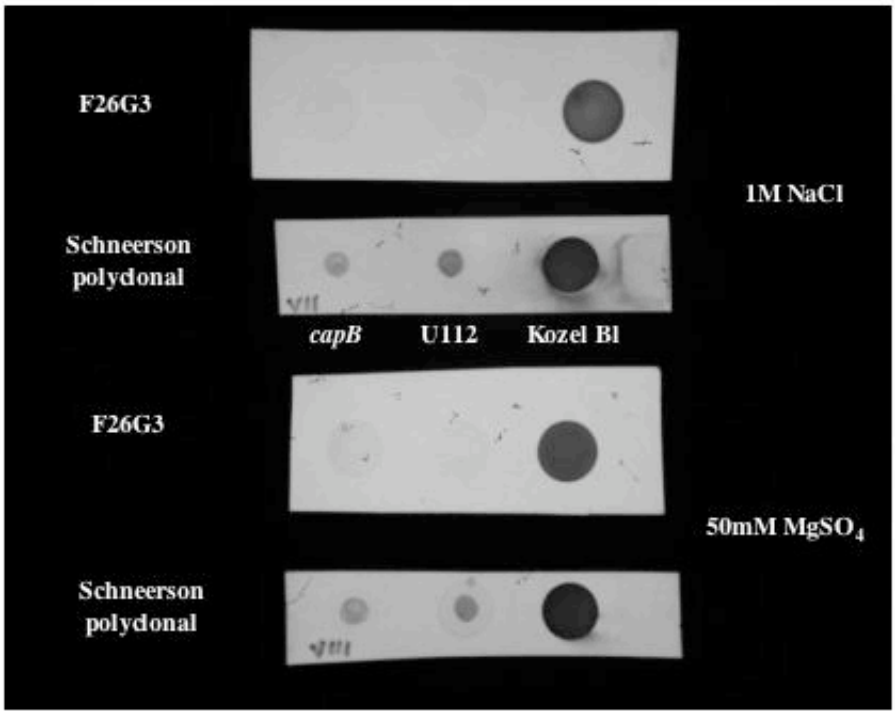


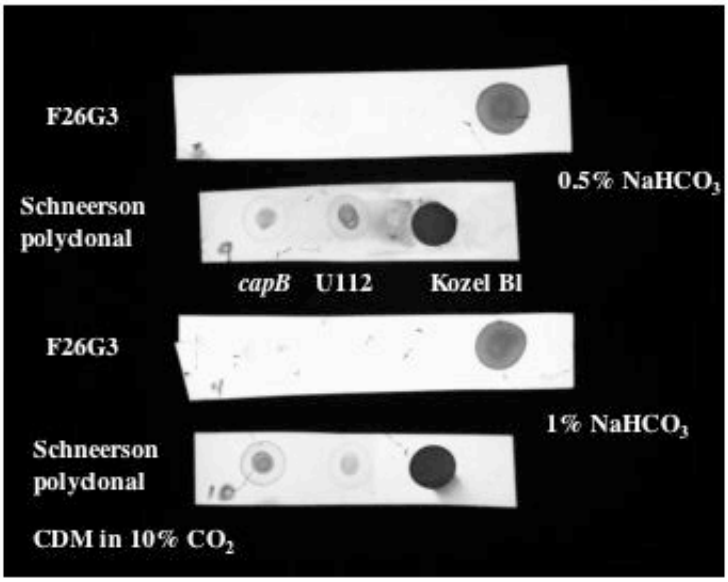
**Figure 17. Immunodot blots of PGA extracts of U112 and the *capB* mutant grown in CDM plus 1M NaCl and 50mM $MgSO_4$.** For the F26G3 blots, no significant signal was seen for both the *capB* mutant and the wildtype U112 under both conditions. Using the polyclonal, signals were seen for both *capB* and U112 at both conditions. There was no differential seen between the mutant and our wildtype extracts.



extracted and blotted (Fig. 17). The monoclonal and the polyclonal reacted well with the positive control. The F26G3 monoclonal only reacted with the *Bacillus* positive control at both conditions. The polyclonal antibody reacted well with the positive control, but only faintly with the wildtype U112 and the *capB* mutant. The signals were of equal intensity between the wildtype U112 and *capB* mutant, suggesting just background reactivity. Control immunodot blot experiments were also run using this same antibody on *S. epidermidis* and its *capB* mutant, which demonstrated no reaction at all with the mutant, but positive for the wildtype (data not shown). The next set of experiments were both the extractions and the supernatants collected after growth of both bacteria in CDM at 10% $CO_2$ and .5 to 1% of $NaHCO_3$. Again, no reaction was seen at all with the monoclonal except for the positive controls (Fig. 18). The polyclonal reacted with both the U112 and the *capB* mutant with equal intensity at these conditions as well, presenting nonspecificity. There was an increase in intensity of the signals for the experimental extractions in the supernatant blot from 0.5% to 1% $NaHCO_3$. The next set of experiments involved bacteria grown overnight in TSB-C and then subjected to a low pH (pH 4) for one hour prior to extraction. An additional experiment was performed in which bacteria, after the pH4 treatment, were centrifuged and lysed in a 2% SDS, 30% glycerol, 250mM Tris hydroxyaminomethane buffer and blotted. This lysis was performed to determine whether our gram-positive extraction perhaps was effective in processing PGA from our bacteria. Similarly, these samples did not react with the monoclonal, and both U112 and the *capB* mutant samples reacted weakly and equally when screened with the polyclonal antibody (Fig. 19). Thus our data suggests that none

**Figure 18 A and B. Immunodot blots for PGA in extracts and culture supernatants of U112 and the *capB* mutant grown in CDM at 10% $CO_2$ with variable concentrations of $NaHCO_3$.** A) Extracts from the strains grown in CDM at 10% $CO_2$ were probed with both antibodies. No signal was seen for *capB* mutant or the wildtype U112 at both $NaHCO_3$ concentrations with F26G3. There was no differential between these two bacteria when probed with the polyclonal antibody at both $NaHCO_3$ concentrations. B) The supernatants from these same cultures were also probed with anti-PGA, yet demonstrated similar results at both $NaHCO_3$ concentrations.

**A**




**B**

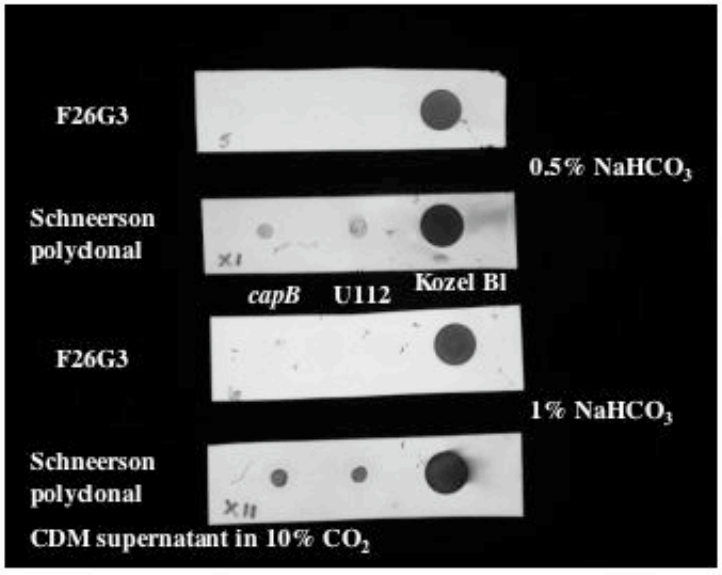
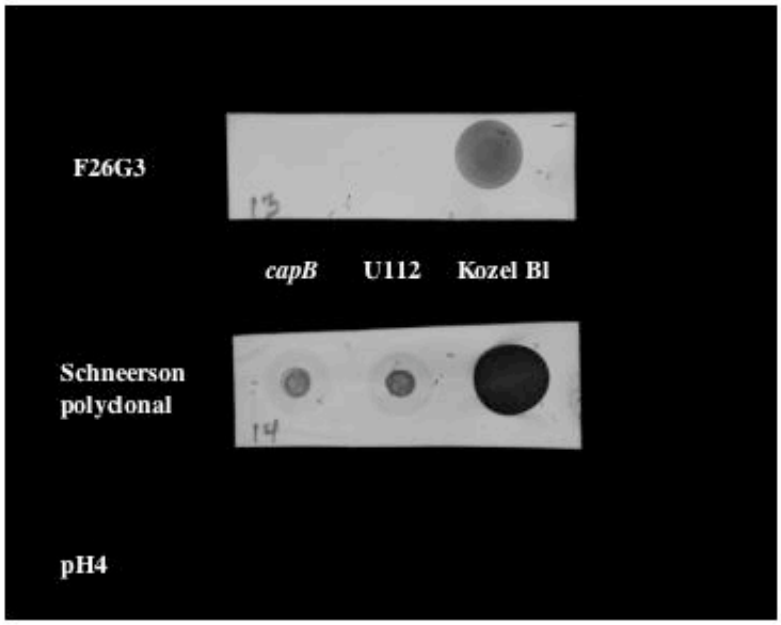
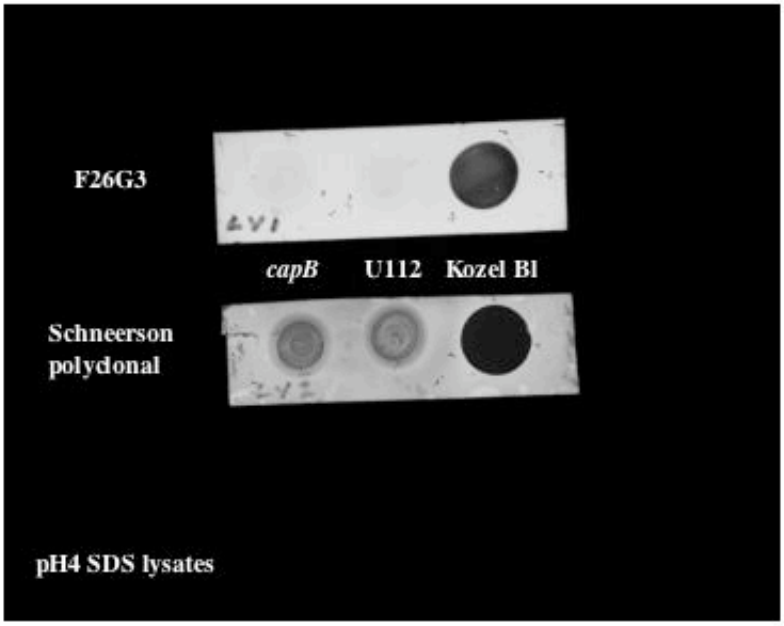


**Figure 19 A and B. Immunodot blots for PGA in extracts from U112 and the *capB* mutant grown in TSB-C, then treated for one hour at pH4, and SDS lysates under the same conditions.** A) The extracts from both strains did not react with F26G3, and there was no differential seen comparing the *capB* mutant to the wildtype U112 with the polyclonal. B) The SDS lysates were performed in case our extraction procedure was not extracting PGA. Again, no signal was seen from both strains with F26G3 and there was no differential seen between the bacteria with the polyclonal.

**A**




**B**



of the above conditions promoted the production of PGA that could be detected immunologically.

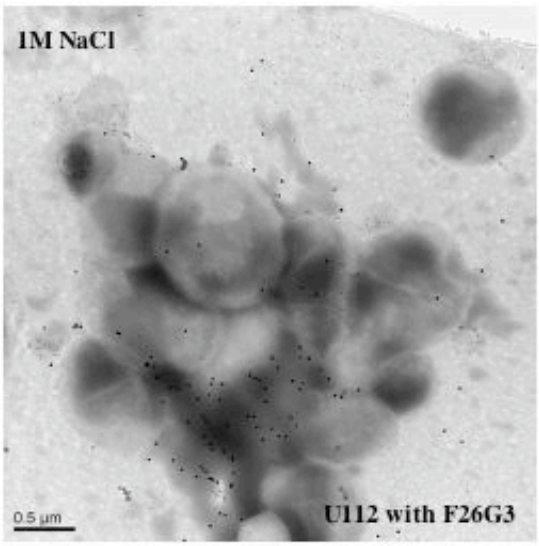
**Immunoelectron micrographs of various environmental conditions**

Treatment of several PGA-producing bacteria such as *S. epidermidis* and *S. haemolyticus* with high concentrations of NaCl has induced PGA production (49 ,79). As I have seen growth differences between our bacteria at 1M NaCl, I chose this condition to test for production of PGA via immunoelectron microscopy. Both strains were grown in TSB-C supplemented to 1M NaCl for overnight culture. Using the described procedure for creating immunoelectron grids, I observed these bacteria using both F26G3 and the Schneerson polyclonal. Although there appears to be gold particles surrounding bacteria of our wildtype U112, similar particles were seen on the mutant grids as well (Fig. 20). Images with the Schneerson polyclonal were also not conclusive, with similar numbers of immuno-gold particles seen and without specificity to the surfaces of the strains (data not shown).

One of the environmental conditions I analyzed was treatment of overnight cultures in TSB-C with HCl. These cultures were acidified to pH 4 to 4.5 after overnight incubation, and shaken for an additional hour prior to addition to the Formvar grids. Samples performed at pHs lower than 3 resulted in degradation and lysis of bacteria, irrespective of genetic background (17). One observation at the lower pH was the level of aggregation by both bacteria (Fig. 21). Secondary gold particles were located throughout the grid on the polyclonal preparations for both bacteria, with seemingly

**Figure 20 A and B. Electron micrographs of U112 and *capB* grown in CDM at 1M NaCl.** Electron micrographs of A) wild-type *F. tularensis* U112 and B) the *capB* mutant grown at 1M NaCl treated with monoclonal antibody F26G3 followed by gold colloidal 10 nm particles attached to protein A. Both images demonstrate no differential of gold particles between the two bacteria. Images were taken at 10,000x on a Jeol JEM-1230 TEM microscope.

