INVESTIGATION OF THE ROLE OF THE CAPBCA LOCUS IN FRANCISELLA NOVICIDA

by

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Bachelor of Science
Trinity College, 2017

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DEDICATION

This thesis is dedicated to my sister, Bailey, for inspiring, nurturing, and guiding my love of science.

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I would like to thank my friends and family for supporting me throughout my degree. My parents, Reed Risteen and Kate Hannon, made earning both my undergraduate and graduate degrees possible. My father and sister especially encouraged my interest in science from a young age. The entire Hannon family cheered me on, and my grandfather Bill Risteen made sure my sister and I had access to an education. Alex Oldershaw supported me endlessly and always had chocolate ready after a long day in lab. The entire Oldershaw family also deserve thanks, as they became my family when my own family is so far away.

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LIST OF ABBREVIATIONS

Poly-γ-glutamic acid ..... PGA

Francisella tularensis subsp. *tularensis* ..... *F. tularensis*

Francisella tularensis subsp. *novicida* ..... *F. novicida*

Francisella tularensis subsp. *holarctica* live vaccine strain ..... LVS

Wild Type ..... WT

Francisella Pathogenicity Island ..... FPI

Colony-forming Units ..... CFU

Tryptic Soy Broth with 0.1% Cysteine ..... TSB-C

Mueller Hinton Broth ..... MHB

Chamberlain's Defined Medium ..... CDM

Phosphate Buffered Saline ..... PBS

Dulbecco's Modified Eagle Medium + 10% fetal bovine serum ..... DMEM

Reverse-Phase Liquid Chromatography ..... RP-LC

Proton Nuclear Magnetic Resonance Spectroscopy ..... ^{1}H NMR

Thin Layer Chromatography ..... TLC

ABSTRACT

INVESTIGATION OF THE ROLE OF THE CAPBCA LOCUS IN FRANCISELLA NOVICIDA

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Gram negative bacterium *Francisella (F.) tularensis* is a highly contagious zoonotic pathogen endemic to the United States of America. Though tularemia cases are relatively rare, the potential for *F. tularensis* to be used as a bioweapon makes studying pathogenesis of this tier 1 biothreat agent important. Previous studies have found the *F. tularensis capBCA* locus to be important for virulence but did not specify the exact role of this locus. Gram positive bacteria *Bacillus anthracis* and *Bacillus subtillus* feature loci with high sequence similarity to the *F. tularensis capBCA* locus. The *B. anthracis capBCA* genes encode proteins that are part of a system responsible for producing poly-γ-glutamic acid (PGA) and building a capsule from the polypeptide. The *B. subtilis pgsBCA* genes similarly encode a system that produces PGA, but the PGA is not attached to the cell surface so the organism does not produce a PGA capsule. Since both the *B. anthracis capBCA* locus and the *B. subtilis pgsBCA* locus are responsible for PGA production, we hypothesized that the *F. tularensis capBCA* locus will perform the same function. We isolated material from a highly related organism, *F. novicida*, a BSL2 model organism for *F.*

tularensis, but could not confirm that the material contains PGA. These results do not support the theory that the *Francisella capBCA* locus synthesizes PGA from glutamic acid monomers.

CHAPTER ONE: INTRODUCTION

Poly-γ-glutamic Acid

Structure of PGA

Poly-γ-glutamic acid (PGA) is a polypeptide consisting solely of glutamic acid monomers linked by bonds between the side-chain carboxyl group and the α-amino group (**Figure 1A**) (Sung, et al., 2005; Ogunleye, et al., 2015). Poly-α-glutamic acid consists of the same monomers, only they are linked by bonds between the backbone amino and carboxyl groups (**Figure 1B**). Most proteins are built linking these two backbone groups – PGA is the exception. PGA therefore is produced differently and has different susceptibility to proteases than other proteins. PGA is not produced by ribosomes because ribosomes can only create α-linkages. This polypeptide is instead built by specialized proteins that are able to create the γ-linkages between glutamic acid monomers. PGA is also unconventional in that it cannot be digested by proteases, which only target α-linkages (Ogunleye, et al., 2015). Instead, PGA is degraded by enzymes that specifically target the α-glutamyl linkage. *B. subtilis* D,L-glutamyl hydrolase is one example of a PGA-degrading enzyme.

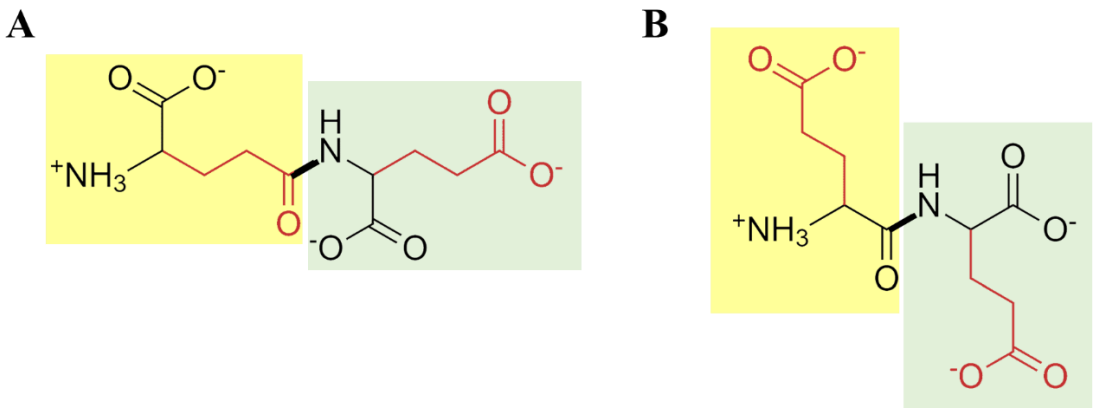


Figure 1. Structure of **(A)** poly-γ-glutamic acid and **(B)** poly-α-glutamic acid. The glutamate side chains are drawn in red, and the monomers are distinguished by the colored boxes. The γ and α bonds are in bold.

PGA can be made up of D-glutamic acid, L-glutamic acid, or a combination of the two enantiomers (Candela & Fouet, 2006; Ashiuchi, 2013). Different organisms produce PGA with different ratios of D- to L-glutamic acid. The average molecular weight of PGA also varies widely between organisms, but it should be noted that molecular weight can also vary between PGA molecules within a single organism (Ogunleye, et al., 2015). Proteins encoded by ribosomes have a set size because they are produced using mRNA blueprints that specify the length and exact amino acid sequence of the protein to be made. PGA is not produced using a blueprint of any sort – glutamate monomers are simply polymerized into a chain. Exact molecular weight is therefore not constant from strand to strand of PGA (Candela & Fouet, 2006; Hsueh, et al., 2017).

The Use of PGA by Microbes

Bacteria benefit from PGA in a variety of ways based on the PGA makeup and whether the PGA is released or capsular. The *B. anthracis* D-PGA capsule is non-immunogenic and facilitates immune evasion by preventing the immune system from detecting it (Leppla, et al., 2002). Furthermore, the PGA capsule enhances the activity of Lethal Toxin – one of the two deadly toxins *B. anthracis* produces (Jang, et al., 2011). The PGA capsule is so important to virulence that unencapsulated strains of *B. anthracis* are non-pathogenic (Ivins, et al., 1986; Scorpio, et al., 2006). *B. anthracis* PGA is attached to the bacterial surface. *Staphylococcus* epidermidis also uses a PGA to evade the immune system, as its DL-PGA capsule protects the bacterium from various antimicrobial peptides and inhibits phagocytosis by neutrophils (Kocianova, et al., 2005). Less is known about the contribution of released PGA towards bacterial fitness. *B. subtilis* PGA contributes to plant colonization, motility, and possibly biofilm formation (Yu, et al., 2016; Wang, et al., 2017; Chagneau & Saier, 2005). Interestingly, *B. subtilis* PGA also appears to limit the effects of *Fusarium graminearum* plant disease.

Cap and Pgs Systems in Bacteria

The systems responsible for producing PGA are fairly well conserved among PGA-producing bacteria. The genes encoding these systems fall within a *cap* locus or a *pgs* locus – the letters "PGS" abbreviating polyglutamate synthase. These genes are generally labeled *cap* genes in organisms that utilize the PGA to produce a capsule – like *Bacillus anthracis* – and *pgs* genes in organisms that release PGA – like *Bacillus subtilis* (Candela & Fouet, 2006). On a most basic level, all PGA producing bacteria feature at least three genes: *cap/pgsB*, *cap/pgsC*, and *cap/pgsA* (**Figure 2**).

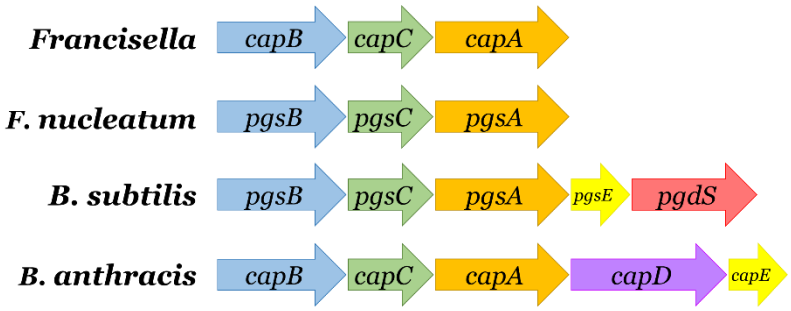


Figure 2. The layout of *cap* and *pgs* genes in their respective loci in *Francisella*, *F. nucleatum*, *B. subtilis*, and *B. anthracis*.

Cap/Pgs Systems in *Bacillus* species

Despite the difference in naming, *Bacillus* Cap and Pgs systems work in a similar manner (**Figure 3**). The proteins that make up these systems associate with each other on the cell membrane, where Cap/PgsB and Cap/PgsC work together to polymerize the glutamic acid monomers within the cytoplasm (Ashiuchi, 2013). Then, Cap/PgsA and Cap/PgsE transport the PGA across the cell membrane. The Cap and Pgs systems diverge at that point; CapD in the Cap system anchors the PGA to the peptidoglycan to form a PGA capsule, while PgdS in the Pgs system breaks down PGA into shorter strands.

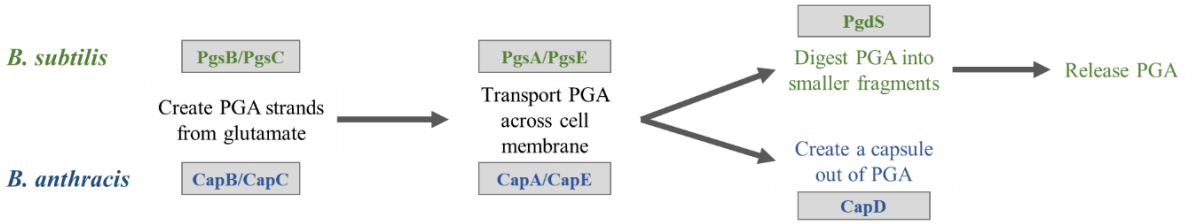


Figure 3. The role of each protein in the Cap and Pgs systems of *B. anthracis* and *B. subtilis*, respectively. Note that *B. subtilis* PgdS is not necessary for PGA release.

