Acetylation as a Regulatory Mechanism of Chitinase Activity in Francisella tularensis subsp. novicida

by

Alexandra Ii

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of
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Bachelor of Science
George Mason University, 2017

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Dedication

To my parents (and cats)

ACKNOWLEDGEMENTS

I would like to thank Dr. van Hoek for giving me the opportunity to work in her lab and trusting me to take on this project. You have been so supportive of me during the past year and patient at every road block. I am so appreciative to be able to continue working with you.

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List of Abbreviations

Acetyl phosphate	AcP
Chitin binding domain	CBD
Chitin binding protein A	CbpA
Chitinase A	ChiA
Chitinase B	ChiB
Chitinase C	ChiC
Chitinase D	ChiD
Fibronectin 3 domain	FN3
Francisella tularensis subsp. *novicida*	FTN
Francisella tularensis tularensis	FTT
Glycosyl hydrolase 18 domain	GH18
Hours	h
In vitro	IV
Isopropyl β-D-1-thiogalactopyranoside	IPTG
Lysine	K
Polymerase Chain Reaction	PCR
Rotations per minute	rpm
Seconds	s

ABSTRACT

ACETYLATION AS A REGULATORY MECHANISM OF CHITINASE ACTIVITY IN FRANCISELLA TULARENSIS SUBSP. NOVICIDA

Alexandra Ii, M.S.

George Mason University, 2019

Thesis Director: Dr. Monique van Hoek

Francisella tularensis is the causative agent of tularemia and is considered a Category A, Tier 1 biological threat agent. We and others have shown that *Francisella* tularensis forms biofilms *in vitro*, and we have previously demonstrated that biofilm formation is negatively regulated by chitinase expression. Given *Francisella*'s classification as a Category A bioterrorism threat and the lack of understanding surrounding environmental persistence, a better understanding of biofilm formation and dispersal is crucial. Our hypothesis is that the activity of chitinase may be regulated by the post-translational modification acetylation, which has been shown to regulate other enzymes and occur in bacteria. A full analysis of the acetylation of the *Francisella* tularensis subsp. novicida proteome has been conducted by the van Hoek lab, resulting in the identification numerous lysine acetylation sites on chitin-binding protein A, Chitinase A, and Chitinase B. Given acetylation's role on enzymatic regulation, we attempted to clone *Francisella tularensis* subsp. *novicida* U112 chitin-binding protein A and chitinases A, B, and D for enzymatic analysis upon chemical acetylation. Chitinase B and

chitin-binding protein were successfully expressed for further experimentation. Concentrated *Francisella* secreted proteins containing chitinases A and B were chemically acetylated using lithium potassium acetyl phosphate, which resulted in a significant decrease in chitinase activity compared to unacetylated protein concentrate and *Trichoderma viride* chitinase (P=0.0067, P=0.000014). Additionally, by treating preformed biofilm with acetylated chitinase limited biofilm dispersal was visualized relative to chitinase treatment (P=0.000017). In conclusion, this study suggests that the inhibition of chitinase activity through acetylation may be one mechanism of regulation of chitinase activity, and thus biofilm formation levels in *Francisella*.

Chapter One: Introduction

Chitinases are glycosyl hydrolases found extensively in both eukaryotes and prokaryotes. They are capable of breaking down the linear β-1,4-linked polymer of N-acetyl-D-glucosamine (GlcNAc) also known as chitin [1]. As the second most abundant polysaccharide on Earth, chitin is a substantial source of naturally available carbon and nitrogen. Chitinases are required for the metabolism of chitin and have therefore been extensively studied in numerous species.

Chitinases are generally regarded as putative virulence factors due to strong upregulation during infection. Studies have documented upregulation of chitinases *in vitro* in infected bodily fluids in *Listeria monocytogenes*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa* [2, 3, 4]. Chitinase transcription was also induced in intracellular infections in macrophage cultures for *L. monocytogenes* and *Salmonella typhimurium*, however this was not found in *Francisella* [5]. Upregulation *in vivo* during infection has been verified in *L. monocytogenes*, *S. typhimurium*, and *Francisella tularensis* [2, 6, 7]. Infections with chitinase mutant pathogens often result reduced murine recovery time as seen in *Legionella pneumophila* and *L. monocytogenes*, however no attenuation was present in *Francisella* infection [8, 9, 10, 7].

Francisella tularensis (*F. tularensis*) is a category A bioterrorism threat and the causative agent of tularemia. Four varieties of chitinases have been found in *Francisella* genomes. Despite over 95% homology between *Francisella* subspecies, chitinases are

genetically differently between subspecies and subpopulations [11, 12]. Additionally, there have been no reports of chitin production in *Francisella*, despite the expression of chitinases [5]. This suggests that chitinases may act on surface chitin sources or other complex carbohydrates produced by *Francisella*. These carbohydrates may act as a structural component of the biofilm, however the biofilm composition of *Francisella* must be fully evaluated before exploring this question.

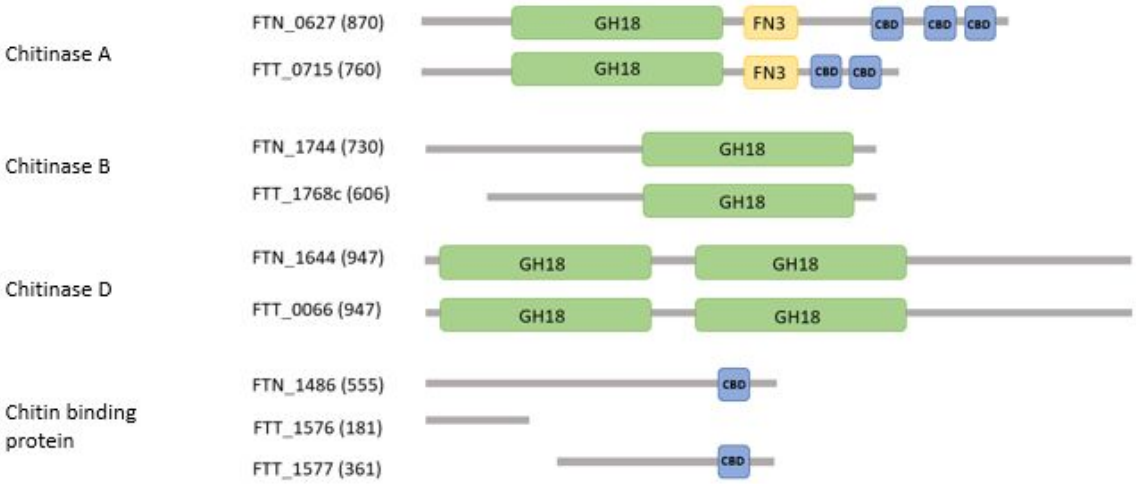


Figure 1. Domain map of chitin binding protein and chitinases A, B, and D. Chitin binding protein and chitinases A, B, and D are present in *Francisella tularensis* subsp. *novicida*, while *Francisella tularensis tularensis* Schu S4 contains genes encoding chitinases A, B, C, and D with a fragmented chitin binding protein gene. (Green boxes= GH18 domain, yellow boxes= fibronectin type 3 domains, blue boxes= carbohydrate binding domains). Amino acid sequences between *Francisella novicida* and Schu S4 subspecies maintain 86.9%, 98.5%, 98.6%, and 98.6% identity, respectively (ChiA, ChiB, ChiD, cbpA). The degree of homology between *Francisella tularensis* subsp. *novicida* and *Francisella tularensis tularensis* Schu S4 and relative enzymatic activities make FN chitinases suitable models for the fully virulent strain. [11]

In *Francisella*, the role of chitinase is not fully understood, however recent studies have shown chitinase to be a substantial regulator of biofilm production [5, 13]. Chitinase has also been found to be upregulated 20 times during *Francisella tularensis* Schu S4 murine infections [7]. As stated previously, chitinases have been identified as virulence factors in many bacteria, however this has been disputed in *Francisella* due to lack of attenuation in mutant infections [14]. Regardless, upregulation data and its role in biofilm regulation have supported the importance of chitinase in the infection cycle of *Francisella*. Chitinase is not believed to have a role in virulence, however the predominant theory predicts that chitinases are largely involved during arthropod infection, where chitinase allows growth on chitin abundant surfaces [13, 15]. Similarly, biofilm regulation through chitinase activity may allow environmental proliferation in aqueous habitats and support water-borne transmission [5].

All chitinases possess one or more glycosyl hydrolase (GH) superfamily domains. In *Francisella* the conserved domain is glycosyl hydrolase 18 (GH18). Non-enzymatic domains commonly found in chitinases such as N-acetylglucosamine-binding protein A, carbohydrate binding, and fibronectin type 3 (FN3) domains facilitate depolymerization or adherence to chitin. The occurrence of these domains differs between both *Francisella* strains and categories of chitinases. Four varieties of chitinases have been identified within *Francisella*: A, B, C, and D. In *F. novicida* the majority of chitinase activity is carried out by chitinases A and B, while chitinase D has been determined to be non-functional through expression in *E. coli* and enzymatic assays [11].