**A**




**B**

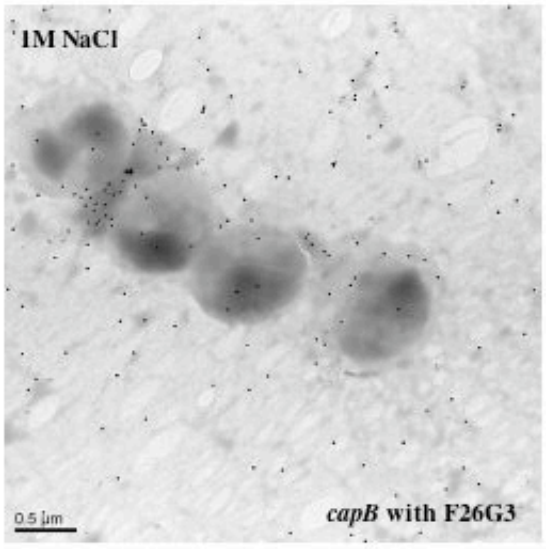
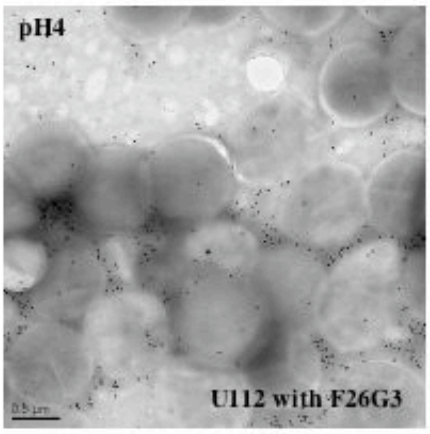
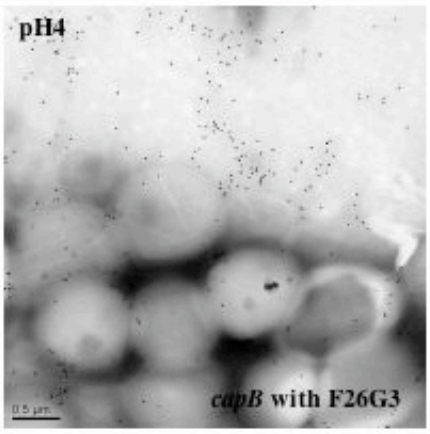


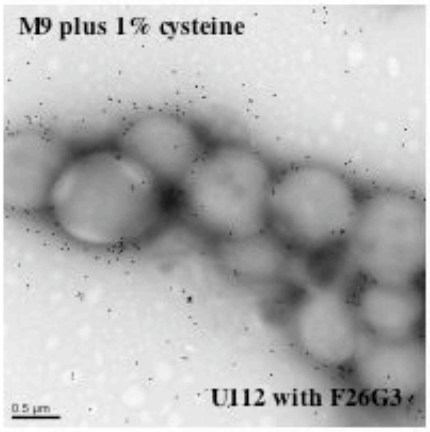
**Figure 21 A and B. Immunoelectron micrographs of U112 and the *capB* mutant at pH4 and in M9 minimal media.** Immunoelectron micrographs of wild-type *F. tularensis* U112 and the *capB* mutant treated with monoclonal antibody F26G3 A) grown in TSB-C, then treated at pH4 and B) grown in TSB-C, then resuspended in M9 minimal media plus 1% cysteine. While no differential was apparent in the reduced pH images between the bacteria, there may be more gold particles in the M9 media image of U112 when compared to its mutant. Images were taken at 10,000x on a Jeol JEM-1230 TEM microscope.

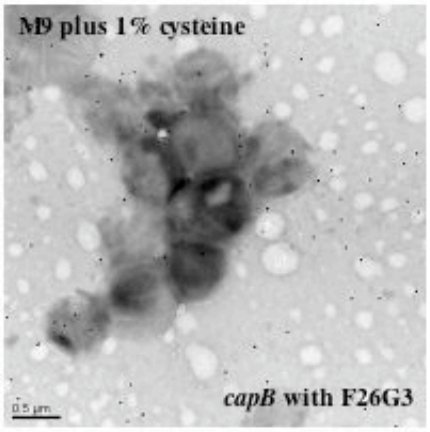
**A**




**B**



similar uniformity. There were beads seen on the bacteria, but just as many beads were located on the matrix itself. It appears that the polyclonal lacked specificity for *Francisella* as there were no concentrated areas seen. The F26G3 monoclonal was localized to diffuse material either on the bacteria or on the matrix, however, the secondary beads recognizing this antibody were seen both on the U112 wildtype preps as well as on the *capB* preps, and in equal number.

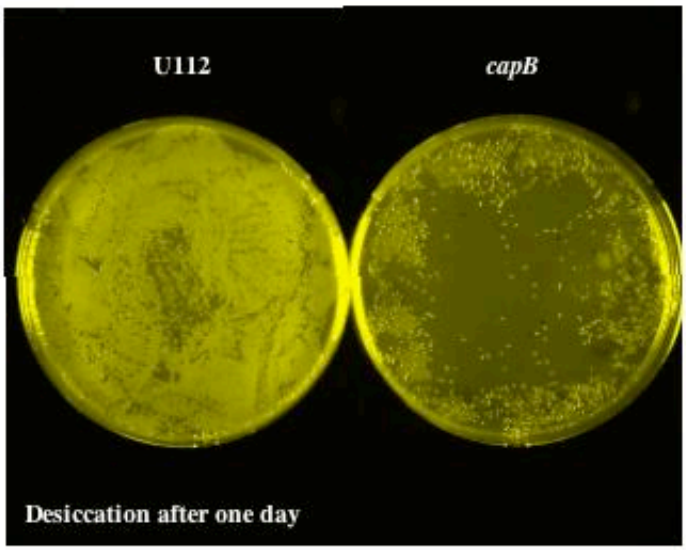
Resuspension of our bacteria in M9 minimal media plus 1% cysteine after overnight growth in rich TSB-C media was used as a starvation-stress response to see if PGA production may be induced under these stringent conditions. Two ml of overnight cultures were centrifuged at 12K, the supernatant decanted, and resuspended in M9 minimal media with incubation at 37°C for four hours prior to deposition on the Formvar grids. Our analysis of these grids (Fig. 21B) showed less diffuse material on the *capB* mutant grids compared to U112, but the location of secondary gold particles from F26G3 anti-PGA was not specific for wildtype. On the capB mutant grids, these gold particles were disseminated throughout and did not associate with this material, very similar to the pattern seen in past grid analyses with the polyclonal.

**Desiccation assays**

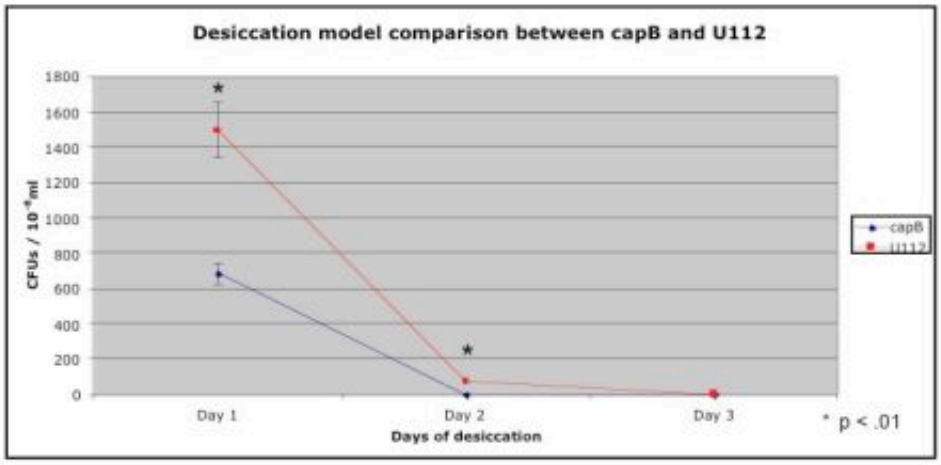
It has been shown that *Azotobacter vinelandii* creates an alginate encystment when placed into a minimal medium with limited carbon sources (23). This aids in the bacteria's persistence in harsh conditions. Persistence of *Francisella* in similar environments was known to occur (e.g. due to infections from contact with animal

**Figure 22 A and B. Desiccation resistance test.** Overnight cultures of U112 and the *capB* mutant were washed and resuspended in M9 minimal media with 1% cysteine, lacking carbon sources. Bacteria (10 µl) were transferred to tubes and incubated open in a dry 30°C incubator. Each day, tubes were resuspended in minimal M9 media, diluted, and plated on to TSB-C plates. A) There was a significant reduction in CFU formed by the *capB* mutant when compared to wild-type control (Day one shown). B) These numbers were tabulated and it was shown that there was a significant difference between these bacteria on day one and two of desiccation, while no colonies were seen with either strain after three days.

**A**




**B**



carcasses that have been dormant for some time), although *Francisella* neither encysts nor sporulates (123, 155). To test the role of capB in desiccation resistance, the U112 wildtype and the *capB* mutant were grown overnight in TSB-C media, centrifuged and decanted, and resuspended in M9 minimal media plus 1% cysteine without carbon sources. 10 µl aliquots were then placed into separate microfuge tubes and incubated in a dry 30°C incubator. At 24-hour intervals, an aliquot would be resuspended in the same M9 minimal media, diluted, and plated to TSB-C plates. These plates were then incubated for CFU counts (Fig. 22). Our results demonstrated that after one day of incubation, the U112 wildtype bacteria were very sensitive to desiccation with a drop of several logs in CFU. However, it was substantially more resistant than the *capB* mutant (mean CFUs 1495 for U112 vs. 682.5 for *capB*, $p<0.05$). By the second day, U112 still had some viability while the *capB* mutant showed no CFUs (mean CFUs 72.5 for U112 vs. 0 for *capB*, $p<0.01$). No colonies were seen on the third day for either bacteria at the $10^{-8}$ dilution. The data demonstrated that the *capB* mutant was less resistant to desiccation than the wildtype.

**Acid resistance assays**

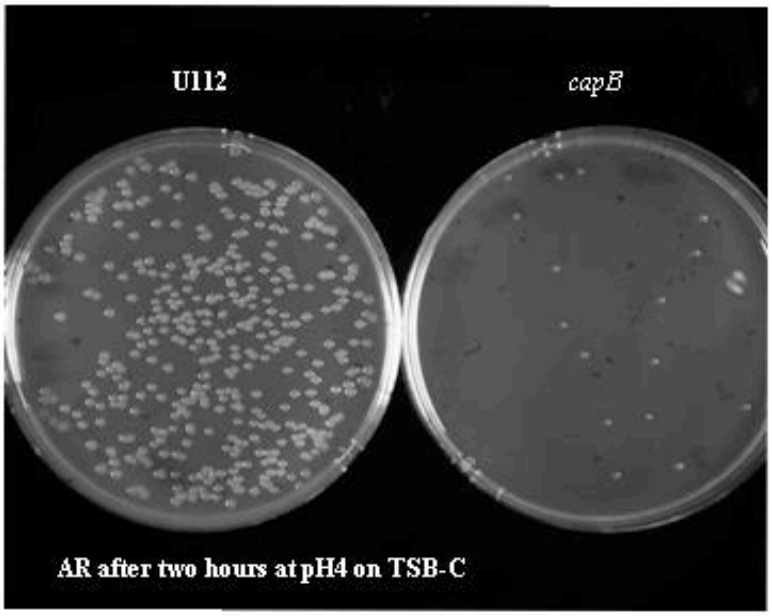
Due to the environmental advantage seen with the wildtype U112 compared to the *capB* mutant, we next compared their acid tolerance and resistance. The definition of several of these terms regarding acid resistance have been confused, even amongst the researchers who have dedicated many years to the study of the mechanisms by which bacteria use to manipulate extracellular and intracellular pH. Acid tolerance was used to

describe mechanisms used by bacteria to grow in acidic conditions whereas acid resistance refers to mechanisms by which bacteria survive in acidic conditions. Acid resistance and tolerance also applies to exposure to fatty acids as well as fermentative acids due to metabolism (17, 28, 38). There were several systems utilized by bacteria, and some of these were dependent on the stage of bacterial growth. Most neutralophiles fare well in a media pH range of 6 to 8. These acid resistance experiments were modeled after similar experiments performed by the Foster lab in which CFUs were counted after acid treatment (133).

*Francisella* strains were grown overnight in either CDM or TSB-C and then normalized aliquots were placed into their respective media that had been acidified to pH 4 by drops of HCl. These inocula were then measured by $OD_{600}$ and placed in the incubator at 37°C with shaking. At timepoints two hours and four hours, aliquots from the inocula were removed, measured by $OD_{600}$ and plated to TSB-C plates for CFU counting. A statistical advantage was seen in the number of CFUs from U112 when compared to the *capB* mutant at both timepoints in the TSB-C experiments (at two hr: mean CFUs for U112 was 352.5 vs. 14 for *capB*, $p<0.05$; at four hr: mean CFUs for U112 was 84.5 vs. 21 for *capB*, $p<0.001$). I also observed a corresponding difference in the means of $OD_{600}$ from the initial inocula reading to the inocula reading at the respective timepoint. The difference in means were as follows: 0.0005 vs. 0.0145 at 2 hours for U112 and *capB*, respectively and 0.008 vs. 0.059 at 4 hours for U112 and *capB*, respectively. The percentage of survival of the *capB* mutant when compared to the U112 wildtype control was 4% at 2 hours and 24.9% at 4 hours. Similar data was collected in

**Figure 23 A and B. Acid resistance test.** Overnight cultures of U112 and the *capB* mutant were then incubated A) two or four hours (data not shown) in TSB-C medium adjusted to pH 4, diluted and plated onto TSB-C plates. A dramatic reduction in the viability of the *capB* mutant was observed after each period of incubation at low pH. B) Survival plots of these data were charted under the same conditions, using overnight cultures of U112 and the *capB* mutant grown in CDM. The survival of the *capB* mutant was 44% of control at two hours and 26% of control at four hours.