Pgs System in *Fusobacterium nucleatum*

Fusobacterium nucleatum (*F. nucleatum*) is the only gram negative organism proven to produce PGA (Candela, et al., 2009). The PGA that is produced by this bacterium is not used to create a capsule. Unlike *Bacillus* species that also release PGA, *F. nucleatum* lacks *pgsE* and *pgdS*. PgdS is not necessary for the release of PGA in *Bacillus* species, but PgsE is necessary (Scoffone, et al., 2013; Candela, et al., 2005; Yamashiro, et al., 2011). *F. nucleatum* is hypothesized to compensate for the lack of a *pgsE* gene with its unique PgsA – also referred to as PgsF – which shows little homology to Cap/PgsA from gram positive bacteria (Candela, et al., 2009). *F. nucleatum* PgsA is thought to carry out the role of *Bacillus* Cap/PgsA and Cap/PgsE combined, and is hypothesized to be specialized for the gram negative cell wall structure.

As discussed below, *Francisella* CapA has very high homology to *F. nucleatum* PgsA and low homology to *Bacillus* Cap/PgsA. Thus, based on sequence comparison alone, the *Francisella* CapA is most similar to PgsA of *F. nucelatum*.

The *capBCA* Locus and PGA Production in *Francisella*

The presence of the *capBCA* locus in *Francisella tularensis* suggests that *F. tularensis* produces PGA. However, there is no research on PGA production in this bacterium. Research on the *Francisella capBCA* locus is limited to studies on the importance of these genes for various bacterial processes including in vitro growth, in vivo growth, and intramacrophage growth.

The Importance of the *capBCA* Locus

The Role of the capBCA Locus in In Vivo Growth. The *capBCA* locus in *Francisella* is involved in murine infection. *Francisella tularensis* subsp. *novicida* (*F. novicida*) *capB* and *capC* mutants were attenuated in C57BL/6 mice (Weiss, et al., 2007). *Francisella tularensis* subsp. *holarctica* live vaccine strain (LVS) *capA*, *capB*, and *capC* mutants similarly demonstrated reduced growth in BALB/c mice, and the *capB* mutant was less virulent overall (Su, et al., 2007; Jia, et al., 2010). As further evidence for the importance of CapBCA in in vivo infections, a *Francisella tularensis* subsp. *tularensis* (*F. tularensis*) *capBCA* mutant was also attenuated in BALB/c mice (Su, et al., 2011). The *cap* locus appears to be particularly important for persistence within the spleens of these mice. The *F. novicida capA* and *capC* mutants and the *F. tularensis capB* mutant showed reduced growth and survival within the spleen (Michell, et al., 2010; Kraemer, et al., 2009). Likewise, the LVS *capB* mutant was cleared faster in the spleen than the wild type (Jia, et al., 2010).

The Role of the capBCA Locus in Intramacrophage Growth. Intramacrophage growth of *Francisella* also appears to be affected by the *capBCA* locus. LVS *capA*, *capB*, and *capC* mutants had inhibited growth in J774A.1 macrophages (Maier, et al., 2007), and whole-locus mutants had reduced growth in MH-S and U937 macrophages (Su, et al., 2011).

The Role of the capBCA Locus in In Vitro Growth. Though the *Francisella capBCA* locus appears to be important for intramacrophage and in vivo growth and survival, its role in in vitro growth is unclear. There was no change in growth of various *F. tularensis* and LVS *cap* mutants in Müller-Hinton broth (MHB) or modified cysteine partial hydrolysate (Michell, et al., 2010; Su, et al., 2007; Su, et al., 2011). Durham-Colleran, Verhoeven, & van Hoek, however, saw inhibited growth of *F. novicida capB* and *capC* mutants in tryptic soy broth with cysteine (TSB-C) (Durham-Colleran, Verhoeven, & van Hoek, 2010). One theory for this discrepancy is that the *capBCA* locus may come into play in less nutrient-rich environments.

The Expected Production of PGA

Though there is no evidence of PGA production in *Francisella*, the sequence homology of *Francisella* CapA, CapB, and CapC proteins to Pgs/CapABC proteins in PGA-producing *Bacillus* and *Fusobacterium* species provides reason to believe that the *Francisella capBCA* locus encodes a system that produces PGA. Thus, this is the fundamental question of my research. Furthermore, the makeup of the gram negative cell wall and the lack of *capD* in *Francisella* suggests that if PGA is produced it is not used to form a capsule. With the evidence leaning towards *Francisella* releasing PGA instead of synthesizing a PGA capsule, I propose that the *Francisella capBCA* genes be renamed *pgsBCA*.

Homology to F. nucleatum Suggests Production of PGA. The CapBC proteins in *F. novicida* and *F. tularensis* are homologous to the Cap/PgsBC proteins in *B. subtilis*, *B. anthracis*, and *F. nucleatum*. However, the *Francisella* CapA protein is only homologous to the *F. nucleatum* PgsA protein (**Table 1**). This homology, in addition to the identical layout of the *cap/pgs* genes between *Francisella* and *F. nucleatum* (**Figure 2**), suggests that *F. nucleatum* is

currently the best model for *Francisella* PGA production. Since *F. nucleatum* does not produce an attached capsule from the PGA it synthesizes, *Francisella* likely does the same. As mentioned above, the proposed dual role of *F. nucleatum* PgsA enables of PGA production despite lacking PgsE. Thus, I hypothesize that the *Francisella* CapA may have the same dual function.

Table 1. The E-values for alignments of *Francisella tularensis* CapA, CapB, and CapC proteins with their homologs in other species of bacteria. Lower E-values indicate greater similarity between two proteins while taking the alignment length into consideration.

	B. anthracis	***B. subtilis***	***F. nucleatum***
Cap/PgsA	0.16	0.48	5e-38
Cap/PgsB	3e-79	5e-76	3e-76
Cap/PgsC	2e-22	2e-19	3e-27

The Gram Negative Cell Wall Conflicts with PGA Capsule Formation.

Francisella does not have a *capD* gene within the *cap* locus, which is essential for building and attaching the PGA capsule to the cell wall of gram positive bacteria. The lack of this gene in the *cap* locus suggests that *Francisella* does not produce a PGA capsule. In addition, the structure of the gram negative cell wall would make producing a PGA capsule near impossible even with the *capD* gene. The cell wall of a gram positive organism like *B. anthracis* and *B. subtilis* consists of only the plasma membrane and an external peptidoglycan layer, so when the PGA is anchored to the peptidoglycan through the action of CapD, it will form a capsule (**Figure 4A**). The peptidoglycan layer in gram negative organisms like *Francisella* and *F. nucleatum* lies between the cell membrane and an outer membrane, and there is no peptidoglycan beyond the outer membrane to which the PGA can be anchored. If the *B. anthracis* CapBCADE system was present

in a gram negative organism, the peptidoglycan would theoretically be anchored to the peptidoglycan between the cell and outer membrane, which would not qualify as a capsule since it is not outside the cell wall (**Figure 4B**). The logistics of producing a PGA capsule conflict with the *Francisella* cell wall structure and the lack of *capD* suggests that this doesn't happen as well.

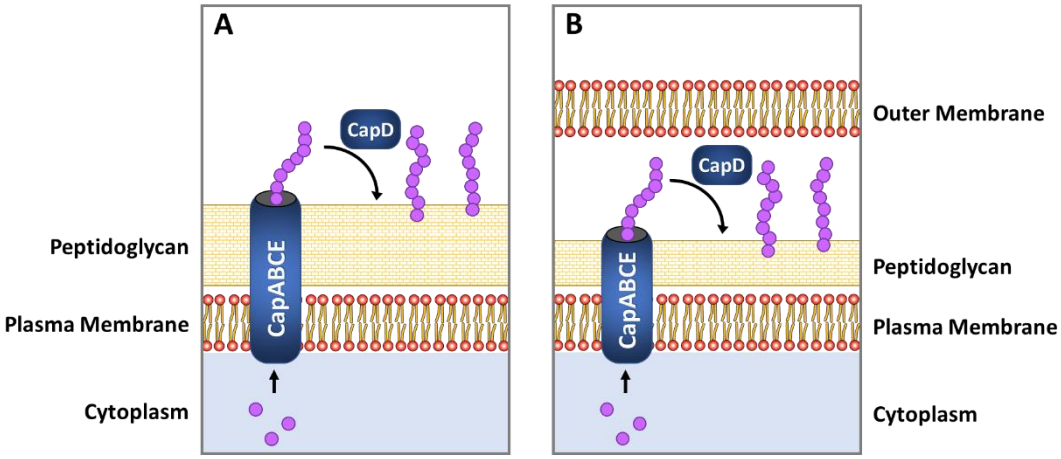


Figure 4. (A) the *B. anthracis* CapBCADE system in its native gram positive organism produces a PGA capsule by anchoring it to the peptidoglycan layer that surrounds the cell. **(B)** If it existed in this form, the *B. anthracis* CapBCADE system in a gram negative organism would not be able to create a PGA capsule because there is no peptidoglycan outside the outer membrane for CapD to anchor the PGA to. Instead, CapD would likely anchor the PGA to the peptidoglycan between the outer membrane and plasma membrane.

Expected activity of Cap Proteins and Other Significant Proteins. Based on the homology of the *Francisella* Cap proteins to known proteins, we can begin to postulate how PGA may be produced in *F. novicida* and *F. tularensis* and what may happen to it afterwards.

CapBCA Complex. A complex consisting of CapB, CapC, and CapA is predicted to be associated with the bacterial membrane. Within the cytoplasm CapB and CapC are thought to catalyze the ATP-dependent reaction responsible for polymerizing glutamic acid monomers. In

other PGA producers, substrate-dependent ATP hydrolysis mediates phosphorylation of the glutamic acid side chain carboxyl, which allows the amino group of another glutamic acid to attack the phosphorylated carboxyl group to create an amide linkage (Sung, et al., 2005; Ashiuchi, et al., 2001). PgsB alone does not have the ability to catalyze ATP-hydrolysis in *B. subtilis*, so the active site in *Francisella* is theorized to lie within the CapBC complex. CapA is situated in the cell membrane, which allows it to fulfil its role in transporting the PGA across the cell membrane and peptidoglycan. CapA may have an additional role in removing the PGA chain from the active site after elongation so that another glutamic acid monomer may be added (Ashiuchi & Misono, 2002). The transport of PGA out of *Francisella* is difficult to predict. CapA/PgsA is widely believed to transport PGA across the cell membrane in PGA producers, but it is unknown if it also transports PGA across the peptidoglycan layer. *Francisella* is gram negative, so the PGA it produces would have to get past both the peptidoglycan layer and outer membrane in order to fully leave the cell. PGA produced by microbes is high in molecular weight, so passing through the peptidoglycan layer and outer membrane is even more of a challenge (Scoffone, et al., 2013; Wu, et al., 2010; Richard & Margaritis, 2003). Therefore, the PGA may simply accumulate in the *Francisella* periplasm instead of being released into the extracellular environment.