Thus far the role of *Francisella* chitin binding protein A has not been extensively evaluated. Previous studies have shown synergistic activities between chitinases and chitin-binding proteins [16]. Chitin binding protein displays a role in virulence in *Vibrio cholera*, suggesting significance for further studies [14]. Additionally, previous studies in the van Hoek lab have noted a 13-fold increase in *cbpA* when treated with signaling molecule *Burkholderia* diffusible signal factor (BDSF). Consistent results were visualized with 26- and 9-fold increases of *chiA* and *chiB,* respectively. These results support the relationship between cbpA, ChiA, and ChiB with other known *Francisella* virulence factors such as IglC and RelA [36].

Previous publications by Dr. van Hoek's lab have established the role of chitinase in the regulation of *Francisella* biofilm [5]. Additionally, acetylation sites have been determined by mass spectrometry for chitin binding protein, and chitinases A and B. Multiple acetylation sites have been found in the catalytic domains of chitinases A and B, however chitinase D was not detectable in the mass spectrometry sample set. Chitin-binding protein was also present in the data set with 9 acetylation sites.

The **goal** of this study is to clone and express the *F. tularensis* subsp. *novicida* chitinases in order to further elucidate the relationship between chitinase activity and acetylation of chitinases and chitin binding protein. Our hypothesis is that acetylation inhibits enzymatic activity of chitinases A and B. We predict that acetylation acts as a sliding scale form of inhibition rather than an on/off mechanism. As determined previously, active chitinase results in the dispersal and inhibition of biofilm; therefore, we

anticipate acetylated chitinase will not be able to disperse or inhibit biofilm, resulting in a buildup of detectable biofilm.

Preliminary Data

Francisella Acetylation

In early 2018, a proteome-wide profiling of N-ε-Lysine acetylation was conducted for *Francisella tularensis* subsp. *novicida* U112 through mass spectrometry of chemically acetylated cell lysate and supernatant. This paper, titled "Proteome-wide profiling of N-ε-Lysine acetylation reveals a novel mechanism of regulation of chitinase activity in *Francisella tularensis*", was submitted to the journal of Molecular and Cellular Proteomics in April 2019. Through this assessment, we identified 280 *Francisella* proteins with 1,178 total acetylation sites. Though previous studies have used chemical acetylation to induce acetylation under circumstances where it may not be detectable, it has not been established whether these are true acetylation sites found in standard growth conditions. However, it has been found in *E. coli* that approximately 90% of acetylation modifications were likely due to chemical acetylation rather than an enzymatic addition, supporting chemical acetylation induction as a method of acetylome studies [17]. Within the Mass Spectrometry data set, we found chitin binding protein and chitinases A and B. Chitinase A was found to have two acetylation sites (K206, K849) in the glycosyl hydrolase 18 and chitin binding domains respectively. These sites were found in standard growth conditions, suggesting that they may have an active role in enzymatic function.

Chitinase B showed numerous acetylation sites in standard growth and chemically acetylated samples. Standard growth samples exhibited 13 acetylation sites on chitinase B (K96, K140, K145, K170, K193, K217, K226, K314, K344, K380, K420, K453, and K542), 3 of which residing the glycosyl hydrolase 18 domain (K420, K452, K542). Chemically acetylated samples presented 7 sites on chitinase B (K197, K226, K344, K420, K453, K582, K589), including 4 sites present in standard growth conditions. Chitin binding protein showed 4 acetylation sites (K225, K258, K465, K555) in standard growth and 5 unique sites (K 232, K373, K400, K411, K430) in *in vitro* samples. Sites located in active domains may serve regulatory purposes, which can be examined through site directed mutagenesis, chemical acetylation, and enzymatic assays.

Chitinase and Biofilm Regulation

In a paper published by the van Hoek lab, it was established that chitinase acts as a negative regulator of *Francisella* biofilm [5]. Though it had been previously shown that without an exogenous sugar source ChiA and ChiB mutants exhibit attenuated biofilm formation on chitin [13], Chung *et al* showed direct dispersal of biofilm when treated with streptomyces chitinase. Previous results were verified by COMSTAT imaging of ChiA and ChiB transposon mutant biofilm (Fig. 2), showing significant decreases in biomass and thickness relative to wild-type *Francisella*. Additionally, when supplemented with SAN or DEQ glycosyl hydrolase inhibitors in conjunction with chitinase, biofilm dispersal was inhibited (Fig. 3). These results support the relationship

between *Francisella* chitinase activity and biofilm regulation.

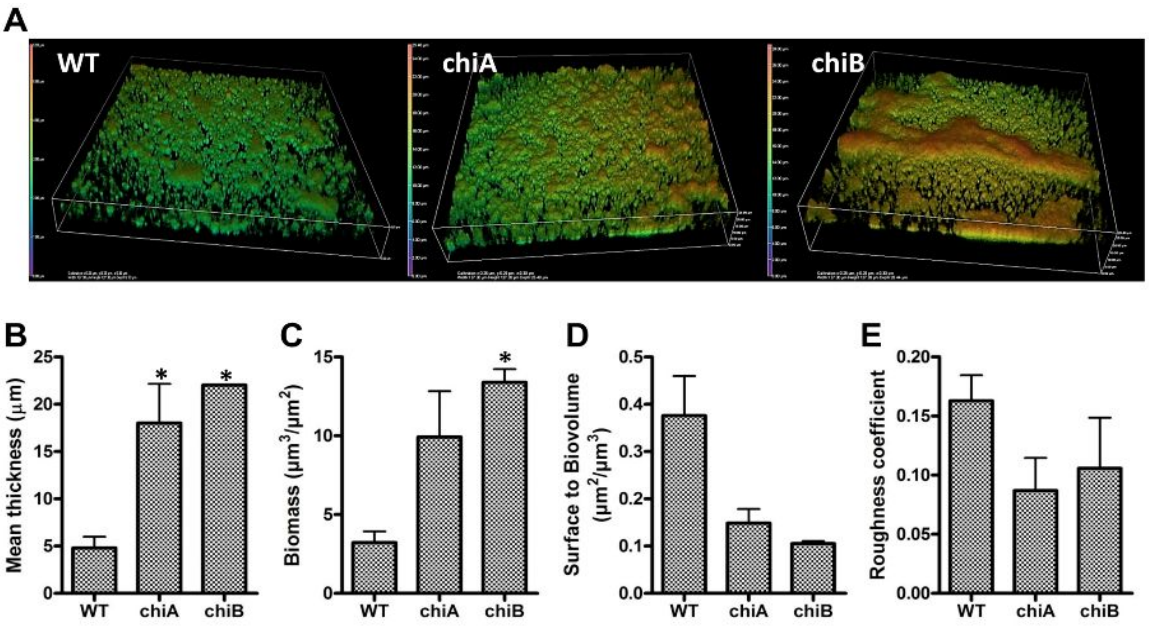


Figure 2. COMSTAT2 analysis of *Francisella* wild type (WT) and Chi transposon mutant biofilms. Both ChiA and ChiB mutants show a significant increase in biofilm thickness. However, the ChiB mutant shows both an increase in mean thickness and biomass, suggesting that chitinase B is a predominant regulator of *F. novicida* biofilm [5].

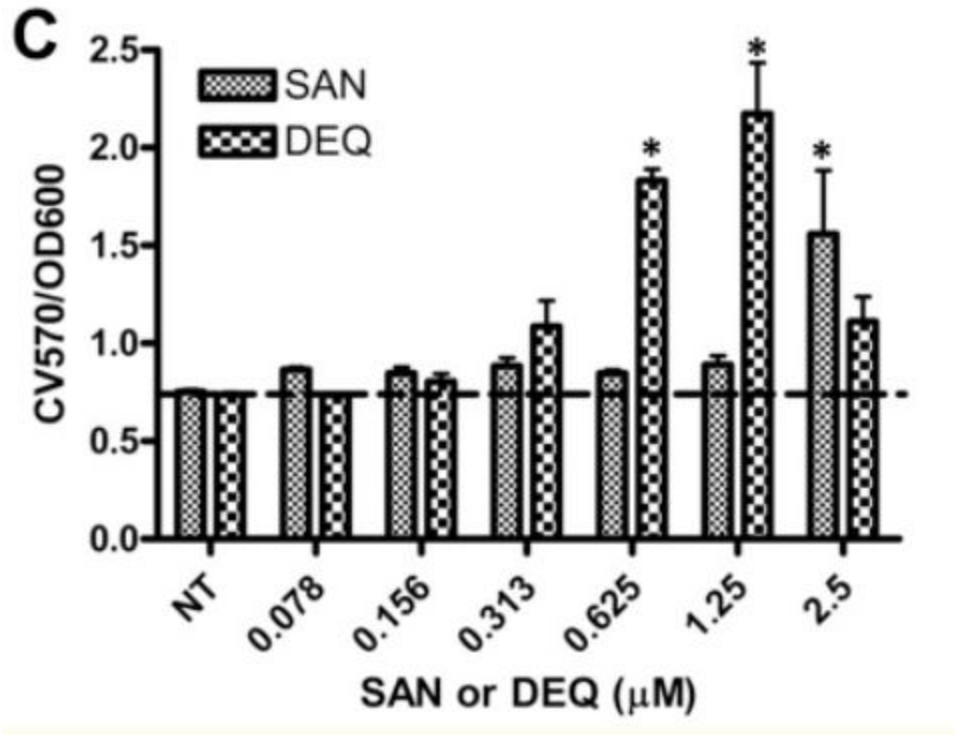


Figure 3. Inhibition of chitinase activity and impact on WT *F. novicida* biofilm dispersal. Inhibition of glycosyl hydrolase activity results in significant lack of biofilm dispersal relative to treatment with only chitinase (no SAN or NEQ) [5].