**A**




**B**

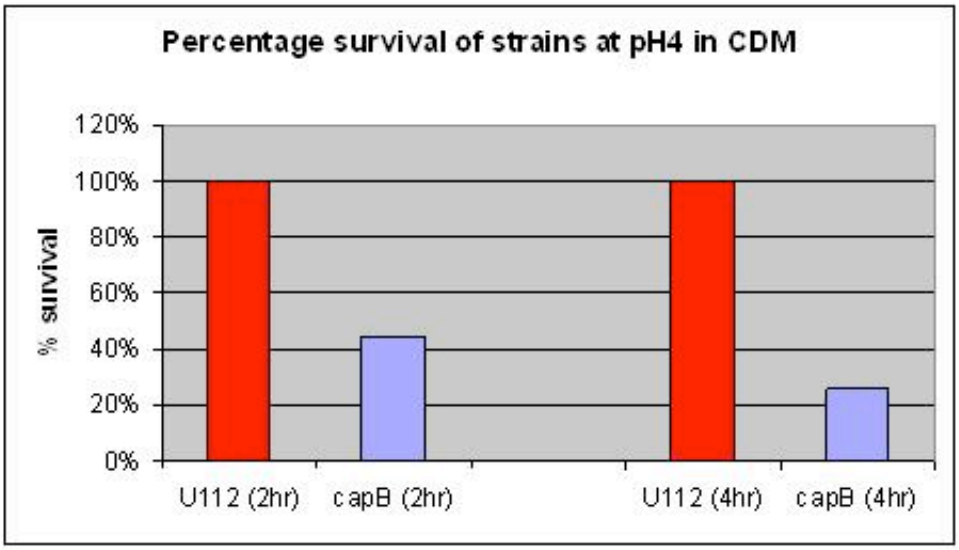
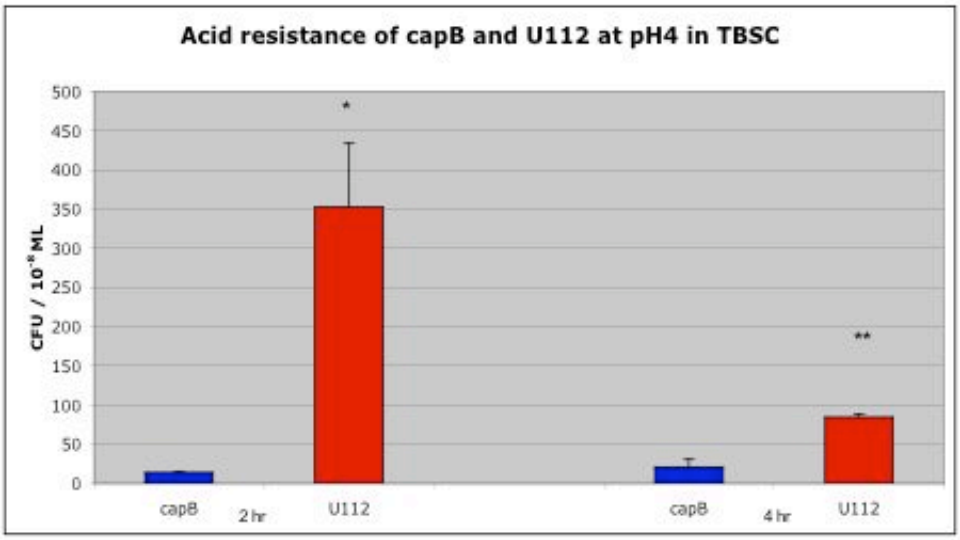


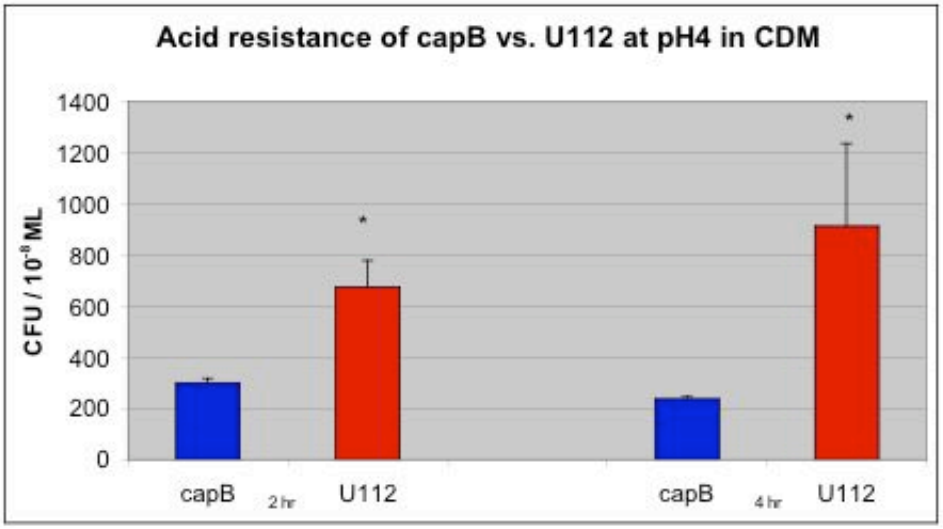
**Figure 24 A and B. Graphical representation of acid resistance data.** Overnight cultures of U112 and the *capB* mutant were incubated two or four hours in A) TSB-C medium adjusted to pH 4, or B) CDM medium adjusted to pH 4. The bacteria were then diluted and plated on to TSB-C plates. Significant reductions in the viability of the *capB* mutant were observed at this low pH in both TSB-C and CDM media.

**A**




*p < .05
**p < .001

**B**




* p < .05

the same experiment except the bacteria were grown in CDM. The number of CFUs for U112 was 675 vs. 300 for *capB* at the two-hour timepoint ($p<0.05$) and 913 for U112 vs. 237 for *capB* at the four-hour timepoint ($p<0.05$). The difference in means were as follows: -0.0025 vs. 0.036 at two hours for U112 and *capB*, respectively and -0.005 vs. 0.0025 at four hours for U112 and *capB*, respectively. The percentage of survival of the *capB* mutant when compared to the U112 wildtype control was 44% at two hours and 26% at four hours. For both experiments, there was little evidence of any change in pH of the media after both timepoints.

A very similar experiment was performed in which overnight culture in TSB-C was used to inoculate fresh media for three hours. These new cultures in log phase were then measured by $OD_{600}$ and then used to inoculate media which was acidified to pH 4. These bacteria were then incubated like the past experiment for two and four hour timepoints, shaking at 37°C (28). When plated after the timepoints onto TSB-C plates, no CFUs were seen for either strain at the same dilution. These data suggest that U112 has an acid resistance mechanism (i.e., survival in acid) which was lacking or inhibited in the *capB* mutant, but this phenomena was only observed after stationary growth by these bacteria. Similar experiments performed with the bacteria in logarithmic growth suggest that *Francisella novicida* U112 may not have an acid tolerance mechanism (i.e., growth in acid) (data not shown).

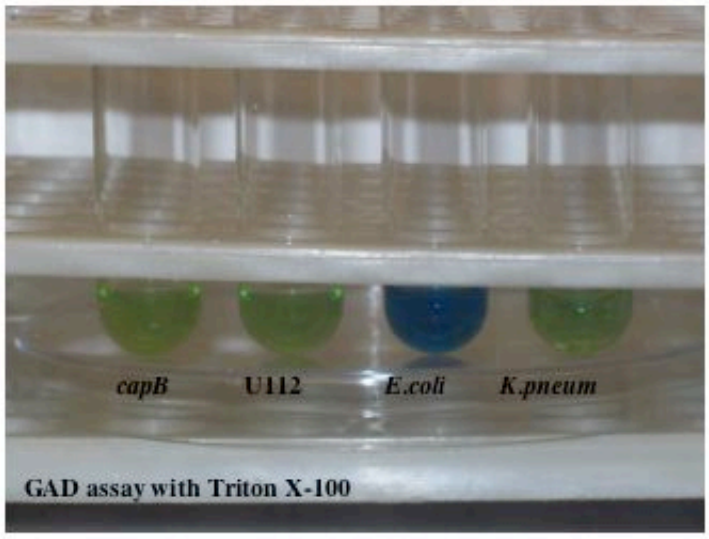
**Intracellular and extracellular glutamate decarboxylase analyses**

Homeostasis of intracellular pH in bacteria has many facets, and their importance in maintaining that balance depends on whether it was gram-negative or gram-positive. Most bacteria use membrane ATPases in which protons were removed from the cytoplasm to initially lower the intracellular pH. These mechanisms have a higher magnitude of importance in homeostasis of gram-positive bacteria (11, 38). Another mechanism shared by both gram-positive and gram-negative bacteria for pH homeostasis was the decarboxylation of certain amino acids. Extracellular amino acids brought into the cell, such as glutamate, lysine, and arginine, can be converted into excreted products after decarboxylation. Intracellularly, this process creates $CO_2$ but more importantly, also consumes a proton which leads to the reduction of intracellular pH. The exchange products for glutamate, lysine, and arginine were γ-aminobutyric acid, cadaverine, and agmatine, respectively. These different amino acid decarboxylases in gram-negatives have different preferences of induction depending on phase of growth and pH of the surrounding media. In *E. coli*, the glutamate decarboxylation system was one of two amino acid decarboxylation sytems utilized at pH 2.5 (130, 131, 133). *Francisella novicida* U112 has genes for encoding glutamate and lysine decarboxylases, but not for arginine decarboxylation, unlike other species of *Francisella* (www.biohealthbase.org).

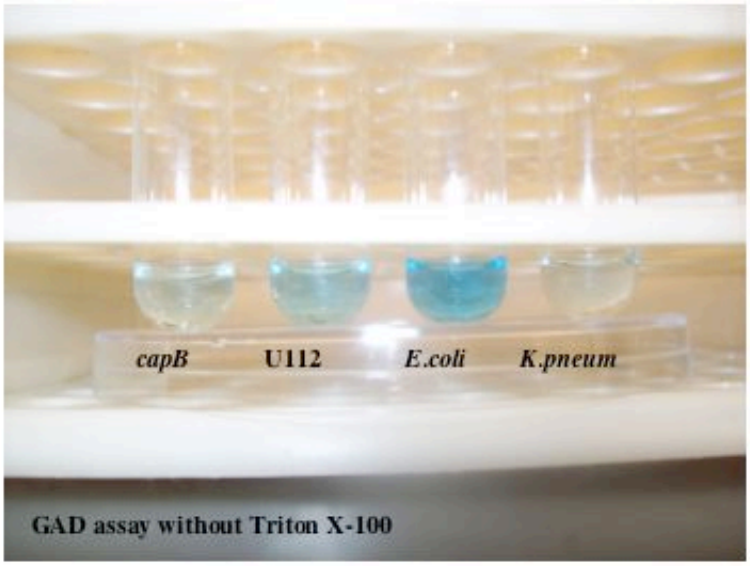
In a traditional glutamate decarboxylation system, the two genes *gadA* and *gadB* encode for isoenzymes responsible for decarboxylation of glutamate. In. *E. coli*, these isozymes were induced separately depending on pH and growth stage, and in some cases, both enzymes were functioning to decarboxylate glutamate. The gene *gadC* encodes the glutamate / γ-aminobutyric acid antiporter responsible for both the import of glutamate

**Figure 25 A and B. The internal and external glutamate decarboxylation assays on *F. novicida* U112 and *capB* mutant.** Rice et al. (130) has described the measurement of glutamate decarboxylation activity using both internal and external GAD assays. The GAD reagent has bromoscerol green which reacts with the level of pH. A) The internal assay (with Triton X-100) was a measure of the activity of glutamate decarboxylases present inside the bacterial cell. Both the *capB* mutant and the U112 wildtype have similar internal glutamate decarboxylase activity, as shown by the light green color. *E. coli* acts as a positive control for the internal measurement. B) The external reaction (without Triton X-100) was a measure of the exchange of glutamate / GABA at the antiporter of the inner membrane. It was here that a difference was seen between the *capB* mutant and the U112 wildtype, as the wildtype was a slightly deeper shade of blue than the *capB* mutant. Again, *E. coli* acts as a positive control and *K. pneumoniae* acts as a negative control.