γ-Glutamyl transferase. Like *B. subtilis* and *F. nucleatum*, *Francisella* encodes a γ-Glutamyl transferase (GGT) enzyme. In *B. subtilis* this enzyme was found to act as an exo-γ-glutamyl hydrolase, which hydrolyzes glutamate at the end of the polypeptide to result in glutamic acid monomers (Yao, et al., 2009). This protein differs from the *B. subtilis* endo-γ-glutamyl hydrolase, PgdS, which instead hydrolyzes PGA in the middle of the polypeptide. *B.*

subtilis GGT appears to rely on PgdS in order to function to its full capacity, only exhibiting measurable hydrolase activity when PgdS reduced the PGA molecular weight to around 100 kDa (Yao, et al., 2009). *F. nucleatum* similarly encodes GGT, but the enzymatic activity of the protein has not yet been studied. It should be noted that *F. nucleatum* and *Francisella* do not have the *pgdS* gene, so their GGT activity either does not rely on PgdS or functions in a limited capacity, if any. Using PSORTb version 3.0.2, subcellular localization of *F. tularensis* GGT was predicted to be within the periplasm (Yu, et al., 2010). The predicted location of the GGT would facilitate its proposed activity of breaking down PGA that has been transported across the inner membrane into the periplasm.

CHAPTER TWO: ROLE OF CAPBCA IN BACTERIAL PROCESSES

Introduction

The *capBCA* locus *Francisella* is expected to be responsible for PGA production. Beyond this main function, the *capBCA* locus may have roles in other cellular processes. The role of *capBCA* genes in biofilm formation, in vitro growth, and intramacrophage growth may suggest the purpose of PGA production in *Francisella* or a contribution by the *capBCA* genes themselves.

Biofilms are adherent communities of one or more species of bacteria enveloped by a protective matrix made up of polymers (van Hoek, 2013). There are several advantages bacteria gain by forming biofilms; horizontal gene transfer is enhanced, the bacteria are protected from harmful substances like oxidative radicals and antibiotics, phagocytosis is inhibited, and adherent bacteria are more able to withstand shear forces (Durham-Colleran, et al., 2010; van Hoek, 2013). There are conflicting reports on the contribution of PGA to biofilms. One study demonstrated that a hybrid *B. subtilis* strain (*B. subtilis* strain JH642 with genes from *B. subtilis* strain RO-FF-1 that are responsible for mucoid colonies) produced significantly less biofilm when the *pgsC* gene was deleted (Stanley, 2005). While this result would imply PGA is involved in biofilm formation, the *B. subtilis* RO-FF-1 *pgsC* deletion mutant produced the same amount of biofilm as the wild type. A different study found a that *B. subtilis* B-1 PGA production was correlated with biofilm production in different concentrations of glycerol and $MnSO_4$, but could not prove that PGA production caused the increase in biofilm (Morikawa, 2006). *F. novicida* and

F. tularensis both produce biofilm, and biofilm production in *F. novicida* especially has been thoroughly documented (Dean & van Hoek, 2015; Dean, et al., 2015; Durham-Colleran, et al., 2010; Chung, et al., 2014). *F. novicida* is therefore an excellent model organism for investigating the role of PGA in biofilm production.

A key virulence factor of *F. tularensis* is its ability to invade macrophages and replicate. *F. tularensis* enters monocytes via phagocytosis, then prevents the lysosome from fusing with the phagosome to form the phagolysosome (Meibom & Charbit, 2010). With the immediate danger of the phagolysosome mitigated, *F. tularensis* degrades the phagosomal membrane to escape into the cytosol where it replicates. *F. tularensis* is protected from the immune system and antimicrobials while inside the cytoplasm of macrophages. Though the intramacrophage life cycle of *F. tularensis* is often linked to in vivo pathogenesis, there is one study that contradicts this paradigm. Researchers created *F. tularensis* subsp. *holarctica* LVS (LVS) and *F. tularensis* SCHU S4 Tn-*pyrF* mutants, which were unable to replicate in primary human macrophages but were as pathogenic as the wild type LVS and *F. tularensis* in chicken embryos and mice, respectively (Horzempa, et al., 2010). On the contrary, mutations in the Francisella Pathogenicity Island (FPI) – a group of genes that are mainly important for phagosomal escape – leads to attenuation in both intramacrophage growth and pathogenicity.

The impact of transposon mutations in the *F. novicida cap* locus on in vitro growth, intramacrophage growth, and biofilm production was investigated.

Materials and Methods

Bacterial Strains and Culture Conditions

F. novicida U112 NR-13 was obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources, Manassas, VA). The *F. novicida* U112 "Two-Allele" transposon mutant library was also obtained from BEI resources. Three mutants were studied – the *capA* mutant (Tn-capA), *capB* mutant (Tn-capB), and *capC* mutant (Tn-capC) (**Table 2**). Cultures were grown in Chamberlain's Defined Medium (CDM) (Chamberlain, 1965) or tryptic soy broth (BD, Franklin Lakes, NJ) supplemented with 0.1% cysteine (TSB-C). The media the mutants were initially cultured in contained 10 μg/mL kanamycin to prevent reversion. Overnight cultures were incubated at 37°C in a shaking incubator for less than 24 hours.

Table 2. The *F. novicida* mutants used in the study. All four mutants feature a T20 transposon type.

Mutant	Strain	Length of ORF	Nucleotide of Insertion in ORF	Direction of Insertion
Tn-capA	tnfn1_pw060418p04q135	1209	772	Forward
Tn-capB	tnfn1_pw060328p02q103	1215	350	Reverse
Tn-capC	tnfn1_pw060328p07q179	462	117	Forward
Tn-iglC	tnfn1_pw060328p06q115	627	217	Reverse

Crystal Violet Biofilm Assays

Overnight cultures of *F. novicida* wild type and *cap* mutants were added to a 96 well plate with 1×10^6 CFU in 200 μL of TSB-C per well. The plates were incubated for two days at 37°C.

After incubating, the OD_{600} of each well was measured at to quantify the bacterial growth. Each well was then rinsed three times with 250 µL phosphate buffered saline (PBS) and fixed with 200 µL of 99% methanol for 15 minutes (Durham-Colleran, et al., 2010). The wells were aspirated and allowed to air dry before staining with 200 µL 0.1% crystal violet for 5 minutes. Tap water was used to gently rinse each well three times, and the 96 well plate was once again allowed to air dry completely. To resolubilize the crystal violet after drying, 200 µL of 33% glacial acetic acid was added to each well. OD_{590} was measured as an indicator of biofilm quantity in each well.

Growth Curves

Overnight cultures of *F. novicida* WT and *cap* mutant strains were diluted to a concentration of 5×10^6 CFU/mL. The starting concentration was estimated using the following previously experimentally derived equation (**Equation 1**).

Equation 1. The equation used to calculate the concentration (CFU/mL) of *F. novicida* using the OD_{600} of the culture.

$$Concentration = (3476342910 \times OD_{600}) - 160763955$$

Each well was filled with 200 µL of diluted culture so that the final concentration was 1×10^6 CFU per well. The 96 well plate was incubated in a spectrophotometer at 37°C, with the machine set to read the OD_{600} every hour for 72 hours with 10 seconds of shaking before each reading. The TSB-C growth curve was carried out in a SpectraMax Plus 384 Microplate Reader

(Molecular Devices, Sunnyvale, CA), while the CDM growth curve was performed using a Cytation 5 imaging reader (Biotek, Winooski, VT).

Gentamycin Protection Assays

A 24 well plate was seeded with 3×10^5 RAW 264.7 cells per well in 425 µL of Dulbecco's Modified Eagle Medium (ATCC 30-2002) with 10% fetal bovine serum (DMEM) and was incubated for 10 to 14 hours at 37°C and 5% CO_2. Each well was inoculated with overnight cultures of *F. novicida* WT, *cap* mutants, or Tn-iglC to achieve a MOI of 100 (3×10^7 CFU per well). The *iglC* gene is known to be important for intracellular replication, so *F. novicida* Tn-iglC was used as a control (Santic, et al., 2005). The 24 well plate was centrifuged for 10 minutes at 300 × g, room temperature, to facilitate infection. The plate was incubated for an hour at 37°C and 5% CO_2 before the media was replaced with DMEM containing 0.1 mg/mL gentamycin to eliminate extracellular bacteria. The plate was incubated for another hour and the media in each well was replaced with 475 µL of DMEM without gentamycin. The plates were incubated for a total of 8 hours and 24 hours before lysing the macrophages to release the intracellular *Francisella* for enumeration. To lyse the macrophages, DMEM was aspirated and 100 µL of 0.1% (wt/vol) sodium deoxycholate in PBS was added to each well. After incubating at 37°C in 5% CO_2 for 5 minutes, 900 mL of PBS was added to each well and enumeration was carried out on BD BBL™ Chocolate II Agar plates (Franklin Lakes, NJ).

Statistical Analyses

For the biofilm assays, the average optical density (OD) of the media was subtracted from the OD of each experimental well to calculate the adjusted OD for each well. Biofilm formation was compared between experimental groups by dividing the average OD_{580} of each

mutant at by the average absorbance of the WT at the same optical density. The mutant biofilms could then be measured as a percentage of the WT. The same process was repeated for the biofilm assay growth measurements, which were measured by OD_{600}. The adjusted OD measurements for all three replicates were pooled before statistical analysis of both growth and biofilm formation data using one-way ANOVA and Tukey Test.

Growth curves parameters were calculated using the Growthcurver package (Sprouffske & Wagner, 2016) in RStudio. For the CDM growth curves, a cutoff at 45 hours was used to improve the fit of the curve. A cutoff at 25 hours was used to improve the fit of the TSB-C curve. The resulting doubling times and maximum growth rates were compared via one-way ANOVA.

Gentamycin protection assays were analyzed using Student's *t* test.

All error bars reflect the standard error, which was calculated using **Equation 2**.