CHAPTER TWO: CHITINASE EXPRESSION

The van Hoek lab has previously attempted to express *Francisella* chitinase A in order to assess its relationship to biofilm and virulence in detail. However prior to this study, attempts have been unsuccessful. Cloning was conducted from a plasmid containing the *Francisella tularensis tularensis* Schu S4 chitinase A coding region and directly from *F. novicida* genomic DNA. Expression of *Francisella* chitinases would allow continued assessment of the impact of acetylation on enzymatic activity and resulting biofilm dispersal.

Expression of FTT Chitinase A

Experimental Setup

A pDEST-17 plasmid containing the *Francisella tularensis tularensis* Schu S4 chitinase A coding region (FLH230430.01X) was purchased from the DNASU Plasmid Repository. FTT ChiA was amplified using designed primers with engineered restriction sites (Table 1). Following restriction, FTT ChiA was ligated into the pQE-30 plasmid and transformed into NEB D5α competent *E. coli*. Plasmids were purified using the QIAprep Spin Miniprep kit following manufacturer's instructions and run on 0.8% agarose gel for verification. Insertion positive clones were sent for sequencing. Insertion verified pQE-30 plasmids were transformed into the NEB Express expression *E. coli* strain. Protein expression was induced using various concentrations of IPTG (0.05mM, 0.2mM, 0.5mM,

1mM) at 30 and 37℃. Samples were collected at 2h, 4h, 6h, 12h, and 24h time points.

Table 1. Primers for amplification from FTT ChiA pDEST-17

Loci	Forward Primer (5'-3')	Reverse Primer (5'-3')
FTT_0715	**GAG CTC** ATG AAC AAA ACA AAA TTA GTC TCA G	**CTG CAG** TTA TTG TTT TTC CCA AAC ATT ACT

Results and Discussion

FTT ChiA was successfully amplified from pDEST17-ChiA purchased from DNASU (Figure 4). Restriction cloning was conducted and FTT ChiA was ligated into the pQE-30 plasmid (Figure 5). Insertion positive plasmids were sequenced, however the complete sequence was not verified due to insertion length. Despite optimization through adjustments in aeration, IPTG concentrations, temperature, and media volume, no expression was visualized by gel or western blot in either secreted protein or cell lysate (Figure 6).

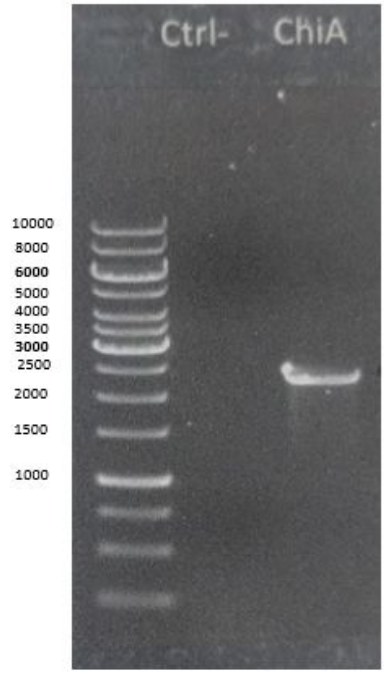


Figure 4. Agarose gel of amplification of FTT ChiA from pDEST clone. PCR amplification was successful using designed primers with SacI/PstI restriction sites, resulting in a 2.3kb band. Negative control showed no amplification.

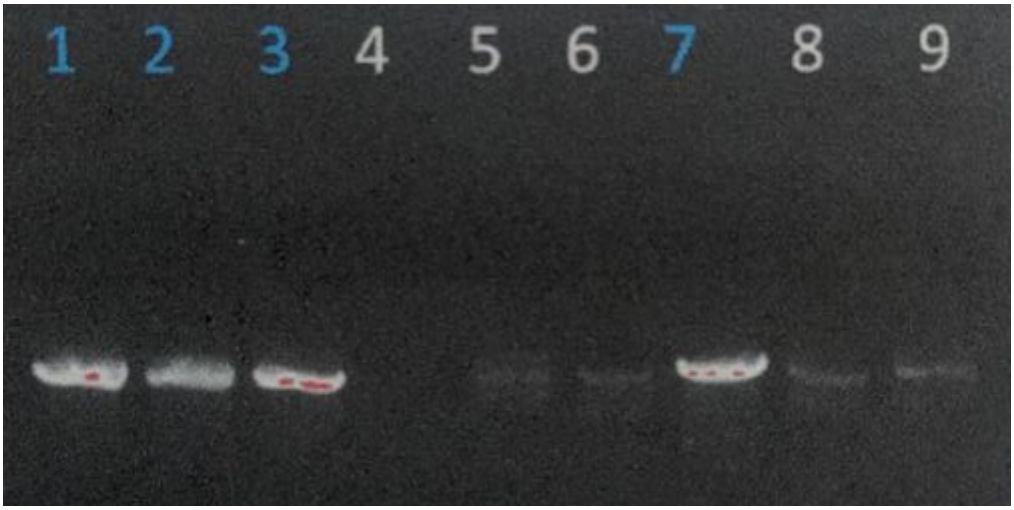


Figure 5. Agarose gel of FTT ChiA pQE-30 ligation. Ligation into pQE-30 was successful as shown by banding in lanes 1, 2, 3, and 7. Positive clones were sent to be sequence verified (blue).

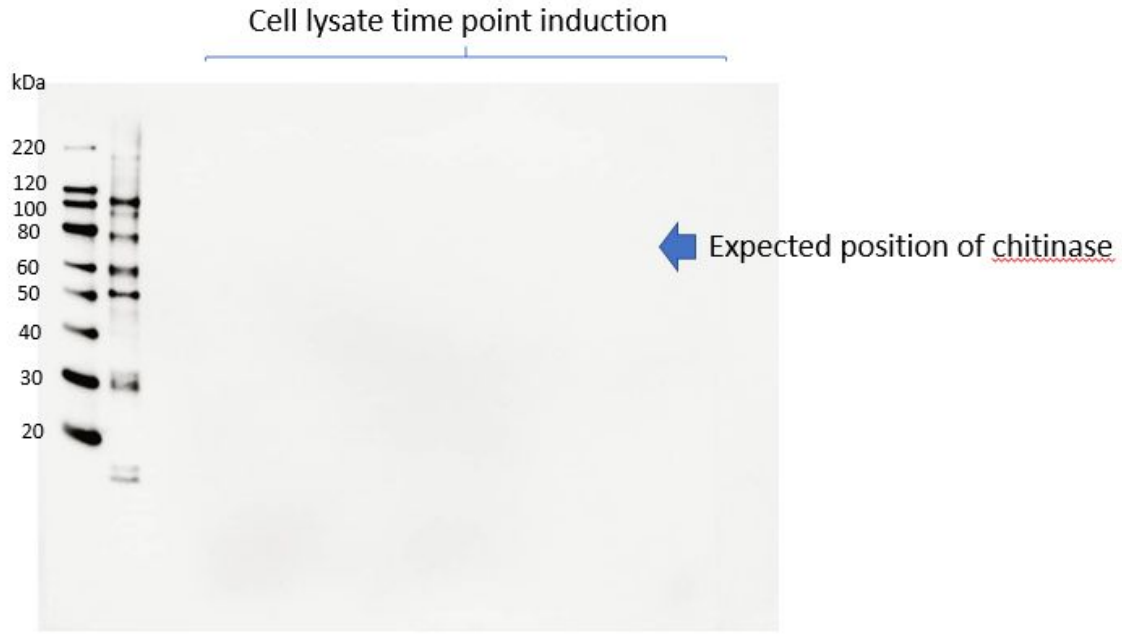


Figure 6. α6his-tag Western blot of IPTG induced ChiA clone. Time point induction with IPTG resulted in no expression, despite optimization. Chitinase A was expected at approximately 80kDa.