**A**




**B**



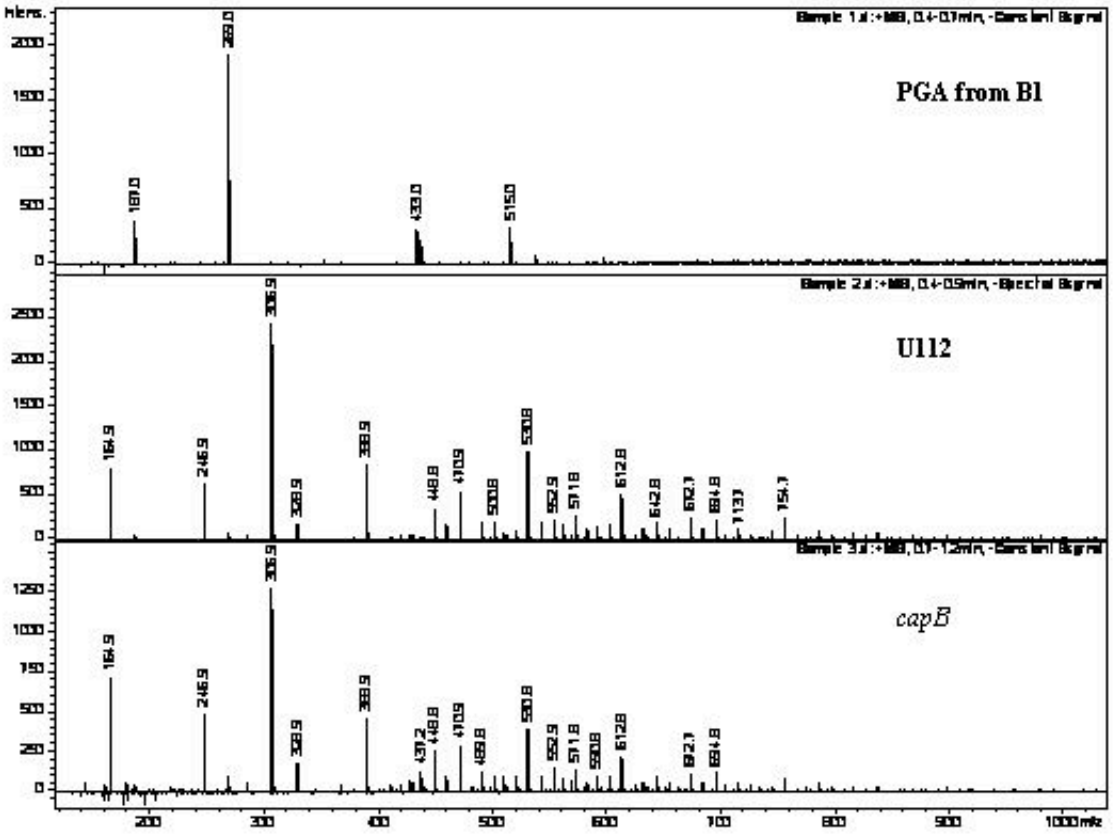
into the cell and the export of γ-aminobutyric acid out of the cell. Rice's GAD assays (130) for measurement of internal and external glutamate decarboxylation were used to determine the relative levels of glutamate decarboxylation in both our wildtype U112 and the *capB* mutant. Overnight cultures (1.8 ml) of both strains were centrifuged and resuspended in 500 µl of GAD reagent and incubated at 37°C for at least four hours. GAD reagent with Triton X-100, which permeates the outer membrane, measures internal glutamate decarboxylation while GAD reagent without Triton X-100 allows analysis of glutamate transport. These mixtures will turn from yellow to blue for a positive reaction and turn clear in a negative reaction. DH5α *E. coli* was used as a positive control and *Kiebsiella pneumoniae* was used as a negative control. Upon mixing and incubation, both the U112 wildtype and the *capB* mutant turned from yellow to green with the GAD reagent plus Triton X-100, demonstrating a slightly positive reaction. With the GAD reagent without Triton X-100, however, the U112 wildtype produced a slight shade of blue while the *capB* mutant was close to clear, similar to the negative control. These data show that while both bacteria seem to have functioning and similar internal glutamate decarboxylation activity, the U112 wildtype has a greater capability for neutralizing the surrounding environment outside the inner membrane than the *capB* mutant.

**Comparison of ESI/MS results from extracts**

Due to the lack of evidence for the presence of PGA in our *Francisella* preparations, I turned to the sensitive method of liquid chromatography / mass spectrophotometry (LC/MS) to detect PGA. As a positive control, PGA from *B.*

*licheniformis* was compared to our samples extracted with sodium acetate / glacial acetic acid. Schneerson et al. (148) provided the details of her analysis of PGA-conjugates that were used to elicit antibodies to the capsule of *B. anthracis*. The analysis of these conjugates was by MALDI-TOF LC/MS (148). Dr. Connell Cunningham, a senior scientist from Rohm and Haas, a subsidiary of Dow Chemical, volunteered to perform ESI ionization MS on three samples: 1) PGA extraction from *B. licheniformis*, 2) PGA extraction of wildtype U112 grown in 1M NaCl, and 3) PGA extraction of our *capB* mutant grown in 1M NaCl. The first image was PGA from *B. licheniformis*, which presents m/z (mass/charge ratios) of 187 and 433, thought to be direct evidence for glutamic acid and polymer (Fig. 26). The peaks of 269 and 515 were thought to be these same structures but with acetyl groups attached (plus 82 m/z units from the original data). This was attributed to the sodium acetate / glacial acetic acid used in the extraction procedure. The second and third images were of wildtype U112 and *capB* mutant, respectively, have many peaks, but none that resemble the actual peaks seen on the PGA standard. There arguably could be small peaks at 187 and at 433 for the wildtype U112, but these same peaks were also seen with our *capB* mutant, and the intensities were very low (<100). Major peaks of the extracts from the wildtype U112 and the *capB* mutant were identical. Many of the peaks in both U112 and the *capB* mutant also have acetyl groups associated with them, some with multiple groups. There may be some peaks unique to both the *capB* (peak 437.2) and the wildtype U112 signatures, or that the corresponding peaks in the opposite graph were at intensities too low to determine in these graphs. This data demonstrates that the extracts produced at 1M NaCl growth with

**Figure 26. ESI mass spectrophotometry on PGA extractions from 1M NaCl.** ESI mass spectrophotometry was performed on a PGA standard from *B. lichenformis* and on bacterial-surface extracts (using a sodium acetate and glacial acetic acid procedure) from wild-type *F. tularensis* U112 and its *capB* mutant grown in CDM at 1M NaCl. The analysis did not show the signature profile of the PGA standard in *F. novicida*, nor were there any obvious differences between U112 and the *capB* mutant.



both the U112 and *capB* mutants were very similar to each other and do not share properties seen in the analysis of the PGA standard from *B. licheniformis*. The analysis of additional samples by Dr. Cunningham would have been helpful, but as these samples were performed free of charge, I was unable to procure his services for additional work. Other PGA extracts were submitted to the VCU Mass Spectrophotometry Center, however, no intelligible data was obtained.

**Amino acid analysis on bacteria samples and extracts**

To further analyze extracts procured from both the wildtype U112 and the *capB* mutant, samples were sent for analysis to the University of California at Davis Amino Acid Laboratory. Initially, our PGA extracts of both bacteria grown in TSB-C, then subjected to pH 4 as well as SDS lysates of both bacteria grown in the same conditions were submitted for free amino acid analysis. These samples were mixed 1:1 with 6% sulfosalicylic acid, which acts to deproteinize the sample, then centrifuged at 14K, filtered through a .45 μm PTFE filter, adjusted to pH 2.2, and then loaded into a Biochrom 30 amino acid analyzer. The PGA extracts from both bacteria only resulted in quantitations for O-phospho L-serine (256 vs. 227 nmol/ml for U112 and *capB*, respectively) (Fig. 27). O-phospho L-serine, also called L-phosphoserine, was a metabotropic glutamate receptor agonist and was structurally very similar to L-glutamate. The SDS lysate samples demonstrated some unusual differences, also no difference seen in L-glutamic acid (4 vs. 3 nmol/ml for U112 and *capB*, respectively). There were, however, some large differences in which the U112 values were higher than the *capB*

mutant (L-glutamine, 43 vs. 20 nmol/ml; L-tyrosine, 194 vs. 43 nmol/ml; and β-alanine, 27 vs. 12 nmol/ml), and others, where the *capB* values were larger (L-aspartic acid, 6 vs. 43 nmol/ml; glycine, 86 vs. 123 nmol/ml; L-leucine, 284 vs. 386, and L-phenylalanine, 117 vs. 170 nmol/ml). The amino acids potentially involved in pH homeostasis were glutamic acid and L-lysine (173 vs. 216 nmol/ml).

Additional free amino acid analysis was performed on samples from both strains grown in TSB-C, but centrifuged, decanted, and resuspended in M9 minimal medium plus 1% cysteine, then incubated for five hours at 37°C with shaking. This was performed due to prior phenotypes seen with this process (e.g. the immunoelectron micrographs) and the lack of additional amino acids in this media, which I hoped would simplify our results. I also prepared these samples by simply centrifuging, boiling the pellet for five minutes, and recentrifuging to collect both the lysate and the pellet. This was done to avoid any interference due to the SDS. As recorded, the pellet volume for U112 was 473 µl and the pellet volume for *capB* was 455.8 µl. As far as L-glutamic acid was concerned, there did not seem to be any differences between wildtype and *capB* in the lysate fraction (4.7 vs. 5.3 nmol/ml) (Fig. 28). There was, however, a difference seen in the supernatant fraction (1.2 vs. 2.5 nmol/ml; wildtype > *capB*) and in the pellet fraction (4.6 vs. 3.3 nmol/ml; wildtype < *capB*). The amino acid profiles for the lysate and the pellet were fairly consistent with each other, except perhaps the L-phosphoserine in the pellet fraction (0.9 vs. 0.1 nmol/ml). Other differences seen in the supernatant fraction were as follows: L-asparagine, 1.8 vs. 5.7 nmol/ml: glycine, 16.9 vs. 30.0 nmol/ml; L-tyrosine, 1.1 vs. 8.9 nmol/ml; and L-lysine, 2.5 vs. 10.4 nmol/ml.

To examine the total amino acid profiles of these M9 treated bacteria, the pellet and supernatant samples were dried slightly in a SpeedVac, then transferred to 10ml ampules for hydrolyzation. Two mls of 6N HCl (containing 200 nmol/ml norleucine as an internal standard) was added, then purged with nitrogen gas for 5 min, and sealed using an open flame. The ampules were then heated at 110°C for 24 hours, cooled, and then purged again with nitrogen gas to dry. 10 ml of 0.2M lithium citrate loading buffer was used to dissolve the samples, filtered through a 0.2 μm filter, and loaded into the Biochrom 30 Amino acid analyzer. The glutamic acid data from the total acid analysis was as follows: supernatant has 113.1 vs. 135.6 nmol/ml (wildtype < *capB*) and the pellet has 5.95 vs. 4.73 μmol/ml (wildtype > *capB*). There were not as many discrepancies in the total supernatant data as in the free amino acid data. However, I did note that L-aspartic acid was 52.0 vs. 67.6 nmol/ml and L-lysine was 23.1 vs. 32.4 nmol/ml. Amino acid data for the pellet fraction that were divergent: L-aspartic acid was 5.00 vs. 3.92 μmol/ml, L-alanine was 4.96 vs. 3.64 μmol/ml, and L-lysine was 4.17 vs. 3.31 μmol/ml. There was some data pointing to an increase in free glutamic acid in the supernatant of the mutant and of total glutamic acid in the pellet of the M9-treated experiment of the wildtype U112. However, there seems to be a decrease in glutamic acid in the total amino acid analysis of the supernatant when compared to *capB*. The glutamic acid data from the SDS-lysate pH 4 subjugation experiment was inconclusive.

**Figure 27. The free amino acid analysis profiles for both PGA extracts and SDS lysates performed under pH 4 condtions.** PGA extractions from bacteria subjected to pH 4 as well as SDS lysates under similar conditions were submitted to the Amino Acid Analysis Facility at UCDavis. There were no significant amino acid profiles seen for the PGA extracts, which only resulted in approximately 250 μM of O-phospho-L serine for both bacteria. Several amino acid differences were seen of the SDS lysates, however, glutamate concentration comparisons were unremarkable (4 vs. 3 nmol/ml for U112 and *capB*, respectively).

## Report of Complete amino acid analysis

**Amino Acid Lab, Haring Hall Rm 1069**

**UC Davis, Davis, CA 95616 (Tel.: 530-752-5058, Fax: 530-752-4698)**

**Cultural media samples from: USArmy (Eric Fleming)**
**Date of arrival: April 22 2009; Date of Report: April 24 2009**

| | SDS - Lysates | | TSB-C pH4 PGA | |
|---|---|---|---|---|
| | Lysate - (nmol/ml) | Lysate + (nmol/ml) | Lysate - (nmol/ml) | Lysate + (nmol/ml) |
| O-Phospho-L-serine | 3015 | 2973 | 227 | 256 |
| Taurine | 219 | 222 | | |
| L-Aspartic Acid | 43 | 6 | | |
| L-Threoine | 64 | 61 | | |
| L-Serine | 10 | 5 | | |
| L-Aspargine | 25 | 18 | | |
| L-Glutamic Acid | 3 | 4 | | |
| L-Glutimine | 20 | 43 | | |
| Glycine | 123 | 86 | | |
| L-Alanine | 50 | 39 | | |
| L-Citrulline | 2 | 4 | | |
| L-α- Amino - n Butryic Acid | 3 | 6 | | |
| L-Valine | 230 | 196 | | |
| L-cystine | | | | |
| L-Methionine | 50 | 40 | | |
| Cystathionine | 321 | 318 | | |
| L-Isoleucine | 116 | 84 | | |
| L-Leucine | 386 | 284 | | |
| L-Tyrosine | 43 | 194 | | |
| β-Alanine | 12 | 27 | | |
| L-Pheylalanine | 170 | 117 | | |
| L-Ornithine | | 4 | | |
| L-Lysine | 216 | 173 | | |
| 1 - Methylhistidine | | | | |
| L-Histidine | 27 | 27 | | |
| Tryptophan | | | | |
| 3 - Methyl-L-histidine | | | | |
| L-Carnosine | | | | |
| L-Arginine | 4 | 1 | | |
| Hproxy-L-proline | | | | |
| L-Proline | | | | |

**Figure 28. The free and total amino acid analysis profile for bacterial lysates, pellets, and supernatants from both bacteria grown in TSB-C, then treated with M9 minimal media plus 1% cysteine.** Although not enough samples were run to perform statistical analysis, the glutamate data for the free amino acid analysis fraction of the wildtype U112 supernatant presented double the amount of glutamic acid seen in the *capB* mutant, but these levels were generally low. Other supernatant differences include L-aspargine, glycine, L-tyrosine, and L-lysine. The only difference seen in the free amino acid pellet profile was O-phospho-L-serine. The total amino acid profiles demonstrated a slight difference in glutamic acid in the pellet (5.95 vs. 4.73 μmol/ml, wildtype > *capB*) and a slight difference in the supernatant (113.1 vs. 135.6 nmol/ml, wildtype < *capB*).