Equation 2. Standard error was calculated using the following equation where n = sample size and $St\ Dev$ = standard deviation.

$$St\ Error\ = \frac{St\ Dev}{\sqrt{n}}$$

Results

Biofilm

Consistent biofilm results could not be obtained using the crystal violet biofilm assay. Significant variation was observed in relative biofilm quantity (as indicated by OD_{580}) between

technical replicates despite several modifications to the procedure. A previous student, Sairah Ahmad, collected data on the biofilms of *capBCA* mutants using confocal microscopy to measure the thickness and the crystal violet assay to measure relative abundance in terms of a biofilm to growth ratio. Her results express that the biofilm to growth ratio of *F. novicida* Tn-capA is significantly lower than that of the WT while that of *F. novicida* Tn-capC is significantly greater (

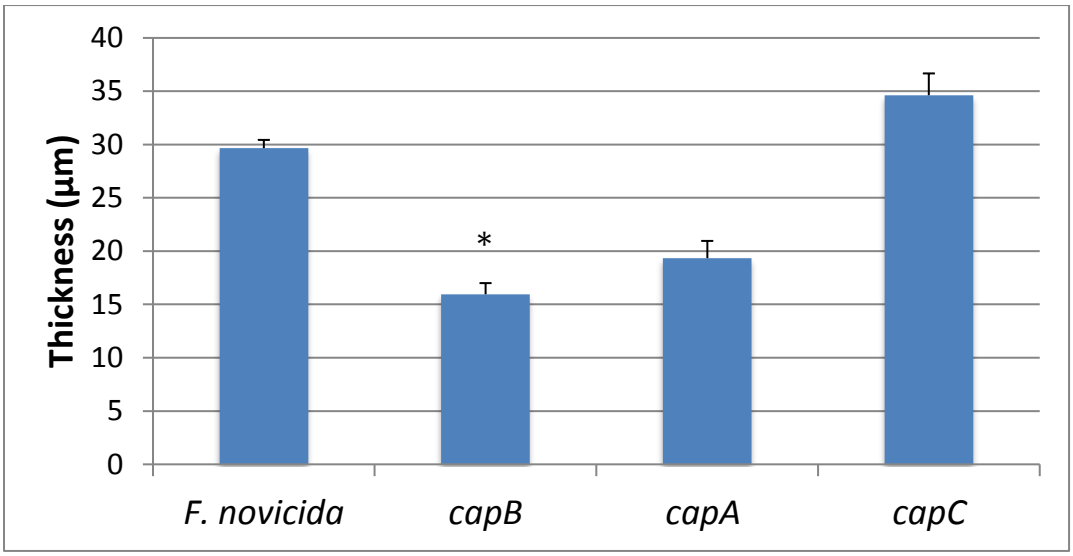


Figure 5**A**). The *F. novicida* Tn-capB biofilm is also significantly thinner than the WT biofilm (

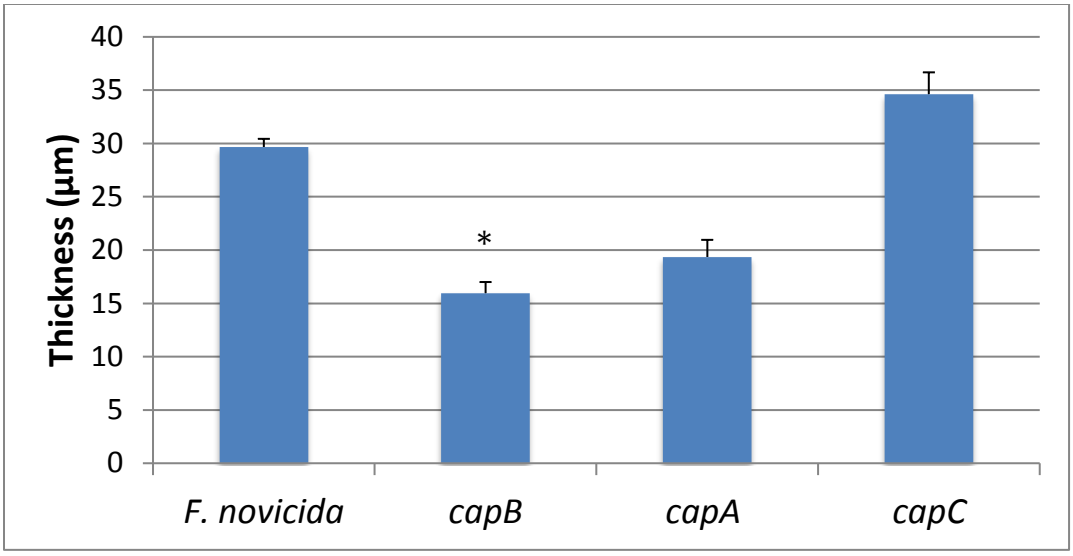
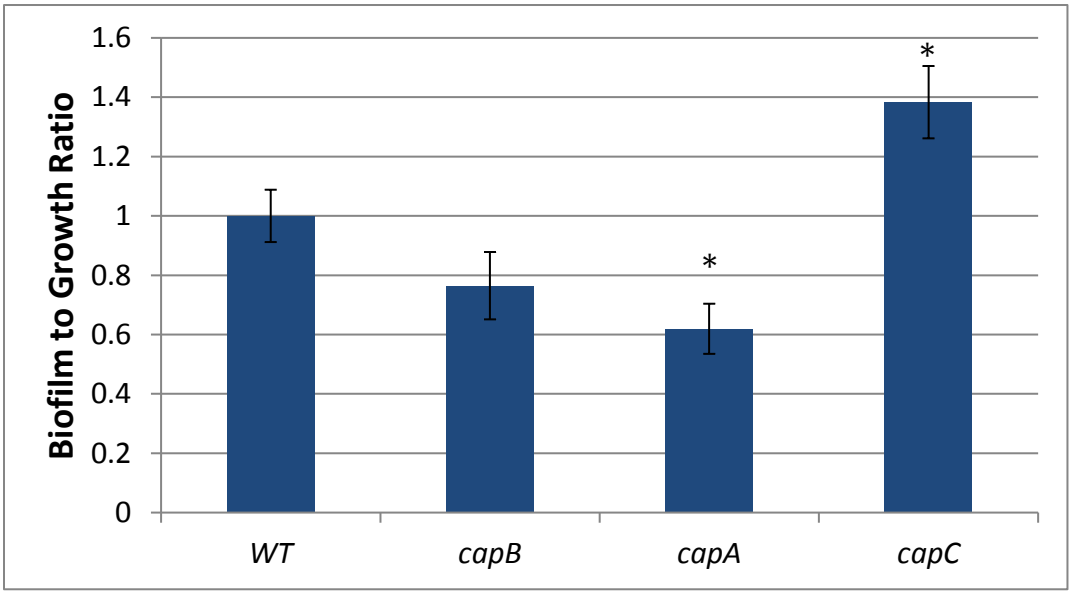

Thickness (µm)
40
35
30
25
20
15
10
5
0
*
F. novicida
capB
capA
capC

Figure 5**B**).


Biofilm to Growth Ratio
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
WT
capB
capA
capC

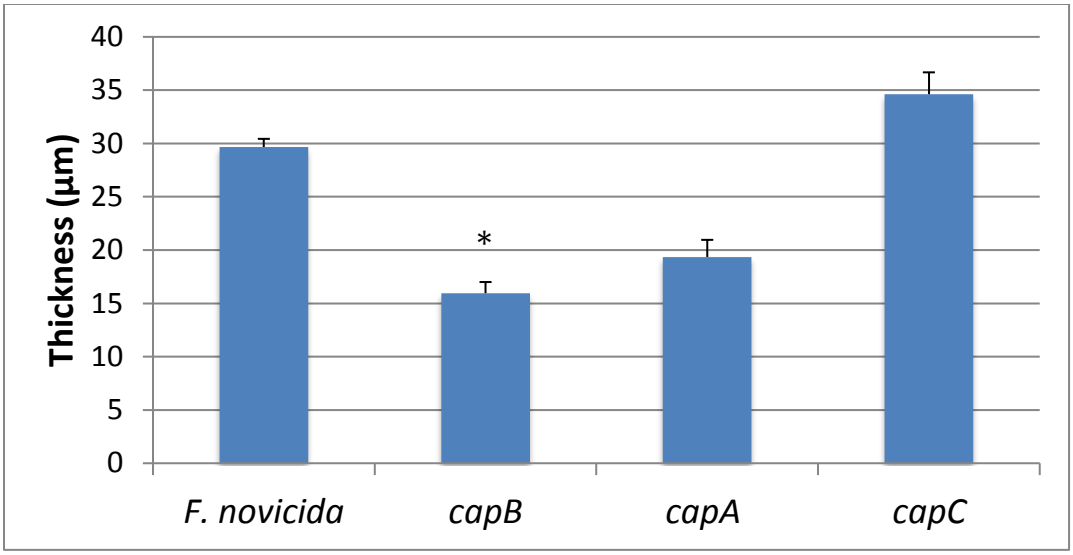


Figure 5. The relative abundance of biofilm as measured by the **(A)** biofilm to growth ratio and **(B)** biofilm thickness. Asterisk indicates significance ($P < 0.05$) as compared with WT. Results were collected by Sairah Ahmad.

The *F. novicida* Tn-capC strain exhibited a unique phenotype after incubation in 96 well plates at 37°C for two days: small white specks floating through the culture (**Figure 6**). This phenotype is not observed under shaking conditions. A BD BBL™ Chocolate II Agar plate (Franklin Lakes, NJ) was inoculated with a two-day *F. novicida* Tn-capC culture from a 96 well plate used in the crystal violet assay, and was incubated for two days at 37°C. Only one colony morphology was seen (data not shown). A gram stain of several identical colonies revealed no visible bacterial cells apart from *Francisella* bacteria.

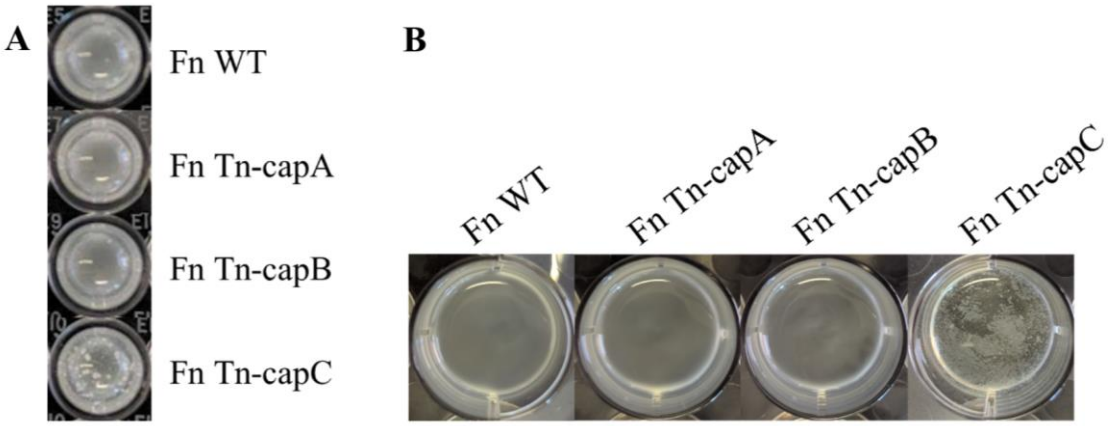


Figure 6. Small clumps of a white substance could be seen floating in a culture of *F. novicida* Tn-capC within **(A)** a 96 well plate and **(B)** a 12 well plate after incubation at 37°C for two days in TSB-C under non-shaking conditions. This phenotype was not present in any other *F. novicida* strains that were subjected to the crystal violet assay in this study.

