IDENTIFICATION AND ANALYSIS OF THE ROLE OF CHITINASE SUBSTRATE IN FRANCISELLA NOVICIDA

by

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Bachelor of Science
George Mason University, 2019

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DEDICATION

This is dedicated to my wonderful PI Dr. van Hoek, my lab mates, and my parents who supported me through all of this.

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I would like to thank my many mentors who made this possible, including Dr. van Hoek, Dr. Polayes, Bonnie Madden, Stephanie Barksdale, and Alexandra Ii. Without all your support and understanding this would not have been possible. Thank you for all the encouragements.

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LIST OF ABBREVIATIONS

Francisella novicida ..... Fn
Francisella tularensis tularensis……Ftt
Chitinase B..... ChiB
Chitinase C..... ChiC
Chitinase A..... ChiA
Chitinase D..... ChiD
Chitin Binding Protein A ..... CbpA

ABSTRACT

IDENTIFICATION AND ANALYSIS OF THE ROLE OF CHITINASE SUBSTRATE IN FRANCISELLA NOVICIDA

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Francisella tularensis is a gram negative facultative intracellular pathogen which is a class A biothreat according to the CDC. This bacteria codes for two chitinases (ChiA and ChiB) and one chitin binding protein (CbpA). Chitin is the most abundant oligosaccharide in marine environments and in the exoskeleton of many insects. In order to digest this material, microorganisms produce chitinases capable of cleaving this polymer. The chitinases in *Francisella novicida* have previously been found by our lab to negatively regulate the bacterial biofilm, most likely cleaving the extracellular polysaccharide substance (EPS) with β-1,4 glycosidic linkages. These enzymes may enable the organism to use the resulting cleaved polymers as carbon and nitrogen sources for growth. Although *Francisella* encodes for several polysaccharide synthases, it has not been found to produce chitin. Therefore, we hypothesized that the chitinase-enzyme substrate that is self-produced in the *F. novicida* EPS and biofilm must be some other molecule also containing β-1,4 glycosidic linkages. The biochemical characterization of

ChiB activity on various substrates will be evaluated and the role of the substrate in the biofilm will be examined.

Based on the-preliminary data from the van Hoek lab and other labs, we think that chitinases play important roles both in *Francisella* ecological persistence and macrophage mediated growth during *Francisella* infection. Identifying and characterizing the substrate for these chitinases will generate information on how *Francisella* uses them to persist in the environment and replicate in human macrophages.

CHAPTER ONE

Francisella (F.) novicida is a gram-negative bacterium and is a model organism for *F tularensis* (SchuS4 strain), the causative agent of tularemia. Identified as a Class A biothreat by the CDC, *Francisella* is the subject of study in both biodefense and biological areas. The lifecycle of *Francisella* includes an ecological niche, a vector, and a eukaryotic host.

The ecological niche of *Francisella* species can be water-associated (Type B tularemia, *F. tularensis holarctica*, *F. philomiragia, F. noatunensis* species), including mud, rivers, brackish water, and hot springs or soil-associated (Type A tularemia, *F. tularensis tularensis,* abbreviated Ftt). The insect vectors include ticks and tabanid flies for Type A tularemia and ticks, tabanid flies, and mosquitos for Type B tularemia (Akimana and Kwaik 2011).

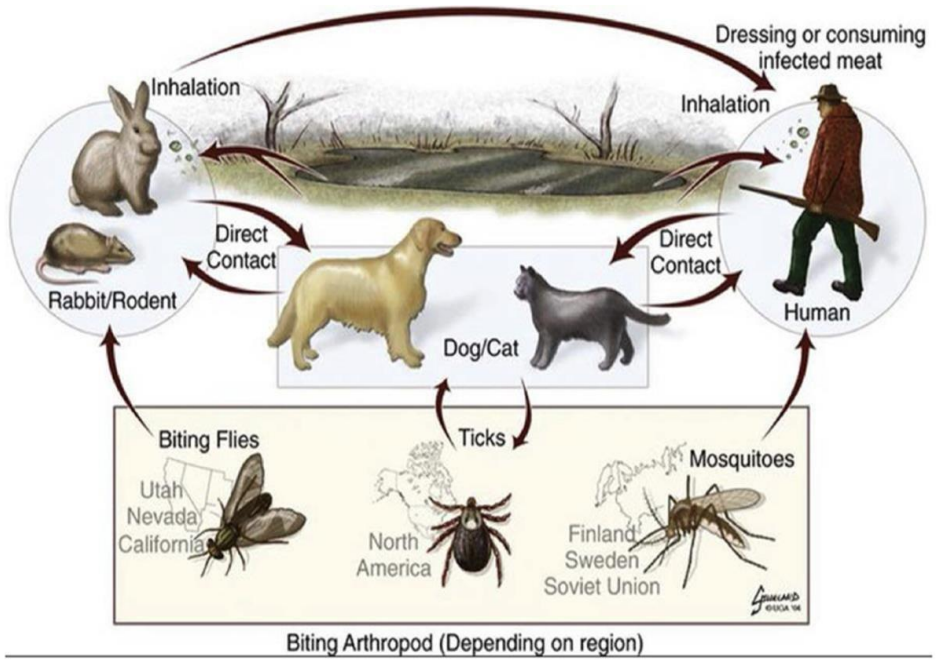


Figure 1: Sylvatic cycle of *Francisella tularensis*, illustrating the transmission cycles and the relevant biting insects depending on the region (Art by Brad Gilleland, UGA College of Veterinary Medicine. © 2004 - 2019 University of Georgia Research Foundation, Inc.). Printed by Permission of the University of Georgia Research Foundation Inc.

F. novicida is used as a model organism for Ftt SchuS4 because there is a greater than 97% homology between *Francisella novicida* and SchuS4 (Siddaramappa et al., 2012) (Challacombe et al., 2017). In addition, immunity to *F. novicida* nonpathogenic mutant "U112Δ*iglB* is the only *F. novicida*-based live attenuated vaccine strain that has been shown to provide heterologous protection against pulmonary LVS and SCHU S4 challenge in the mouse model" (Signarovitz et al. 2012). *F. novicida* also is non-infectious in humans, so it is safer to use in lab, with results reflecting those in the human virulent strain Ftt SCHU S4 for the most part (Gallagher et al. 2007). The limitations of

F. novicida as a model for *F. tularensis* SchuS4 include genetic differences in operons resulting from genomic rearrangement (Rohmer et al., 2007)(Kingry & Petersen, 2014) relative lack of pseudogenes and coding for only one copy of the pathogenicity island. *Francisella novicida* only has one copy of the FPI (pathogenicity island) while *Francisella SCHU S4* and *F. holarctica* have two copies (Nano and Schmerk 2007). The *F. novicida* mouse model is also somewhat limited due to the high mortality rate in the mice versus the low mortality rate in humans. This is due to the higher bioactivity of the *F. novicida* LPS, showing *F. holarctica LVS* to be a better mouse model (Kieffer 2003).

Francisella chitinase genes.

Francisella encodes a class of extracellular enzymes called chitinases that cleave the ß (1,4)- glycosidic linkages in chitin *in vitro* (van Hoek 2013). Since *Francisella* does not produce chitin, but the enzyme degrades *Francisella* biofilms (Chung et al. 2014) the substrate cleaved by these chitinases is likely to be another *Francisella*-produced polysaccharide that contains the same bonds but remains unidentified. Chitin is the most abundant polymer in the marine environment, similar to how cellulose is the most abundant polymer on land. We are interested to know what other polysaccharide is cleaved by these enzymes and if it is produced by *F. novicida*.

Francisella species encode up to 4 chitinase genes and up to 2 chitin binding proteins. These genes are summarized in **Table 1**. The fully virulent strain Ftt SCHU S4 encodes ChiA (FTT_0715), ChiB (FTT_1768c), and Chitinase binding protein (FTT_1577) (**Table 1**). *F. novicida* contains ChiA (FTN_0627), ChiB (FTN_1744), and

Chitinase binding protein A (FTN_1485) (**Table 1**). ChiC is not present in *F. novicida* and is either a pseudogene or a fragment in the other strains. It can be seen from the table that all species of *Francisella* have ChiA, ChiB and ChiD. The environmental organism *F. philomiragia* seems to express all 4 chitinase genes. The chitin binding proteins (Cbp) are distributed more unevenly between species. *F. tularensis* SCHU S4 has encoded CbpA between two genes, compared to the one gene found in *F. novicida*, see below, while this protein is fragmented and encoded in 3 genes in LVS and not present in *F. philomiragia.* Cpb21 is found in *F. novicida, F. tularensis* Live Vaccine Strain and *F. philomiragia*, with fragments found in *F. tularensis* SCHU S4 again.

	F. novicida U112	***F. tularensis SCHU S4***	***F. holarctica Live Vaccine Strain (LVS)***	***F. philomiragia***
ChiA	FTN_0627 ABK89520 870aa	FTT_0715 AJI68664 760 aa	FTL_1521 764aa	Fphi_0215 AJI55264 892 aa
ChiB	FTN_1744 ABK90596 730 aa	FTT_1768c* AJI68365 606 aa	FTL_0093 730 aa	Fphi_0864 AJI74803 740 aa
ChiC	Not present in *F. novicida* U112.	FTT_1593/FTT_1592* Pseudogenes	FTL_1635 Fragment, 704aa	Fphi_0209 ABZ86427 785 aa
ChiD	FTN_1644 ABK90502 947 aa	FTT_0066 CAG44699 947 aa	FTL_1793 947 aa	Fphi_0964 950 aa
CbpA	FTN_1485 ABK90352 555 aa	FTT_1576/FTT_1577* AJI68602 181aa/361 aa	FTL_0530 FTL_0531 FTL_0532 97 aa/85 aa/156 aa	Can't identify AJI53618 (Sort of FSC454_07655)
Cbp21	FTN_1192 ABK90078 596 aa	FTT_0815c/FTT_0816c CAG45449 82 aa/297 aa	FTL_1408 574 aa	Fphi_0111 595 aa

Table 1**: Francisella chitinase and chitin-binding protein genes.** Locus and accession numbers obtained through Uniprot protein search and BLAST analysis from original *F. novicida* U112 proteins. Homologs are placed in the same rows with the shared name. Grey boxes represent missing genes and * represents pseudogenes. The number of amino acids in the encoded protein(s) are shown for comparison.

ChiA: ChiA is a large protein (870aa) with multiple domains including a Glycosyl Hydrolase 18 domain, a fibronectin type 3 domain, a chitodextrinase domain, a chitin-binding domain of chitinase C, and an aromatic chitin/cellulose binding site residue.

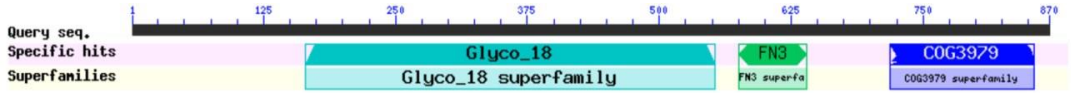


Figure 2**: Chitinase A domain graphic.** Obtained through PubMed protein conserved domains.

ChiB: ChiB is a shorter protein (730aa), with multiple domains including a glycosyl hydrolase 18 domain, a chitin/cellulose binding domain, chitin/cellulose binding residues and Nacetylglucosamine-binding domain.

Figure 3**: Chitinase B domain graphic.** Obtained through PubMed protein conserved domains.

ChiD: ChiD is a large protein (947aa) that contains an N-acetylglucosamine-binding protein domain, as well as having GH_18 characteristics.

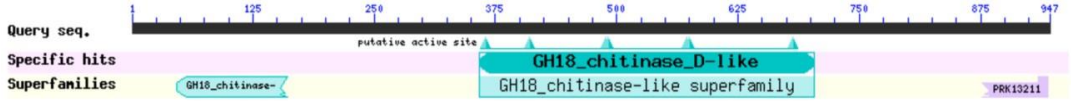


Figure 4**: Chitinase D domain graphic.** Obtained through PubMed protein conserved domains.