## Report of Complete amino acid analysis

**Amino Acid Lab, Haring Hall Rm 1069**
**UC Davis, Davis, CA 95616 (Tel.: 530-752-5058, Fax: 530-752-4698)**

**sample from: Eric Fleming**

**Date of arrival: May 19, 2009; Date of Report: May 22, 2009**

| Free amino acid | Measured concentration | | | | | |
|---|---|---|---|---|---|---|
| | Lysatc- (nmol/ml) | Lysatc+ (nmol/ml) | Supnt- (nmol/ml) | Supnt+ (nmol/ml) | Pellet- (nmol/ml) | Pellet + (nmol/ml) |
| O-Phospho-L-serine | 2.0 | 2.7 | 2.7 | 3.3 | 0.9 | 0.1 |
| L-Aspartic Acid | 8.1 | 9.4 | 12.9 | 12.6 | 6.6 | 7.6 |
| L-Threoine | 4.2 | 3.9 | 6.4 | 9.2 | 2.3 | 2.4 |
| L-Serine | 11.4 | 10.8 | 12.2 | 9.3 | 12.0 | 12.3 |
| L-Aspargine | 5.4 | 5.7 | 1.8 | 5.7 | 1.3 | 0.9 |
| L-Glutamic Acid | 4.7 | 5.3 | 1.2 | 2.5 | 4.6 | 3.3 |
| Glycine | 11.0 | 11.8 | 16.9 | 30.0 | 4.0 | 4.9 |
| L-Valine | 10.0 | 17.0 | 42.9 | 38.7 | 2.7 | 4.1 |
| L-cystine | 189.0 | 197.0 | 499.0 | 483.0 | 49.1 | 64.5 |
| L-Isoleucine | 10.0 | 9.1 | 22.1 | 31.0 | 2.1 | 2.2 |
| L-Leucine | 32.0 | 33.8 | 68.4 | 77.9 | 8.0 | 11.8 |
| L-Tyrosine | 4.2 | 6.2 | 1.1 | 8.9 | | |
| L-Pheylalanine | 9.6 | 12.6 | 21.9 | 31.5 | 2.1 | 3.4 |
| L-Lysine | 9.5 | 6.2 | 2.5 | 10.4 | 2.1 | 2.5 |
| L-Histidine | 3.8 | 3.8 | 4.8 | 5.1 | 1.2 | 1.1 |

*** 1) Pellet- has final volume of 455.8ml. 2) Pellet+ has final volume of 473ml

| Total amino acid | Bacteria Pellet | | Supernatant | |
|---|---|---|---|---|
| | P- µmol/ml | P+ µmol/ml | Supnt- nmol/ml | Supnt+ nmol/ml |
| L-Aspartic Acid | 3.923 | 4.998 | 67.6 | 52.0 |
| L-Threoine | 2.097 | 2.613 | 32.4 | 24.0 |
| L-Serine | 2.229 | 2.850 | 45.9 | 40.4 |
| L-Glutamic Acid | 4.730 | 5.953 | 135.6 | 113.1 |
| Glycine | 3.151 | 3.569 | 62.2 | 63.6 |
| L-Alanine | 3.642 | 4.956 | 37.4 | 26.1 |
| L-Valine | 2.887 | 3.543 | 80.4 | 70.2 |
| L-cystine | 0.254 | 0.245 | 488.4 | 547.6 |
| L-Methionine | 1.071 | 1.311 | 32.1 | 26.1 |
| L-Isoleucine | 2.703 | 3.290 | 56.5 | 51.7 |
| L-Leucine | 3.247 | 4.059 | 92.4 | 91.1 |
| L-Tyrosine | 1.308 | 1.666 | 21.5 | 18.2 |
| L-Pheylalanine | 1.632 | 2.072 | 48.0 | 41.5 |
| Ethanolamine | 0.702 | 0.989 | 13.3 | 13.3 |
| L-Lysine | 3.308 | 4.169 | 32.4 | 23.1 |
| L-Histidine | 0.667 | 0.913 | 12.3 | 11.4 |
| L-Arginine | 1.325 | 1.717 | 7.2 | 6.4 |
| L-Proline | 1.378 | 1.751 | 67.9 | 63.6 |

# CHAPTER FOUR: DISCUSSION

As stated earlier in this work, the capsule of *Francisella* has been an enigmatic entity for some time, and many researchers have attempted to discern its existence, its characteristics, and more importantly, its role in pathogenesis. *Francisella* was one of the most infectious organisms due to its infectivity and information regarding its unique ability to invade and persist in host cells, as well as its ability to evade both the innate and the adaptive immune system, would be valuable. Genetically, information regarding virulence genes of the pathogenicity island has vastly improved our knowledge of potential regulators as well as genes essential to intracellular survival. There was, however, a paucity of information on effectors and structural components of the outer membrane of *Francisella* (7, 16). Such information could be used to aid in the design of potential vaccine targets as well as potential antimicrobial peptides. Although the weaponization of infectious agents has been prohibited by most governments, the impact of such a bioweapon attack on any civilization would be catastrophic, and the consequences of being ill-prepared could alter the way we live. Use of other bioweapons in the recent past have been limited in scope, however, as the anthrax attacks in Washington D.C. of 2001 demonstrated that although prophylaxis would not be available to all, subsequent treatment of those potentially infected proved effective and that although the dissemination was not extreme, the radius of exposure was greater than initially predicted.

There was limited data on the structure of the outer membrane of *Francisella*. Extractions have been performed on the lipopolysaccharide of *Francisella novicida* and these experiments have uncovered many aspects that vary from the structure of *E. coli* LPS. It was known that the LPS from *Francisella* does not elicit the intense cytokine response from target cells via interaction with the TLR-4 receptor as other gram-negatives do, which has been attributed to differences to the absence of a phosphate group on the lipid A molecule as well as differences in glycosylation. It was known that this lack of phosporylation at the lipid A molecule gives *Francisella* resistance to polymyxins (62). It has also been determined that most of the lipid A molecules of the *F. novicida* membrane were without core or O-antigen polysaccharides (180, 184). These free lipid A molecules could be the source of other structural moieties of the outer membrane. Differences have also been noted in the acyl composition of the lipid A molecule, with *Francisella* not only missing an acyl group on the third hydroxyl but also having longer fatty acid chain groups (12-14 for *E. coli* vs. 16-18 for *Francisella*) (179, 181). Another structural component not fully understood was the aspect of phase variation of O-antigen polysaccharides. Blue variants of LVS have been described that have demonstrated greater virulence in animal models. These colonies, under certain conditions, such as passage through particular cells or continued culture, can appear to change morphology to what was known as the grey variant, with reduced virulence in animal models. It has been determined that this phase variation from blue to grey can be a change in the composition of the O-antigen chains of the LPS or modifications to the lipid A molecule itself, or potentially both. It was believed that *Francisella novicida* was

not subject to this phenomenon (40, 65). Such variations can influence intracellular survival and responses to both innate and adaptive immune processes (62). It has been shown that *Francisella* with certain O-antigen profiles were able to promote greater induction of nitric oxide, thus increasing intracellular killing.

The other known structural form on the outside of *Francisella* bacteria was the polysaccharide capsule, which has been characterized as possessing mannose, rhamnose, galactose, and two forms of fatty acid (68). These sugars were of a different composition than the O-antigen chains, and were sure to alter the outer moieties seen by aspects of the immune system (111). Although this encapsulation can be created in vitro via passage of bacteria in Chamberlain's defined media, neither the ingredients nor the in vivo conditions in which encapsulation predominates were known. It was thought that this capsule was an extracellular phenomenon and influences virulence as has been shown by both Chamberlain and Golovliov (30, 57). It has been difficult to attribute the characteristics of this structure, as capsule mutants have not been well characterized. Sandstrom's detailed work with *Francisella* mutagenized via acridine orange treatment as capsule mutants were believed to have been O-antigen mutants by several researchers. It was thought that the polysaccharide encapsulation enables these bacteria to be phagocytosed easier. It was believed that the mechanism of action for *Francisella* engulfment into monocytes was the polysaccharide: scavenger or mannose receptor interaction (35). Perhaps the polysaccharide moieties of this capsule enable these bacteria to be easily recognized and thus, engulfed by macrophages. It has been suggested that this capsule itself may have a role in the escape of these bacteria from the

phagolysosome and may act as a cascade signal to initiate intracellular growth. Clemens et al. (35) demonstrates that *Francisella* requires both complement and complement receptor interaction for the engulfment of bacteria by macrophages. There may be some interaction between complement and the capsule of *Francisella* which still eludes us.

The properties of polyglutamic acid (PGA) would make it a superb virulence factor, and as it has been shown in several gram-positive bacteria to aid in minimizing immune reactions, avoiding phagocytosis, effectively protect bacteria in stringent periods, and perhaps act as a source for trophism. The fact that the genes responsible for PGA synthesis were present and working in *Francisella* was great evidence for the existence of a PGA encapsulation as does many of the phenotypes presented by the absence of one or several of these genes. The generation of minicell *E. coli* with exogenously expressed *cap* genes was used to determine that the proteins of *cap A, B,* and *C* were all isolated from membrane fractions after trypsinization (107). This data, as well as the evidence of PGA production from *E. coli* reengineered with exogenous *cap* genes, demonstrates that PGA can be produced from bacteria other than gram-positives (9, 72). The *cap* operon itself from the pOX2 plasmid of *B. anthracis* was flanked by *IS231* insertion sequences, and theorhetically may be able to move from genome to genome. This initially may be how this sequence has ended up in so many different, unrelated bacteria.

As stated before, there have been many different transposon systems which have been developed for use in *Francisella*, including those used by the Kawula, Nano, and Weiss labs. Many of these, however, frequently were limited in genomic coverage and

have low transformation efficiencies (sometimes 1 mutant per $10^8$ CFU). There also have been problems with the stability of many of the *Tn5* based systems (54, 76, 104). As for our purposes, I was not interested in the creation of a mutant library and the genetic targets in which I was interested were already known. Therefore, I decided to investigate methods of site-directed mutagenesis to create our mutants in genes thought to be responsible for PGA capsule biosynthesis. Traditionally, many researchers have used the Maier series of plasmids (105) as models for suicide vectors for use in mutagenesis of *Francisella*. These vectors were initially designed as shuttle vectors between *Francisella* and *E. coli* and were still predominately used in most complementation of allelic exchange experiments in *Francisella*. These plasmid insertions were thought to be stable once in *Francisella*, but were limited in the methods of transformation. It was also found that working with these plasmids was not as easy as planned, as insertions of *Francisella* genomic DNA could periodically become reorganized upon propagation (105). Also, in order to use these plasmids as a suicide vector, several modifications were necessary such as the removal of ORFs responsible for plasmid replication in *Francisella*. Dr. Michael Harwich was credited with the addition of an *oriT* sequence in the pFNLTP7 plasmid so that it could be used in conjugation experiments for transformation. Two elements missing from these series of plasmids was a *Francisella* promoter to drive the kanamycin resistance cassette in single copy and a levansucrase gene (*sacB*) used as a counterselection marker so that resolution of the integrated plasmid sequence out of the *Francisella* genome would be possible, resulting in our unmarked deletion of our target gene (19). Due to problems attempting to correct for these changes however, I acquired

the targeting construct pGIR457 from Dr. Ramakrishnan through a material transfer agreement (MTA). She used the construct to create her mutant of the *fslA* gene in LVS. This construct contained both the *Francisella nuo* promoter which could be used to drive the kanamycin antibiotic cassette as well as the *sacB* gene as a counterselection agent. Growth of the merodiploid colonies on sucrose-containing media will select for excision of the plasmid DNA. Correct recombinants were then screened for loss of the antibiotic resistance but loss of the gene of interest. Simple insertion of our *Francisella* homologous DNA for *capB* via ligation of our PCR products into pGIR457 minus the *fslA* DNA also was difficult (162).

At this time, I acquired both *Francisella novicida* U112 and plasmids pKEK 887, 898, 923, and pKEK 1118 from Dr. Karl Klose via an MTA. The first three plasmids were basic target insertion constructs with the antibiotic cassettes erthryomycin, kanamycin, and chloramphenicol, respectively. The plasmid pKEK1118 was the construct used to ablate the *iglB* gene in *Francisella novicida*. All of these constructs have the FTN_1451 promoter driving their respective antibiotic cassettes as well as the universal primer sites, UniUP and UniDN, flanking the antibiotic cassettes. These constructs were capable of nonpolar mutations of any targeted gene, given the correct flanking sequences. As *F. novicida* is easier to manipulate genetically than its peers, I began to customize flanking regions of *capB* for insertion. Looking at the plasmid sequence of pKEK series, I determined that I could amplify the erythromycin cassette from pKEK887 with the universal primers and using PCR SOEing, link the antibiotic cassette with the *capB* flanking regions to create a single sequence. Once this sequence

was created, I used a basic *E. coli* vector such as pBlueScript SK + as our backbone construct to create the suicide vector pEF25. This also allowed us to utilize blue / white screening of recombinants (94).

In the past, I had attempted many methods for transformation of our vector DNA into *Francisella*, to include triparental conjugations, cryotransformation, and electroporation (54). The triparental conjugation experiments were time-consuming and not very effective, although other site-directed mutants have been created using this technique. It seems, however, that conjugation was a more effective method of transformation for species such as *F. holarctica* and *F. tularensis*. Cryotransformation was also attempted with limited success using transformation buffers with both magnesium sulfate and rubidium chloride to create *Francisella* competent cells. Liquid nitrogen as well as dry ice in ethanol was used in these processes (14, 120). Shuttle plasmids were transformed, but I had no successful attempts with suicide vectors. I ultimately used the electroporation technique of LoVullo et al. (97) to correctly transform *F. novicida* with pEF25. Electroporation easily was the more effective method of transformation of *Francisella*, although it does present problems of potential aerosolization of bacteria, which may not be an option if using type A organisms. It involves the creation of competent *Francisella* via washes in 0.5M sucrose, and these cells can be stored at –80°C for extended periods of time without losing their competence (97). Our electroporation efficiency for the creation of our *capB* mutant was approximately 10%, which was lower than what was experienced by Klose using cryotransformation in his creation of the *iglB* mutant (94).

The mutant was verified through RT-PCR of isolated RNA for primers specific for *capB* as well as positive controls of other neighboring genes, including the immediate downstream transcript of *capC*. Its presence in both the wildtype U112 and the *capB* mutant demonstrated that the mutation created was indeed nonpolar, so that ideally, any change in phenotype seen could be attributed to the loss of *capB*. To also ensure that plasmid DNA had not inserted elsewhere in the genome, or that there may be an inadvertent insertion of our antibiotic cassette someplace else in the genome, Southern blotting of the *capB* DNA was performed and validated by the singular presence of our antibiotic cassette in the *capB* gene (data not shown).