Growth Curves

No significant difference was observed in doubling time or growth rate between the wild type and *capBCA* mutants in CDM (

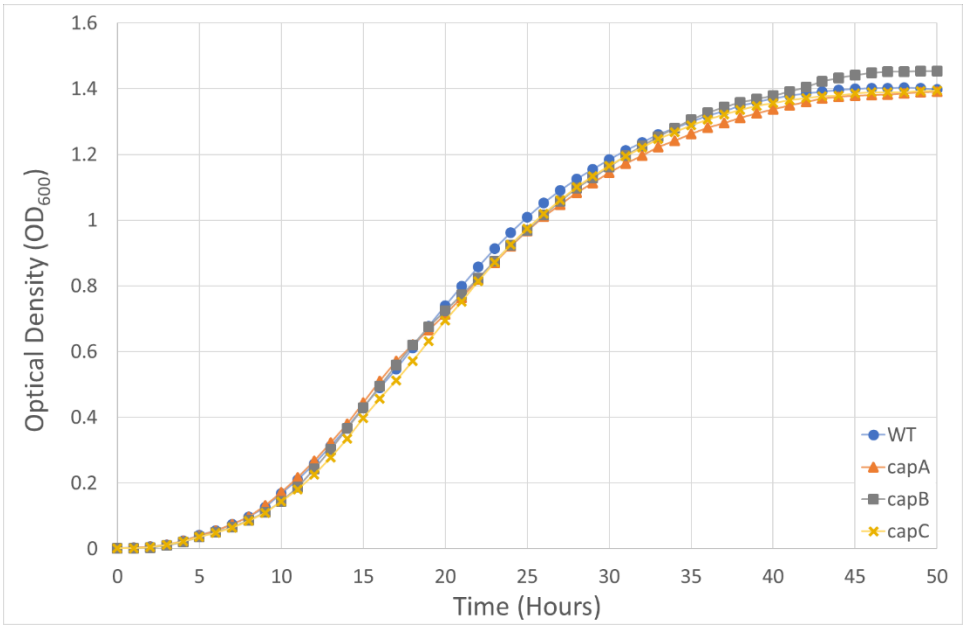


Figure 7) or TSB-C (

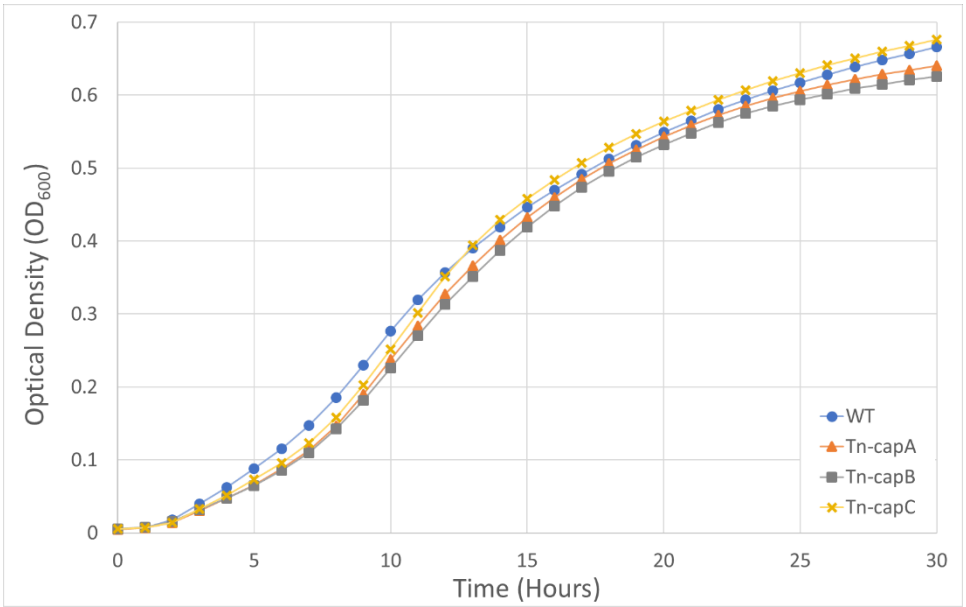


Figure 8).

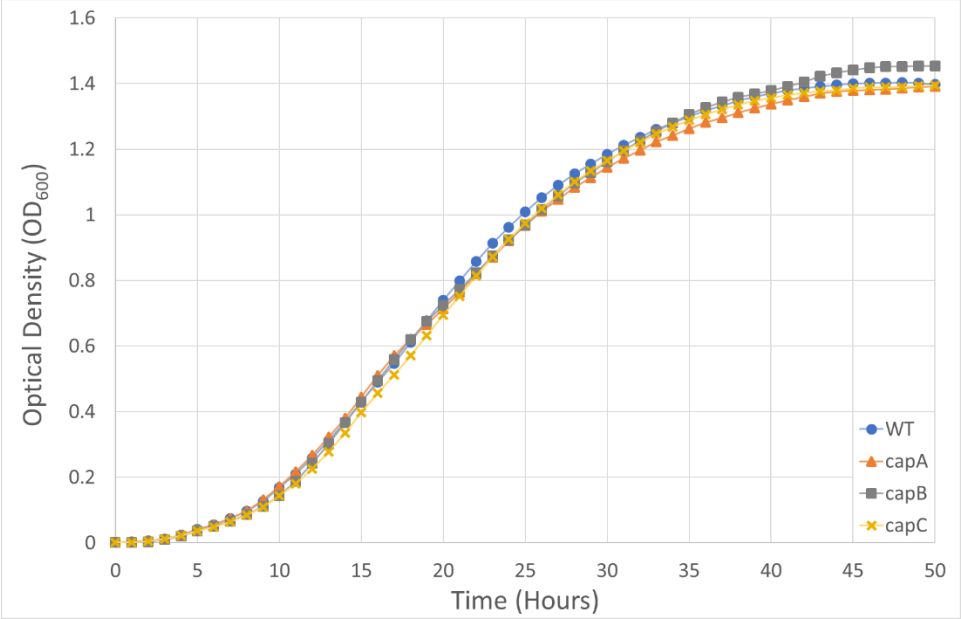


Figure 7. Growth of *F. novicida* WT and *cap* mutants in CDM at 37°C as measured by OD_{600}.

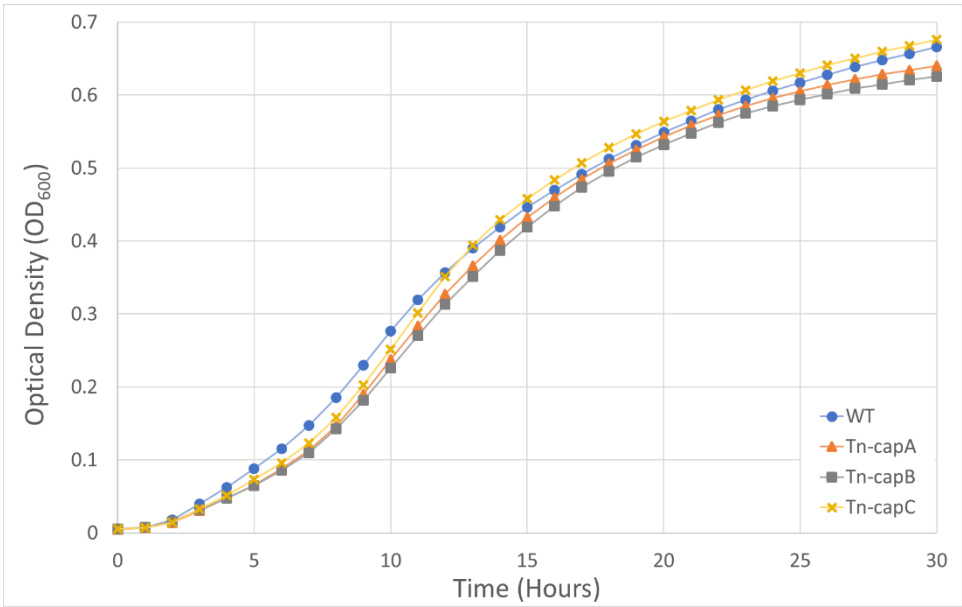


Figure 8. Growth of *F. novicida* WT and *cap* mutants in TSB-C at 37°C as measured by OD_{600}.

Intramacrophage Growth

After 8 hours *F. novicida* Tn-capB showed non-significant deficiency in intramacrophage replication as compared to the wild type (

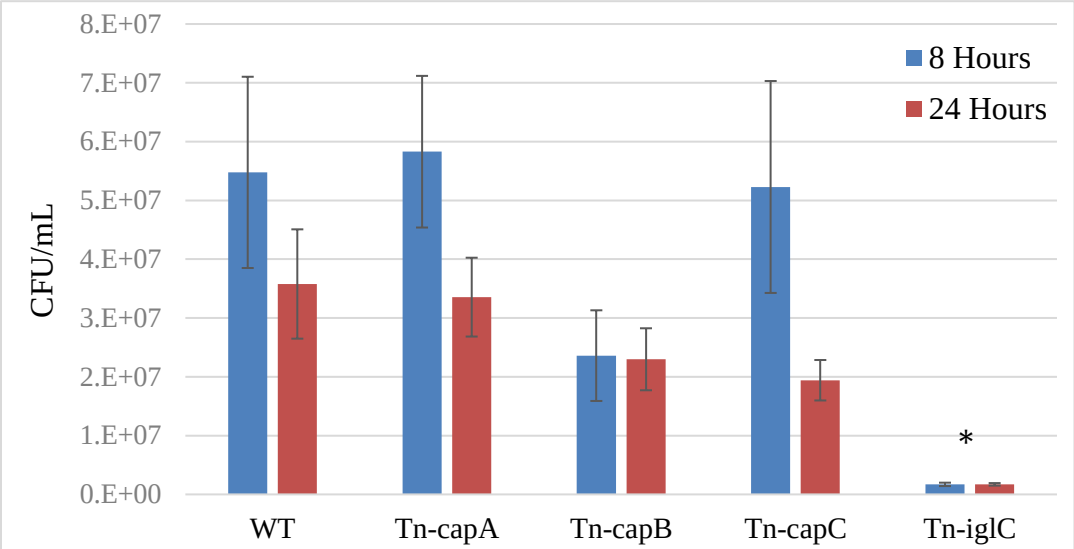


Figure 9). It should be noted that this mutant does not have data from the 8-hour time point for one of the three replicates. When the missing data is replaced with the average of the previous two replicates, the deficiency becomes significant (data not shown).