One limitation of *F. novicida* as a model for *F. tularensis* in the study of chitinases is that there is only a 90% homology between ChiA genes in both species (**Table 2**). Because of

this, we have focused on ChiB for this study, which is much more similar between the strains (see **Table 3**). ChiA and CbpA both have N-terminal chitin binding domains while ChiB has a C-terminal binding domain. This might be a benefit when adding an N-terminal tag in the cloning of ChiB. ChiB only has a 28.07% homology to CbpA and ChiA has 50% homology to CbpA, mostly due to the location of the chitin binding domains.

Table 2**: Chitinase A homology among Francisella species.** Values obtained by running BLAST analysis through PubMed protein on each pair of ChiA homologs.

	F. novicida ChiA	**Ftt ChiA**	**F_{LVS} ChiA**	**Fphil ChiA**
F. novicida ChiA	100%	90.62%	90.41%	73.94%
Ftt ChiA		100%	98.69%	71.60%
F_{LVS} ChiA			100%	71.48%
Fphil ChiA				100%

To ensure that results will transfer from *F. novicida* to Ftt SCHU S4, we examined whether ChiB and Chitin binding protein will have similar binding sites and domains between the strains. We found that they were very similar in their sequence conservation in Ftt SCHU S4 (98.51%) and FtLVS (97.95%) (**Table 3**). Interestingly, *F. philomiragia* ChiB is the most different from *F. novicida* ChiB. This difference may reflect some adaptation of *F. philomiragia* to its environmental habitat. Thus, due to the high conservation of ChiB sequence across the human relevant *Francisella* species

(**Table 3**) and our prior results (Chung et al., 2014) confirming an important role for ChiB in *F. novicida* biofilm formation, we chose to focus on ChiB for this study.

Table 3: Chitinase B homology among *Francisella* species. Values obtained by running BLAST analysis through PubMed protein on each pair of ChiB homologs.

	F. novicida ChiB	Ftt ChiB	Flvs ChiB	Fphil ChiB
F. novicida ChiB	100%	98.51%	97.95%	55.69%
Ftt ChiB		100%	99.17%	50.71%
Flvs ChiB			100%	55.69%
Fphil ChiB				100%

Chitin Binding Proteins

We are also interested in the potential role of the chitin-binding proteins, and so compared the conservation of this protein across *Francisella* species (**Table 4 and 5**). We observed that *F. novicida* U112 has two chitin binding proteins (CbpA and Cbp21) that only share 32% homology between each other, and SCHU S4 has genes encoding parts of two chitin binding proteins (CbpA and Cbp21) that share 30.91% homology (See Appendix for Cbp21). CbpA in *F. novicida* is an extracellular, secreted protein that is 555 amino acids long, and 62,553 Da. This protein is characterized by having two carbohydrate binding domains, CBM5, Carbohydrate-Binding Module Family 5 and CBM73, Carbohydrate-Binding Module Family 73. CBM5 in CAZY is defined as "Modules of approx. 60 residues found in bacterial enzymes. Chitin-binding described in

several cases. Distantly related to the CBM12 family. Note: Previously known as cellulose-binding domain family V (CBD V).” CBM73 is defined as “Modules of approx. 65 residues found on various enzymes active of chitin (Forsberg et al. 2016). Distantly related to CBM5.” No other catalytic activity is noted in the annotations.

The LVS strain appears to have three fragmented genes that overlap *F. novicida* CbpA FTN_1485, see **Figure 5** below, and seems likely to be inactive.

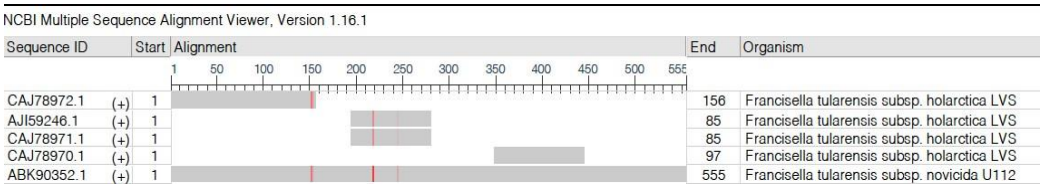


Figure 5**: Alignment of CbpA in *Francisella novicida* and *Francisella holarctica*.** Alignment obtained from NCBI alignment viewer.

For *F. tularensis* SchuS4, there are two genes broken up (FTT_1576 and FTT_1577) that overlap the sequence of FTN_1485. Shared functional domains include the chitin-binding domain of chitinase C. FTT_1577 contains a CBM_5_12 domain from aa 331-357 , which is a Carbohydrate binding domain. FTN_1485 contains the same domain CBM_5_12 from amino acid 525-551, which is also a Carbohydrate binding domain.

Table 4: Chitin-binding protein CbpA homology among *Francisella* species. Values obtained by running BLAST analysis through PubMed protein on each pair of CbpA homologs.

	***F. novicida* CbpA**	**Ftt CbpA**	**Flvs CbpA**	**Fphil CbpA**
***F. novicida* CbpA**	100%	98.34%	42.22%	69.56%
Ftt CbpA		100%	42.22%	68.44%
Flvs CbpA			100%	36.00%
Fphil CbpA				100%

Francisella biofilm formation

Francisella novicida and *Francisella tularensis* are both able to form biofilms under certain conditions. SCHU S4 can form a biofilm at around 10 days of incubation (Champion et al., 2019) whereas *F. novicida* can form biofilm within 2-3 days of incubation (Durham-Colleran et al. 2010). The SCHU S4 biofilm is not substantial but can be formed on polystyrene 96-well plates (van Hoek 2013) and chitin surfaces (Margolis et al. 2010). There is a difference in the structures of the biofilm produced by *F. novicida* vs Ftt. The Ftt biofilm is made up of O-Ag structures that consist of 4-glucose units of two internal carbohydrate residues (α-D-GalNAcAN–α-D-GalNAcAN) and two peripheral residues (β-DQui4NFm and β-D-QuiNAc), whereas the *F. novicida* O-Ag has the same internal residues but distinct terminal residues (α-D-GalNAcAN and α-D-QuiNAc4NAc) (Champion et al., 2019).

There are several components found to be important in the formation of *F. novicida* biofilm including the two component QseBC system (Colleran et. al, 2009). Important genes in the regulation of *F. novicida* biofilm are FTN_1465 (QseB), FTN_1617 (QseC), and FTN_0451- FTN_0456 (Gene cluster for c-di-GMP) (Santic et

al., 2005). QseB is a “biofilm mediating response-regulator” (van Hoek, 2013). The biofilm was highly attenuated in each of these mutants.

The dispersal of the biofilm is regulated by *cis*-11-methyl-2-dode-cenoic acid (DSF) dispersal factors that induce the production of endo-B-(1,4)- mannosidase, which degrades the polysaccharides in the biofilm (Dean et al., 2015).

The expression of chitinases in biofilms is strongly upregulated as a result of the BDSF factors. The chitinases are known to cleave the polysaccharides in this biofilm so upregulation of the chitinases reduces biofilm formation through cleavage of these extracellular polysaccharides. Thus, chitinases are negative regulators of *F. novicida* biofilm, and the expression of chitinases and CbpA is highly upregulated by BDSF, suggesting that these enzymes are the mechanism by which BDSF disperses biofilm in this organism.

Table 5: Fold increase of ChiA, ChiB and CbpA expression following BDSF treatment of *F. novicida*. Values obtained from Dean et al. 2015 Table 2.

Gene	**Locus**	**Fold change in expression RNA-seq**	**qRT-PCR fold change in expression**
CbpA	FTN_1485	20.61	13.36
ChiA	FTN_1744	52.62	26.08
ChiB	FTN_0627	23.22	9.29

Possible Role of Chitinases

The van Hoek lab's research on the chitinases encoded by *Francisella novicida* shows that they are negative regulators of *F. novicida* biofilm formation *in vitro* and possess glycosyl hydrolase activity (Chung et al 2014). This may be particularly helpful for the aquatic element of the bacteria's survival, as well as in insect or environmental reservoirs, to break down chitin available in these environments. The role of chitinases was also explored in Margolis et al, who showed that biofilm formation was higher in Ftt SCHU S4 than *F. novicida* LVS in static conditions. It was also shown that chitinase mutants were present as single bacteria or only small clusters when grown on crab shells or chitin films compared to the wild type. The conclusion from these mutant studies showed that chitinase enzymes are required for biofilm formation in the absence of the chitinase monomer GlcNAc (Margolis et al. 2010).

There is limited study on the role of these chitinases in *Francisella* infection *in vivo*. One study (Twine et al. 2006) showed that ChiA was upregulated *in vivo* in mouse spleen tissue during infection. It was also found that *F. tularensis* A1 strains produced ChiA *in vivo* not for pathogenesis, but for growth (Chandler et al. 2011). However, when injected intradermally, it was found that deletion of ChiA did not cause a change in mouse survival (Kadzhaev et al. 2009). ChiB deletion mutant was shown to increase attachment but decrease intracellular replication rate in A549 cells (Chung et al. 2014).

Table 6**: Summary of prior *in vivo* and cell-based studies on *Francisella* Chitinase related genes.** Information obtained from papers cited in the reference column.

Gene	**Effect of mutation & Species of *Francisella***	**Reference**

ChiA	1. highly upregulated *in vivo* Ftt A1 2. upregulated in mouse spleen cells 3. expressed in vivo SCHU S4	Chandler et al. 2011 Twine et al. 2006
ChiB	1. Decreases replication rate of *F. novicida* in host cells 2. Decreased CFU of F. novicida in host cells.	Chung et al. 2014
CbpA	1. Attenuates bacterial virulence in mice (other bacteria)	Frederiksen et al. 2013
Cbp	None found	None found

Table 7: Summary of gene expression and proteomics studies on *Francisella* Chitinase related genes. Information obtained from papers cited in the reference column.

Gene	**Gene Expression or Proteomics result**	**Reference**
ChiA/ ChiB	1. Secreted by T2SS 2. Biofilm formation 3. Nutrient acquisition	Chandler et al. 2014, Chung et al. 2014
CbpA	1. Degradation of chitin 2. Enhance substrate affinity 3. Increase catalytic ability 4. Secreted protein	Frederiksen et al. 2013 Dean, Chung and van Hoek, BDSF paper.
Cbp	None found	None found

Francisella extracellular carbohydrates

While the exact identity of the chitinase substrate has yet to be elucidated as part of this project, there are known roles for other extracellular carbohydrates in *Francisella* biology. It has been shown that carbohydrates play a role in *Francisella* pathogenicity. *In vivo, Francisella* has been shown to produce LPS O-antigen polymers, OAg capsules and high molecular weight carbohydrates and glycoproteins (CLC) as a form of host-adaption

(Holland et al. 2017). The absence of O-antigen leads to a loss of virulence for *Francisella* A and B strains. Mutants lacking this O-antigen were more sensitive to lysis and were unable to replicate as efficiently (Freudenberger Catanzaro and Inzana 2020). This formation is able to effectively shield from macrophages and host antibodies. These evasive measures showed a 60-95% reduction in antibody recognition (Zarrella et al. 2011). High MW carbohydrates have also been shown to be highly immunogenic in the host response to *Francisella* (Chaves et al. 2013).

CHAPTER TWO

Chitinase enzymes have been proven to cleave multiple substrates in previous studies in other bacteria. ChiS and ChiL from *B. pumilus* were proven to degrade the chitin-rich fungal cell wall and the peptidoglycan cell wall in several species of bacteria (Ghasemi et al. 2011). Along with bacteria, these multi-functional enzymes have been found in several species of plants and fish as defense mechanisms. Hevamine, an endochitinase found in *Hevea brasiliensis,* shows functionality of both a chitinase and lysozyme. It is a family 18 glycosyl hydrolase found in plants as a defense against both fungi and bacteria (Terwisscha van Scheltinga et al. 1994). Thus, the substrate of chitinase may not be chitin *per se*.

The family 18 glycosyl hydrolase (GH18) has the property of cleaving β-1,4 glycosidic linkages. GH18 class enzymes have been characterized in *Francisella* (CAZY website) and the following genes are annotated as having this activity (**Table 9**).

Table 8**: Glycosyl transferases in *Francisella novicida* and *Francisella tularensis*.** Genes obtained from CAZY database.