It was seen with the reengineered *E. coli* that the PGA production was much lower than what was seen with other *Bacillus* species, in the range of 24 mg/L. These genes were under the direction of the pTrc promoter of the expression plasmid (9). Jiang et al. (72) was able to produce greater amounts of PGA (3.7 g/L) with his reengineered *E. coli* expressing the PGA genes of *B. subtilis* by maximizing culture conditions and using the constitutive promoter of the D-amino acid aminotransferase gene of *Geobacillus toebii*. There has been no detailed characterization of the promoter region of the PGA genes in *Francisella*, and although it has been shown that there was active expression at this location via microarray data, there was no knowledge of the type of regulation of these genes. I used many of the culture conditions known to aid in the biosynthesis of polysaccharide production as well as traditional conditions known to increase PGA production in bacteria such as *Bacillus* and *Staphylococcus epidermidis*.

Our initial visual analysis for the production of PGA via classical light microscopy with nigrosin was negative as well as attempting to use colony morphology growth on Congo Red plates (91, 128). It was apparent from our nigrosin stains of wildtype bacteria that I would not be able to use this technique as a diagnostic for the loss of PGA. This was due to the relative small size of the bacteria, the lack of specificity of the technique (PGA vs. polysaccharide), as well as the potential low production of PGA. Due to the fact that *F. novicida* does not appear to form as many O-antigen chains as LVS, *F. novicida* was able to bind greater amounts of the dye than LVS and presents a deep red, crimson color vs. the lighter pink of LVS (179). Variable shades of pink were also possible due to changes in O-antigen composition as was seen with phase variation. It was thought that if there was extracellular membrane production of PGA that its polyanionic nature would prevent the binding of this dye, resulting in lighter color colonies of the wildtype U112 in comparison with the *capB* mutant. This, however, was not seen as both bacteria were similar in both color and colony morphology, even at the highest magnification possible. Even in experiments with both 1M NaCl and 50mM L-glutamic acid, there still was no apparent morphological difference between these two bacteria.

Our experiments with sodium deoxycholate, however, pointed to there being an ionic exchange difference at the membrane between the wildtype U112 and the *capB* mutant. As stated before, this detergent has been used to elucidate ionic barrier differences in O-antigen mutants of *Francisella* (39, 91). With the statistical difference seen between these two bacteria at the higher sodium deoxycholate levels, there appeared

to be ionic differences. It was unclear whether this difference was due to lack of polymer at the cell surface allowing greater permeability for the detergent or perhaps a subcellular difference in charge responsible for this phenomenon. Measurement of the voltage potential between the membranes of these bacteria may uncover whether this occurrence was strictly due to charge differences in the bacteria. Other experiments such as the acid resistance assays and the growth advantage seen with culturing in high NaCl media may be explained by differential properties of ion exchange between the two bacteria. There could be many possibilities for this potential electrical potential difference (131). It was well established that modifications to the murein lipoproteins, which act as a bridge between the peptidoglycan layer and the outer membrane, can cause changes in membrane permeability and electrical potential. Alterations to selective porins could also cause such changes. Perhaps additional membrane permeability experiments could be performed to clarify this hypothesis.

The results from our serum sensitivity assays suggest that there was no protective difference between the wildtype U112 and our *capB* mutant when treated with preimmune serum. Serum sensitivity was usually a measurement of complement's ability to first bind to the bacterial surface and then become activated to form MAC complexes. Complement's ability to bind to the surface was usually blocked by the O-antigens of the LPS, although base components of the LPS were known to help activate complement. Neither the interaction nor the effects of the binding of complement and the capsule of *Francisella* were known. It was known, however, that complement as well as complement receptors have an integral role in opsonization and phagocytosis of these

bacteria (35). The capsule mutants of Sandstrom were shown to be serum-sensitive and many of these mutants survived by being phagocytosed prior to MAC complex formation (140). As stated above, however, these mutants were now thought to be O-antigen mutants. A recent finding was that *Francisella* was capable of binding factor H, which helps in the inactivation of the alternative pathway. It was believed that *Francisella* intrinsically has defense mechanisms against both the classical and alternative pathway for complement activation (18, 34). It was not known whether encapsulation of the bacteria either by PGA or polysaccharides would influence the level of complement killing or the mechanism of action via factor H. In *B. anthracis*, PGA capsule mutants were shown to be phagocytosed in the absence of complement, suggesting that the binding of complement was not essential for phagocytosis (25). It has been shown, however, that PGA does protect against some aspects of the innate immune system such as protection of *S. epidermidis* from defensins and neutrophil granules, which tend to be cationic in nature (79).

Our data demonstrated a decrease in intracellular replication of our *capB* mutant vs. the wildtype U112 when infecting RAW 264.7 murine macrophages at both two hour and 24 hour timepoints. Although I saw an increase in CFU of our wildtype bacteria at MOI 100 after two hours, I believed the overall difference in CFUs was due to properties of these bacteria in regards to their intracellular replicative abilities, not a difference in binding and engulfment by macrophages. In theory, the PGA encapsulated wildtype bacteria should have reduced numbers of bacteria engulfed by the macrophages (79). This was seen with the lower amount of PGA production of *S. epidermidis* in comparison

to that of *B. anthracis*. If both bacteria had equal intracellular replicative potential, there should be lower numbers of bacteria in our wildtype preparations when compared to the *capB* mutant, due solely to the reduction in engulfment. *CapB* mutants of LVS have been shown to have lower bacterial burdens in infected animal models, both in co-infections as well as direct comparative studies between wildtype and mutant bacteria. Catheter infections of *capB* mutants in *S. epidermidis* resulted in reduced bacteria isolated from these sites in in vivo murine subcutaneous models when compared to wildtype. These reductions were independent of any biofilm formation. Similar *Francisella* mutants of intracellular growth have also displayed both reduced bacterial burdens in animal models as well as reduced in vitro macrophage infection. There were, however, some *Francisella* mutants which display in vivo attenuation without differences in intracellular replication (186). Differences in dissemination potential could also cause a difference between these two variables. It should be noted that purified PGA was found to disseminate to the liver and spleen, specifically to Kupffer cells, sinusoidal endothelial cells, and splenic macrophages, all known targets of *Francisella* infection (163, 164). Therefore the reduced bacterial burden in animal model experiments could be the result of both reduced intracellular replication as well as reduced dissemination.

The reduced level of PGA production in *S. epidermidis* may be an excellent start to determine whether *Francisella* was capable of producing a PGA capsule. This bacteria has already shown that it produces PGA at a much lower level than most of the *Bacillus* species (1.2 ug/L vs. 5-10 g/L), and most of our initial work shows that if PGA was being made by *Francisella*, it may be in an amount less than *S. epidermidis*. Our initial

immunoelectron micrographs with both bacteria grown in either TSB-C or CDM media have no specific reactivity when using the antibodies against PGA. There was diffuse material with gold particles localized seen in these immunoelectron micrographs, which I believe was capsular material. However there was no loss of either the gold particles or the diffuse material in the mutant preparations. Gold particle localization to the diffuse material seems more specific in the F26G3 antibody preparations than the polyclonal to *B. pumilus*. There were many different media supplementations as well as culture conditions used to try to induce PGA production in *Francisella*. Many of these conditions presented secondary phenotypes seen which would suggest the production of PGA. These conditions include growth at high NaCl concentrations (1M+), stationary growth at low pH (pH 4), and resuspension in minimal media plus 1% cysteine. However, for most of these conditions, there was no differential seen in immunoelectron micrographs between the wildtype and the *capB* mutant. It was unlikely that any polysaccharide encapsulation of *Francisella* would render a PGA capsule undetectable by immunological methods as the PGA capsule of *S. haemolyticus* was still visible with or without polysaccharide encapsulation (49). It also provides support for the biosynthesis of two very different polymers for encapsulation in the same bacteria, depending upon culture or environment. It was not known how much PGA was produced from *S. haemolyticus*, but it was stated that it was on the level of its relative *S. epidermidis*.

It was shown that our *capB* mutant had growth defecits when grown in 1M NaCl or higher in both TSB-C and CDM. This was very similar to the data seen with *S. epidermidis*, in which the PGA capsule was suggested to aid in the binding of water

molecules due to its anionic nature. However, it was also suggested that there may be other methods of protection provided by PGA, due to the limited amount made by this bacteria. One simple aspect was that in the gamma linkage reaction by the amide ligase between glutamate residues, a water molecule was generated. This water molecule would, in itself, increase the turgor pressure inside the cell (25, 48). There were instances of polyglutamylation of various proteins and other molecules in order to make them more hydrophilic. Certain tRNAs have been shown to undergo such modification as well as eukaryotic microtubules. In the case of microtubules, the addition of several glutamate residues to an existing glutamate residue of α- or β-tubulin takes place under certain conditions. Polyglutamylation of tubulin was thought to increase the affinity of tubulin for specific MAPS (microtubule-associated proteins) and has been shown to important in centriole stability as well as axonemal motility. This phenomena, however, has not been shown in prokaryotic bacteria, only in eukaryotes. Nor has any clear consensus sequence for polyglutamylation been discovered, although sites of polyglutamylation usually occur at acidic stretches of amino acid sequence containing glutamate (178). Another instance of polyglutamylation to stabilize molecules was the polyglutamylation of folate derivatives by folyl-polyglutamate synthease. It has been shown that four to seven glutamate residues were added to tetrahydrofolate to increase the transport properties of this important coenzyme (64). Such modification of enzymes could be an additional component to surviving growth in high salt and other harsh environments and may be direct or indirect in which chaperone proteins could be modified to protect against the denaturation of other proteins.

There were many facets to osmoprotection which should be discussed. Most enterbacteriaceae, as non-halophilic bacteria, have a salt tolerance up to 1.5 M. Several of the gram-positive bacteria can withstand slightly higher salt concentrations due to increased peptidoglycan. Also bacterial membrane integrity was partially dependent on cations interacting with both the negative components of LPS as well as the anionic amino acids of the peptidoglycan layer (38). For most gram-negative bacteria, as the environment becomes more hypertonic, the bacteria initially attempts to compensate for the solute imbalance by increasing the intracellular potassium ion concentration. In response to this influx of potassium, the cell induces creation of glutamate to mainly act as a counterion to the rise in potassium (21). Potentially, our *capB* mutant may have deficiencies in this arena as well, perhaps unable to import or create this level of glutamate internally, or unable to maintain a steady pool of glutamate to counteract the potassium. Deficits in internal glutamate during periods of hyperosmolarity have resulted in reduction of growth. The internal pool of glutamate was maintained by the action of three enzymes: glutamate synthetase, glutamine synthetase, and glutamate dehydrogenase. The cycle between glutamine and glutamate has been described as glutamine being the internal barometer of nitrogen availability and glutamate being responsible for up to 88% of the cell's nitrogen (189). In addition, high levels of intracellular potassium were not healthy for the cell, and upon long periods of high osmolarity, different molecules were used as substitute counterions, for the potassium was then effluxed. Different compatible solutes can be used by the same bacteria and some of these include glycine betaine, ectoine, choline, and trehalose (77). It was

believed that the uptake and synthesis of these molecules can be dependent on potassium-glutamate levels. It was not known whether polyglutamylation has any effect on this form of regulation. Cyclic ionic amino acids, such as proline, tend to increase in concentration during these stressful periods and act as compatible solutes (98). Could the amide ligase be responsible for a cyclic form of glutamate, similar to β-glutamate by being metabolically inert, yet functions as a counterion? In marine methanogens, salt tolerance was maintained by using both α- and β- forms of glutamate as counterions to potassium. The β-glutamate was unique in that it was not incorporated into other proteins due to its configuration. It has been described as a compatible solute that was metabolically inert (135, 136).

Very little information has been obtained regarding iron limitation and its effects on *Francisella*. The discovery of siderophoric molecules for *Francisella* was a fairly recent find and it was still uncertain how these siderophore-iron complexes were acquired by the bacteria, as only one potential iron transporter gene has been discovered upon genomic analysis. It has been shown that iron-starvation of LVS in Muller-Hinton broth minus iron requires an overnight incubation prior to analysis for iron-deplete growth, whereas *F. novicida* did not require such prestarvation. It has been suggested that different species of *Francisella* may have various methods for retaining iron (42, 90, 162). The anionic nature of PGA would suggest that perhaps it was used to sequester or even attract cationic molecules. It was known that ferric iron binds to PGA better than ferrous, and that this binding was primarily based on the oxidation state of the iron ion, although aerobic conditions also favor ferric binding (109). Studies have demonstrated

that, of the various cations usually present in media, the ferric iron has the highest affinity for PGA compared to its peers, followed by calcium. Another phenomenon seen with cation-capsule mixtures from *B. licheniformis* was the reduction in pH without the capsule. The interaction between the anionic amino acid and the metal ion would release a proton, lowering the pH of the mixture (110). Overnight cultures of both our strains would usually result in the *capB* mutant having a slightly lower pH than its wildtype counterpart. Further work was required to determine whether this was due to cation-capsule interactions. Additionally, metal-binding to anionic capsule may alter its structure and other properties inherent to PGA. As far as magnesium was concerned, there was no difference seen in the growth curves between our wildtype U112 and the *capB* mutant. It has been shown that increases in $MgCl_2$ have increased PGA production in species such as *Bacillus*. The magnesium was used by the ATPase in the formation of the polymer. Concentrations as low as 1mM of $MgCl_2$ have been shown to be adequate for PGA production in *B. anthracis* and in the reengineered *E. coli*. I tested incremental concentrations from 0.5 to 200 mM of both $MgCl_2$ and $MgSO_4$ and no evidence of PGA production was evident, nor was there any difference in growth between the two bacteria. Although manganese has been shown to be an adequate substitute for magnesium at a much lower concentration (μM vs. mM), and, in some cases, produces more PGA than $MgCl_2$, similar experiments performed with $MnCl_2$ were as unproductive (6, 9).