Though FTT ChiA clones were successfully produced, we were unable to express recombinant ChiA from these clones. Several issues may have resulted in the lack of expression or detection of expression. The 6his-tag incorporated using the pQE-30 plasmid is located on the N-terminus of the secretion signal peptide found on ChiA. It is possible that chitinase was produced following IPTG induction, however the 6his-tag was cleaved, resulting in a lack of detection by western blotting. However, in this scenario there may be a degree of visualization in the cell lysate, which was not found experimentally. Additionally, we were not able to verify the accuracy of the full sequence

due to the length of the insertion. Because the beginning and end of the sequence showed no errors or frameshifts, it was assumed that the protein would be able to be expressed correctly and potential internal mutations would have negligible impact on protein function. Finally, the pQE-30 plasmid utilizes the T5 promoter, which has been shown to have weaker strength relative to the T7 promoter. These issues were acknowledged and addressed during the second phase of cloning.

Expression of FTN CbpA and ChiA, ChiB, and ChiD

Considering the high degree of amino acid similarity between the chitinases in the fully virulent Schu S4 strain compared to *F. novicida* (Fig. 1), the *F. novicida* chitinases and chitin-binding protein were cloned. Previous studies had verified comparable chitinase activities between the two strains [11].

Experimental Setup

Primer design and vector verification. The method of cloning was based on a *Francisella* chitinase activity study conducted by Chandler *et al*. Slight adjustments were made during primer design to avoid nonspecific binding during PCR. Primers were designed using Thermo Fisher OligoPerfect and altered manually to minimize dimer potential and ensure appropriate GC content. Forward and reverse primers had restriction sites incorporated for restriction cloning into the pET23b+ expression vector. Unique restriction sites were verified by NCBI BLAST. Prior to cloning, the open reading frame

(ORF) were verified by VectorNTI software.

Table 2. Primers for amplification of *Francisella tularensis* subsp. *novicida* U112 from genomic DNA (GCT AGC= NheI, CTC GAG= XhoI)

Protein class	Loci	Amplicon Size	Forward primer (5'- 3')	Reverse primer (5'-3')
ChiA	FTN_0627	2624	**GCT AGC** GGA TGA ACA AAA CAA AAT TA	**CTC GAC** TTG TTT TTC CCA AAC
ChiB	FTN_1744	2204	**GCT AGC** GGA TGA AAT ACA AAA AGT TAT T	**CTC GAG** TTT ATC ATT TAT AGG ATA AAA CTC
ChiD	FTN_1644	2855	**GCT AGC** GGA TGA GAA AAC TTT TTA TAA AT	CCC **CTC GAG** TTT ACT ATC TAT TTT TGT
cbpA	FTN_1485	1793	**GCT AGC** GGA TGA AGA AAC TTA TTG	CCC **CTC GAG** CTT AAC TAT ATT CCA AG

Table 3. Primers for amplification from PCR product (GCT AGC= NheI, GTC GAC=SalI, CTC GAG= XhoI)

Protein class	Loci	Amplicon Size	Forward primer (5'- 3')	Reverse primer (5'-3')
ChiA	FTN_0627	2622	**GCT AGC** ATG AAC AAA ACA AAA TTA	**GTC GAC** TTG TTT TTC CCA AAC
ChiB	FTN_1744	2202	**GCT AGC** ATG AAA TAC AAA AAG TTA TT	**CTC GAG** TTT ATC ATT TAT AGG ATA AAA CTC
ChiD	FTN_1644	2853	**GCT AGC** ATG AGA AAA CTT TTT ATA AAT	CCC **CTC GAG** TTT ACT ATC TAT TTT TGT
cbpA	FTN_1485	1791	**GCT AGC**	CCC **CTC**

			ATG AAG AAA CTT ATT G	**GAG** CTT AAC TAT ATT CCA AG

PCR of target Francisella novicida amplicons. *Francisella tularensis* subsp. *novicida* U112 was grown in tryptic soy broth supplemented with 1% cysteine at 37℃ (TSB-c) with shaking at 180rpm for 24 hours. Bacterial genomic DNA was harvested from whole cell lysate using the Qiagen DNeasy UltraClean Microbial kit. PCR was conducted using 15ng genomic DNA, 0.5U NEB Q5 High Fidelity DNA polymerase, 1x Q5 reaction buffer, 200uM dNTPs, and 1μM designed primers to amplify chitin binding protein A (ABK90352.1) and chitinases A (ABK89520.1), B (ABK90596.1), and D (ABK90502.1) amplicons. PCR settings were optimized at 98℃ for 30s, followed by 25 cycles of 98℃ for 10s, 55℃ for 30s, and 72℃ for 80s, and final extension at 72℃ for 2 min. PCR product clean-up was conducted using the QIAquick PCR purification kit prior to blunt end cloning.

Blunt end cloning. Based on methodology established by Chandler *et al*, plasmids containing the targeted amplicon were created using the Zero Blunt™ TOPO™ PCR Cloning Kit following standard protocol. Cloning product was transformed into the NEB D5α competent using the 42℃-heat shock protocol of insert protocol. After 18-22 hours of growth on kanamycin selective plates, individual colonies were used to inoculate kanamycin media. Following 18-22 hours of growth, plasmids were isolated using the QIAprep Spin Miniprep kit. Amplicon insertion was verified by running purified plasmid

on 0.8% agarose gel supplemented with ethidium bromide and comparing relative plasmid size to a negative control (TOPO plasmid without insertion). Plasmids with insertion were sent for sequencing to a Functional Biosciences with designed internal primers to ensure complete sequencing (Table 4).

Table 4. Internal primers to verify full sequences of ChiA, ChiB, ChiD, and cbpA.

Amplicon	Internal Primer (5'-3')
ChiA	CGC TAG ATC AAT GAC TGT AGC TAG TG
ChiB	ACA ACT GGC TTA CCT CAA ACT AT
ChiD	GTA GTT CTG ATA TGC CTA AGA ATG AT
cbpA	GAA GAT GCT AAT AAA GCC T

Restriction cloning and transformation. Upon sequence verification, insertion positive vectors were digested using enzymes specific to engineered primers. Corresponding restriction sites were present in the pET23b+ vector as verified by Vector NTI. The reactions were conducted using, 200-300ng/μL insertion positive TOPO plasmid, 10uL 10X Restriction buffer, 10uL 10X BSA, 15-40U restriction enzyme 1, 15-40U restriction enzyme 2, and appropriate volume of nuclease free water. Reagent were mixed thoroughly and incubated for 1 hour at 37℃. Restriction product was run on an 0.8% agarose gel following previous method and appropriate bands corresponding with insertions were excised. DNA was purified from the gel using the QIAquick Gel Extraction kit following manufacturer's instructions. Inserts were ligated into the

pET23b+ vector at a plasmid to insert ratio of 1:5 with appropriate ligation controls. Plasmids were run on agarose gel for insertion verification. Positive plasmids were transformed into NEB D5α competent strains using the 30s 42℃ heat shock protocol, verified by agarose gel, and stored at -80℃ in 20% glycerol stocks until further use.

Expression and protein purification. Positive pET23b+ vectors with insertions were transformed into the BL21(DE3)/pLysS cell line and grown for 18 hours in selective media. Plasmids were isolated and run on an agarose gel for verification. After 18-hour growth in selective LB, expression was induced overnight using Magic Media. Supernatant and cells were separated by centrifugation after overnight induction. Cells were lysed by sonication. Samples were run on Tris-Bis gel and processed by Coomassie and western blot verification. Expression in the supernatant was verified using anti-6histidine antibodies. Expressed proteins were purified using 6xhis-tag Dynabeads (Invitrogen) following standard protocol.

Results and Discussion

CbpA, ChiA, ChiB, and ChiD amplicons were successfully amplified from *Francisella tularensis* subsp. *novicida* U112 genomic DNA using the initial designed primers (Table 2). However, additional nucleotides in the forward primers would have induced a frameshift following ligation into the pET23b+ vector, therefore a secondary set of primers was created (Table 3). Amplification from genomic DNA was unsuccessful

using the second primer set, however PCR was successful by using the primary PCR product as the template DNA as opposed to genomic DNA (Figure 7). This adjustment resulted in the removal of the nucleotide insertion.