***Francisella novicida* U112**		***Francisella tularensis* SCHU S4**	
Protein name	**Family**	**Protein name**	**Family**
AW25_1484	GT2	BZ14_1112/FTT_1629c	GT2
AW25_792	GT107	BZ14_1184/FTT_1568c	GT19
FTN_0130	GT4	BZ14_1193/FTT_1561	GT30
FTN_0300	GT2	BZ14_1308/FTT_1461c	GT4
FTN_0453	GT2	BZ14_1312/FTT_1457c	GT4
FTN_0516	GT5	BZ14_1317/FTT_1452c	GT2

FTN_0517	GT35	BZ14_1339/FTT_1433	GT2
FTN_0538	GTnc	BZ14_1570/FTT_1237	GT8
FTN_0546	GT83	BZ14_1572/FTT_1235c	GT4
FTN_1195	GT28	BZ14_2060/FTT_0811c	GT28
FTN_1212	GT4	BZ14_2077/FTT_0799	GT4
FTN_1213	GT2	BZ14_2078/FTT_0798	GT2
FTN_1214	GT2	BZ14_2079/FTT-0797	GT2
FTN_1215	GT107	BZ14_390/FTT_0455c	GT83
FTN_1218	GT4	BZ14_391/FTT_0454	GT2
FTN_1253	GT4	BZ14_4/FTT_0792	GT4
FTN_1255	GT8	BZ14_434/FTT_0417	GT35
FTN_1422	GT4	BZ14_435/FTT_0416	GT5
FTN_1423	GT4	BZ14_887	GT4
FTN_1427	GT4	BZ14_720/FTT_0158c	GTnc
FTN_1469	GT30	BZ14_719/FTT_0159c	GTnc
FTN_1477	GT19	BZ14_732/FTT_0146	GT5
FTN_1554	GTnc	BZ14_731/FTT_0147	GT35
FTN_0545	GT2		
FTN_1403	GT2		
WbtD	GT4		
WbtG	GT4		
WbtQ	GT4		

Table 9: Glycosyl hydrolase family 18 enzymes in *Francisella* species *F. novicida* U112 and SCHU S4. Locus numbers obtained through PubMed protein BLAST search. (*=pseudogene)

***F. novicida* U112**	Chitinase A (ChiA; FTN_0627)	Chitinase B (ChiB; FTN_1744)	FTN_1644* (ChiD)

***F. tularensis* SCHU S4**	BZ14_1157	BZ14_825	BZ14_94/BZ14_941
	FTT_0715 / FTT0715 (ChiA)	FTT_1768c / FTT1768c (ChiB)	FTT_0066* / FTT0066 (ChiD)

Table 10 shows that *F. novicida* U112 encodes the same chitinases that SCHU S4 does in multiple strains. This homology supports our hypothesis that results from *F. novicida* U112 could be applicable to human virulent strains.

Known substrates of Chitinases

Chitinase's ability to cleave different substrates has been tested previously in several studies. Obviously, they can cleave chitin. They can also cleave other ß-1,4 linkages. Chitinases purified from *B. circulans* showed enzymatic activity similar to that of lysozymes using a double displacement mechanism. These chitinases were able to cleave chitotetraitol (Armand et al. 1994). Chitinases have mostly been tested with trimeric chitin derivatives (Pleban et al. 1997) and lipochitooligosaccharides, to which chitinases show cleavage specificity (Schultze et al. 1998). These studies provide some clues and new directions for this study to identify potential *Francisella* chitinase substrates.

Chitinases are important to study because they break down the second most abundant polysaccharide in nature. However, *Francisella* does not appear to make chitin. Thus, the role of chitinases and their substrate in *Francisella* microbial physiology remains unclear.

Understanding the *Francisella*-produced substrates that *Francisella* chitinase ChiB can cleave will be important not only for *Francisella* microbial physiology

research, but for biotechnology research as well. The breakdown of both cellulose and chitin has implications for being renewable carbon sources (Yan and Fong 2015), in addition to their importance in microbial physiology.

CHAPTER THREE

Preliminary analysis of the chitinase genes as well as polysaccharide gene expression pathways was done to begin working to address my hypothesis.

STRING analysis to identify co-expressed or associated proteins:

Analysis of the Chitinase proteins and their potential regulators, co-expressed and associated proteins, using STRING analysis was performed. String analysis demonstrates that proteins or genes are connected via experimental data, literature co-citation or neighboring gene expression. STRING analysis of ChiB in *F. tularensis* SchuS4 (**Figure 6**) demonstrates that ChiA, ChiB and CbpA are co-expressed (see orange circled nodes) and closely associated via multiple modes of interaction captured by STRING. In addition, this node was connected to an EamA transporter (BZ14_1712) and a glycosyltransferase (BZ14_398) that "catalyzes membrane lipid-linked oligosaccharides" (Rearick et al. 1981). Also, an MFS protein was linked to the Chitinase node. These proteins are involved in membrane transport of several substances including simple sugars and oligosaccharides (Pao et al. 1998). Interestingly, the chitinases are shown to be associated with several proteins that are known or proposed drug targets in Ftt. In this first level of interaction, there is no interaction with CbpA, or a carbohydrate synthase gene, although there is a glycosyltransferase gene linked to the ChiB node.

The table below is adapted from the STRING output data but also translates those proteins into FTT locus numbers and gene/protein names. Connected nodes are illustrated with colored circles around the dots. Interestingly, no carbohydrate synthase genes were

identified as being in association with ChiB, so the identity of the putative substrate synthase system is still undefined by this analysis.

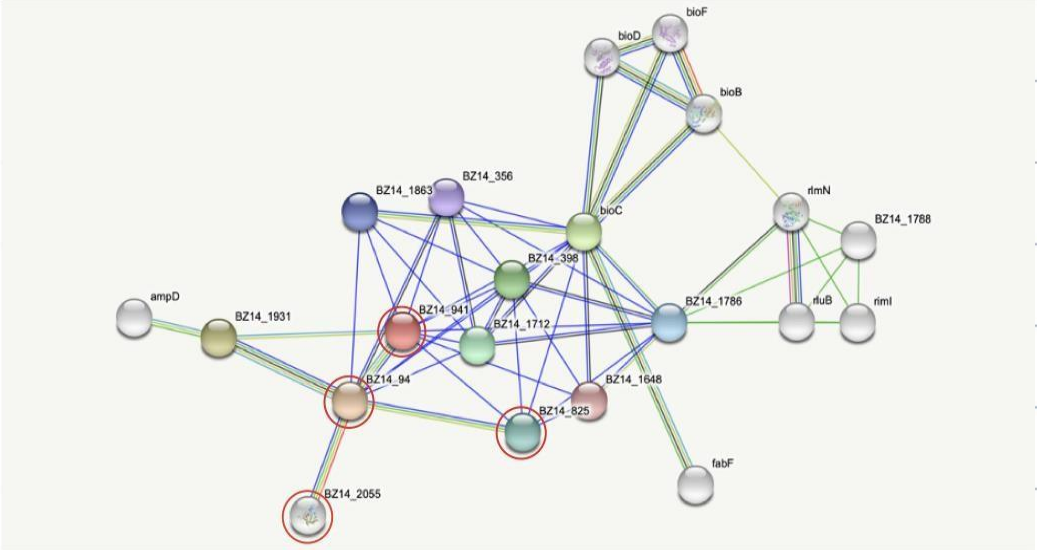


Figure 6**: Ftt ShuS4 STRING.** Figure obtained using STRING analysis (string-db.org) to examine the protein of interest and associated proteins. Dark blue lines represent gene co-occurrence, red lines represent gene fusions, green lines represent gene neighborhood, yellow lines represent text mining, black lines represent co-expression, and pink lines are experimentally determined.

Table 10: Annotation of the nodes, gene numbers, and functions of the Ftt SchuS4 STRING picture shown in Figure 6. Data obtained from string-db.org using proteins annotation export centered around **ChiB.**

Node	**Gene**	**Function**
BZ14_825	FTT_0066	GH18 protein **(ChiD)**
ampD	FTT_0162	N-acetylmuramoyl-L-alanine amidase family protein
BZ14_398	FTT_0447c	Dolichyl-phosphate-mannose-mannosyltransferase family protein
BZ14_356	FTT_0487	Major Facilitator Superfamily protein
BZ14_94	FTT_0715	Fibronectin type III domain protein **(ChiA)**
BZ14_2055	FTT_0816c	Chitin-binding protein **(Cbp21)**
BZ14_1931	FTT_0928c	Beta-N-acetylhexosaminidase

bioD	FTT_0934c	ATP-dependent dethiobiotin synthetase BioD
bioC	FTT_0935c	Biotin synthesis protein BioC
bioF	FTT_0936c	Aminotransferase class I and II family protein
bioB	FTT_0937c	Biotin synthase
BZ14_1863	FTT_0989	Similar to Q9I271 Hypothetical protein PA2044 from *Pseudomonas aeruginosa*
riml	FTT_1054c	Acetyltransferase domain protein
BZ14_1788	FTT_1055c	Uncharacterized protein
rluB	FTT_1056c	Pseudouridine synthase
BZ14_1786	FTT_1057c	Tetratricopeptide repeat family protein
rlmN	FTT_1058c	Dual-specificity RNA methyltransferase RlmN
BZ14_1712	FTT_1118c	EamA-like transporter family protein
BZ14_1648	FTT_1170	Sel1 repeat family protein
fabF	FTT_1377	3-oxoacyl-[acyl-carrier-protein] synthase 2
BZ14_941	FTT_1768c	Glycosyl hydrolases 18 family protein **(ChiB)**

BZ14_941 (FTT_1768c) is chitinase B in the SHUS4 strain of *Francisella* and is the central protein of this network. BZ14_94 (FTT_0715) is Chitinase A, BZ14_825 (FTT_0066) is Chitinase D, and BZ14_2055 (FTT_0816c) is Cbp21. As shown in **Figure 6** by four red circled nodes, Cbp21 is only connected to Chitinase A, but the 3 chitinases are all connected with each other. BZ14_1931(FTT_0928c) has a glycosyl hydrolase 3 (GH3) domain and is in the same gene neighborhood and has beta-N-acetyl-hexosaminidase activity and N-acetyl-beta-D-galactosaminidase activity.

In the second shell of interactions, the most notable is the biotin synthase family. Biotin gene cluster (BioCBDF) is associated with the Chitinase cluster. It has been shown

that biotin synthesis is a virulence factor for *Francisella* (Feng et al. 2014). Biotin is a vitamin (B7) that is involved in the metabolism of carbohydrates (Ohrui et al. 1978).

Another notable interaction is AmpD, which has involvement in the degradation of peptidoglycan. AmpD has been found to be essential in SHUS4 for cell wall recycling (Bachert et al. 2019). FabF is a drug target in *Francisella* due to its involvement in the fatty acid synthesis pathway. The pathway is different from the mammalian pathway and fabF inhibitors (thiolactomycin and cerulenin) have been tested (Kingry et al. 2013). Triclosan is a fabL inhibitor.