Our overnight growth curve experiments with various concentrations of sodium bicarbonate and 10% carbon dioxide caused increases in the pH of the media as expected, which was the probable cause for the differences seen in the amount of growth. It was

reasonable to believe that the increase in bicarbonate made the media too alkaline for optimal growth. Interesting, the pH of the *capB* mutant was consistently lower than the pH of the wildtype U112. These findings will be addressed in the acid resistance section of this document. The increase in bicarbonate also increased the viscosity of the supernatant for both bacteria, which was collected for further study. The carbon dioxide was used in anaplerotic $CO_2$ fixation reactions by *Bacillus* to maintain the balance of many precursors to nucleotides and amino acids, such as oxaloacetate and α-ketoglutarate. These were both intermediates in the tricarboxylic acid cycle and α-ketoglutarate was one of the key components in the synthesis of glutamate. It was believed that *Francisella* could possibly use similar anabolic pathways for the genesis of glutamate for PGA biosynthesis. It seems, however, that the viscous material isolated from the supernatants was mostly sugars from the polysaccharide encapsulation. Both the wildtype U112 and the *capB* mutant had similar viscosities and anthrone assay measurements.

As described earlier, many different capsule isolation techniques were attempted to procure PGA from our strains under different culture conditions. The majority, however, of these procedures required multiple ethanol precipitation steps, in which most ionic polymers would aggregate including polysaccharides and PGA. It was not known whether PGA production in *Francisella*, if possible, was secreted or attached to the outer membrane or peptidoglycan layer (24, 26). Many of the PGA isolation procedures for bacteria such as *B. licheniformis* were techniques applied solely to the supernatant. Conversely, there also were procedures in which the supernatant was removed and PGA

isolation was performed as washes to the bacteria itself. And without an inexpensive and accurate measure for PGA, our selection criteria for an extraction were limited. However, after narrowing our selection down to the *B. anthracis* PGA isolation of Dr. Chabot and the *B. anthracis* PGA isolation of Dr. Kozel, I isolated our samples using the Kozel technique. This sodium acetate / glacial acetic acid procedure would allow us to collect material from both the supernatant and washes from the bacteria, and as stated previously, our limited analyses from these samples were consistent. These samples were stored at –20°C as several papers cited the degradation of PGA polymer without frozen storage.

As with many of the immunoelectron micrographs performed under various conditions, larger scale inoculations of bacteria under similar conditions were used in order to isolate PGA from these bacteria. Immunoblots were performed on bacterial extracts at 1M NaCl, 50mM $MgCl_2$, 10% $CO_2$ plus sodium bicarbonate, stationary growth at pH4, and controls. None of the wildtype U112 extracts produced positive signals with the monoclonal anti-PGA F26G3, which was shown to have reactivity with *B. licheniformis*, but not *B. subtilis*. There were signals seen for the wildtype U112 at most of these conditions with the polyclonal, but usually there was no differential between the wildtype and the mutant. This polyclonal was against *B. pumilus* and was used in similar experiments in immunblots with *S. epidermidis*. It has been stated that this bacteria only produces a fraction of the PGA seen in other *Bacillus* species, yet the polyclonal was able to discern PGA production between a *capB* mutant and wildtype *S. epidermidis* (79). The control blot demonstrated reactivity of the polyclonal with PGA

from both *B. licheniformis* and *B. subtilis*. With the following data, there may be limited specificity with the polyclonal as it seems to bind slightly to all of our extracts (controls, wildtype, and deletion mutants). More information about these antibodies would be beneficial to being able to discern the data presented, as no crossreactivity experiments with these antibodies has been performed against various forms of PGA. Another bit of information which would be useful was the extent to which these antibodies can bind and the specific epitope profile. The PGA formed from the control bacteria was usually composed of polymer of larger MW, such as 100-1000 kDa. Candela and Fouet (25) show antibody-PGA interaction at intervals of 36-38 nm. It was not known what length of polymer was necessary for stable antibody interactions. Was it possible that *Francisella* was capable of short polymer production, which would provide many of the properties discussed so far, yet unable to interact with antibodies designed against larger structures? Antibodies to *B. licheniformis*, which produces (D+L) PGA filaments, recognizes PGA produced from *B. anthracis* (D), but would it recognize the rarer (L) PGA filaments produced by some organisms? Greater information was needed regarding the crossreactivity abilities of these antibodies and the extent of interaction depending on polymer length.

Most of the protection mechanisms described for protecting bacteria during periods of high osmolarity will apply to desiccation as well. Due to the prolonged nature of desiccation, the dynamics of potassium-glutamate should only apply to short-term exposure. Long exposure to slow desiccation under aerobic conditions would involve oxidative stresses and protection of necessary proteins. The accumulation of compatible

solutes highly benefits this situation and work by the suggested hypothesis of preferential exclusion. The thought was that the build-up of these preferably metabolically inert molecules, which can be at low molar levels of concentration, were not in contact with essential proteins and molecules. This "exclusion" creates a water layer of the remaining water molecules around proteins so that it is energetically favorable for the protein to maintain its conformation and configuration (123). *Francisella* does not possess genes for sporulation yet has a substantial desiccation tolerance when compared to other persistent bacteria such as *Mycobacterium tuberculosis*, *Yersinia pseudotuberculosis*, and *Moraxella bovis*. Indeed, *Francisella* has been shown to have comparable desiccation tolerance to *Streptococcus pyogenes* and *Corynebacterium diphtheriae* (155). There was a lack of dipicolinic acid in *Francisella*, however it was believed that there were other aspects to *Francisella* which give it unique persistence characteristics. In our experiments, I did not see CFU formation at our standard dilution of $10^{-8}$ after three days. These results were seen after incubation in minimal media plus 1% cysteine. Reproduction of this experiment in a further reduced media may increase our desiccation potential, or perhaps a lower dilution. It has been shown that for *Francisella*, persistence was greater when the level of relative humidity was low. In fact, these bacteria have been shown to have the lowest survival rate at high relative humidity in aerosol experiments when compared with several of its peers (155). Although our experiments were performed in a dry incubator at 30°C, perhaps further timepoints could be achieved by reducing the ambient humidity. Another aspect of desiccation was the extent of nutrient starvation that can occur. It is known that glutamate was usually the source of most

nitrogen in the bacterial cell. Could short oligmers of PGA act as a storage stockpile for additional nitrogen and carbon in times of nutrient starvation? Another gene which has shown a similar importance as far as virulence in animal studies was cyanophycinase, which in cyanobacteria was responsible for the breakdown of cyanophycin in times of nutrient starvation as a nitrogen reserve (55). As can be expected, there was a paucity of information on desiccation of any the bacteria on the select agents list. The resuscitation of *Francisella* after particular stresses was another factor to consider, as passage through an animal system has been shown in several types of bacteria to be necessary after experiencing stringent conditions for viability. The plating of the desiccation-stressed bacteria onto rich media agar may not provide the appropriate conditions necessary for colony growth. Additional CFUs may have been demonstrated on another agar type, such as cysteine heart agar with defibrinated blood.

One of the interesting phenotypes I experienced with the *capB* mutant was the acid resistance of the wildtype U112. Most neutralophiles can tolerate growth in media within a range of pH from 5 to 8. Some of these bacteria have developed systems to exist at a slightly greater range, however, the internal pH must remain between 6.5 and 7.5 to avoid damage to organelles and macromolecules (11). Potentially, I learned that several of these responses to external stimuli could be related. There was evidence, in the case of *E. coli*, that changes in external sodium levels may have an effect on pH homeostasis (132). There were many integrated systems involving passive and facilitated ion flux at the membrane, external and internal stimulation resulting in induced or repression at the transcription level, proton movement at the membrane, and potential post-translational

modifications. The decrease in external pH initially results in influx of potassium, thus resulting in the efflux of protons via the potassium / $H^+$ antiport system in an attempt to increase the intracellular pH. It was important to remember that the cell will attempt to maintain the proton motive force so that protons will flow down its gradient into the cell in order to produce ATP. Also, the influx of potassium will create a similar scenario to high osmolarity, as internal glutamate production will be induced as well as increased influx of this amino acid to counteract the positive potassium. As the media decreases in pH, most enteric bacteria use amino acid decarboxylation systems to maintain its intracellular pH. The *E. coli* model was the standard for these systems as it utilizes three different amino acid decarboxylation mechanisms to maintain pH homeostasis. These systems include arginine, lysine, and glutamate decarboxylation. Each of these amino acids was decarboxylated to agmatine, cadaverine, and γ-amino butyric acid (GABA), respectively. Each of these systems has different methods of induction and was preferentially used in different stages of growth (17 ,28). *F. novicida* has only two of these systems, lysine and glutamate decarboxylation. The glutamate decarboxylation system was the most robust of the three and was used in periods of low pH (<3) and in stationary phase. The decarboxylation reaction itself consumes an intracellular proton and all of these systems rely on substrate exchange, so that in the case of glutamate decarboxylation, glutamate was converted to GABA, which was then exported out of the cell in exchange for fresh glutamate.

There were also several different regulatory factors which were thought to be involved in controlling these reactions. Regulatory elements such as RpoS, PhoP, and

Fur have been shown to modulate and induce acid shock proteins which were used to physically deal with damage to macromolecules from exposure to low pH (88). It was interesting to note that the Fur protein appears to have both the ability to repress iron-related genes as well as activate acid shock genes independent of iron. The PhoP / Q system was a two-component system which has been demonstrated to be important in intracellular survival, but that PhoP can also act as an acid shock protein. It was not known whether a *Francisella* analog has been annotated for this two-component regulatory system (139). RpoS, on the other hand, was responsible for regulating a host of genes necessary for stationary phase, low pH, oxidative stress, and heat tolerance. RpoS has shown increases in translation but not in transcription in response to changes in concentration of inorganic fatty acids, which was directly related to the internal:external pH gradient. It was believed that these inorganic acids as well as anionic molecules such as polyamines can be directly responsible for increases in the amino acid decarboxylation system (27, 73). Polyamines were polycationic molecules such as spermine, cadaverine, and putrescine, which can be generated de novo or taken in from the environment, and can reach millimolar concentrations inside the cell. Some of these polyamines were potential byproducts from the amino acid decarboxylation reaction themselves. Jung and Kim (73) have published research on *E. coli* which indicates that polyamines have a negative effect on cAMP levels, which in turn, has a negative effect on glutamate decarboxylation. Polyamines have also been shown to scavenge oxygen free radicals which were created due to the modifications in pH and aeration. Therefore, they were involved in the protection of both nucleic acids and proteins. It has been demonstrated in

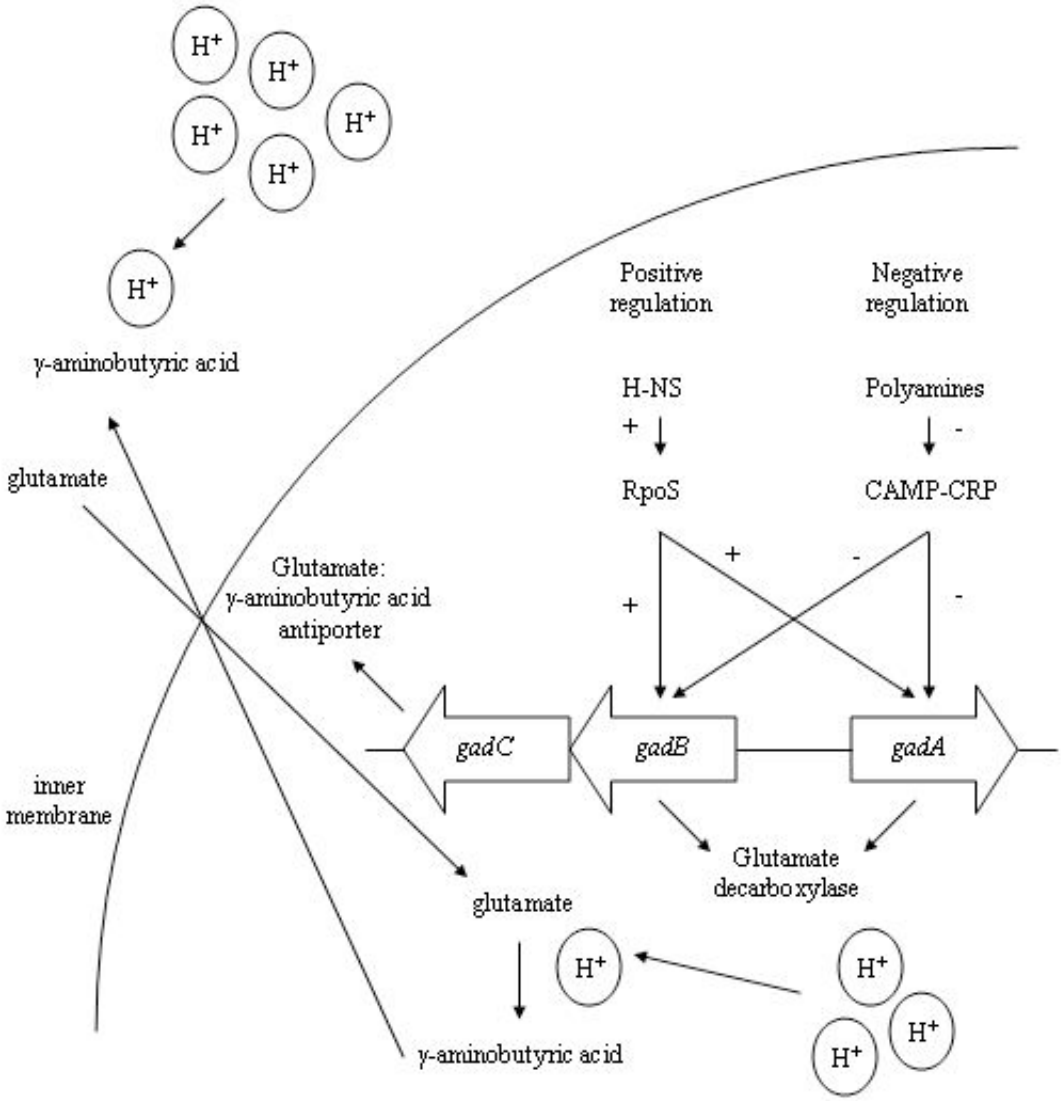
*Streptococcus pneumoniae* that uptake of polyamines increases due to increased production of polyamine transporters during periods of oxidative stress. Polyamines have also been implicated in decreasing outer membrane permeability, enhancing phagolysosome stability, and promoting apoptosis of polymorphonuclear cells (151).