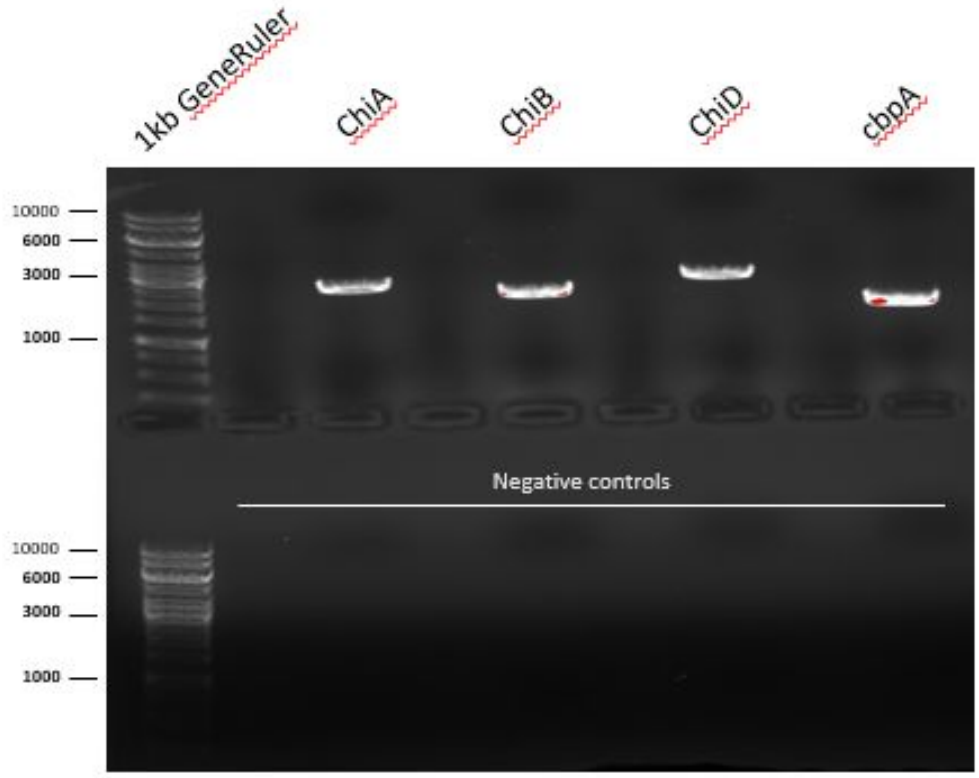
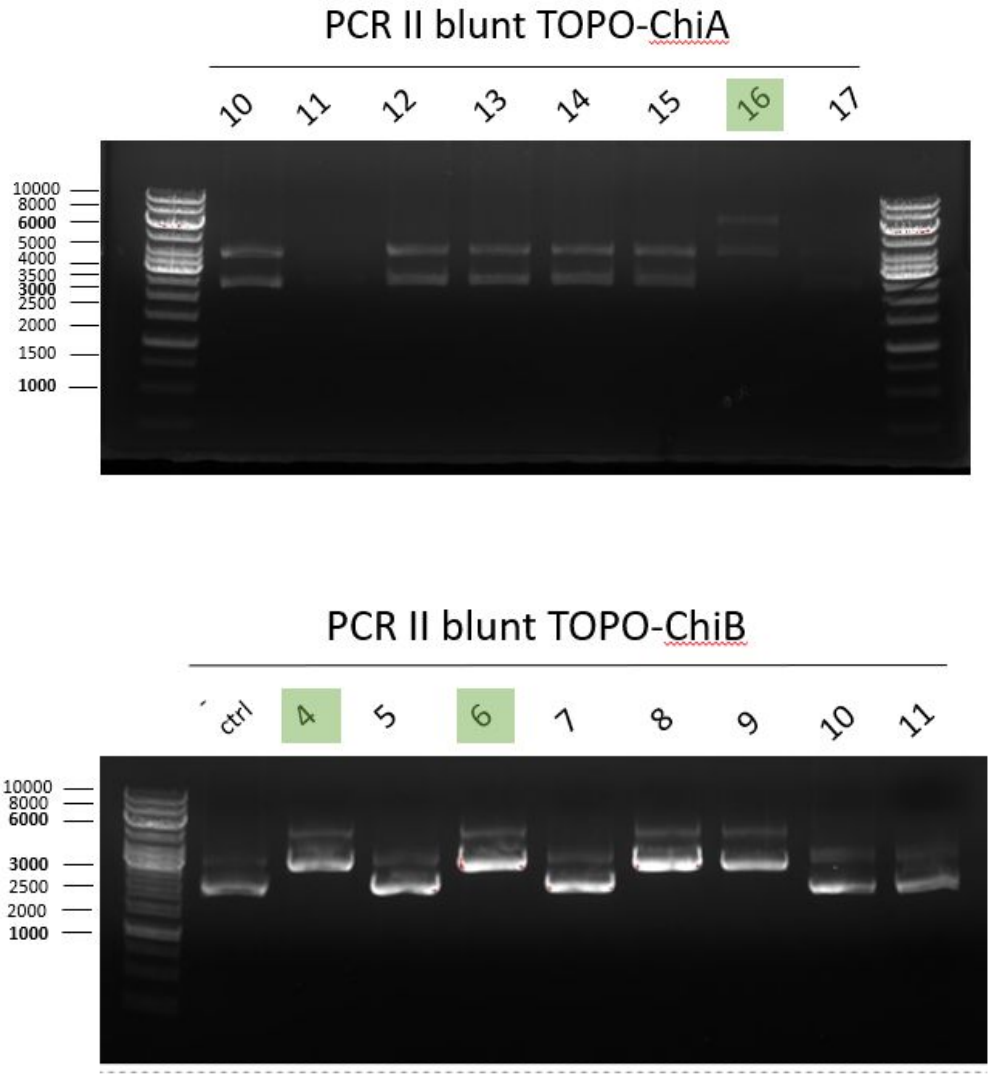


Figure 7. PCR from *F. novicida* genomic DNA. Amplification resulting in appropriate band sizes for ChiA, ChiB, ChiD, and cbpA at 2624, 2204, 2855, and 1794bp, respectively. Primers showed a high level of specificity given the lack of secondary banding. Additionally, negative controls were clear.

Following PCR purification, amplicons were inserted into the PCR II TOPO vector using blunt end cloning and transformed into NEB DH5α competent E. coli. Insertions were verified by agarose gel and clones (ChiA16, ChiB4, ChiB6, ChiD3,

cbpA7) were sequenced verified using internal primers (Fig. 8, Table 4).



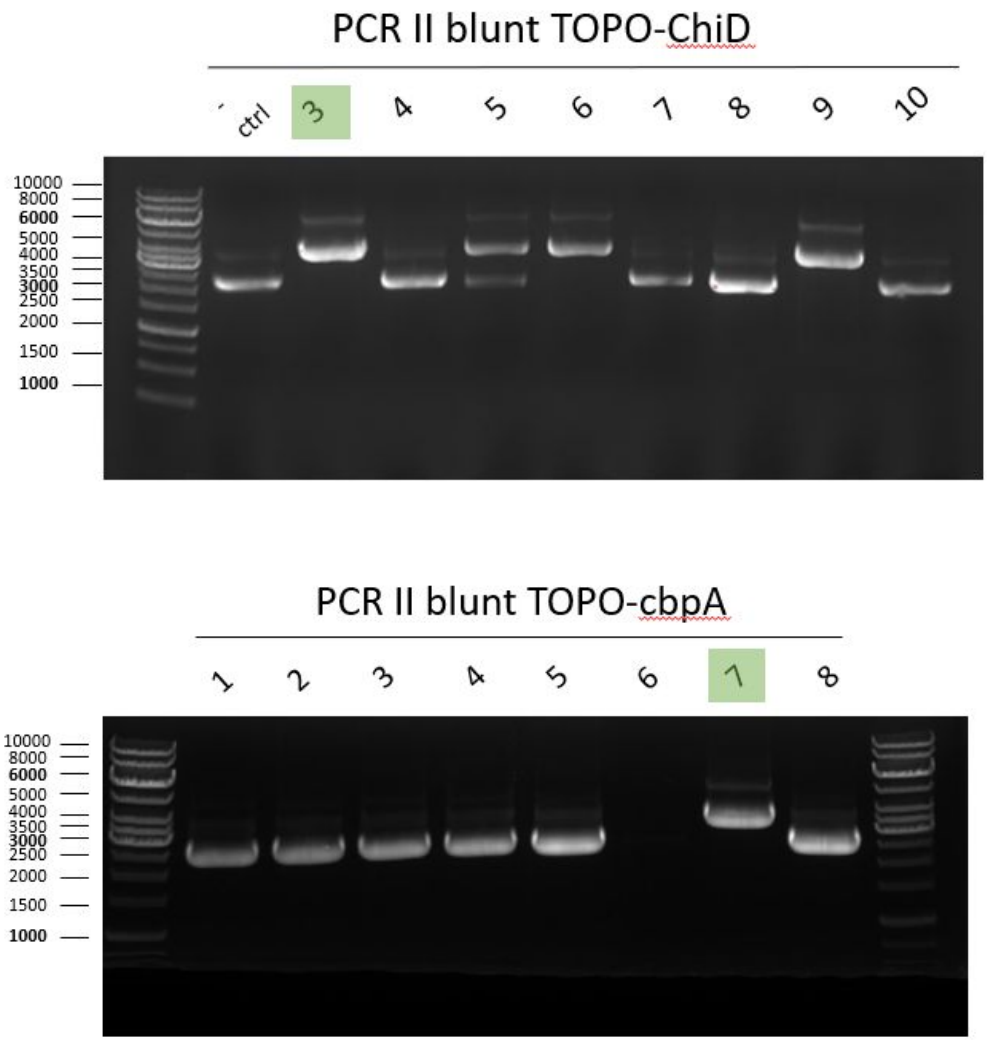


Figure 8. Agarose gel of blunt cloning into TOPO vector. Plasmids were run on the gel without restriction, resulting in inaccurate size representations. However, insertions were assumed by heavier band relative to the negative TOPO (empty) control. Clones highlighted in green were sent for sequencing.