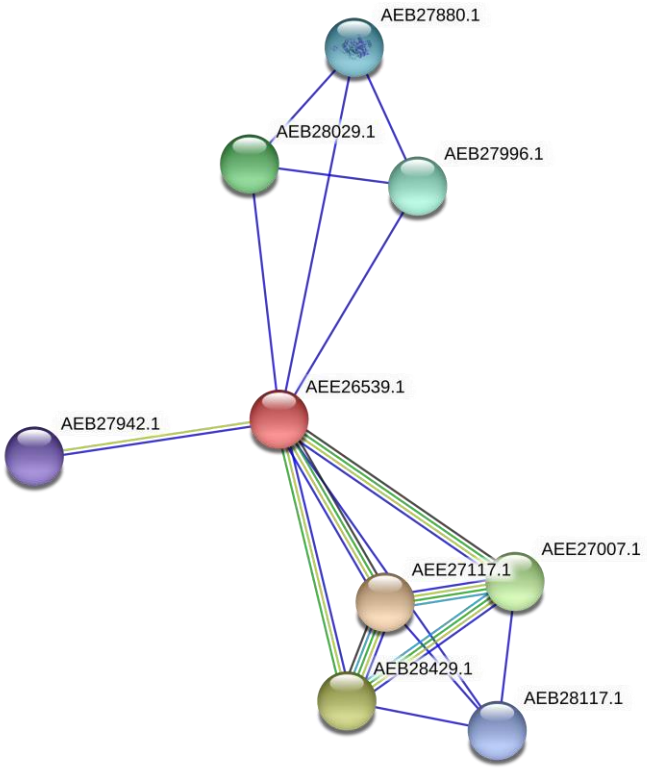
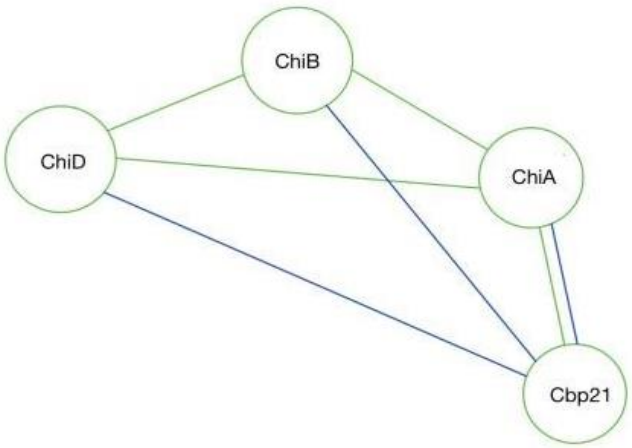


Figure 7: STRING interactions in *F. hispaniensis* around ChiB. Figure obtained using STRING analysis (string-db.org) to examine the protein of interest and associated proteins. Lines have same representations as in Figure 6.

Table 11**: The interactions shown for** *F. novicida* **chitin binding protein.** Accession numbers obtained from PubMed protein and BLAST results obtained from PubMed protein BLAST.

	Blasts to which protein in *F. novicida* U112	Blasts to Locus #	Blast results
AEE26539.1	Chitin Binding Protein 21	FTN_1192	97.82%
AEE27117.1	ChiB	FTN_1744	92.48%
AEB28429.1	ChiA	FTN_0627	94.64%
AEE27007.1	ChiD	FTN_1644	88.58%
AEB28029.1	nicotinamide ribonucleoside (NR) uptake permease (PnuC) family protein	FTN_0188	94.24%
AEB27996.1	ComEC/Rec2-related protein	FTN_0155	90.92%
AEB27880.1	histidine acid phosphatase	FTN_0022	95.99%
AEB28117.1	sel1 repeat family protein [*Francisella tularensis subsp. novicida* U112]	FTN_0275	93.47%
AEB27942.1	Glyco_hydro_129	FTN_0103	85.14%

In this STRING interaction analysis in *F. novicida*, ChiB is shown to interact with Cbp21, ChiA ChiD and FTN0103 a glycosyl hydrolase. The interaction of ChiB with



Cbp21 suggests that our idea for a connection between the chitinases and chitin binding proteins may have some support from this data.

Figure 8: Simplification of the STRING analyses for *Francisella tularensis, holarctica,* and *hispaniensis*. This figure shows the interaction between the chitinases and Cbp21. The blue lines represent *F. holarctica* and *F. hispaniensis,* while the green line represents *F. tularensis*.

The STRING analysis for the *Francisella holarctica* was similar to the SHUS4 strain, so the STRING was not included.

CHAPTER FOUR

Another goal of this project is to study Chitin binding protein A (CbpA) and determine a role for this protein. This protein is a known secreted protein (Hager et al. 2006), and is often found coexpressed along with ChiA and ChiB (Dean et al. 2015), Type IV pili act as Type II secretion system (Forsberg and Guina 2007), but its function is undetermined. *F. novicida* encodes for two Chitin binding proteins annotated as CpbA (FTN_1485) and Cpb21 (FTN_1192). Biochemically, CbpA contains N-terminal chitin-binding domain and an aromatic chitin/cellulose binding residue and is predicted to have chitin binding activity. Thus, we performed STRING analysis on CbpA to identify co-associated proteins.

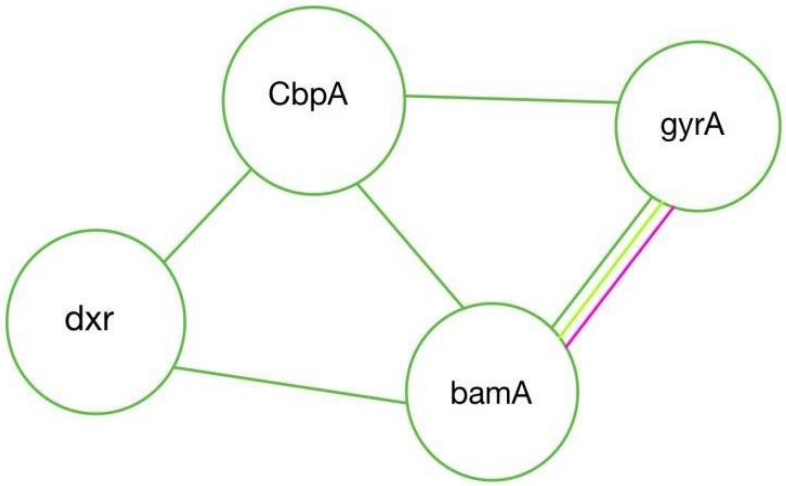


Figure 9**: STRING analysis of CbpA (*Francisella tularensis subsp. Holarctica* and *Francisella tularensis)*.** Figure obtained using STRING analysis (string-db.org) to examine the protein of interest (CbpA) and associated proteins (gyrA, bamA and dxr). Green lines represent gene neighborhood assocation, pink lines represent experimentally determined connections, and yellow lines represent text mining association.

In the String analysis of interactions, shown in **Figure 9**, BamA, an outer membrane assembly factor, plays a role in the outer membrane vesicles that are released in stress conditions (Klimentova et al. 2019), and is required for autotransporter biogenesis (Rossiter et al. 2011). GyrA is DNA gyrase subunit A that has importance in the antibiotic treatment of tularemia. DNA gyrase is the target of fluoroquinolone and single point mutations in either GyrA or GyrB can lead to antibiotic resistance (Caspar et al. 2017). Dxr is also a target for antimicrobial drugs. The methylerythritol phosphate (MEP) pathway is very important to *Francisella* because mutations in this pathway have been shown to be lethal. The MEP pathway produces isoprenoids (hydrocarbons essential for many cellular processes) and it has been shown that fosmidomycin inhibits MEP synthase in *F. tularensis* LVS (McKenney et al. 2012). Unfortunately, no hint of the Chitinase substrate synthase genes are observed in this STRING analysis. STRING analysis was not performed for the *F. novicida* U112 genes as they are not present in the STRING database, so a different *Francisella* strain was used (**Figure 9**).

Chitin Binding Protein A in *Francisella holarctica* is DA46_156. The *F. novicida* U112 equivalent is FTN_1485 (CbpA). In Holarctica, DA46_154-156 are fragments of CbpA in *F. novicida*, as shown in **Figure 10** below, and each have 100% sequence identify with the sections of CbpA in *F. novicida*. Whether these three proteins DA46_154-156 can interact to perform the same functions as the intact CbpA is not known, or whether the function is destroyed by the interruption of the genes. Interestingly in this analysis, these CbpA fragments do not interact with ChiA or ChiB.

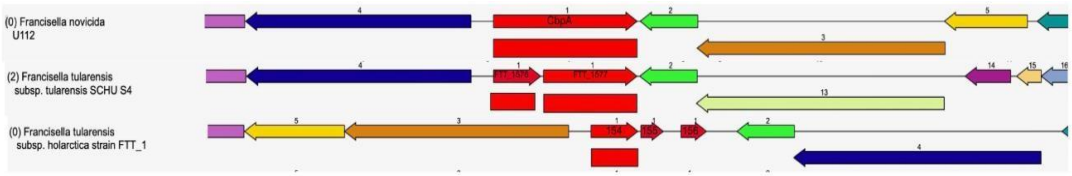


Figure 10**: PATRIC alignment of the CpbA genes.** *F. tularensis holarctica* genes DA46_154156 are shown in red with genes in *F. tularensis* SchuS4 (FTT_1576 and FTT_1577) and *F. novicida* (FTN_1485) CbpA.

In SCHU S4, CbpA is known as FTT_1577 and has two fragments of the *F. novicida* CbpA between BZ14_1175 and BZ14_1176. As shown in **Figure 10,** these two proteins represent two "fragments" of CbpA in *F. novicida*, with 100% identity for each with the respective sections of *F. novicida* sequence of CbpA. It is not known whether these two genes can produce proteins with the functional equivalent of CbpA in *F. novicida* or if the function is destroyed by the gene interruption. Again, these Cbp fragments are not found associated with ChiA or ChiB in this analysis.

SCHU CpbA also has interactions with dxr, bamA and gyrA, as seen in the string analysis for CbpA of *F. holarctica*. This consistent interaction with these three proteins between strains suggests that they may be of importance in the function of the chitin binding proteins in *Francisella.*

CHAPTER FIVE

The following analysis was done of the relevant Chitinase genes discussed in this thesis (**Table 1**) to identify neighboring genes and potential operons and shared promoters. We used the program Biocyc Genes (biocyc.org) and the locus numbers from the Table above.

Chitinase A Operon: The first analysis is of Chitinase A gene from *F. novicida* and *F. tularensis* Schu S4. From these figures (**Figure 11A, Figure 11B**), it can be seen that Chitinase A most likely has its own promoter both in *F. novicida* and *F. tularensis subsp. SCHU S4*. This similarity in gene organization is desirable in the application to the virulent strain from the study of the avirulent strain. Also, the gene does not appear to be in an operon, and so likely has its own promoter. The genes on each side of ChiA are consistent in *F. novicida* and *Ft* (ATGC138).

A. ChiA *F. novicida*

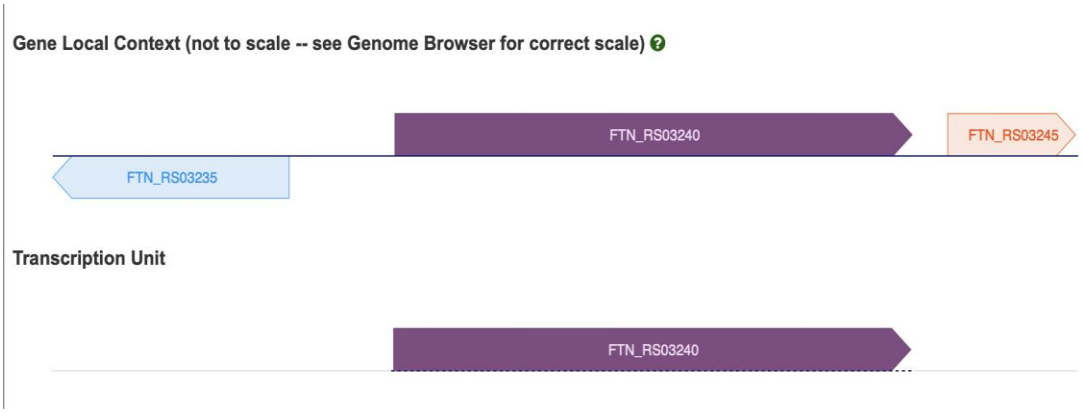
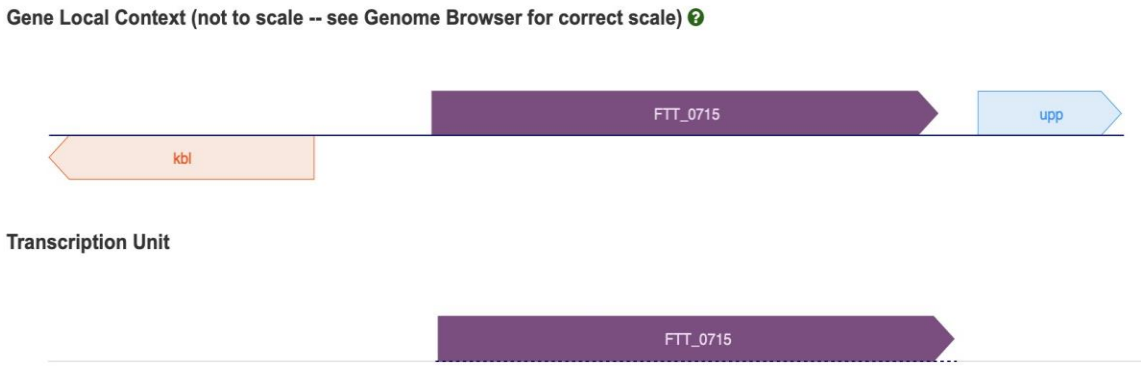


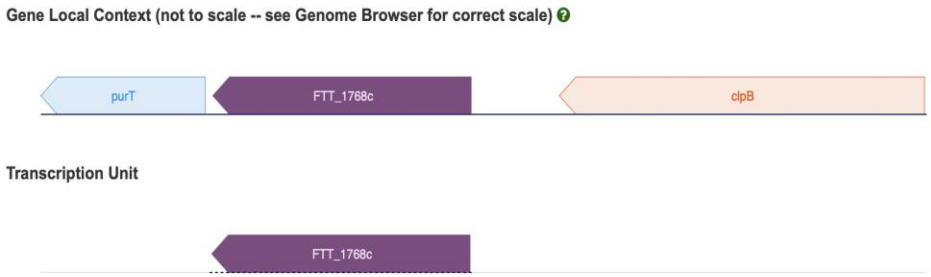
Figure 11**: Gene organization of ChiA in (A)** *F. novicida* and **(B)** *F. tularensis***.** Figure obtained using Biocyc Genes (biocyc.org) and the locus numbers to examine genes and potential operons.