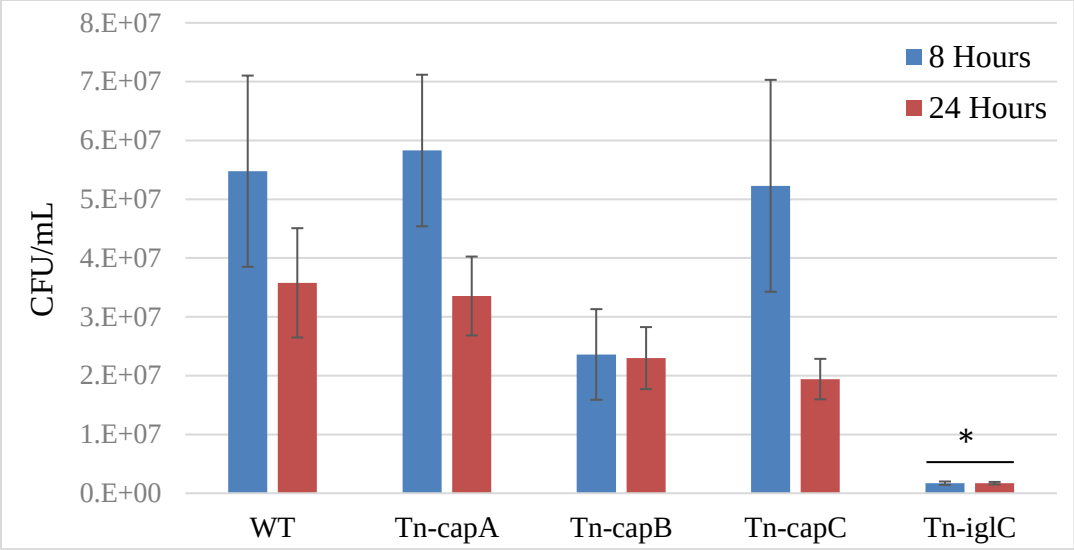


Figure 9. Bacterial concentration in cultures of RAW 264.7 macrophages after incubation for 8 hours and 24 hours post-gentamycin treatment. Asterisk indicates significance ($P < 0.05$) as compared with WT.

Discussion

Biofilm

Most research on the contribution of *cap*/*pgs* genes to biofilm production has been carried out in *B. subtillis* and offers conflicting conclusions. A PGA-producing strain of *B. subtillis* did not produce significantly less biofilm when *capB* (previously referred to as *ywsC*) was deleted (Stanley & Lazazzera, 2005). Chagneau & Saier carried out a similar study in a strain of *B. subtillis* that does not produce PGA and found no difference in biofilm production between the wild type and Δ*capB* mutant (Chagneau & Saier, 2005). On the other hand, two studies found that Δ*pgsB* mutants of multiple *B. subtillis* isolates produced biofilms that were less complex or structured than their wild type counterparts (Yu, et al., 2016; Liu, et al., 2010). This impaired

biofilm structure may be caused by reduced adhesion to surfaces since PGA is thought to aid in colonization of surfaces, as demonstrated on apples (Liu, et al., 2010).

The research on the relationship between *F. novicida cap* genes and biofilm formation is just as inconclusive as the research in *B. subtillis*. Durham-Colleran *et al*. found that mutations in *capB* and *capC* have no significant effect on biofilm production (Durham-Colleran, et al., 2010). Meanwhile the results in this thesis show that Tn-capB and Tn-capA mutants exhibit reduced biofilm formation, while the Tn-capC mutant produces more biofilm than the wild type (

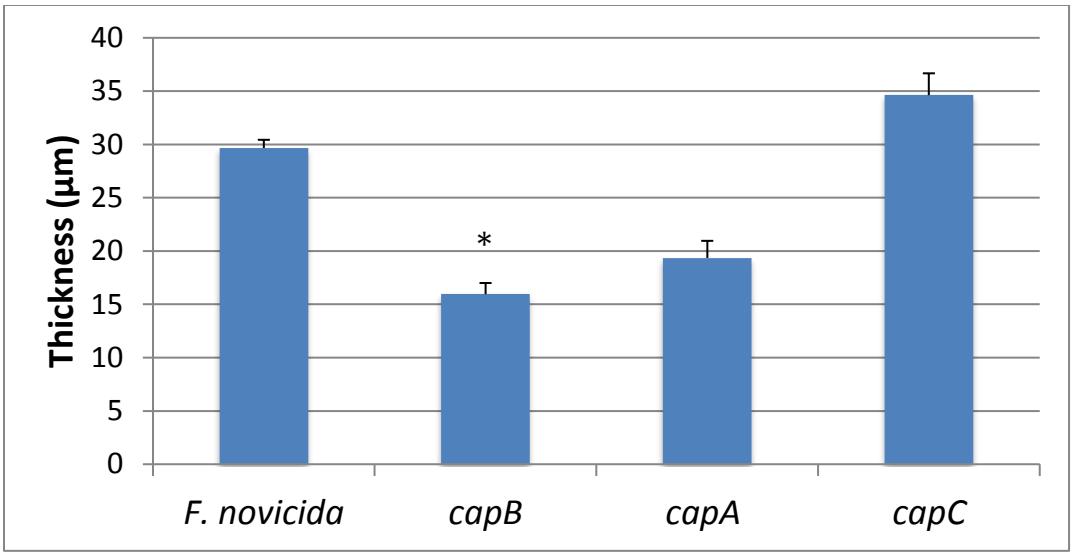


Figure 5).

The crystal violet assay can be used to measure relative biofilm formation for adherent biofilms but may not accurately account for non-adherent biofilms. The *F. novicida* Tn-capC mutant appears to produce small white particles that float freely, which could potentially be bits of pellicle (**Figure 6**). The discrepancy between the results of Durham-Colleran *et al*. and our

results on biofilm production in the *F. novicida* Tn-capC mutant may be due to minor differences in technique that result in this pellicle not being accounted for.

Growth Curves

No difference in in vitro growth was observed in the *F. novicida cap* mutants in CDM (

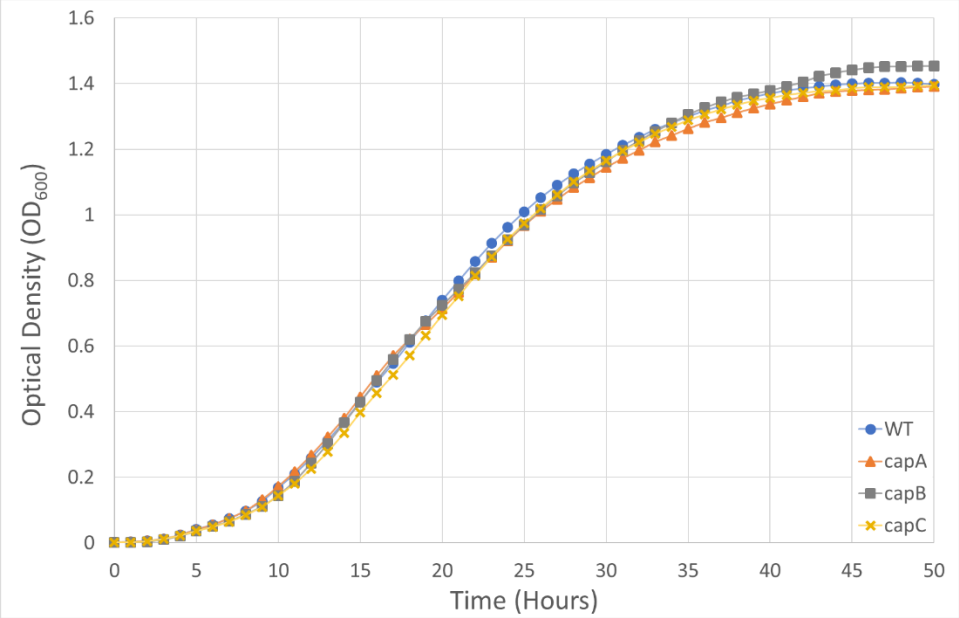


Figure 7) or TSB-C (

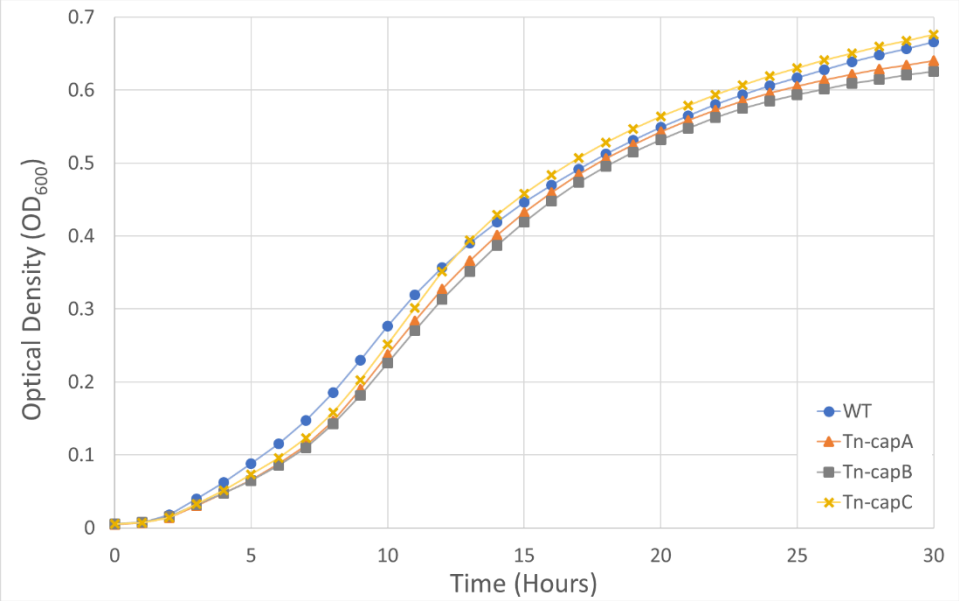


Figure 8). These results agree with the previous research on the *capBCA* locus in *F. tularensis* and LVS. LVS *capBCA* mutants did not demonstrate impaired growth in MHB, and neither did the *F. tularensis capBCA* mutants (Su, et al., 2007; Su, et al., 2011). A *F. tularensis capB* mutant did not exhibit reduced growth in modified cysteine partial hydrolysate broth either (Michell, et al., 2010). Though Durham-Colleran *et al* did find that *F. novicida* Tn-capB and Tn-capC were inhibited in growth according to OD_{600} after two days of incubation, they did not collect data at multiple time points and were therefore unable to calculate doubling time or growth rate (Durham-Colleran, et al., 2010). It is therefore evident that the *capBCA* locus does not affect in vitro growth of *Francisella.* The *capB* gene is only expressed at relatively low levels in LVS grown in vitro, so it is unsurprising that the *cap* locus has little effect under these conditions (Su, et al., 2011).

Intramacrophage Growth

Unlike in vitro growth, intramacrophage growth appears to be inhibited in some *Francisella cap* mutants. LVS with a *capBCA* mutation or a *capB* mutation showed impaired replication in both murine macrophages and human macrophages (Su, et al., 2011), and LVS *capB*, *capC*, and *capA* mutations were associated with reduced growth in J774A.1 macrophages (Maier, et al., 2007). Upon further examination of the LVS *capA* mutant, Maier *et al.* found that the mutant was able to enter J774A.1 macrophages as well as the wild type but was deficient in intramacrophage replication after the entry. These results suggest that CapA – and possibly CapB and CapC by association – is involved the later stages of LVS intramacrophage growth.