Restriction digestion was conducted based on engineered restriction sites in forward and reverse primers. Restriction products were run on agarose gel and the bands

containing the target amplicons were excised and purified (Fig. 9). CbpA, ChiB, and ChiD were visualized and excised; however, ChiA was not detectable visually or by nanodrop following band purification. Purified 5' overhang insertions were ligated into correspondingly digested pET23b+ vector and transformed into NEB DH5α competent *E. coli* for verification. CbpA and ChiB transformations resulted in colonies, while ChiD transformation showed no growth. CbpA and ChiB insertions were verified by agarose gel (Fig. 10).

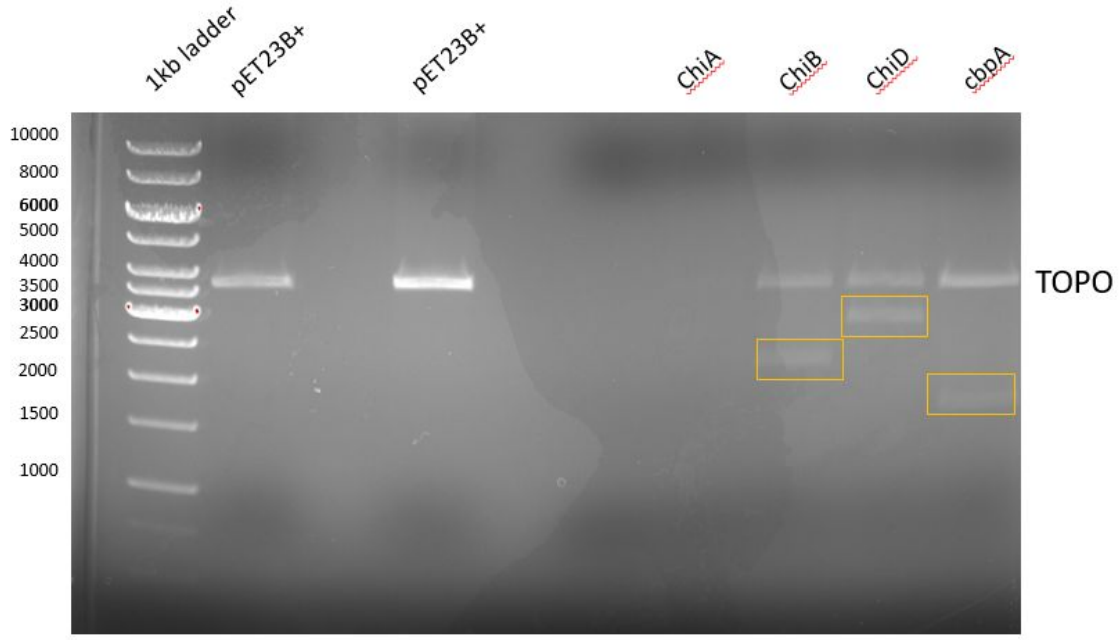


Figure 9. Agarose gel of restriction of TOPO clones. Appropriate bands were visualized for ChiB, ChiD, and cbpA at 2200, 2851, and 1789bp respectively (yellow). Band were not detectable in the ChiA lane following restriction.

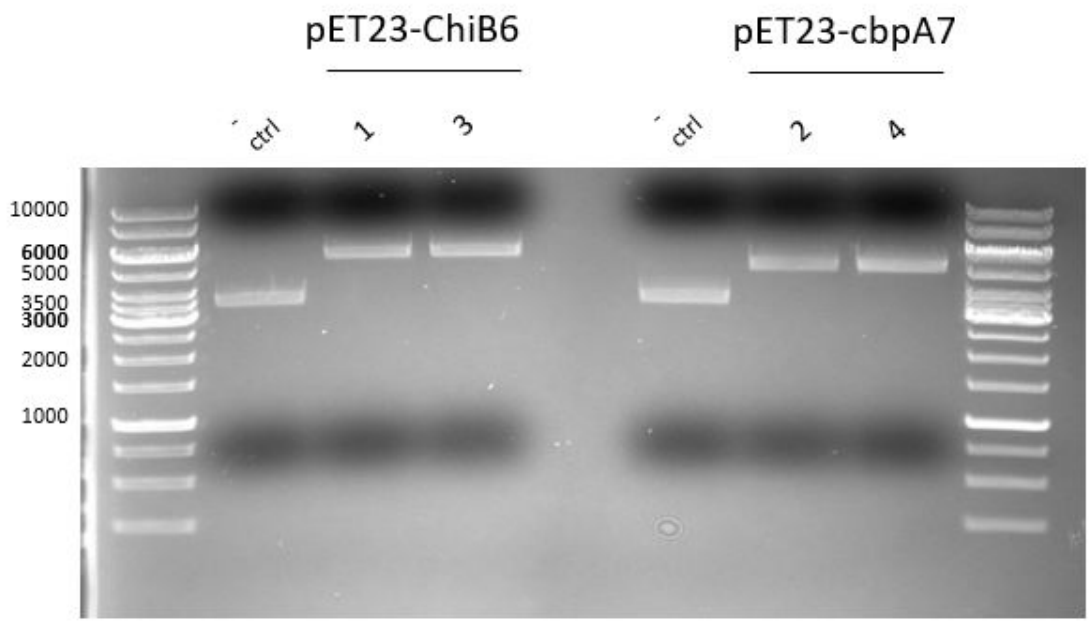


Figure 10. Agarose gel of pET23b+ restriction clones. ChiB and cbpA insertions can be visualized by the 5.8kb and 5.3kb bands relative to the 3.7kp pET23b+ negative control band (no insertion).

CbpA and ChiB positive insertion pET23b+ plasmids were transformed into the BL21(DE3)/pLysS expression *E. coli* strain. Following overnight growth in selective media, protein expression was induced overnight using MagicMedia. Cell lysate and supernatant were run on protein gels and 6his-tagged protein expression was found in both (Fig. 11).

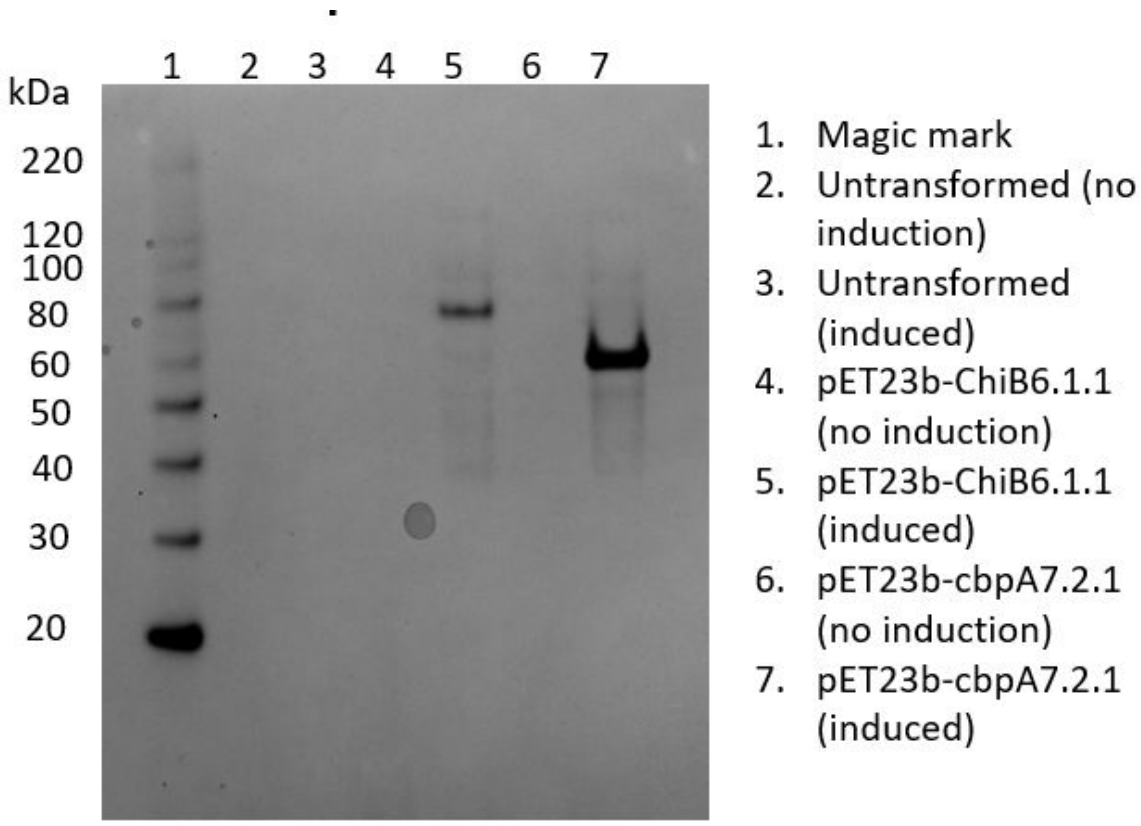


Figure 11. Coomassie gel stain of expression induction. Expression is detectable by the 79kDa ChiB and 62kDa cbpA bands, relative to uninduced lanes (even).