B. ChiA SCHU S4



These Chi A genes have over 90% homology and seem to have the same promoters in both *F. tularensis* and *F. novicida*, allowing for results from *F. novicida* to be applicable to *F. tularensis*.

A. ChiB SCHU S4



B. ChiB *F. novicida*

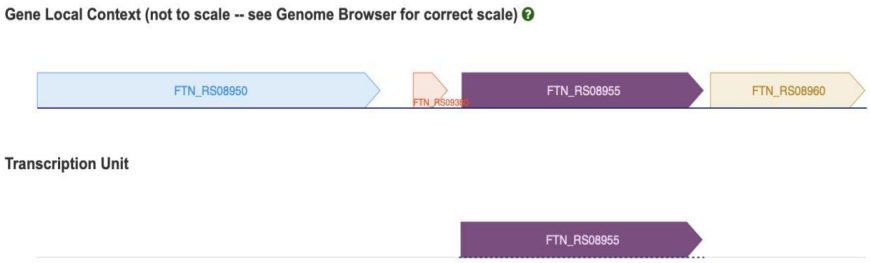


Figure 12: Gene organization of ChiB in (A) *F. novicida* and (B) *F. tularensis*. Figure obtained using Biocyc Genes (biocyc.org) and the locus numbers to examine genes and potential operons.

The second analysis is of Chitinase B gene from *F. novicida* and *F. tularensis* Schu S4. From these figures (**Figure 12A, Figure 12B**), it can be seen that Chitinase B most likely has its own promoter both in *F. novicida* and *F. tularensis subsp. SCHU S4* similar to ChiA. The genes on either side are consistent as with ChiA, with the exception of clpB being different between *F. novicida* and *F. tularensis.* This has implications for what signals may activate ChiB and/or ChiA, as they are not in the same operon, and may be differentially regulated. The promoter region of ChiB may be important to study further to understand its regulation.

CHAPTER SIX

Next, we analyzed the Chitin binding protein genes in both strains. Unlike ChiA and ChiB, the chitin binding protein genes of these two organisms are divergent in their gene arrangements (*F. novicida* contains one gene where the Cbp of SCHU S4 is split into two) (**Figure 13**). In addition, their organization in the genome appears to differ. (**Figure 14**). Unlike its chitinase counterparts, Chitinase binding protein A (CbpA) does not show as high a level of homology in *F. novicida* and *F. tularensis subsp. SCHU S4.* The gene (FTT_1576/FTT_1577) is CbpA split into two genes with the same regions seen in *F. novicida* across FTT_1577 (**Figure 14**). FTT_1577 and FTN_1485 both have chitinase C binding domains and chitin/cellulose binding sites whereas FTT_1576 does not show any of these regions. Potential implications are that findings about CbpA from *F. novicida* may not translate to the human virulent strain SCHU S4. Additionally, we do not know whether the protein products of the two FTT genes could have the same biological function as the CbpA protein in *F. novicida*. The ClustalOmega alignment of the sequences is shown in **Supplemental Figure S1** in Appendix 1.

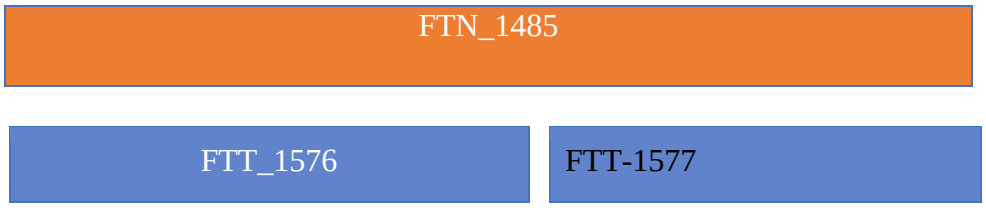
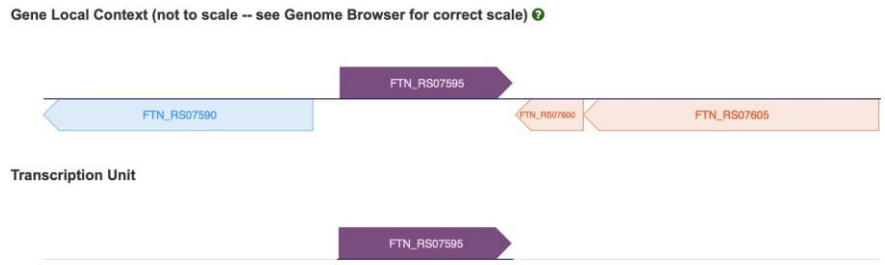


Figure 13: Alignment of CbpA from *F. novicida* with SchuS4.

Each of these species also contains a second chitin binding protein, called Cbp21, which differs from CpbA in binding domains and regions. In *F. novicida*, CbpA has the chitin binding domain of chitinase C and chitin/cellulose binding domains where Cbp21 has N-acetyl-glucosamine binding protein A and chitin binding domain 3 (**Figure 14**). ChiC and CbpA were run through BLAST to see their homology due to their binding domains, and they share 42.22% of 25% coverage, so they are not alike. Alignment of Cbp21 with Chitinase C suggests that Cbp21 may be the 'vestigial ChiC' in *F. novicida*.

A. CbpA *F. novicida*



B. Cbp SCHU S4

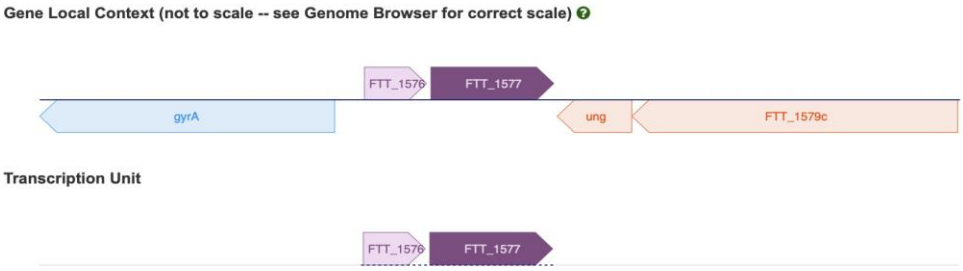


Figure 14: Gene organization of Cbp in (A) *F. novicida* and (B) *F. tularensis* SCHU S4. Figure obtained using Biocyc Genes (biocyc.org) and the locus numbers to examine genes and potential operons.

In summary, the literature suggests that Chitinase B in *Francisella* is an important extracellular enzyme and that the chitin-binding protein CbpA may also be important. The additional chitinase enzymes and chitin binding proteins were discussed. Specifically, ChiB is required for nutrient acquisition and negative regulation of *F. novicida* biofilm production (Chung et al. 2014). In SchuS4, ChiB mutants showed increased attachment but decreased replication rates. Specifically, CbpA was found to be co expressed and co-secreted with ChiA and ChiB (Hager et al. 2006). In addition, mutation of CbpA led to decreased attachment to chitin and lack of enzymatic activity in other bacteria (Frederiksen et al. 2013). Finally, under biofilm dispersal conditions, ChiA, ChiB and CbpA were all very highly overexpressed 20-50-fold (Dean et al., 2015) due to BDSF treatment. Examination of the *Francisella* genome identifies no chitin synthase genes. Together, the literature and the preliminary analysis done here support my approach and the importance of my main hypothesis.

Methods

Protein production. *F. novicida* ChiB was cloned into an expression vector (pQE-30) for recombinant protein expression in DHα *E. coli* cells.

PCR amplification of ChiB DNA was done from *F. novicida* U112 cDNA and the product was inserted into pTrcHis II TOPO (TOPO TA cloning kit, Invitrogen) and

transformed into *E. coli* One Shot® TOP10 Chemically Competent cells (Life Technologies, Invitrogen).” (Dean et al. 2020).

Clones already made by Alexandra Ii were used for expression of ChiB and CbpA. These cells were grown for 8 hours in Amp LB broth, centrifuged for 10 minutes at 6000 g, and resuspended in Magic Media. The cells were grown for 24h in the Magic Media and centrifuged for 10 min at 6000g. The supernatant was collected, filtered using a .22um vacuum filter and then aliquoted for single freeze thaw cycles. To purify the ChiB and CbpA from the supernatant, Ni-NTA spin columns were used to capture and concentrate the proteins according to the manufacture protocol. Once the final elution was collected, the protein concertation was measured using the Nanodrop One and frozen for downstream use.

Protein detection by Western Blotting. Once the protein was purified from the bacterial supernatant, it was run on a 4-12% Bis-Tris denaturing gel at 200V for 35min. The gel was then transferred to a membrane using the iBlot 2 and IVF ministacks. The membrane was then blocked with milk for 1 hour, then incubated overnight with the primary antibody (Goat anti-His). After the primary antibody was washed off with TBS-T, a secondary antibody was added and incubated with the membrane for 2 hours. Post incubation with the secondary antibody, the membrane was washed 3x with TBS-T and then saturated in Super signal and imaged.

Biofilms. Biofilms used in this study were all grown on 96-well polystyrene plates. 200ul of 1:30 diluted bacteria were plated and grown for 24h. For the addition of protein, the cells were measured before the addition of protein and then after overnight incubation at OD600.

The heat fix protocol was used to quantify the biofilm, which consists of rinsing the plate 2x with tap water, drying for 1 hour at 70C, adding 200ul of 0.1% crystal violet for 15 minutes, rinsing 3x with tap water, and resuspending the biofilm in 33% acetic acid to be measured at OD590.

Protein concentration. Since ChiB was only able to be expressed in small quantities, protein was concentrated from the supernatant as well. Bacterial cultures grown for 48h were spun down and the supernatant was collected. After filtration with .22um vacuum filter, the supernatant was spun with a 50k concentrator and eluent was collected for downstream analysis. The eluent from the concentrator was run on a 4-12% Bis-Tris denaturing SDS gel, stained with Coomassie blue for protein visualization and then saved for MS analysis along with small aliquots of the eluent. Bands from the SDS page gel were excised and sent for MS analysis to confirm protein banding.

Substrate Assays. Eluted protein was quantified on the Nanodrop one and added accordingly to the chitinase (Sigma Chitinase Assay Kit CS0980) and cellulose assays (Thermo Enzcheck cellulose substrate) according to the manufactures protocol.

Biofilm attachment assay. Wild type and mutant cultures were grown (180 rpm) overnight at 37 °C to stationary phase. Cultures were transferred (200 µl) to 96-well polystyrene plates that had been coated with poly-L-lysine (Lab-Tek II/Thermo Fisher Scientific, Waltham, MA) and allowed to adhere for 1, 2, or 4 hours statically at 37 °C. The medium was removed, and the wells stained with CV as described above (Champion et al. 2019).