While the *cap* locus likely plays a role in intramacrophage replication for LVS, these genes do not appear to have the same importance in *F. tularensis*. A *F. tularensis capB* mutant was not deficient in replication within J774A macrophages (Michell, et al., 2010). Furthermore, *capC* and *capB* appear to be downregulated in *F. tularensis* within primary murine macrophages (Wehrly, et al., 2009). One possible explanation for the differing importance of the *cap* locus in intramacrophage replication is that *F. tularensis* encodes more proteins that aid in intramacrophage replication than LVS, thereby compensating for the low levels of *capC* and *capB* transcription.

F. novicida seems to fall between LVS and *F. tularensis* regarding the contribution of the *cap* locus to intramacrophage replication. While *F. novicida* Tn-capA and Tn-capC do not exhibit impaired replication within RAW 264.7 after 8 hours post-infection, Tn-capB intramacrophage

replication is slightly reduced (

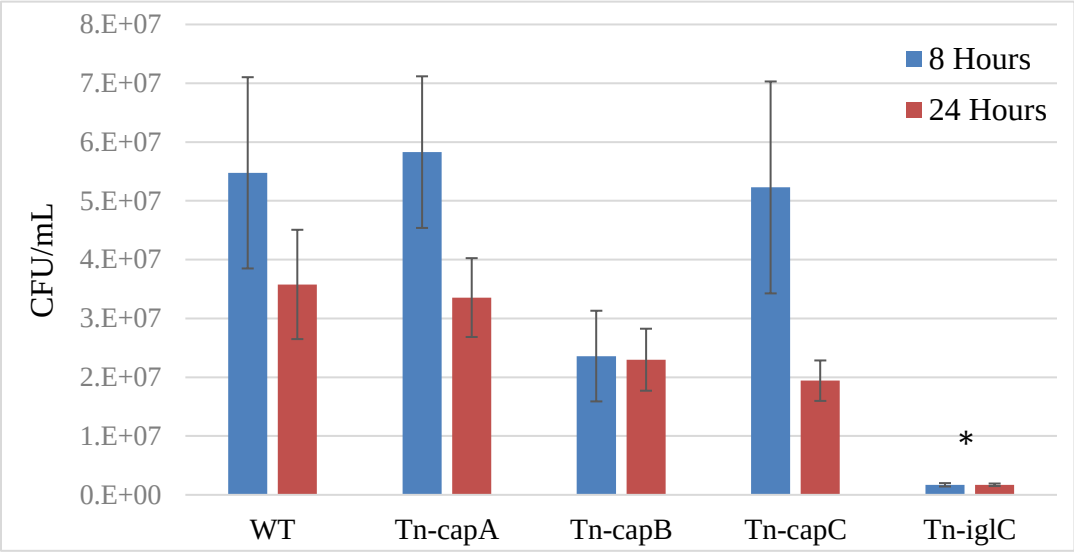


Figure 9). Though this difference in intracellular replication is non-significant, the lack of significance is probably due to the absence of data for a single 8-hour time point for Tn-capB. If this assumption is correct then the results would suggest that CapB can function without CapC in *F. novicida*, unlike the expected codependent relationship.

Cap/PgsC and Cap/PgsB have the same function, yet both have been shown to be necessary for PGA production in multiple *Bacillus* species (Candela, et al., 2005; Sawada, et al., 2018). These two proteins have therefore been theorized to work together in synthesizing PGA molecules. The potentially differing contribution to intracellular growth and biofilm formation between CapB and CapC in *F. novicida* could be explained by one of two theories; CapB alone is able to produce PGA that aids in intracellular replication and biofilm formation, or CapB serves a purpose unrelated to PGA synthesis. The gentamycin protection assay should be repeated to clarify if Tn-capB exhibits significantly reduced intramacrophage replication after 8 hours of

infection. An additional 2 hour time point may be useful in this case to determine if *F. novicida* CapB – like CapA in LVS – only contributes to later stages in intramacrophage replication.

CHAPTER THREE: PGA ISOLATION AND ANALYSIS

Introduction

PGA is a material of commercial interest with proposed applications in the medical, beauty, food, and bioremediation industries (Luo, et al., 2016; Sung, et al., 2005). PGA production through microbes on an industrial scale therefore has been the subject of several studies. Since the actual isolation of PGA from these microbes is an essential part of creating commercially available PGA, isolation and purification has been studied extensively in the microbes of commercial interest.

The procedure used to isolate PGA from *F. novicida* in this study was based on a method used to isolate PGA from a candidate commercial PGA-producer, *Bacillus licheniformis* ATCC 9945A (Yoon, et al., 2000). This PGA isolation procedure is relies on the solubility of DL-PGA in water and ethanol. PGA is soluble in water, so contaminants that are insoluble in water can be removed through centrifugation (Candela & Fouet, 2006). Ethanol solubility is less defined for PGA; PGA containing equimolar amounts of D-Glu and L-Glu monomers is insoluble in ethanol, while PGA consisting of only one enantiomer is soluble in ethanol (Candela & Fouet, 2006). Ethanol precipitation can therefore be used in DL-PGA isolation, but not D-PGA or L-PGA isolation. *B. licheniformis* synthesizes DL-PGA, so the procedure involves ethanol precipitation. Though there is no evidence that would suggest of *F. novicida* produces DL-PGA (instead of D-

PGA or L-PGA), the isolation procedure was attempted because most PGA-producers synthesize DL-PGA (Candela & Fouet, 2006; Ashiuchi, 2013).

Materials and Methods

Bacterial Strains and Culture Conditions

F. novicida U112 NR-13 was grown in 100 mL of TSB-C within a 500 mL Erlenmeyer flask for approximately four days at 37°C in a shaking incubator.

PGA Isolation

PGA isolation was attempted using the procedure outlined by Yoon *et al*. with some alterations (Yoon, et al., 2000). The culture was centrifuged for 20 minutes at 12,000 × g and 4°C to remove the bacterial cells. The supernatant was transferred to clean tubes before centrifuging again to ensure that all of the cells were removed. The supernatant was combined with four volumes of reagent alcohol (Fisher Chemical, Pittsburgh, PA) and left at room temperature overnight on a benchtop rocker. The precipitated material was separated from the alcohol through centrifugation at 12,000 × g and 4°C for 40 minutes and removal of the supernatant. Water was added to dissolve the presumed PGA, and water-insoluble impurities were removed by centrifuging the material for 20 minutes at 25,000 × g and 4°C and collecting the supernatant. Aqueous material was desalted through dialysis in 3.5K MWCO SnakeSkin™ Dialysis Tubing (Thermo Fisher, Waltham, MA) against deionized water for 24 hours at room temperature with two water changes. The desalted solution was lyophilized before reconstituting in about 4 mL of ultrapure water so that it could be dialyzed once again to

remove contaminating proteins. The dialysis was carried out in a 300K MWCO Float-A-Lyzer® G2 (Spectrum Labs, Rancho Dominguez, CA), which is much greater than the molecular weight of any proteins *F. novicida* encodes. The aqueous material was once again lyophilized to produce a white fluffy powder.

Proton Nuclear Magnetic Resonance Spectroscopy (^{1}H NMR)

^{1}H NMR analysis of the isolated material was performed with a Bruker Ascend 400 MHz NMR spectrometer (Bruker, Switzerland) using D_2O as the solvent.

Hydrolysis and Amino Acid Analysis

Isolated material was sent to Bio-Synthesis Inc. (Lewisville, Texas) for hydrolysis and amino acid analysis. Hydrolysis was carried out in 6M HCL at 110°C for 24 hours (Yao, et al., 2015). After hydrolysis, acid was removed through rotary evaporation. The hydrolyzed material underwent amino acid analysis using reverse-phase liquid chromatography (RP-LC) with pre-column derivatization and fluorescence detection.

Thin Layer Chromatography (TLC)

Hydrolyzed Batch F material was dissolved in distilled water at a concentration of about 5.6 mg/mL. Standard solutions of glutamic acid, asparagine, phenylalanine, and lysine were produced in distilled water at a concentration of 1 mg/mL. All amino acid standards had an approximate pH of 7.

TLC was performed using a previously published procedure (Ponder, et al., 2003). High performance silica gel TLC plates (Whatman, Maidstone, UK) were fully developed in a solution of dichloromethane and methanol (1:1 v/v) to clean the plates. After air drying, 10 µl of each

sample was applied to the plate. The plate was then dried in a hybridization oven at 40-45°C before being developed in a small twin-trough TLC chamber, using n-butanol−acetic acid−water (3:1:1 v/v/v). The plate was air-dried after developing. Detection was carried out by saturating the plate with ninhydrin, air drying for 30 minutes, and heating at 100-105°C for 10 minutes in a hybridization oven. A digital caliper was used to measure the distance travelled by the solvent front and spots for each lane so that R_f could be calculated.

Results

Proton Nuclear Magnetic Resonance Spectroscopy (^{1}H NMR)

The ^{1}H NMR results of Batch F do not indicate the presence of PGA based on the ^{1}H NMR results previously published (

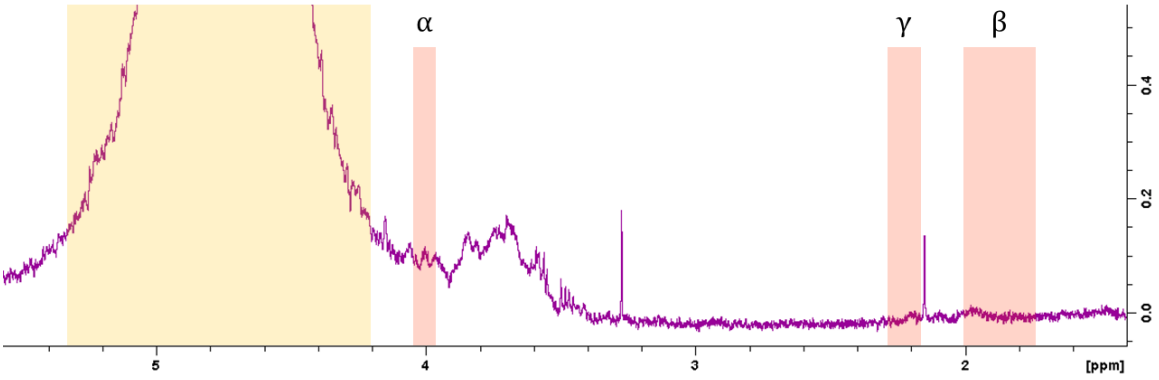


Figure 10) (Khalil, et al., 2016). A sample of Batch E before the second round of dialysis was similarly analyzed, but there were so many peaks from impurities that it would be impossible to differentiate PGA peaks from contaminant peaks.