Recombinant CbpA and ChiB protein purification was conducted from sonicated cell lysate using 6his-tag Dynabeads. Western blotting using 6his-tag antibodies showed nonspecific binding, however bands were present at the appropriate protein mass, showing elution of recombinant protein (Fig. 12). Elution may be optimized through purification from supernatant or repeated elutions to provide a clean recombinant product for further experimentation. Though ChiA and ChiD pET-23b clones were not produced and verified, intermediate PCR blunt II TOPO clones were sequenced and may be ligated

into pET-23b for future work.

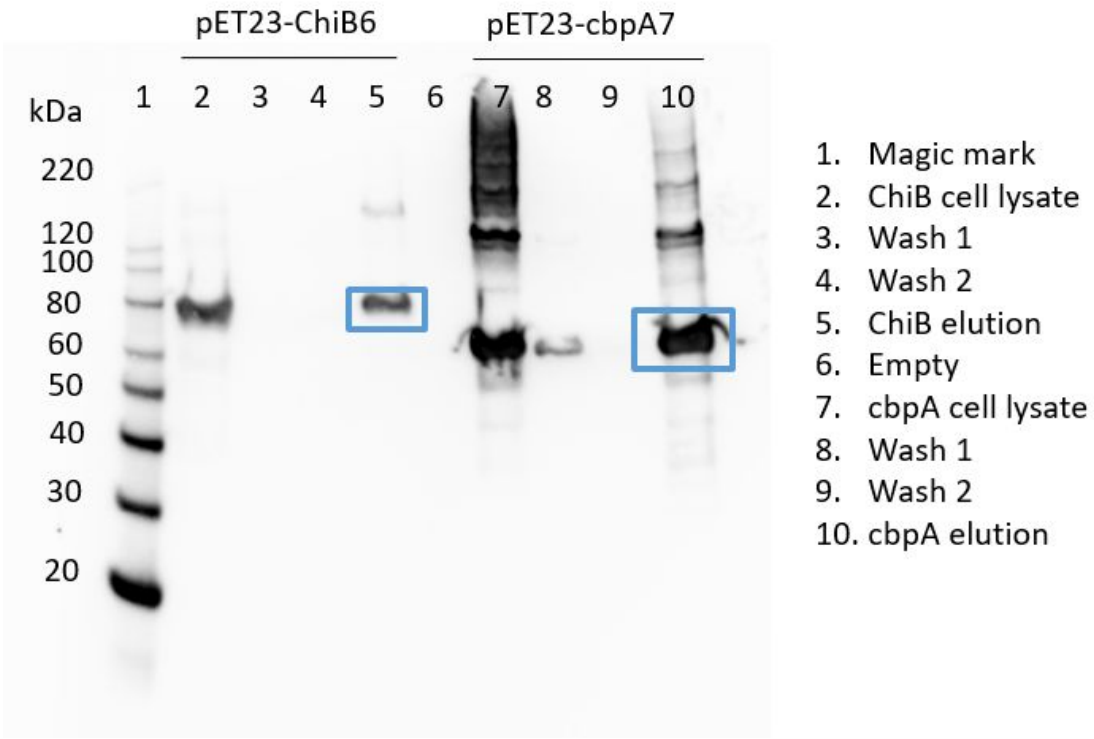


Figure 12. α6his-tag western blot of ChiB and cbpA Dynabeads elution. ChiA and cbpA were successfully expressed, however elution by Dynabeads did not result in clean purification. This is evident by nonspecific binding found in final elution lanes 5 and 10.

Chapter Three: N-ε-Lysine Acetylation of chitinases

Acetylation in Bacteria

Acetylation is a post-translational modification where an acetyl group is covalently added to the ε-amino group of a lysine. Acetylation has been established as a regulatory mechanism in both eukaryotes and prokaryotes, which is extensively involved in metabolism, transcription, translation, and enzymatic regulation [18]. Full acetylation proteome studies have been conducted for *Escherichia coli* [19], *Mycobacterium tuberculosis* [20], *Bacillus subtilis* [21], *Streptomyces griseus* [22], and other bacteria. These protein modulations allow for bacteria to appropriately respond to environmental changes and involve regulation of virulence.

Though a study in *E. coli* reports the majority of acetylation occurs non-enzymatically via acetyl phosphate, enzymatic acetylation is facilitated by acetyltransferases [17]. Nonenzymatic acetylation occurs through the donation of an acetyl group from acetyl phosphate (acetyl CoA) to the deprotonated ε-amino group of a lysine. Deacetylases enzymatically remove acetyl group, regardless of acetylation mechanism (enzymatic or nonenzymatic addition). The extent of enzymatic acetylation in bacteria has been largely unexplored and serves as a topic for future research.

It has been previously determined that acetylation is capable of regulating enzymatic activity in bacteria. This has been established in studies of MbtA in

Mycobacterium tuberculosis, NhoA and adenosylmethionine synthase (MAT) in *E. coli*, and others [17]. Though activation of enzymatic activity has been described in eukaryotes, this has not been found in prokaryotes.

Acetylation of FTN Secreted Proteins

It has been established previously through proteomic data that *F. tularensis* has seven secreted proteins, including chitinase A, chitinase B, and chitin-binding protein A [23]. In order to evaluate the impact of acetylation on chitinase activity, *F. tularensis novicida* supernatant containing secreted proteins was chemically acetylated and concentrated. Presence of acetylation sites within the glycosyl hydrolase 18 domain of both chitinases A and B suggested that glycosyl hydrolase activity would be turned off by chemical acetylation. As determined by Chung *et al*, lack of glycosyl hydrolase activity results in a failure to disperse biofilm. It was therefore hypothesized that acetylation of concentrated chitinases would result in lack of activity and limited biofilm dispersal.

Experimental Setup

Protein concentration. Francisella tularensis subsp. novicida U112 was grown in tryptic soy broth supplemented with 1% cysteine at 37℃ with shaking at 180rpm for 48 hours. Supernatant was separated from cell pellet by centrifugation at 5000rpm for 10 minutes and filtered through a 0.22μM filter. Concentration was conducted using Vivaspin20 30kDa MWCO, following manufacturer's instructions. Supernatant was supplemented with EDTA-free protease inhibitor prior to concentration. Protein

concentration was measured using BCA assay or nanodrop and stored at -80℃ until further use.

In vitro acetylation. Acetylation was induced using lithium potassium acetyl phosphate at 50mM or 200mM for 2 hours at 37℃ with mixing. Acetylation of secreted proteins was conducted prior to concentration by Vivaspin20.

Chitinase Assay. Chitinase assays were conducted from concentrated FTN supernatant using the Sigma-Aldrich chitinase assay kit following standard protocol. Endochitinase activity was measured exclusively, due to previous studies showing negligible exochitinase activity [11]. Endochitinase activity was measured using 4-Nitrophenyl β-D-N,N′,N″-triacetylchitotriose substrate with a 0.01 mM p-Nitrophenol standard and *Trichoderma viride* positive control. Fluorescence was quantified at OD405. Two technical duplicates were conducted. Statistical significance was measured via one-way ANOVA using Graphpad Prism 6.

Biofilm Assay. Biofilm assays were conducted using the standard crystal violet method. Overnight cultures of FTN were diluted 1:30 in TSB-c and 200uL were distributed into each well of a 96-well plate. Following overnight growth at 37c, growth was measured at OD600. Liquid culture was removed and biofilm in wells was chemically fixed using 200uL of methanol and allowed to air dry. Biofilm was stained for 15 minutes using 0.1% crystal violet and washed three times using deionized water. After drying, dye was resolubilized using 30% acetic acid and read at OD590.

Biofilm dispersal assay. Dispersal was measured using the crystal violet staining method following treatment with acetylated or nonacetylated concentrated FTN secreted protein. Following chemical fixation, each well was treated with 200uL concentrated protein at known concentrations (2mg/mL, 1mg/mL, 0.5mg/mL, 0.25mg/mL, 0.125mg/mL) and incubated at 37c for 1 hour. Treatment was removed and the standard biofilm protocol continued as described previously. Data were analyzed by Student's T-test using Graphpad Prism 6.

Results and Discussion

Acetylation resulted in a significant decrease in endochitinase activity in concentrated supernatant (P=0.0067) (Fig. 13). Given that numerous acetylation sites exist on chitin binding protein and Chitinases A and B found in the concentrated secreted protein, acetylation within active sites likely inhibited enzymatic activity. Additional experiments using recombinant clones and site directed mutagenesis should be conducted to determine the importance of individual acetylation sites.