Results

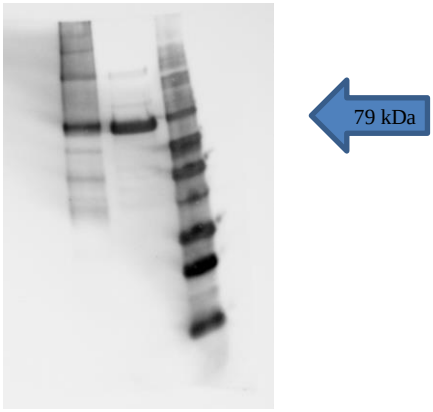


Figure 15: Western blot confirming presence of 6xHis rChiB. Western blot using anti-His tag antibody.

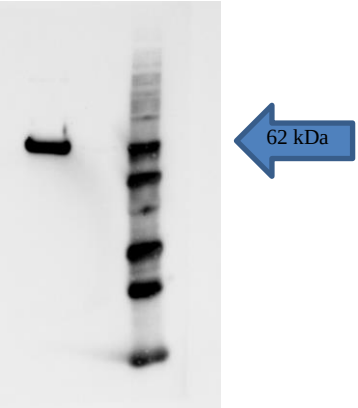


Figure 16: Western blot confirming presence of 6xHis rCbpA. Western blot using anti-His tag antibody.

As shown in **Figure 15 & 16**, Western blots confirm the presence of rChiB and rCbpA in the supernatant of the DHα *E. coli* cells after processing and purifying of the 6x His tagged proteins.

Chitinase Activity

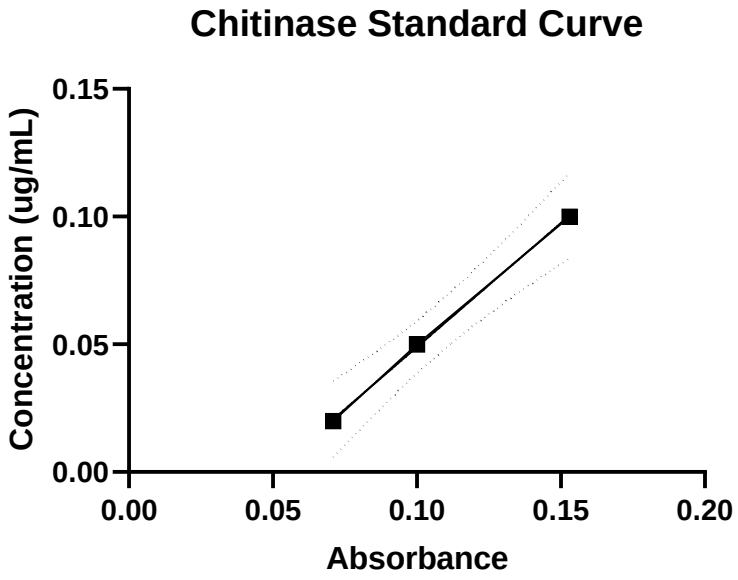


Figure 17: Chitinase assay confirming enzymatic activity of the expressed rChiB. *F. novicida* rChiB protein was expressed and purified and assayed for activity as described below.

The chitinase standard curve confirms enzymatic activity of the purified rChiB as seen in Figure 17. The substrate used was 4-Nitrophenyl β-D-N,N′,N″-triacetylchitotriose (Sigma N8638) which is annotated as a chitinase and lysozyme substrate. Activity with this substrate was highest out of all tested and was used for all chitinase assays.

Biofilms

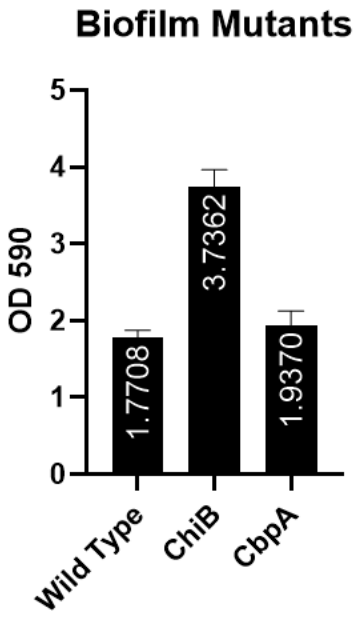


Figure 18**: Graph of the average biofilm of Wild Type U112 *Francisella novicida*, ChiB mutant and CbpA mutant.** All error bars are standard deviation.

Previous data on ChiB was confirmed when testing the transposon mutants. ChiB mutants showed a statistically significant increase in biofilm production with a p value < 0.05, confirming that they are negative regulators of the biofilm. CbpA did not show a statistically significant increase in biofilm with a p value > 0.05 (**Figure 18**).

Mass Spec Data

Table 12: Composition of partially purified supernatants of *F. novicida* by Mass Spectrometry.

Protein	Sample 1	Sample 2	Sample 3
Chitinase	5%	6%	5%
Chitin Binding Protein	5%	7%	4%
GroEL	8%	9%	7%
DnaK	7%	7%	6%

Samples are biological replicates from the same *F. novicida* 48h TSB-C grown culture. These results of Mass Spectrometry show that ChiB and CbpA are present at high levels in the sample and thus these samples are suitable for down-stream use in enzymatic assays (**Table 12**).

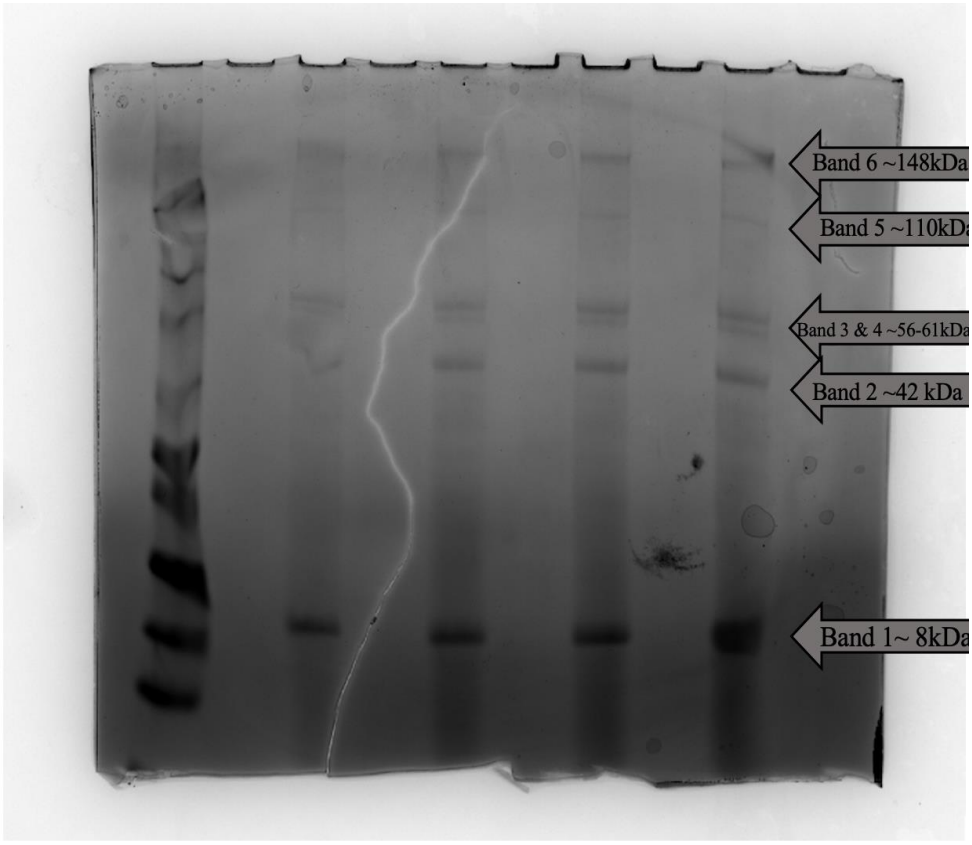


Figure 19: Protein gel showing the contents of the concentrated supernatant from 48h *Francisella novicida* culture.

Table 13: Top 5 proteins for each band sent out for Mass Spectrometry analysis.

Protein	Band 1	Band 2	Band 3&4	Band 5	Band 6
Most abundant	Elongation factor TU	GroEL	ChiB	FTN_0715	FTN_0714
2nd	AhpC/TSA family protein	Chitin binding protein	DnaK	FTN_0714	FTN_0715
3rd	FTN_0715	ChiB	M13 family metallopeptidase	carbamoyl phosphate synthase large subunit	

4th	iron/manganese superoxide dismutase family protein	FTN_0715	FTN_0714	aconitate hydratase	
5th	ThiJ/PfpI family protein	FTN_0714	FTN_0715	isoleucyl-tRNA synthetase	

The samples from the 48-hour supernatant were run on a 4-12% Bis-Tris gel (Figure 19) and the bands were excised and sent for analysis by Mass Spectrometry. The data from the samples confirms that ChiB and CbpA were seen in high concentrations in all three of the samples and the two bands corresponding closest to ChiB and CbpA did contain the proteins in high abundance as shown in **Table 13**.

rChiB Cellulose assays

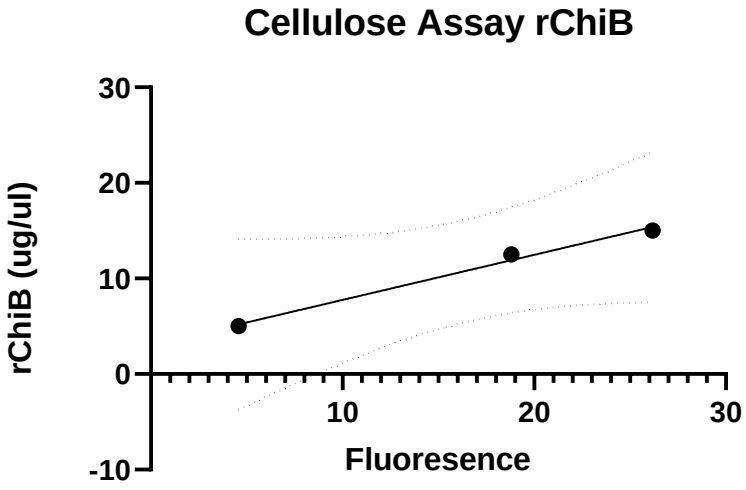


Figure 20: Cellulose assay with *F. novicida* rChiB.

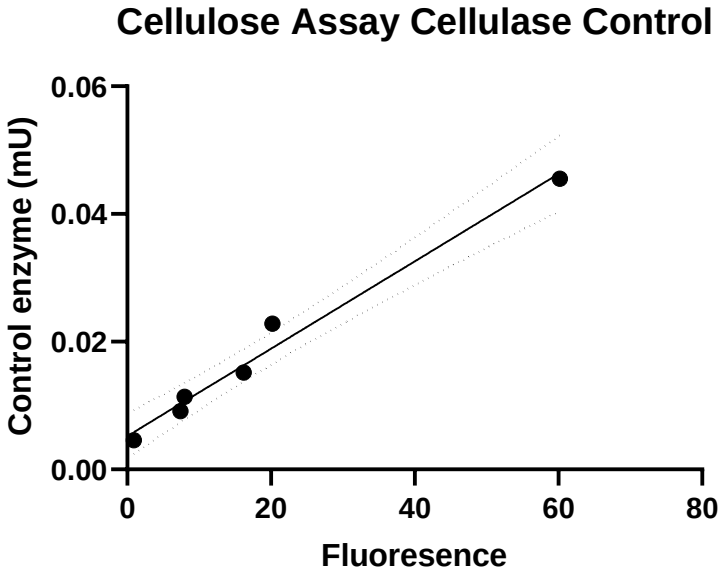


Figure 21: Cellulose assay positive control standard curve (*Trichoderma reesei* cellulase, Sigma C2730). for recombinant protein comparison.