Specifically, the glutamate decarboxylation system was made up of enzymes responsible for the decarboxylation event and the antiporter which effluxes the byproduct of the reaction as well as influx of fresh glutamate at the inner membrane. In *E. coli*, two genes encode two versions of glutamate decarboxylase (*gadA, gadB*) and the antiporter (*gadC*). In *F. novicida*, only one gene has been annotated as a glutamate decarboxylase (FTN_1701) and the antiporter has been suggested to be encoded by FTN_1529. Mutations in these types of genes produce organisms that have limited acid resistance. Also in *E. coli*, it has been shown that the sodium ion concentration has influence on *gadX* and *gadW*. These two gene products were known to regulators of *gadE*, which was a direct activator for the glutamate decarboxylation system. The sodium ion influence on these two regulators appears to be posttranscriptional. Therefore, the same influx of sodium experienced during exposure to 1M NaCl or the indirect sodium increase due to desiccation could also potentially modify the activity of glutamate decarboxylase or the exchange of glutamate for GABA (100, 102, 132). Dr. Foster's experiments with the GAD assay provides two types of information. First, by sampling bacteria with the GAD reagent with Triton X-100, one was able to determine the relative level of glutamate decarboxylation intrinsic to that bacteria via the cytosolic process, ie. *gadA, gadB*. The decarboxylation of glutamate results in the consumption of a proton, the generation of

$CO_2$, and GABA. Sampling of the bacteria with the GAD reagent without Triton X-100 measures the ability of the bacteria to exchange the substrates, ie. *gadC*. It was thought that GABA exchange also will help to neutralize the surrounding media (28, 130). Our data demonstrated that while both the wildtype U112 and *capB* had the capability to decarboxylate glutamate, the *capB* had a reduced ability to neutralize acidity outside the inner membrane. This reduced ability may explain the mutant's reduced growth in our acid resistance studies as well as the difference in pH of inoculations between the two bacteria. Could the reduced ability for *capB* to exchange substrates in the glutamate decarboxylation process result in reduced intracellular glutamate during these periods of acid stress? Mutants of the reengineered *E. coli* without the *cap* operon demonstrated a reduced amount of radioactive internal glutamate when compared to *E. coli* with the *cap* operon (107). If there was a differential in glutamate / GABA exchange, it may be interesting to determine whether the lack of influxing glutamate or lack of effluxing GABA or both was the source of acid sensitivity seen. It has also been shown in the *E. coli* system that synthetic glutamate analogs have acted as inhibitors to this system as well (185). Our data from the acid resistance experiments demonstrated that *Francisella* does not seem to have a functioning log phase acid resistance system. Although some of these systems have been implicated in log phase protection, other mechanisms apparently have greater importance (perhaps ATPases or a functioning arginine decarboxylation system).

Another aspect of pH homeostasis should be considered and discussed as well. There have been experiments performed in which the conversion rate of glutamate to

**Figure 29. Model of glutamate decarboxylation in *E. coli*.** This image demonstrates the relationship between glutamate decarboxylation activity and the exchange of fresh glutamate for GABA at the antiporter of the inner membrane. Many different control mechanisms were thought to be involved in the regulation of glutamate decarboxylation, such as polyamines, cAMP, and RpoS involvement.




adopted from Jung I. L. and Kim I. G. Journal of Biological Chemistry 278:22846-52

GABA was compared to conversion of arginine to agmatine, resulting in a 3:1 ratio. It has been proposed that this greater ratio was necessary due to the chemistry involved in the decarboxylation reactions. The site of decarboxylation for glutamate was the carboxyl side chain which has a $pK_a$ of 4.25. Simply stated, this group will remain protonated at pH < 4.25. One of the largest objects of discussion amongst acid resistance researchers was that as glutamate enters the cell in a low pH media, this R-group will be protonated, only to lose that proton once inside the cell, as the pH will probably be higher than 4.25. It was believed that decarboxylation then occurs, consuming that proton in the conversion to GABA. Many feel that there was some aspect to the system which was missing, as there wasn't a net loss of protons upon GABA exchange. Proponents of the utility of this system believe that the ratio of decarboxylation was the key to this conundrum, or perhaps, additional utilization of de novo glutamate which was protected from deprotonation prior to decarboxylation. Still another theory was that glutamine can also participate in this exchange and that glutamine dehydrogenase occurs first to create intracellular glutamate, which was then decarboxylated. The $pK_a$ of the R group of glutamine was 8.18, which means that this group would probably be protonated at most physiological conditions.

The capability of acid resistance for many pathogenic bacteria provides them with the ability to pass through harsh environments such as the host's stomach in the case of *Salmonella* and hemorrhagic *E. coli*, to survive in acidic foods such as *Listeria monocytogenes*, or to survive phagolysosomic acidification in macrophages or PMNs (11, 92). It has been thought that the passage of *Francisella* in the pathogenesis of

macrophages was that, after looping phagocytosis by the cell, the bacteria escape from the early endosome shortly after lysosome fusion. It has been shown that no acidification of these vesicles was necessary for escape and that this acidification was arrested (35, 144). These transitions were observable due to specific markers used for identification the maturation stages of phagolysosomes and visualization by immunoelectron microscopy. Early endosomal markers such as EEA-1 were seen in infected vacuoles very early in infection and subsequent turnover of these markers for LAMP-1 does occur, but at a fairly low frequency, with less than 20% of infected bacteria inside vacuoles positive for LAMP-1 by one hour of infection. The majority of bacteria were found in the cytoplasm, where for the next four to 24 hours, replicate in the cytoplasm. It was believed that the multiplied bacteria then signal for apoptosis of the host cell and escape to infect other cells. However, a seminal paper by Checroun provided evidence of entry of these bacteria into autophagic vacuoles hours after infection and that these vacuoles were acidified, staining positive for LAMP-1 and cathepsin D. Both of these markers were indications of lysosome fusion and acidification (31). These bacteria-filled vacuoles were also positive for MDC or monodansylcadaverine, an autophagic probe, and LC3, an autophagosomal membrane-associated protein. Additional proof of acidification of these autophagic vacuoles was the accumulation of LysoTracker Red DND-99 dye, which permits estimation of the pH of the vacuoles. It has been suggested that this method of intracellular trafficking may be dependent on IFN-γ activation. In addition, autophagic processes have been observed to occur as early as one hour after infection.

The knowledge of this transition, however, may provide insight into the virulence of the wildtype U112 when compared with the *capB* mutant. I have experienced several phenotypes which have demonstrated the acid resistance of the wildtype. This resistance has been shown to occur after stationary phase growth as there was no growth seen at low pH (pH 4) during log phase growth. These conditions would be very similar to the pathogenesis of *Francisella* in macrophages. It was thought that *Francisella* did not require genes necessary for acid resistance due to the fact that the bacteria were found to escape the phagolysosome prior to lysosome fusion. This premise must now be reexamined as these genes now may be necessary to allow *Francisella* to survive residence in these autophagosomes. *Francisella* does possess lysine decarboxylation genes in addition to the glutamate decarboxylation machinery and could potentially use both of these mechanisms to protect itself while in the autophagosome. As *Francisella* does not seem capable of growth at these low pHs, it would make sense to escape such exposure early in infection, but once the bacteria have replicated to a suitable number (ie. after cytoplasmic replication), survival at low pH would give these bacteria an appreciable advantage. This information would help explain the reduction of bacteria in the *capB* mutant in our macrophage infection model as well. With the inability to withstand the low pH of the autophagosome, replicated bacteria of the *capB* mutant would be subject to hydrolases once entering the autophagic pathway. It was not known how the lack of an amide ligase would result in reduced glutamate exchange, thus reduced acid resistance. It was also not known whether the autophagic process was mediated by the bacteria or by the host, as examples of both scenarios do exist (41).

Another question that requires investigation was iron acquistion for the replicating bacteria. It has been shown that acidification promotes the release of free iron and was a must for many intracellular pathogens in order to acquire iron. Perhaps the siderophores produced by *Francisella* were transiently required during intracellular replication and other, less efficient iron acquistion methods were necessary during autophagosome residence (162).

Our ESI/MS submissions suggest that the 1M NaCl data for our wildtype U112 and *capB* extracts did not resemble the mass/charge profile for PGA from *B. licheniformis*. Limited identification of the source of the peaks for the wildtype U112 and *capB* can be made and comparison to MS data from LPS samples from the literature was not informative (181). Additional separation of our extracts via the use of ionic columns does not seem to have been necessary for comparative analysis. Perhaps additional submissions of other environmental conditions may have shed some insight into the structures of the components in these extracts as well as additional controls for PGA. In the Schneerson paper, the PGA samples were derivitized with heptafluorobutyryl isobutyl esters after hydrolysis with HCl, and were separated by HP-5 0.32x30 mm glass capillary column (148). Perhaps separation and derivitization was necessary for accurate analysis of PGA polymers. It seems that the derivitization allowed for the determination of the different enantiomers of glutamic acid. There again, there was a lack of information on mass spectrophotometry of PGA for us to draw any conclusions from our previous data, other than the extract samples of our two bacteria were very similar and that they do not resemble the PGA profile of *B. licheniformis*.

Our survey of the free amino acid demonstrates no glutamic acid or any typical amino acid for that matter in the PGA extract of either bacteria at pH 4. There were, however, substantial amounts of L-phosphoserine found in these extracts, which was structurally similar to L-glutamate. Analogs to glutamate have been shown to inhibit the glutamate-dependent acid resistance system. It has been shown that 1.5mM of L-*trans*-pyrrolidine-2, 4-dicarboxylic acid (L-PDC) can significantly inhibit the in vitro glutamate decarboxylation of *E. coli* (185). This blockage takes place at the glutamate / GABA exchange antiporter (*gadC*) and was found to be strictly competitive. Perhaps L-phosphoserine was having a similar effect in our acid resistance studies, acting as a competitive inhibitor to glutamate and disrupting the glutamate decarboxylation cycle. Additional tests could be performed to determine if this was a likely scenario. There was limited information on the specificity of the *gadC* antiporter but such information could be useful to identify potential glutamate analogs that may obstruct this system. I consistently see a difference in L-phosphoserine in favor of the wildtype U112, at pH 4 and with the minimal media. The SDS samples show an increase in glutamine when comparing the U112 to the *capB* mutant, which may be an indication of glutamine utilization for decarboxylation. Again, a greater understanding of *Francisella's* glutamate / GABA antiporter was necessary. The minimal media data presented both a difference in free glutamic acid when comparing the supernatants and the pellet fractions. If PGA were part of the supernatant fraction, one would expect the glutamic acid content to favor the wildtype. The pellet fraction presents a lower glutamic acid amount for the mutant when compared to the control, however, this may be due to PGA, another source

of polyglutamylation, or just a difference in free glutamic acid. The other amino acid potentially involved in acid resistance was lysine, in which I consistently see higher amounts in our mutant supernatant samples than in the wildtype. This trend may also warrant additional attention as this resembles what was seen with glutamate in the supernatant. Analysis of the total amino acid data also demonstrates higher amino acid content of glutamate and lysine in the supernatant fraction for the mutant, as the pellet information paralleled the free amino acid profile for glutamate. As with the MS data, additional studies were needed to draw more substantive conclusions regarding PGA production.

Many different experiments have been performed by various researchers, both *Bacillus* and *Francisella* alike, to determine whether *Francisella* was capable of producing a capsule consisting of polyglutamic acid. Under the conditions tested, it seems that the existence of a PGA capsule for *Francisella* was still enigmatic, although these studies may have clarified some of the idiosyncrasies which make *Francisella* such an efficient pathogen of cells such as monocytes and macrophages. It was still unclear how the loss of a gene such as an amide ligase can result in a reduced potential for replication in macrophages as well as reduced potential to survive desiccation. It may be possible that gene has an additional function other than the γ-bridging of glutamic acid. Further tests should be able to discern this, as radioactive studies using isotopic glutamate or glutamine may not elucidate the existence of a PGA capsule, but may clarify the glutamate:glutamine equilibria of these bacteria. Studies into the glutamate / GABA antiporter should also be performed to determine whether there actually was a differential

in activity of that inner membrane protein. As mentioned before, a greater knowledge and repetorie of antibodies specific for different forms of PGA would be useful, as visualization immunologically may be still be possible. And it could stand to reason that the appropriate conditions for polymerization have not been discovered. Investigation into the expression levels of the genes potentially responsible could also elucidate which conditions favor expression as would access to steady mass spectrophotometry. As isolation of these membrane complexes was rather difficult, some proteomics research of recombinant proteins may uncover affinities and relationships with other inner membrane proteins, which may clarify the poly-γ-glutamate synthetase complex's potential role, if any, in *Francisella*.

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# VITAE

MAJ Eric Fleming was born in Baltimore, Maryland on July 18, 1970. He was raised in Fredericksburg, VA and graduated from Courtland High School in 1988. MAJ Fleming earned his bachelor's degree in Biology at the University of Virginia in 1992 and earned his master's in Genetics and Molecular biology at the University of North Carolina at Chapel Hill in 1995. He then enlisted in the U.S. Army becoming a combat medic for 1-5 Infantry in the 25th Infantry Division, Ft. Lewis, WA serving two years before being direct commissioned to 1st Lieutenant. 1st Lieutenant Fleming was sent to the United States Army Medical Research Institute of Infectious Diseases at Ft. Detrick, MD as a microbiology augmenter to screen environmental samples from the senate offices during the D.C. anthrax attacks of October 2001. As a Captain, he managed the Leishmania Diagnostic Laboratory at the Walter Reed Army Institute of Research, located in Silver Spring, MD which was responsible for diagnosis of the Leishmania clinical samples from Soldiers located in both Iraq and Afghanistan from 2003 to 2005. In 2005, he was one of three Army officers selected to earn his Ph.D. in microbiology and began his dissertation research under Dr. Dennis Ohman in 2006. He was promoted to the rank of Major in early 2009.