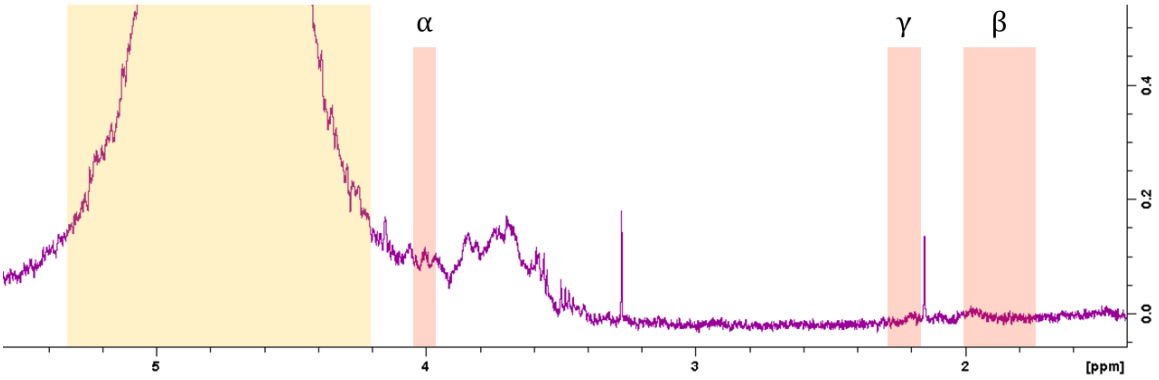


Figure 10. The ^{1}H NMR spectrum of the material isolated from *F. novicida* in D_2O. The peak highlighted in yellow is from the D_2O, while the regions highlighted in red are where the peaks from PGA are expected to be according to a previously published spectrum of PGA **(Khalil, et al., 2016)**.

Amino Acid Analysis

Of the 1 mg sample of Batch F sent for analysis, only 364.06 µg consisted of amino acids. Amino acid analysis by RP-LC with fluorescence detection revealed that glutamic acid and glutamine made up only 13.55% of the amino acids in the sample of Batch F (**Table 3**). Glutamine is converted into glutamic acid during the hydrolysis procedure, so it is impossible determine the quantity of glutamine versus glutamic acid in the original sample when using this method. A pure PGA sample should contain only glutamic acid. Therefore, PGA could not be identified in the Batch F material isolated from *F. novicida*.

Table 3. The relative quantity of each amino acid present in the Batch F material, given as a percent of the total amount of amino acids recovered from the sample.

Amino Acid	*Percent*

Aspartic acid & asparagine [a]	14.06
Glutamic acid & glutamine [b]	13.55
Lysine	10.69
Leucine	7.66
Valine	6.40
Alanine	6.28
Isoleucine	5.90
Threonine	5.66
Arginine	4.79
Serine	4.56
Phenylalanine	4.41
Glycine	4.40
Tyrosine	3.85
Proline	3.40
Histidine	2.10
Methionine	2.10
Cysteine	0.19
Tryptophan [c]	0.00

[a] asparagine in the original material is detected as aspartic acid due to the hydrolysis procedure

[b] glutamine in the original material is detected as glutamic acid due to the hydrolysis procedure

[c] the method of amino acid analysis employed has low rates of tryptophan recovery

Thin Layer Chromatography (TLC)

TLC of the previously hydrolyzed isolated material (Batch F) showed that at least five amino acids were found in the material (**Figure 11**). More specifically glutamic acid (**c**), phenylalanine (**a**), and lysine (**e**) could be identified within the hydrolyzed material. Asparagine may also be present (**d**). These results are not indicative of PGA in the material isolated from *F. novicida.*

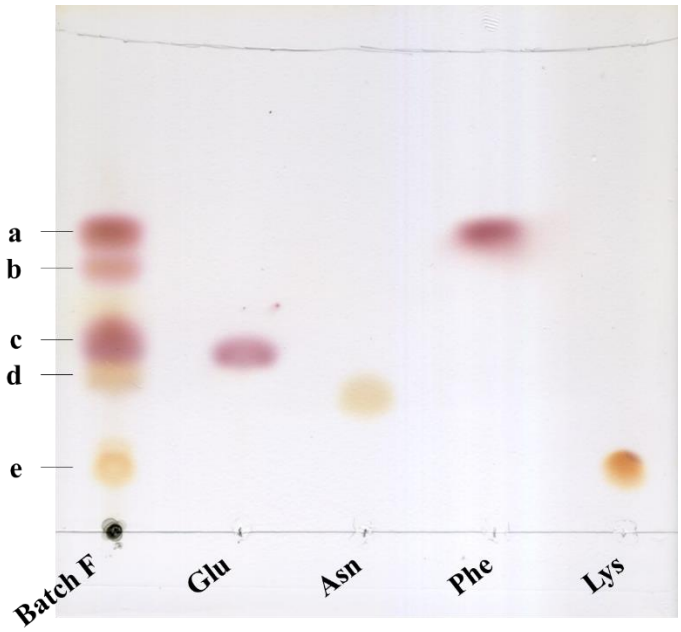


Figure 11. A silica gel TLC plate of hydrolyzed isolated material and four amino acid standards. The amino acids were visualized with ninhydrin.

Discussion

F. novicida PGA isolation was attempted using a protocol developed for the isolation of PGA from fed-batch *Bacillus licheniformis* cultures (Yoon, et al., 2000). Commercial amino acid analysis, thin layer chromatography, and ^{1}H NMR detected no PGA in the material isolated from *F. novicida*. It is possible that *F. novicida* does not produce PGA despite the presence of *capBCA* genes. Previously a *B. subtillis* strain (JH642) was found to be unable to produce PGA despite encoding PgsB, PgsC, PgsA, PgsE, and PgdS (Stanley & Lazazzera, 2005). However, this strain was able to produce PGA when altered to contain a copy of *swrA* (a regulator of flagella biosynthesis) and *degQ* (a transcriptional regulator) from a hybrid *B. subtillis* strain that produces PGA. *Bacillus* DegQ was later found to be an essential part of a signal transduction

cascade that activates *pgs* gene transcription (Hsueh, et al., 2017), so the change in ability of *B. subtillis* strain JH642 to make PGA is likely related to the transcription of these genes. Likewise, this phenomenon could be explained in *F. novicida* by non-functional or missing regulatory genes and proteins. Su *et al* observed that CapB is produced at low levels in LVS growing in vitro (Su, et al., 2011), suggesting that *F. novicida* may not produce PGA under these conditions. Investigating the transcription of *F. novicida cap* genes may provide answers to why PGA could not be isolated.

Although it is possible that *F. novicida* does not produce PGA, it is also possible that it does produce PGA but under different conditions. PGA production is greatly affected by culture conditions including media, temperature, incubation time, and aeration (Ashiuchi, 2013; Buescher & Margaritis, 2007). The PGA isolation procedure used in this research was based off of a procedure intended for fed-batch cultures of *B. licheniformis*, in which the bacteria was grown in a bioreactor that controlled dissolved oxygen content, pH, and nutrient flow into the culture (Yoon, et al., 2000). The conditions in which *F. novicida* was cultured for PGA isolation differed greatly and could not be optimized in the same way due to the lack of a bioreactor. *F. novicida* PGA may be produced under different conditions than described in this paper, as only one culture method was attempted. Media in particular may be important to PGA production in *F. novicida*. Microbes that produce PGA fall into two categories: L-glutamate dependent PGA producers and L-glutamate independent PGA producers (Luo, et al., 2016). *F. novicida* may produce PGA in a L-glutamate-dependent manner, in which case the addition of L-glutamate to the culture medium would stimulate PGA production.

The PGA isolation procedure may also be inadequate for this particular research. Most research on PGA isolation is focused on *B. subtillis* and *B. licheniformis* due to their high yield of PGA and is directed towards improving industrial-scale production of this commercially relevant polymer (Ashiuchi, et al., 2001; Wu, et al., 2010; Richard & Margaritis, 2003; Scoffone, et al., 2013; Richard & Margaritis, 2003). If PGA is released on a significantly larger scale than other extracellular material, purification methods employed for these organisms could therefore be less thorough and still result in detectable PGA. There are incredibly small peaks – barely above the baseline – on the Batch F ^{1}H NMR spectrum in the regions where the β and γ PGA peaks are expected (**Figure 10**). Likewise, the amino acid analysis would not rule out a very small – and therefore undetectable – quantity of PGA. Additionally, *F. novicida* may produce D-PGA or L-PGA as opposed to the expected DL-PGA. The purification procedure is not able to isolate L-PGA or D-PGA, as it would remain in the alcohol after the alcohol is removed.

It is likely for these reasons that PGA isolation was not attempted in *F. nucleatum*. Instead, Candela *et al*. used a dot blot with anti-PGA antibodies to prove that *F. nucleatum* produces PGA (Candela, et al., 2009). This method would be the most direct method to determine if *F. novicida* produces PGA and would allow for *F. novicida* grown in several culture conditions to be assayed for PGA production. For this reason, I propose replicating this experiment in *F. novicida* before ruling out PGA production.

CHAPTER FOUR: CONCLUSION

Though the production of PGA has been thoroughly investigated in *Bacillus* species and other gram positive organisms, *Francisella* PGA production has never been studied. In fact, only one gram negative organism has been found to produce PGA (Candela, et al., 2009). The *Francisella capBCA* locus has been attributed to aiding virulence and in vivo growth (Kraemer, et al., 2009; Su, et al., 2007; Su, et al., 2011; Michell, et al., 2010; Jia, et al., 2010; Weiss, et al., 2007), but the exact function of the CapBCA proteins has not been examined despite their homology to PGA-producing proteins in *B. anthracis*, *B. subtillis*, and *B. licheniformis*. Since there is evidence that the *capBCA* locus is medically relevant in *Francisella*, I aimed to further investigate the role of these genes in infection and growth and determine if they produce PGA in *F. tularensis* model organism, *F. novicida*.

Crystal violet biofilm assays demonstrated that all of the *cap* genes impact biofilm production in *F. novicida*, with mutations in *capB* and *capA* reducing biofilm production and mutations in *capC* increasing biofilm production. CapB and CapC also appeared to have different roles in infecting RAW 264.7, as shown by the Tn-capC mutant having no deficiency in intramacrophage replication and the Tn-capB mutant exhibiting reduced replication. None of the capBCA genes impacted in vitro growth in TSB-C or CDM.

The isolation of PGA from *F. novicida* was attempted to determine if this organism produces PGA, but no PGA could be definitively isolated. The isolation procedure resulted in a

small amount of white, water-soluble material. Analysis of this material using RP-LC with fluorescence detection, TLC, and ^{1}H NMR did not identify PGA within the material. A more straightforward PGA-detection method that does not require PGA isolation should be attempted to confirm that *F. novicida* does not produce PGA.

These results indicate that the role of *Francisella* CapB in virulence and biofilm production may be unrelated to PGA-production – the originally hypothesized function of this protein. Further investigation into the function of *F. novicida* CapB may reveal a potential drug target or vaccine candidate for biothreat agent *F. tularensis*.

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