Cellulose assays were performed according to the manufacturers protocol (Enzcheck cellulose substrate) and after background was subtracted, the data was analyzed for activity against the positive control cellulase enzyme, *Trichoderma reesei* (Sigma C2730). The cellulase activity was confirmed with this assay. With rChiB, 5 ug/ul is equivalent to about .008 mU of activity when directly compared to activity from the positive control as seen in Figure 20 & 21.

Supernatant cellulose assays

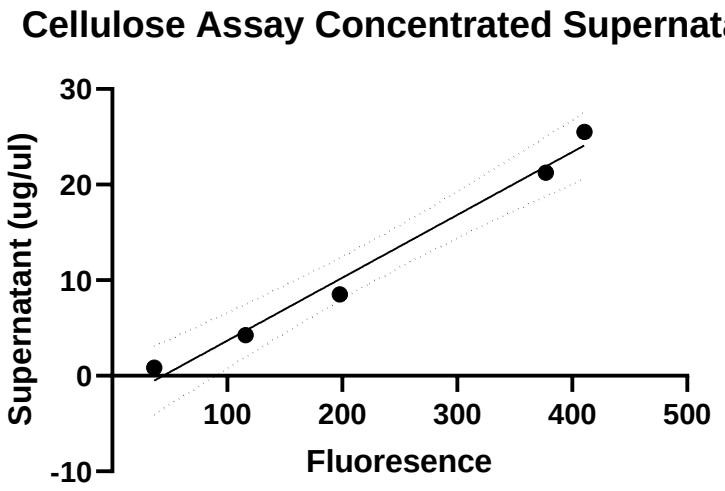


Figure 22: Cellulose assay with the addition of concentrated bacterial supernatant.

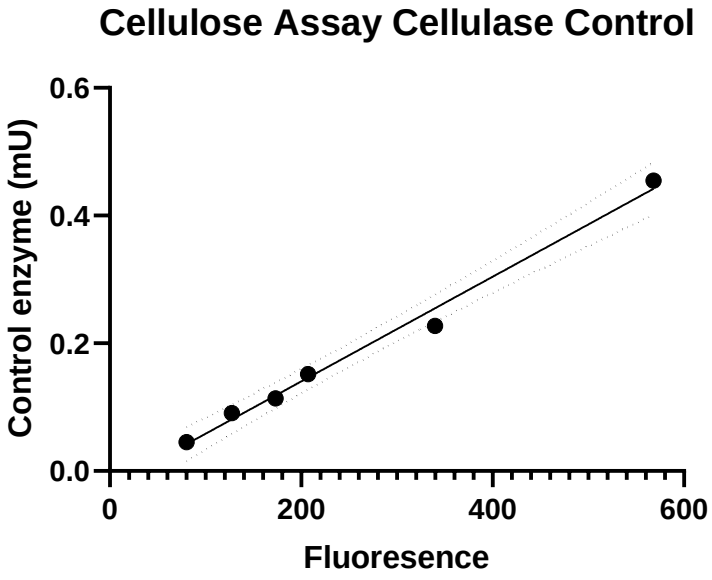


Figure 23: Cellulose assay positive control standard curve (*Trichoderma reesei*, Sigma C2730). for supernatant comparison.

The concentrated supernatant of Wild Type Francisella novicida was added to the same cellulose assay and was found to have activity against the substrate as seen in Figures 22 & 23. After the background was subtracted, it was found that 8.5ug/ul is equivalent to about .13 mU of activity when directly compared to the positive control.

Mutant biofilms and attachment assays

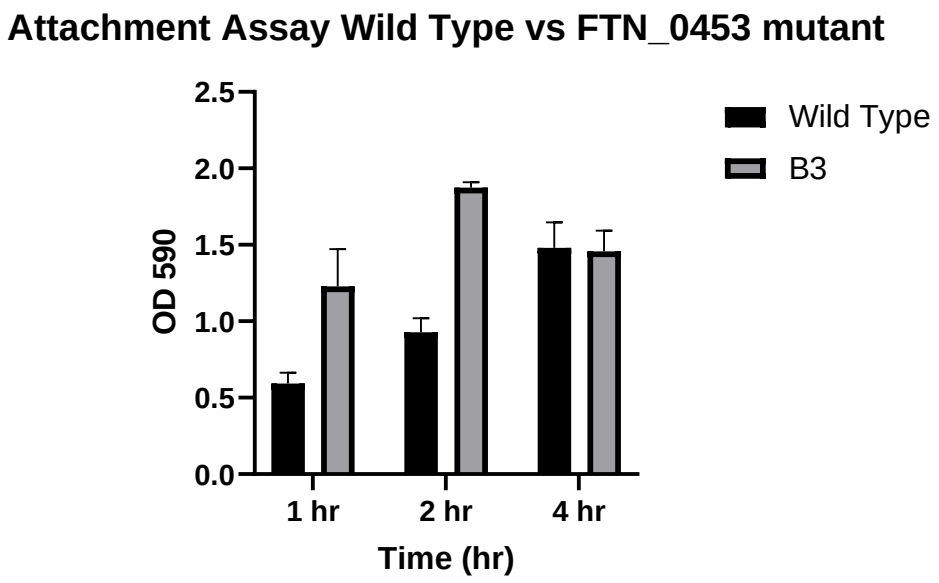


Figure 24: Attachment assay of FTN_0453 mutant and the wild type.
The FTN_0453 transposon mutant shows statistically significant better attachment at 1hr and 2hrs with a p value < 0.05, while the 4hr attachment shows that there is no statistical difference between the two for attachment.

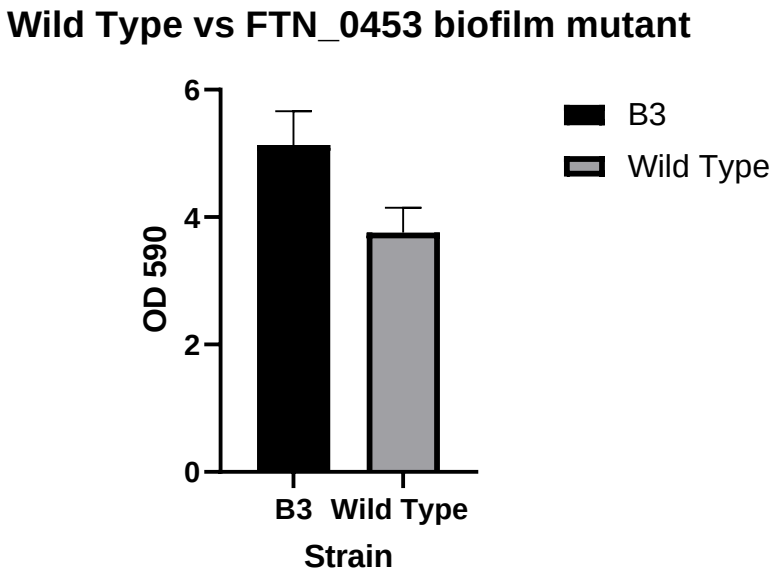


Figure 25: Biofilm assay of the wild type vs the FTN_0453 mutant at 37C for 24h. There is a statistically significant increase in the amount of biofilm produced by the FTN_0453 transposon mutant compared to the Wild type biofilm with the p value < 0.05.

The results of these two assays suggest that the B3 transposon mutant did not knock out activity of the gene and instead either increased activity of this transferase or disrupted the ability of the gene cluster that regulates biofilm to do so effectively. Both of these assays (**Figure 24 & 25**) confirm that some change has been made to the regulation of biofilm, but it is unlikely that only one factor is playing into the polysaccharide synthesis that contributes to the biofilm mass.

Cellulase Addition Wild Type Biofilm

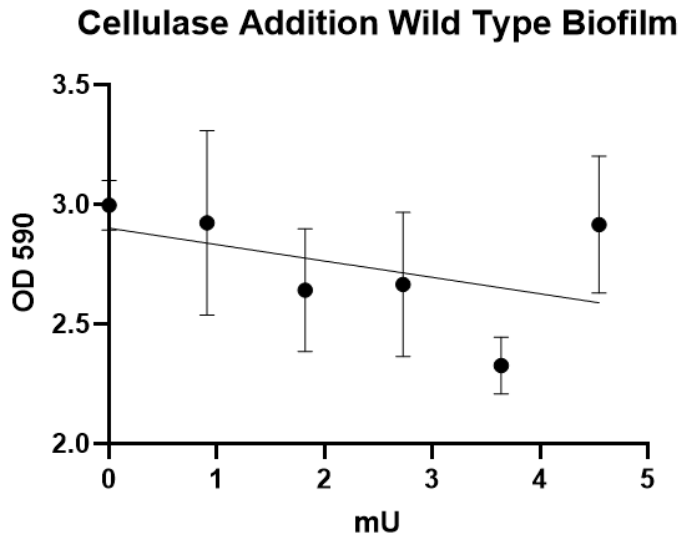


Figure 26: Wild Type Biofilm with addition of cellulase from *Trichoderma reesei* (Sigma C2730).

Several concentrations of cellulase (mU) were added to a 24h biofilm and then the biofilm was grown for another 24h statically at 37C (**Figure 26**). The amount of untreated biofilm (0) remaining was compared to the amount of treated biofilm remaining. The cellulase is affecting the biofilm crystal violet staining, suggesting that the biofilm contains cellulose or another polysaccharide containing β, 1-4 glycosidic linkages. Although the decrease in biofilm seen is modest, the concentration of cellulase added was similar to the concentration added to the cellulose assays, and the decrease is not statistically significant. This data supports our previous data showing cellulase decreases biofilm density.

CHAPTER SEVEN

Discussion

In this work we have shown that ChiB is able to cleave both chitin and cellulose and may be able to cleave lysozyme substrates as well. Along with this, it was shown that CbpA did not have an effect when paired with ChiB for the chitinase assays. This could be due to many factors including the lack of necessity for CbpA to tag a substrate when the substrate is already present in such high quantities for the enzyme. It is also possible that CbpA does not actually tag the substrate, in which case more study needs to be done on the role of CbpA in contributing to enzymatic activity.

Since *Francisella novicida* does not produce chitin, it was hypothesized that there is another gene responsible for producing a similar substrate that would make up a portion of the EPS. Since it has been shown that ChiB is able to cleave cellulose, that may be the substrate of interest in the biofilm. Several glycosyl transferases have been identified in the *Francisella novicida* genome and one, FTN_0453, contains a cellulose synthase superfamily domain and a DXD motif which is part of the catalytic domain and indicates activity. When this protein is run on BLAST through PubMed Protein, there are several proteins that share high homology with the *Francisella* protein that serve as cellulose synthase proteins.

In order to test the theory that cellulose could be the substrate in question in the biofilm, several tests were performed on the transposon mutant for FTN_0453. Several biofilms were performed on both transposon mutants for FTN_0453, D7

(tnfn1_pw060420p01) and B3 (tnfn1_pw060328p03). D7 did not appear to lack biofilm formation activity, while B3 showed both an increase in biofilm and attachment. This gene is part of a cluster of genes (FTN_0451-FTN_0457) that regulates the novicida biofilm. It is possible that knocking out this gene disrupted the bacteria's ability to regulate biofilm, thus generating more biofilm. It is also possible that the transposon mutant increased the expression of this gene. Future studies are required to understand the interactions with this gene cluster.

Finally, cellulase was added to the wild type biofilm which did show a decrease in biofilm post incubation. This is consistent with previous data that shows cellulase can decrease the biofilm density. Although this supports the theory that cellulose could make up a portion of the biofilm, it is possible that there is one or more substrates that contain the same β, 1-4 glycosidic linkages, able to be cleaved by cellulose and chitinases. Further studies including biofilm mass studies are required to identify which of these substances is contributing to biofilm mass.

Future directions

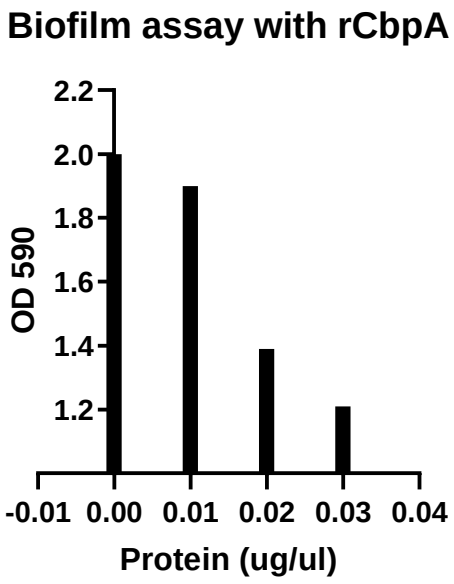


Figure 27: Addition of rCbpA to a WT *Francisella novicida* biofilm.