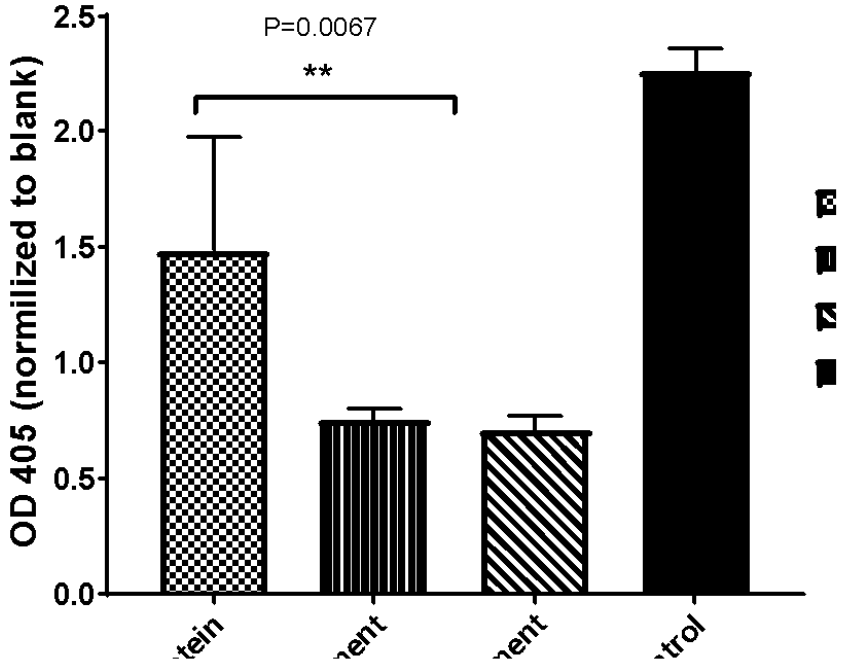


Figure 13. Chitinase assay of Francisella tularensis subsp. novicida concentrated secreted proteins. The activity was measured with 4-Nitrophenyl β-D-N,N′,N″-triacetylchitotriose substrate (endochitinase activity). Positive control: chitinase from *Trichoderma viride*. Standard: 0.01 mM p-Nitrophenol. Statistical significance was calculated by ANOVA test.

Biofilm dispersal was significantly inhibited by acetylation in the 2mg/mL concentrated supernatant treatment (P=0.000017) (Fig. 14). No significant difference was visualized between acetylated and nonacetylated treatments in lower concentrations. As supported by Chung *et al*, inactivation of chitinase activity results in failure to disperse biofilm. Acetylated recombinant chitinase may be used in the future to verify these

results.

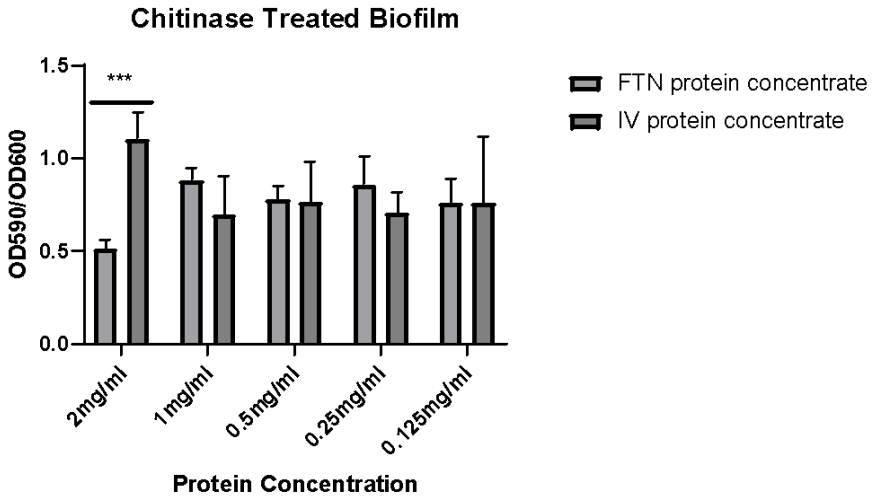


Figure 14. Dispersal assay of *Francisella tularensis* subsp. *novicida* biofilm treated with concentrated secreted proteins. *In vitro* (IV) acetylation was conducted using 50mM lithium potassium acetyl phosphate prior to protein concentration. Biofilm values (OD590) were normalized to total growth (OD600) prior to biofilm treatment. Statistical significance was calculated by student's T-test.

Chapter Four: Conclusions and future directions

Experiments using concentrated protein have supported that acetylation of chitinase results in enzymatic inactivation, resulting in the inability to disperse biofilm compared to the nonacetylated form. Though the chitinase assay is highly specific and would likely not be disrupted by other secreted proteins, recombinant enzymes must be studied in isolation in order to further support our hypothesis. We have successfully expressed *F. novicida* chitinase B and chitin-binding protein A and have provided the foundation for expression of chitinases A and D in the future. The established *F. novicida* chitinase clones can be used to further our understanding of chitinases and their role in the life cycle of *Francisella*. Though we have established acetylation turns off enzymatic activity of chitinases, the specific mechanism of regulation is unclear and should be emphasized in the future.

Acetylation and Biofilm

As established previously, chitinase is a negative regulator of biofilm and it has been determined that biofilm dispersal is directly induced by glycosyl hydrolase activity. Given the expression of *F. novicida* chitinases, chitinase assays following acetylation of recombinant enzymes should be conducted. Chitinase assays may be supplemented with acetylated/nonacetylated chitin-binding protein to determine if an augmentation in

activity occurs. Additionally, biofilm dispersal assays should be repeated using acetylated recombinant protein. Though experimentation using acetylated concentrated secreted protein supported our hypothesis, utilizing purified enzyme would provide more direct evidence and follow standard scientific practice.

Site Directed Mutagenesis

To assess the importance of individual acetylation sites towards enzymatic function, site directed mutagenesis should be conducted using the pET23b clones. Primary targets for mutagenesis are lysines located within the conserved regions of chitinases such as the glycosyl hydrolase 18, fibronectin 3, and chitin binding domains. Mass spectrometry data showed two acetylation sites for chitinase A, both of which in the glycosyl hydrolase 18 (K206) and chitin binding (K849) domains. Both of which would be ideal candidates for site directed mutagenesis. Chitinase B exhibited 16 acetylation sites, where 13 sites were found non-chemically acetylated samples and 7 following chemical acetylation. Site directed mutagenesis should be pursued for the 5 lysines sites found within the glycosyl hydrolase 18 domain (K420, K453, K542, K582, K589). Due to the high endochitinase activity of Chitinase B, site directed mutagenesis and expression may show the importance of multiple acetylation sites within the active region of the enzyme. Additionally, multiple mutations may be induced into the GH18 domain to support this claim. Following the production of mutated clones, recombinant enzymes can be expressed, then acetylated and nonacetylated chitinase activity can be measured.

This may quantitatively show the importance of individual acetylation sites for chitinase inhibition. Similarly, biofilms can be treated to verify the relationship between acetylation inhibition of chitinase activity and biofilm dispersal.

Glycosyl Hydrolase Activity

The glycosyl hydrolase 18 domain is associated with numerous glycolytic activities. Though the *Francisella* chitinases are referred to as "chitinases", recombinant expression allows for further analysis and potential substrate identification. FTN Chitinase D in particular showed no endo- or exochitinase activity, despite possessing two intact GH18 domains. Similarly, glycosyl hydrolases in *Enterococcus faecalis* and *Streptococcus pyogenes* display no chitinolytic activity but promote hydrolysis of N-linked glycoproteins [24, 25, 26]. Enzymes containing the GH18 domain have been known to possess chitinase [27], lysozyme [28], endo- β -N-acetylglucoaminidase [29], peptidoglycan hydrolase [30], and Nod factor hydrolase activity [31], and may act non-catalytically as a xylanase inhibitor. However, it should be emphasized that the diverse GH18 domain is not limited to bacterial species and is exhibited throughout the kingdoms of life. Regardless, assays may be conducted using recombinant enzymes to determine non-chitinolytic activities, which may elucidate substrates chitinase may act on during biofilm regulation.

Chitinase Synergy

It has been determined that chitinases often work synergistically with other chitinases and chitin-binding proteins [32]. These effects have been studied in numerous bacteria including, *Alteromonas* [33], *Bacillus thuringiensis* [34], and *Serratia proteamaculans* [35]. Because *Francisella* secretes more than one chitinase, it could be hypothesized that they can augment each other's activity. This could be easily assessed through a standard chitinase assay. Additionally, this process may be repeated using non-chitin-based assays if additional substrates are determined.

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BIOGRAPHY

Alexandra Ii graduated from South Lakes High School, Reston, Virginia, in 2012. She received her Bachelor of Science from George Mason University in 2017.