A titration of rCbpA was added to a Wild Type *Francisella novicida* biofilm (**Figure 27**) to see its effect on biofilm production. A decrease was seen with an increase in rCbpA added to the biofilm. This does suggest a role for CbpA in biofilm regulation and will require further study to understand the interactions between the chitinases, CbpA and the biofilm substrate.

Possible Role of Biotin

In the studied protein interactions between the chitinases and chitin binding protein, the biotin synthase gene cluster was closely associated with the chitinases. This could be an important interaction within the role of biofilm regulation and could be studied alongside the chitinases to further understand the role of synthases.

Collection and characterization of Extracellular polysaccharide (EPS)

In order to fully understand the composition of the *Francisella* biofilm, collection and characterization of the biofilm could be done and sent out for classification of the polysaccharides that are made in the biofilm. This characterization could lead to further understanding and identification of the true substrate or substrates for the chitinases in the francisella biofilm.

Future directions for biofilm substrate

In order to fully understand the role of FTN_0453, a deletion mutant should be tested using the attachment and biofilm assays to fully see the role of the protein in relation to biofilm activity.

The gene cluster of FTN_0450-0457 that regulates the biofilm is not in *Francisella tularensis* and therefore is most likely not applicable to the virulent bacteria's biofilm or virulence. Other glycosyl hydrolases may form possible substrates for ChiB in *Francisella tularensis* and therefore can be studied further for a more applicable role of the substrate.

APPENDIX

Supplemental Figure S1. Clustal Omega alignment of CbpA in *Francisella novicida* and *Francisella tularensis*. FTT_1576/FTT_1577 and FTN_1485.

```
tr|Q5NEP3|Q5NEP3_FRATT      ------------------------------------------------------------      0
tr|Q5NEP4|Q5NEP4_FRATT      MKKLIVSSIIASIMLPATTFANNKQLNQGEVAYHNTFPISQSEAYDIFITNTDYANTVIM      60
tr|A0Q7Y7|A0Q7Y7_FRATN      MKKLIVSSIIASIMLPATTFANNKQLNQGEVAYHNTFPISQSEAYDIFITNTDYANTVIM      60

tr|Q5NEP3|Q5NEP3_FRATT      ------------------------------------------------------------      0
tr|Q5NEP4|Q5NEP4_FRATT      SNTIAGVMYGYLIKQKYPKMKFNKDYLYGSLIGQLMQESDMSSQLAQNFNPNDPDQLIHN      120
tr|A0Q7Y7|A0Q7Y7_FRATN      SNTIAGVMYGYLIKQKYPKMKFNKDYLYGSLIGQLMQESDMSSQLAQNFNPNDPDQLIHN      120

tr|Q5NEP3|Q5NEP3_FRATT      ------------------------------------------------------------      0
tr|Q5NEP4|Q5NEP4_FRATT      STYANILLQAGQGGPYQINDYSKRLPSAEAKGDLSLVNYNAVAAILGYSIQDQDDGSQTK      180
tr|A0Q7Y7|A0Q7Y7_FRATN      STYANILLQAGQGGPYQINDYSKRLPSAEAKGALGLVNYNAVAAILGYSIQDQDDGSQTK      180

tr|Q5NEP3|Q5NEP3_FRATT      --------------MITAFYHFNDINRMNVNSTNDWYVYHDQWNKCLENSQKNTAGGLTF      46
tr|Q5NEP4|Q5NEP4_FRATT      K-----------------------------------------------------------      181
tr|A0Q7Y7|A0Q7Y7_FRATN      KIGPEALDNIYSGPMITAFYHFNDINRMNVNSTNDWYVYHDQWNKCLENSQKNTAGGLTF      240

tr|Q5NEP3|Q5NEP3_FRATT      PFVMNIVYNAGDYSPVLKTYLNICAEGSTNTKEYKNINNWLLETINYQTIIGAEKPNTGE      106
tr|Q5NEP4|Q5NEP4_FRATT      ------------------------------------------------------------      181
tr|A0Q7Y7|A0Q7Y7_FRATN      PFVMNVVYNAGDYSPVLKTYLNICAEGSTNTKEYKNINNWLLETINYQTIIGAEKPNTGE      300

tr|Q5NEP3|Q5NEP3_FRATT      ATYYRYPRQVSFYINQIFNNNNKKLEDTGITVNNQVTFSISRLKQVFAMVMHKLSYRDNN      166
tr|Q5NEP4|Q5NEP4_FRATT      ------------------------------------------------------------      181
tr|A0Q7Y7|A0Q7Y7_FRATN      ATYYRYPRQVSFYINQIFNNNNKKLEDTGITVNNQVTFSISRLKQVFAMVMHKLSYRDNN      360

tr|Q5NEP3|Q5NEP3_FRATT      DDLHFISIEDANKAFDQAINQITTADGQIPEDLSFSNTQKTTNQQRLLIYKIIQKAISNL      226
tr|Q5NEP4|Q5NEP4_FRATT      ------------------------------------------------------------      181
tr|A0Q7Y7|A0Q7Y7_FRATN      DDLHFISIEDANKAFDQAINQITTTDGQIPEDLSFSNTQKTTNQQRLLIYKIIQKAISNL      420

tr|Q5NEP3|Q5NEP3_FRATT      AQNNDIDFTKTYDESKSSPEPGPTPEPQYTYPDGIGSYLNGTVVKAGDSEYKCKEANWCN      286
tr|Q5NEP4|Q5NEP4_FRATT      ------------------------------------------------------------      181
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tr|Q5NEP3|Q5NEP3_FRATT      NPAYTPLGNFSDAAWENLNPQPKPTPVGTWDPTKEYVAGDTVEINGVKYAAQWWNKGVNP      346
tr|Q5NEP4|Q5NEP4_FRATT      ------------------------------------------------------------      181
tr|A0Q7Y7|A0Q7Y7_FRATN      NPAYTPLGNFSDAAWENLNPQPKPTPVGTWDPTKEYVAGDTVEINGVKYAAQWWNKGVNP      540

tr|Q5NEP3|Q5NEP3_FRATT      VENHTQYQDPWNIVK     361
tr|Q5NEP4|Q5NEP4_FRATT      ---------------     181
tr|A0Q7Y7|A0Q7Y7_FRATN      VENHTQYQDPWNIVK     555
```

Supplemental material for Cbp21, Cellulose-degrading Lytic Mono-oxygenase:

Supplemental Table 1: Accession numbers, description, and alignment of Cbp21 in *F. novicida* and *F. tularensis*. Accession numbers obtained from PubMed protein and alignment obtained from NCBI alignment viewer.

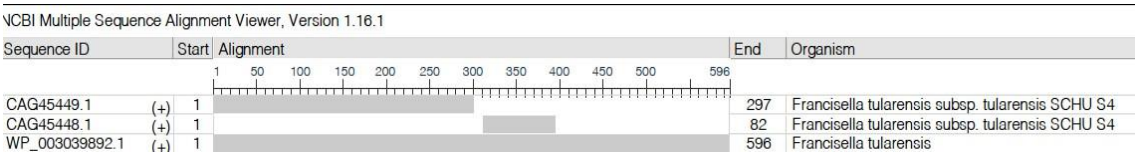
NCBI Multiple Sequence Alignment Viewer, Version 1.16.1

Sequence ID		Start	Alignment	End	Organism
			1 50 100 150 200 250 300 350 400 450 500 555		
CAG46209.1	(+)	1		181	Francisella tularensis subsp. tularensis SCHU S4
CAG46210.1	(+)	1		361	Francisella tularensis subsp. tularensis SCHU S4
ABK90352.1	(+)	1		555	Francisella tularensis subsp. novicida U112

Accession	Description

CAG46209.1	hypothetical membrane protein [*Francisella tularensis subsp. tularensis* SCHU S4]
CAG46210.1	hypothetical protein FTT_1577 [*Francisella tularensis subsp. tularensis* SCHU S4]
ABK90352.1	chitin-binding protein [*Francisella tularensis subsp. novicida* U112]

Supplemental Figure 2: Alignment of *F. tularensis* and *F. novicida* Cbp21. Alignment obtained from NCBI alignment viewer



The conclusion from this analysis is that *F. novicida* and *F. tularensis* LVS share Cbp21, and potentially so does *F. philomiragia*, although with lower homology, as it has shown for most of these comparisons. *F. tularensis* SchuS4 has a fragment set of genes that span part of the Cbp21 sequence in the other organisms, albeit with high identity. Whether these fragments are functional or not in *F. tularensis* SchuS4 is unknown. The domains in Cbp21 include the CBM domain noted below.

Cpb21 has recently been annotated as a cellulose-degrading lytic mono-oxygenase, hydrolase activity, hydrolyzing O-glycosyl compounds. However, *F. tularensis* LVS has a very different CBP that is likely not of the lipid monooxygenase type. Cp21 is annotated to have a Carbohydrate-Binding Module Family 5 from amino acids 225-267, which includes a 60 aa chitin binding domain, previously annotated as a cellulose binding domain. http://www.cazy.org/CBM5.html. In addition, these proteins contain Auxiliary Activity family 10 domains, which are defined as follows in CaZy. "AA10 (formerly CBM33) proteins are copper-dependent lytic polysaccharide monooxygenases (LPMOs); some proteins have been shown to act on chitin, others on cellulose; lytic cellulose monooxygenase (C1-hydroxylating) (EC 1.14.99.54); lytic cellulose monooxygenase (C4-dehydrogenating)(EC 1.14.99.56); lytic chitin monooxygenase (EC 1.14.99.53); lytic xylan monooxygenase / xylan oxidase (glycosidic bond-cleaving) (EC 1.14.99.-) Note: AA10 (formerly CBM33). The enzymes in this family were originally classified as chitin-binding proteins (CBM33); Vaaje-Kolstad et al. have shown that these proteins are in fact oxidative enzymes [PMID - 20929773]. They are now reclassified in the AA category of CAZy. Because a significant literature is associated with the old name CBM33, we recommend to describe these enzymes as "AA10 (formerly CBM33)." http://www.cazy.org/AA10.html The Mechanism of these enzymes is to be a monooxygenase. A similar conclusion is found after performing a PFAM and InterPro analysis of this protein.
http://pfam.xfam.org/family/PF03067
https://www.ebi.ac.uk/interpro/entry/InterPro/IPR036573/.

Because of the lytic mono-oxygenase activity and the classification of it as GH_61 (Glyco_hydro_61) of this protein, it will not be the focus on this study. GH_61 family members are reported to be more likely to be accessory proteins than hydrolases.

"Although weak endoglucanase activity has been demonstrated in several members of this family [1-3], they lack the clustered conserved catalytic acidic amino acids present in most glycoside hydrolases. **Many members of this family lack measurable cellulase activity on their own, but enhance the activity of other cellulolytic enzymes.** They are therefore unlikely to be true glycoside hydrolases [4]. The substrate-binding surface of this family is a flat Ig-like fold [5]." http://pfam.xfam.org/family/Glyco_hydro_61

Additional identified domains include from amino acids 1..499, a PRK13211 domain, which is an N-acetylglucosamine-binding protein A-like domain (CDD:237309). In addition, the CBM_3 domain was identified from amino acids 23-191, Chitin binding domain; pfam03067, (CDD:281112) and from amino acids 251-252, an aromatic chitin/cellulose binding site (CDD:213178) is identified.

Supplemental Table 2: Chitin-binding protein 21 homology among *Francisella* species. Values obtained by running BLAST analysis through PubMed protein on each pair of Cbp21 homologs.

	***F. novicida* Cbp21 FTN_1192**	**Ftt Cbp21 FTT_0816c**
***F. novicida* Cbp21**	100%	98.98%
Ftt Cbp21		100%

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